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Cloning, characterisation and identification of several polymorphisms in the promoter region of the human α_{2B}-adrenergic receptor gene☆

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Abstract

Screening of a foetal brain genomic DNA library allowed to isolate a 10-kb fragment of the gene encoding the human α_{2B}-adrenergic receptor, that contained 5.5 kb of the 5′-flanking region, the open reading frame and 2.9 kb of the 3′-flanking region. The 1-kb fragment upstream from the start codon was rich in GC, lacked consensus TATA or CAAT box, but contained several Sp1-binding sites. Other potential *cis*-regulatory elements found in the 5′-flanking region included AP2, USF, Stat-6, NFκB and Olf-1. A single canonical polyadenylation signal (AATAAA) was found at position +3252/+3257 and the polyadenylation site was 3274 nucleotides downstream from ATG. Transfection experiments with chimeric luciferase contructs containing various truncated fragments of the 5′-region showed that the fragment −3160/+3 exhibited promoter activity in all tested cell lines and permitted the definition of a minimal 200-bp promoter (−603/−411) containing three putative Sp1-binding sites and two initiator elements. Transcriptional activity of this region was inhibited by the addition of mithramycin, a specific inhibitor of Sp1 binding to GC-rich sequences. The search for sequence variants within a fragment covering 1.7 kb of 5′-flanking region and the coding region allowed us to identify five novel single nucleotide polymorphisms. Interestingly, the G/C substitution at position −98 relative to the start codon was common and in complete linkage with a previously identified insertion/deletion polymorphism in the coding region which was showed to affect α_{2B}-adrenergic receptor function. Based on transfection data and computer-assisted sequence analysis, the −98 G/C single nucleotide polymorphism was located within a portion of the 5′-UTR (−127/+3) affecting luciferase activity and it created additional putative binding site for Sp1. However, G/C substitution had no significant incidence on promoter activity in BHK-21 or HeLa cells.

Keywords: Adrenergic receptor; Catecholamine; *α2C2*; Promoter; Polymorphism

1. Introduction

α_2-Adrenergic receptors (α_2-ARs) are widely distributed in the organism and participate in the control of a variety of physiological processes, such as regulation of blood pressure, lipolysis and insulin release [1]. Three distinct α_2-AR subtypes (namely α_{2A}, α_{2B} and α_{2C}), have been characterised and cloned [2]. All α_2-AR subtypes preferentially interact with $G_{i/o}$ proteins and share common pathways of signal transmission, including inhibition of adenylyl cyclases, closure of voltage-gated Ca^{2+}-channels, activation of inwardly rectifying K^+-channels and stimulation of the MAPKs, Erk1 and Erk2 [3,4]. Subtypes exhibit similar affinity for the endogenous catecholamines, epinephrine and norepinephrine, but diverge in many aspects of their pharmacology, biochemistry and regulation. Studies on

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Abbreviations: AR, adrenergic receptor; SNP, single nucleotide polymorphism; SSCA, single strand conformation analysis; I/D, insertion/deletion.

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several human populations have indicated that all three α_2-ARs are polymorphic. The consequences of variations occurring in their third intracellular loops have been analysed in transfected cells. They result in a gain of agonist-stimulated α_{2A}-AR function [5], impaired α_{2B}-AR desensitisation [6] or impaired α_{2C}-AR coupling to G_i [7].

The α_{2B}-AR is encoded by the *α2C2* gene in human [8] and by the RNG gene in rat [9]. Studies on knock-out mice indicate that α_{2B}-AR plays an important role in the regulation of blood pressure [10,11]. However, the tissue expression pattern of this subtype is not known in detail. RNase protection assays and RT–PCR experiments showed the presence of substantial amounts of *α2C2* transcripts in human heart, aorta, liver and spleen [12,13], but the expression of the α_{2B}-AR protein still remains to be established in these tissues. On the other hand, radioligand binding studies have demonstrated that α_{2B}-AR is densely expressed in rat kidney, placenta and foetal reticulocytes [14,15], but this is not the case in human. The divergence in expression patterns between species may be the result of differences in the functional characteristics of the flanking regions of the genes encoding the α_{2B}-AR subtype.

The promoter regions of the genes encoding the α_{2A}- and α_{2C}-AR subtypes have been sequenced and partially characterised in both human and rat [16–19]. The organisation of the mouse α_{2B}-AR gene has also been studied [20]. Transcription is initiated 415 bases upstream from the ATG codon and is controlled by a GC-rich promoter region lacking a TATA box but containing a GC box and a single CAAT box. As a first step toward the elucidation of the molecular mechanisms governing the expression of the α_{2B}-AR in human tissues, we have cloned and sequenced the 5′- and 3′-flanking regions of the *α2C2* gene. The site of polyadenylation was determined and the promoter activity of the 5′-region was analysed by transfecting different cell lines with chimeric constructs containing luciferase as reporter gene. Based on the analysis of genomic DNA from 138 unrelated individuals, the present study also establishes that *α2C2* gene has several single nucleotide polymorphisms (SNPs) in its coding and 5′-non-coding regions. A guanine to cytosine substitution at position −98 was common and in complete linkage with a previously identified insertion/deletion (I/D) polymorphism [21]. The possible incidence of this SNP was investigated in transfected cells.

2. Materials and methods

2.1. Screening of a human genomic library

A human foetal brain genomic DNA library in the cosmid vector SuperCos 1 (Stratagene) was screened using the *Acc*I–*Acc*I fragment of *α2C2* (nucleotides +571/+1532) as a probe [8]. Titration, hybridisation and washes were carried out as recommended by the manufacturer.

2.2. DNA sequencing and analysis

Restriction fragments of the *Eco*RI–*Kpn*I fragment of a positive cosmid were subcloned and sequenced in both orientations with an automated DNA sequencer (ABI 373, Perkin Elmer Applied Biosystems). The nucleotide sequence reported in this paper has been deposited in GenBank with the accession number AF005900. The 5′- and 3′-flanking regions were analysed using the computer programs available at the WWW server of the Human Genome Center at the Baylor College of Medicine (http://searchlauncher.bcm.tmc.edu/). Through the whole manuscript, nucleotides were numbered according to their position relative to the translation start site of *α2C2* gene; a negative sign thus refers to nucleotide upstream from ATG.

2.3. PCR and single strand conformation analysis (SSCA)

Human genomic DNA was isolated from whole blood. The region of interest was amplified in four parts. Primer pairs 1 and 2 amplified the 5′-flanking region and pairs 3 and 4 amplified the coding region of the human α_{2B}-AR gene (Table 1). The pairs overlapped to cover the region from nucleotide −1730 to nucleotide +1416. PCR conditions were optimised using a PCR optimiser kit (Invitrogen). Reactions were conducted in 5 μL volume containing

Table 1
PCR primers, product sizes and restriction enzymes used for SSCA

Pair number	Sense primer/antisense primer	Product size (location)	Restriction enzyme for SSCA
1	S1: 5′-TGTCAGAGGTGGATTGGTGCTTAT-3′; AS1: 5′-GCGCGCAGCTGTGGAGGGGTCGGT-3′	1071 (−1730/−660)	*Bse*RI
2	S2: 5′-GGTTGTGGTGTATTGCGACC-3′; AS2: 5′-TAGGGGTCCTGGTGGTCCAT-3′	887 (−867/+20)	*Bsi*EI, *Bsa*WI
3	S3: 5′-GGGGCGACGCTCTTGTCTA-3′; AS3: 5′-GGTCTCCCCCTCCTCCTTC-3′	878 (−95/+783)	*Dde*I, *Dra*III
4	S4: 5′-GCAGCAACCGCAGAGGTC-3′; AS4: 5′-GGGCAAGAAGCAGGGTGAC-3′	815 (+602/+1416)	*Alu*I, *Hin*cII

Table 2
Identified polymorphisms and restriction enzymes used for genotyping

SNP	Enzyme	Band sizes in wild type and variants[a]	Primer pair	Amino acid	Frequency (%)
−98 G/C	*Ava*I	63, 55, 137, 65, 273, 65, 229 (110, 119)	S2 + AS2		46
−87 G/T	*Hin*fI	887 (779, 108)	S2 + AS2		1
+36 A/G	*Bsa*JI	42, 511 (84, 427), 160, 1, 6, 11, 35, 35, 77	S3 + AS3	12 (silent)	2
+632 G/C	*Aci*I	175 (152, 23), 7	S4 + AS3	211 (Gly → Ala)	2
+1182 C/A	*Nar*I	578, 208 (786), 28	S4 + AS4	394 (silent)	46

[a]Bands that are present in the wild type are underlined. Their substituting counterparts in variants are in parenthesis.

100 ng DNA, 0.5 μM of each primer, 0.2 mM deoxy-NTPs, 30 nM [^{33}P]dCTP and 0.25 U Amplitaq DNA polymerase (Perkin Elmer). PCR products were digested with restriction enzymes for SSCA analysis (Table 1). The digested samples were mixed with SSCA buffer (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue), denatured for 5 min at 95° and loaded on Mutation Detection Enhancement (MDE) high resolution gel (FMC Bioproducts). Gel electrophoresis was performed twice using two different running conditions, 6% MDE gel at 4° and 3% MDE gel at room temperature, both at constant power (4 W) for 16 hr. The gels were dried and autoradiography was performed by apposing to Biomax MR film (Eastman Kodak Co) for 24 hr at room temperature. DNA samples migrating at different rates in SSCA were sequenced either with an automated DNA sequencer or with a Thermo Sequenase Cycle Sequencing Kit (Amersham Life Sciences). Suitable restriction enzymes were used for genotyping (Table 2). The α_{2B}-AR I/D genotypes were determined as previously described [21]. Briefly, DNA amplification using 5′-AGGGTGTTTGTGGGGCATCT-3′ as sense primer and 5′-CAAGCTGAGGCCGGAGACACT-3′ as antisense yields amplicon sizes of 112 bp for the insertion allele and 103 bp for the deletion allele. PCR products were loaded onto 4% agarose gel with ethidium bromide staining and the different alleles were identified based on their different electrophoretic mobility.

2.4. Reporter gene constructs

Luciferase constructs are numbered according to the translation start site of the *α2C2* gene and were generated from the promoterless vector pGL3-Basic (Promega). The construct pGL3C2 −603/+3 was obtained by PCR using as template a pKS plasmid (Stratagene) containing the *Not*I/*Hin*dIII fragment (−603/+1540) of *α2C2* gene. The −603/+3 fragment was amplified using an antisense primer 5′-GTCCTGGTGGTTCATGACGG-3′, which creates a *Bsp*HI site at position +3, and M13–40 as a sense primer. The PCR product was then cloned into pKS, cut with *Sac*I and *Bsp*HI and inserted into pGL3-Basic digested with *Sac*I and *Nco*I. All other pGL3 constructs ending at position +3 (pGL3C2 −3160/+3, pGL3C2 −1874/+3, pGL3C2 −1034/+3, pGL3C2 −411/+3 and pGL3C2 −127/+3) were obtained by cloning the corresponding fragments using pGL3C2 −603/+3 as recipient. The constructs containing nucleotides −1792/−127 were generated by inserting the *Nco*I/*Nco*I fragment of *α2C2* into pGL3-Basic cut by *Nco*I, producing reporter constructs in sense (pGL3C2 −1792/−127) and antisense orientation (pGL3C2 −127/−1792). pGL3C2 −1034/−127, pGL3C2 −603/−127 and pGL3C2 −411/−127 were obtained by cloning the fragments *Sac*II/*Nco*I, *Not*I/*Nco*I and *Pst*I/*Nco*I into pGL3-Basic digested with the corresponding enzymes. Finally, pGL3C2 −3089/−1999, pGL3C2 −603/−411, pGL3C2 −603/−545 and pGL3C2 −712/−545 were obtained by subcloning these fragments into pKS to create restriction sites compatible with the polylinker of pGL3-Basic. The constructs containing fragments with a cytosine at position −98 (pGL3C2 −127/+3 mut, pGL3C2 −411/+3 mut, pGL3C2 −603/+3 mut, pGL3C2 −1034/+3 mut and pGL3C2 −1874/+3 mut) were generated by site directed mutagenesis using S-98C (5′-CGCCGCCCGAGGGGC-GACGCTCTTGTCTAGC-3′) and AS-98C (5′-AAGAG-CGTCGCCCCTCGGGCGGCGCCGAGG-3′) as primers and the corresponding pGL3C2 constructs as template. The pGL3-CMV was generated from pGL3-SV40 by replacing SV40 by CMV promoter. All the constructs were verified by sequencing using RV3 and GL2 as primers (Promega).

2.5. Cell transfection and measurement of reporter gene activity

HeLa (human cervix adenocarcinoma), SK-N-MC (human neuroblastoma), BHK-21 (baby hamster kidney fibroblast), CaCo2 and HT29 (human colon adenocarcinoma) were routinely subcultured in Dulbecco's modified Eagle's medium supplemented with 5% foetal calf serum. Cells were transfected using the ProFection Mammalian Transfection System (Promega) or FuGene-6 Transfection Reagent (Roche Diagnostics). Briefly, cells were seeded at appropriated density in 6-well plates and transfected with 4.5 μg of luciferase construct plus 0.5 μg of β-galactosidase plasmid, pCMVβ (Clontech). Cells were harvested 48 hr after transfection, disrupted in lysis buffer (100 mM potassium phosphate, 0.2% Triton X-100, pH 7.5) and reporter gene activity was measured using the luciferase assay reagent (Promega) and *o*-nitrophenyl β-D-galactopyranoside (Sigma) as substrate.

Fig. 1. Nucleotide sequence of the 5′-flanking region of *α2C2* gene. Nucleotides are numbered according to their location relative to the translation initiation codon. Elements resembling transcriptional initiator sequences are in brackets, Sp1-binding sites are boxed and AP2-binding sites are bold, underlined. The sites for initiation of transcription predicted by computer analysis are indicated by arrows. Other putative binding sites for *trans*-acting factors include Stat-6 (underlined with open circles), USF (underlined), NFκB (underlined with a dashed line) and Olf-1 (underlined with solid circles). Overlapping glucocorticoid and progesterone response elements are indicated by double underlines.

3. Results

3.1. Sequence analysis of the flanking regions of the α2C2 gene

A cosmid containing a 30-kb insert was isolated by screening a human genomic library with a 0.96-kb probe (corresponding to the *Acc*I–*Acc*I fragment of the *α2C2* coding region). The insert was characterised by restriction enzyme digestion and Southern blot analysis, allowing the isolation of an *Eco*RI–*Kpn*I fragment containing 5.5 kb of the 5′-flanking region, the open reading frame and 2.9 kb of the 3′-flanking region of the human *α2C2* gene. The nucleotide sequence of the entire fragment has been deposited in GenBank with the accession number AF005900.

The nucleotide sequence of the portion of 5′-flanking region spanning from *Xba*I restriction site to translation initiation codon is presented in Fig. 1. The region is rich in GC, this is particularly true for the 1 kb upstream from ATG where the mean GC content is 78%. Computer-assisted search for potential *cis*-acting elements indicated that neither a TATA box nor a CCAAT box is present. However, two initiator elements (YYANWYY, at positions −461/−455 and −424/−417), and numerous consensus binding sites for the transcription factor Sp1 were predicted. Several putative sites for other factors were also detected. Of those with high identity scores, binding sites for AP2 are located at positions −635/−624, −844/−834 and −2366/−2356. A consensus sequence for USF was found at position −1811/−1798. Sites for Stat-6, NFκB and Olf-1 were predicted at positions −2523/−2515, −1742/−1735 and −753/−743. Finally, overlapping glucocorticoid and progesterone response elements were found at position −1542/−1533. Analysis of the 5′-region using the mammalian promoter prediction program TSSG [22] indicated the existence of one TATA-less promoter with a single transcription initiation site at position −553. A similar prediction, but with a transcription initiation site located at position −472, was obtained with the TSSW program which uses a different database.

Examination of the 3′-flanking region revealed the existence of a single canonical polyadenylation signal (AATAAA) at position +3252/+3257. The functionality of this signal was tested on mRNA prepared from HT29, a cell line previously demonstrated to contain trace amounts of the *α2C2* transcript [23]. RT–PCR experiments with a series of primers located in the 3′-flanking region of *α2C2* gene indicated that the end of the *α2C2* transcript is located between positions +3219 and +3299 (not shown). In agreement with this conclusion, a BLAST search for sequence homology against this region permitted the identification of a human cDNA clone (IMAGE:1500825 3′) with perfect identity with *α2C2*. This clone ends 17 bases downstream to the AATAAA signal showing that the polyadenylation site is located at position +3274.

3.2. Characterisation of the promoter region

Because of the inherent difficulty to design convenient primers and to reverse transcribe regions that are extremely rich in GC, all attempts to map the 5′-end of *α2C2* transcript using RT–PCR or RACE methods on RNAs prepared from HT29 cells or human kidney were unsuccessful. In order to get information regarding the promoter region, we therefore sought to measure the transcriptional activity of the 5′-flanking region by means of cell transfection. Preliminary experiments demonstrated that the construct containing the entire 5′-flanking region (pGL3C2 −3160/+3) is able to drive luciferase expression in various cell lines, including CaCo2, SK-N-MC, HT29, BHK-21 and HeLa (not shown), suggesting that the region spanning from nucleotide −3160 to ATG does not contain the elements responsible for cell-specific expression of the *α2C2* gene. The promoter activity was further studied by transfecting HeLa and BHK-21 with constructs carrying serially truncated segments of the 5′-flanking region (Fig. 2). In HeLa cells, the luciferase activity of pGL3C2 −3160/+3 was 18-fold that of pGL3-Basic and represented approximately 25% of that of pGL3-SV40. The comparison of luciferase activity between deletion constructs indicated that the progressive removal of 5′-fragments stretching between nucleotides −3160 and −603 did not significantly affect the transcriptional activity. Further deletions, however, resulted in a sharp decrease. Indeed, the constructs pGL3C2 −411/+3 and pGL3C2 −127/+3 exhibited luciferase activity not significantly different from that of the promoterless construct pGL3-Basic. With the exception that activity of all constructs was lower and that removal of the −1043/−603 region provoked a slight decrease, similar observations were done when BHK-21 were used as host cells. Thus, data on both cell types suggested that elements crucial for transcriptional activity of the α_{2B}-AR promoter are located upstream from position −410. This conclusion was confirmed by transfection of BHK-21 cells with other truncated constructs (Fig. 3). The region spanning nucleotides −3089/−1999 was devoid of transcriptional activity. The construct with the −1792/−127 fragment in sense orientation (pGL3C2 −1792/−127), but not in antisense (pGL3C2 −127/−1792), had high activity, demonstrating that this region behaves as a true promoter with orientation-dependent activity. Of note, the constructs containing fragments −1792/−127, −1034/−127 and −603/−127 exhibited twice more activity than their corresponding counterparts ending at position +3. The same was true in HeLa (not shown), indicating that the removal of the GC-rich region from nucleotide −127 to ATG provoked a significant increase in luciferase activity. As previously found with constructs ending at position +3, more extensive 5′-deletion to position −411 resulted in a sharp decrease in activity, since pGL3C2 −411/−127 was 4-fold less efficient than pGL3C2 −603/−127. By contrast, the construct containing sequence from

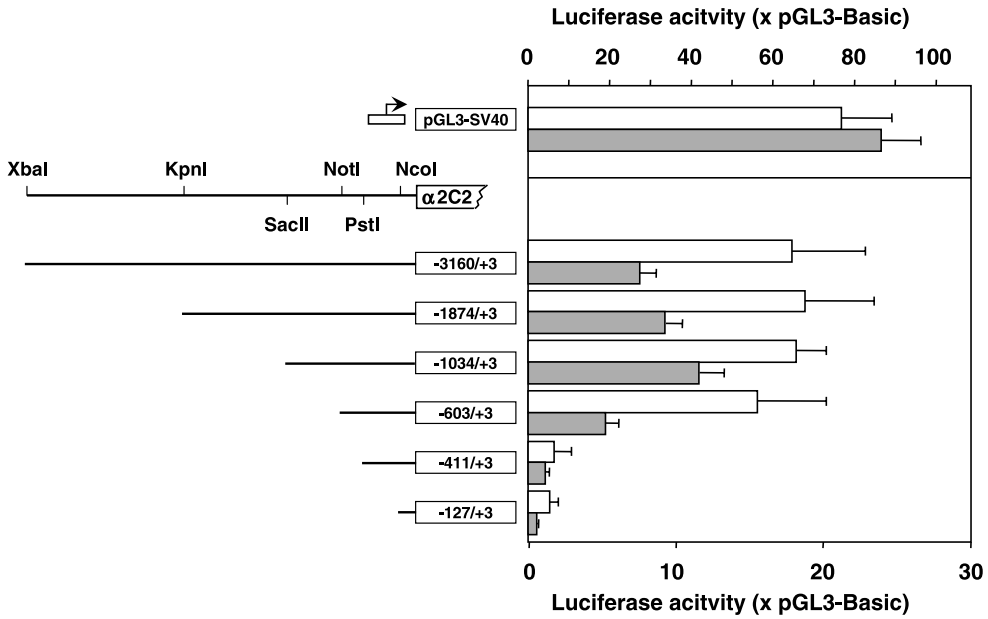

Fig. 2. Transcriptional activity of constructs containing various 5′-deleted fragments of the *α2C2* gene promoter. A series of luciferase constructs containing promoter fragments with various 5′-ends but a common 3′-end (+3) were cotransfected with pCMVβ into HeLa (open bar) or BHK-21 (grey bar). After 48 hr, the cells were lysed and assayed for luciferase and β-galactosidase activity. The promoter activity is normalised for variations in transfection efficiency using β-galactosidase activity as internal standard and is expressed relative to the activity of the promoterless construct pGL3-Basic. Reported values are means ± SEM from four independent experiments performed in triplicate. The constructs are numbered according to their position relative to the translation initiation codon.

−603 to −411 had high activity indicating that the *cis*-elements required for optimal transcriptional activity are present in this region. Because two putative transcription start sites at positions −553 and −472 were predicted in this region, the activity of two constructs, in which the proximal site is eliminated by truncation at position −545, was measured. The two constructs (pGL3C2 −603/−545 and pGL3C2 −712/−545) were totally inactive (Fig. 3), strongly suggesting that the transcription start site predicted at position −553 is inoperant. To validate the importance of Sp1 binding for the activity of the *α2C2* promoter, the effects of mithramycin were investigated on luciferase activity of different constructs. This drug is a well-established inhibitor of Sp1 binding to its correspond-

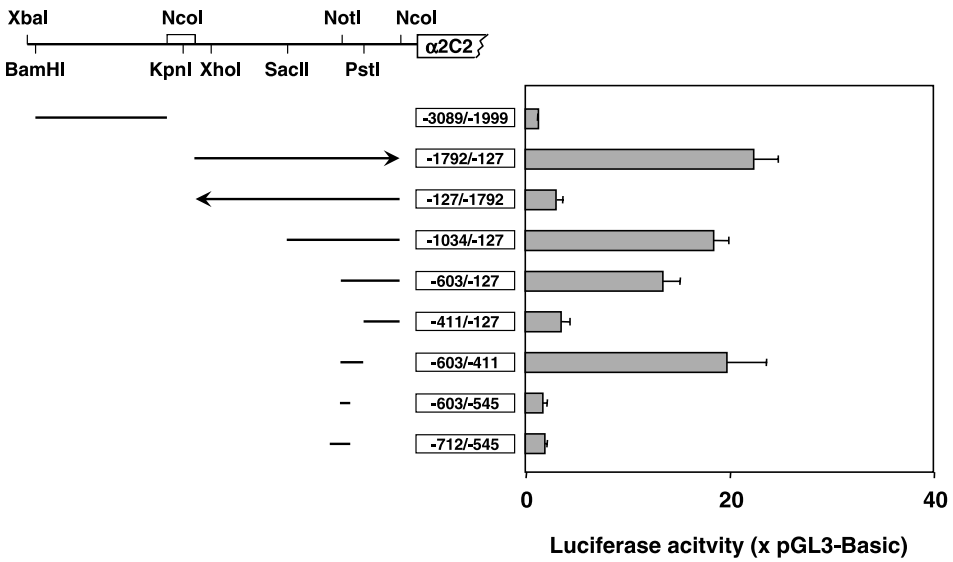

Fig. 3. Transcriptional activity of truncated fragments of the *α2C2* gene promoter. Luciferase constructs containing truncated fragments of the human α_{2B}-AR gene promoter were cotransfected with pCMVβ into BHK-21 cells. After 48 hr, the cells were lysed and assayed for luciferase and β-galactosidase activity. The luciferase constructs are numbered according to their position relative to the translation initiation codon. Their activity is normalised for variations in transfection efficiency using β-galactosidase activity as internal standard. Results are expressed as fold-increase of luciferase activity of the promoterless construct pGL3-Basic. Reported values are means ± SEM from five independent experiments performed in triplicate.

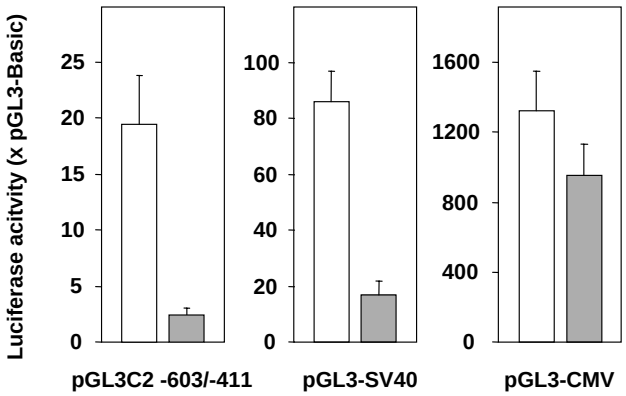

Fig. 4. Effect of mithramycin on the transcriptional activity of *α2C2* gene promoter. BHK-21 cells were transfected with pGL3C2 −603/−411, pGL3-SV40 or pGL3-CMV. Cells were allowed to recover for 8 hr and then treated for 16 hr (grey bar) or not (open bar) with 100 nM mithramycin. Luciferase activity is expressed as fold activity of the promoterless vector pGL3-Basic. Reported values are means ± SEM from three independent experiments performed in triplicate.

ing GC box [24]. Results in Fig. 4 demonstrated that the activity of pGL3C2 −603/−411 was strongly attenuated (10% of control) in BHK-21 cells treated with 100 nM mithramycin. As expected, a large reduction of activity was also observed with pGL3-SV40, but not with pGL3-CMV.

3.3. Identification and analysis of SNPs

The five SNPs that were identified in the present study are presented in Table 2. Two SNPs (−98 G/C and −87 G/T) were located in the 5′-non-coding region while the three others (+36 A/G, +632 G/C, +1182 C/A) were in the coding region. Of 138 subjects that were genotyped for the −98 G/C polymorphism, 36 (26%) had the G/G genotype, 76 (55%) had the G/C genotype and 26 (19%) had the C/C genotype. Interestingly, the −98 G/C polymorphism was in complete linkage with the I/D polymorphism in the coding region [21], G with insertion and C with deletion (Fig. 5). Also the SNP at position +1182 was in complete linkage (N = 16) with the −98 G/C and I/D polymorphisms. This SNP has been published in GenBank (accession number NM000682). The second SNP in 5′-non-coding region (−87 G/T) was found in only one individual and was therefore not studied further. Finally, of the two other SNPs in the coding region, the first (+36 A/G) is a silent mutation; whereas the second causes a glycine to alanine substitution at the start of the third intracellular loop (amino acid 211). In this study population, the alanine allele was rare (allele frequency 2%) and no homozygous individual was identified.

The −98 G/C polymorphism being located within a portion of the 5′-UTR (−127/+3) that affects luciferase activity (see Figs. 2 and 3) and computer-assisted analysis indicating that it results in the creation of an additional putative Sp1-binding site which may affect transcriptional activity of the core promoter, a series of luciferase constructs with G/C substitution were generated. When transfected in BHK-21, constructs containing a C at position −98 (pGL3C2 −1874/+3 mut, pGL3C2 −1034/+3 mut, pGL3C2 −603/+3 mut, pGL3C2 −411/+3 mut and pGL3C2 −127/+3 mut) exhibited identical reporter activ-

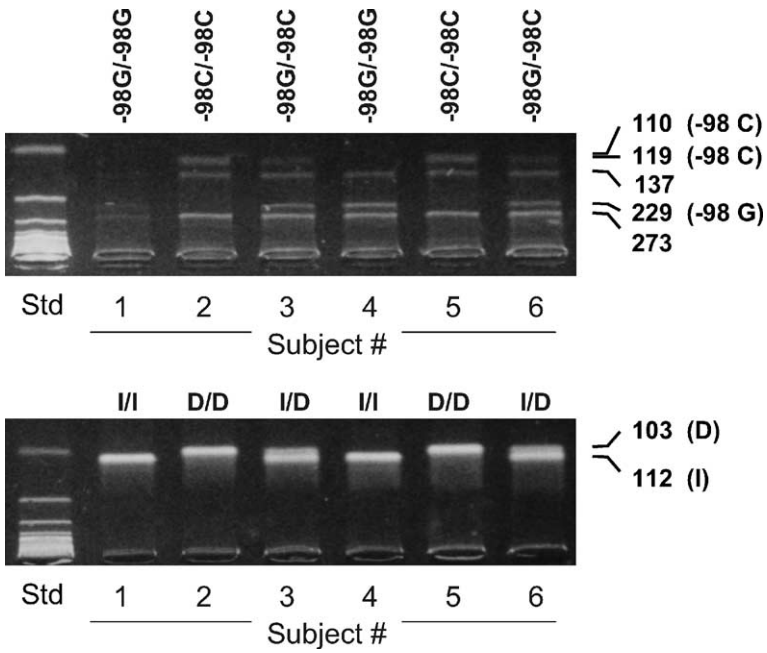

Fig. 5. Genotyping of the −98 G/C and insertion/deletion variants of the human α_{2B}-AR gene. The same subjects were genotyped for both −98 G/C (upper panel) and insertion/deletion (lower panel) polymorphisms. Human genomic DNA was isolated from whole blood. The −98 G/C polymorphism was identified by amplification of DNA with primer pair 2 and digestion of PCR product with *Ava*I. The insertion (I) and deletion (D) variants were also genotyped by PCR and the two alleles were identified on the basis of their difference in size. Std = 100-bp DNA ladder.

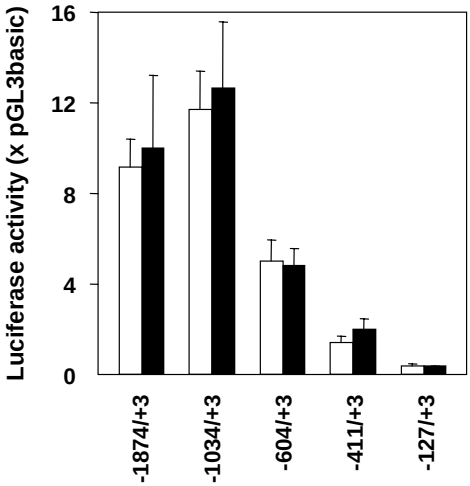

Fig. 6. Transcriptional activity of various constructs with G/C substitution at position −98. Luciferase constructs containing promoter fragments without (open bar) or with G/C substitution at position −98 (black bar) were cotransfected with pCMVβ into BHK-21 cells. After 48 hr, the cells were lysed and assayed for luciferase and β-galactosidase activity. The luciferase constructs are identified by their position relative to the start codon. Their activity is normalised for variations in transfection efficiency using β-galactosidase activity as internal standard. Results are expressed as fold-increase of luciferase activity of the promoterless construct pGL3-Basic. Reported values are means ± SEM from four independent experiments performed in triplicate.

ity as those containing a G (Fig. 6). The same was true when HeLa was used as host cell (data not shown).

4. Discussion

Although the promoter regions of the genes encoding the α_{2A}- and α_{2C}-AR subtypes in human and rat α_2-ARs have been the subject of several studies [16–19], the upstream regions of the genes encoding α_{2B}-AR have so far remained unknown. The present work was thus carried out as an initial approach to characterise the organisation of the human α_{2B}-AR gene and to define the elements accounting for its expression. Screening of a human genomic library allowed us to isolate an *Eco*RI–*Kpn*I fragment containing 5.5 kb of the 5′-flanking region, the open reading frame, and 2.9 kb of the 3′-flanking region of the *α2C2* gene.

Pairwise comparison of the 5′-flanking regions of the human, mouse, and rat α_{2B}-AR genes with BLAST (BLAST 2 sequences) [25] indicates that rat and mouse exhibit considerable sequence homology (GenBank accession numbers AF366899 and M94583) whereas human is almost completely divergent. The identity between sequences was restricted to short portions close to the beginning of the open reading frame and spanning from nucleotide −249 to −154 for the mouse and from nucleotide −262 to −142 for the rat. Also, comparison of the three human subtypes did not reveal significant similarities in their 5′-non-coding regions. Such divergence in promoter regions may explain the differences in the patterns of α_{2B}-AR tissue distribution observed between human and rat or mouse. Our data from cell transfection with luciferase constructs showed that the region from nucleotide −3160 to the ATG codon contains neither the sequences responsible for cell-specific expression of α_{2B}-AR nor enhancer/silencer elements. Further studies with constructs containing larger fragments will be necessary to identify such motifs. It is remarkable that the human gene is, in this respect, different from the murine gene. The 5′-flanking of the mouse α_{2B}-AR gene was indeed shown to direct transcription in cell lines derived from the kidney (BHK-21, OK) or the nervous system (NG108-15, PC12) but not in insulinoma, neuroblastoma or glioma cell lines [20]. The situation for the human α_{2B}-AR is also different from that for the rat α_{2C}-AR, since a 94-bp fragment of the RG10 gene promoter was demonstrated to contain the *cis*-acting elements capable to direct transcription in permissive host cells [18]. Using a series of luciferase constructs with serial 5′-deletions, we identified the sequence fragment located between −603 and −411 as the core promoter. There is no TATA box, but three Sp1 sites and two initiator elements in this region. The implication of Sp1 sites is strongly suggested by experiments with mithramycin, however, the functionality of these elements awaits definitive demonstration, especially because the precise location of the transcription start site was not yet firmly identified. A TATA-less promoter consisting of a GC box and an initiator element was previously described in the mouse α_{2B}-AR gene [20], whereas a TATA-like box was found in the promoter region of human, but not rodent, α_{2C}-AR gene [18,19]. Of the three subtypes, the α_{2A}-AR promoter is therefore the only one with a canonical TATA box [16]. In contrast with other subtypes, the size of the human α_{2B}-AR transcript is presently unknown. According to our data, the polyadenylation site is located 1.93 kb downstream to TGA. With also the mouse gene having a very long 3′-UTR (2.3 kb), such a feature appears a characteristic of α_{2B}-AR mRNA. All our attempts to precisely locate the 5′-end of the α_{2B}-AR mRNA remained unsuccessful. There are at least two reasons for this failure. First, the lack of a suitable source of RNA (i.e. human tissue or cell line) containing substantial amounts of the target transcript to perform RNase protection assays. Second, the very high GC content of the 5′-UTR which makes extremely difficult reverse transcription based approaches, such as primer extension or RACE. According to computer prediction and transfection experiments, the transcription start site would be approximately located 500 bases upstream from ATG. The removal of the proximal part of the 5′-UTR (−127/+3) resulted in a 2-fold increase in luciferase activity, indicating that this region negatively influences expression. This is probably due to an effect on translation rather than transcription. Indeed, this region not only may form strong secondary structures but also contains a micro-ORF (position −126/−76) with an ATG in a relatively good initiation context and may thus

interfere with α_{2B}-AR translation. Whatever it is, assuming that the length of the 5′-UTR is 0.5 kb, the size of the human α_{2B}-AR transcript would be about 3.8 kb. This size is similar to that of the human α_{2A}-AR mRNA, a factor that may have contributed to the difficulty to identify α_{2B}-AR mRNA by Northern blot in tissues containing a mixture of both subtypes.

In this study, we identified five novel SNPs that are located in the 5′-non-coding and the coding region of the gene. Interestingly, the guanine to cytosine substitution at position −98 and a silent cytosine to guanine substitution at position +1182 were in complete linkage with the previously reported I/D polymorphism. According to previous studies, the D variant (Del 301–303) of the human α_{2B}-AR, which consists in the deletion of three Glu residues in an acidic motif located in the third intracellular loop, exhibits depressed GRK2-mediated phosphorylation and is refractory to agonist-promoted desensitisation [6]. Furthermore, the D/D genotype is associated with augmented risk for acute myocardial infarction [26,27] but not with hypertension [28]. As suggested by a study of haemodynamic response to acute adrenaline infusion in healthy young men [29], the most plausible mechanism explaining the association between D/D genotype and myocardial infarction is a blunted increase of coronary blood flow due to exaggerated α2B-AR-mediated vasoconstriction. Because sequence variation in the 5′-UTR region may interfere with expression through altered assembly of the transcription pre-initiation complex at the core promoter, modification of mRNA decay or change in translation rate, the possible functional consequences of the −98 G/C polymorphism were investigated. Despite the presence of a new putative binding site for the transcription factor Sp1 in the −98C sequence, no change in the activity of luciferase constructs was observed. Elements in a promoter region can function differently depending on the cellular environment. For example, a promoter segment of the rat α_{2A}-AR gene has a positive effect on transcription in HT29 cells, but has a negative effect in RINm5F cells [30]. The possibility that the −98 G/C polymorphism appears silent because HeLa or BHK-21 cells do not allow discrimination between the two variants cannot be definitively ruled out. Further studies will be necessary to elucidate whether the −98 G/C variation or the I/D variation of the third intracellular loop, or both are causally involved in the observed clinical associations with the D form of the human α_{2B}-AR.

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