Functional studies of the 5'-untranslated region of human 5-HT₄ receptor mRNA

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The serotonin 5-HT₄ receptor (where 5-HT stands for 5-hydroxytryptamine) is a member of the seven transmembrane-spanning G-protein-coupled family of receptors and mediates many cellular functions both in the central nervous system and at the periphery. In the present study, we isolated and characterized the 5'-flanking region of the h5-HT₄ (human 5-HT₄) receptor. We demonstrate the existence of a novel exon that corresponds to the 5'-untranslated region of the h5-HT₄ receptor gene. RNase protection analysis and reverse transcriptase-PCR experiments performed on human atrial RNA demonstrated that the major transcription start site of the h5-HT₄ receptor gene is located at –3185 bp relative to the first ATG codon. In addition, a 1.2 kb promoter fragment which drives the transcription of the 5-HT₄ receptor was characterized. The promoter region lacks TATA and CAAT canonical motifs in the appropriate location, but contains putative binding sites for several transcription factors. Transient transfection assays re-

vealed that the (–3299/–3050) gene fragment possesses the ability to promote the expression of the luciferase reporter gene in human cell lines. In contrast, the promoter was silent in monkey COS-7 cells, indicating the requirement of specific factors to initiate transcription in human cells. In addition to the promoter element, enhancer activity was found in a region (–220/–61) located in the long 5'-untranslated region. Mutational analysis, gel shift and transfection assays identified an Nkx2.5 (NK2-transcription-factor-related 5)-like binding site as a regulatory sequence of this enhancer. Our results suggest a complex regulation of the h5-HT₄ receptor gene expression involving distinct promoters and non-coding exons.

Key words: cAMP, 5-hydroxytryptamine (serotonin), mRNA, Nkx2.5, transcriptional regulation, 5'-untranslated region.

INTRODUCTION

Serotonin (5-HT, 5-hydroxytryptamine) is a major neurotransmitter in both the central nervous system and peripheral tissues. Of the 14 mammalian 5-HT receptor subtypes identified so far, all are G-protein-coupled receptors, with the exception of the 5-HT₃ receptor that belongs to the superfamily of ligand-gated ion channels [1]. The 5-HT₄ receptor was first described as a potent 5-HT receptor capable of inducing cAMP production in primary neuronal cultures from mouse colliculi [2]. Subsequent pharmacological studies have demonstrated the existence of 5-HT₄ receptor responses in the heart, adrenal gland, bladder and digestive tract of various species, including human [3,4]. In mammalian brain, activation of the 5-HT₄ receptor increases memory and learning [5]. Accordingly, the 5-HT₄ receptor stimulates acetylcholine release in rat frontal cortex [6,7] and regulates the processing of the amyloid precursor protein [8,9]. In the peripheral tissues, the 5-HT₄ receptor has been shown to regulate gastrointestinal tract motility, intestinal electrolyte secretion and bladder contraction [4].

In the heart, 5-HT₄ receptors exert strong positive chronotropic, inotropic and lusitropic effects in human and pig atria. However, unlike β -adrenergic receptors, the 5-HT₄ receptor exerts its effects exclusively on the atrial tissue and has no functional effects on ventricles [10,11]. In addition, the cardiac effects of the 5-HT₄ receptor are restricted to human and pig atria and are absent from a large number of laboratory animals, such as rat, guinea pig, rabbit and frog [12,13]. Regarding 5-HT₄ receptor-associated cardiac

disorders, there are *in vivo*, *in vitro*, and clinical evidence that these receptors can trigger atrial arrhythmia [14]. Therefore the regulation of 5-HT₄ receptor expression is of particular importance to understand their involvement in this cardiac disorder.

Several groups have isolated and sequenced C-terminal splice variants of the h5-HT₄ (human 5-HT₄) receptor. A total of eight C-terminal isoforms and one internal splice variant have been currently identified and are named as h5-HT_{4(a)}, h5-HT_{4(b)}, h5-HT_{4(c)}, h5-HT_{4(d)}, h5-HT_{4(e)}, h5-HT_{4(f)}, h5-HT_{4(g)}, h5-HT_{4(h)} and h5-HT_{4(hb)} [15–19]. These receptors are heptahelical transmembrane receptors that are positively coupled with adenylate cyclase. The h5-HT₄ receptor gene is located on chromosome 5, bands 5q31–q33 [20] and contains five exons encoding the common transmembrane domains of the h5-HT₄ receptor as well as eight alternatively spliced cassettes that code for the internal and C-terminal splice variants [17]. The 5'-UTR (5'-untranslated region) of the h5-HT₄ receptor gene has been recently characterized from human placental cDNA. The 5'-UTR is extremely long in this tissue, since it reached more than 5100 bp upstream from the translation start site and consists of 25 exons and a part of exon 26, which includes the first methionine (Figure 1A) [21]. Thus according to the present study the entire h5-HT₄ receptor gene spans more than 700 kb and is divided into at least 38 exons.

To date, there is no detailed information on the identification of the *cis*-acting elements, which may be involved in the regulation of h5-HT₄ receptor expression. Therefore, to understand further the regulation of the h5-HT₄ receptor gene and its potential implication in cardiac disorders, such as atrial arrhythmia, we

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; Ets-1, E26 transformation-specific-1; HEK cells, human embryonic kidney cells; 5-HT, 5-hydroxytryptamine; h5-HT₄ receptor, human 5-HT₄ receptor; MEF-2, myocyte enhancer factor-2; Nkx2.5, NK2-transcription-factor-related 5; Oct-1, octamer-binding factor-1; RPA, RNase protection assay; RT, reverse transcriptase; 5'-UTR, 5'-untranslated region; WT, wild-type.

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Figure 1 5'-Flanking region of the h5-HT₄ receptor in human atria

(A) 5'-Gene organization of the h5-HT₄ receptor in human atria. An arrow indicates the ATG translation initiation codon. (B) Nucleotide sequence (GenBank[®] accession no. AC008627.7) of the 5'-upstream flanking region of the h5-HT₄ receptor gene showing the position of various putative transcription factor binding sites (underlined). Bases relative to the ATG translation initiation codon (in boldface, designated as +1) are numbered on the left and on the right. The intron sequence is shown in lower-case letters and the coding sequence in bold letters.

characterized its 5'-UTR in human atrial RNA. RT (reverse transcriptase)-PCR experiments and RNA protection assay showed a major transcription start site located within intron 25 at -3185 bp relative to the first ATG codon. In addition to the promoter element, enhancer activity was found within intron 25 but in a region (-220/-61) located in the 5'-UTR. Mutational analysis, gel shift and transfection assays identified an Nkx2.5 (NK2 transcription-factor-related 5)-like binding site as a regulatory sequence of this enhancer.

MATERIALS AND METHODS

Surgery

All methods for obtaining human tissue were approved by the Ethics Committee of the Hôpital de Bicêtre and Université de Paris-

Sud. Specimens of right atrial appendages were obtained from patients undergoing heart surgery for coronary artery diseases or valve replacement at the Institut Hospitalier Jacques Cartier (Massy, France).

Cell culture and products

The human neuronal neuroblastoma cell line IMR32, HEK (human embryonic kidney) and human cervix epitheloid carcinoma (HeLa) cells were purchased from A.T.C.C. (Manassas, VA, U.S.A.) and were grown at 37°C in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) foetal calf serum, 1% non-essential amino acids and antibiotics in humidified 5% CO₂. COS-7 cells were cultured as described previously [15]. Primary cultures of neonatal rat ventricular myocytes were prepared from hearts of 2-3-day-old Sprague-Dawley rat pups. Briefly, hearts were digested with collagenase

and pancreatin. Myocytes were then purified over a Percoll gradient and were plated at a density of 1×10^6 cells per 35 mm dish and cultured overnight in DMEM containing 5% foetal calf serum and 10% horse serum. All media, media supplements and sera were obtained from Life Technologies. PCRs were performed with High Fidelity Taq DNA polymerase (Roche Diagnostics, Meylan, France).

Oligonucleotides

Numbering is relative to the first ATG codon. Primers used for the different experiments were designed based on the sequences of the 5-HT₄ receptor cDNA (GenBank® accession no. Y12507) and the BAC clone CTB-160022 (GenBank® accession no. AC008627.7). Sense primers: –2106 (positions –2106/–2084); –2340 (positions –2340/–2318); –2711 (positions –2711/–2689); –2762 (positions –2762/–2740); –2837 (positions –2837/–2815); –2994 (positions –2994/–2972); –3093 (positions –3093/–3071); –3134 (positions –3134/–3112); –3192 (positions –3192/–3170); –3299 (positions –3299/–3277); antisense primers: +40 (positions +40/+20); +131 (positions +131/+113); –2166 (positions –2166/–2188); –2318 (positions –2318/–2341); –2392 (positions –2392/–2415); –2970 (positions –2970/–2947). The sequences of S124, S125, S137, S138 and β -actin primers have been described previously [15,21].

RT-PCR

Total RNA was prepared from human atrial tissues and cell lines using the TRIzol® RNA purification system (Life Technologies). After purification, RNA was treated with DNase I (Life Technologies) and 5 μ g of total RNA was then hybridized with oligo(dT) primer and reverse-transcribed using Superscript RT II (Life Technologies). The resulting single-strand cDNAs were used as templates in two successive PCRs using the different primers indicated in this paper. Both PCRs were performed using the following cycle conditions: denaturation for 1 min at 94 °C, annealing for 30 s at 58 °C, and extension for 1 min 30 s at 72 °C, with a final extension step for 8 min at 72 °C. The PCR products were electrophoresed on 1–2% agarose gel containing 0.01% ethidium bromide and photographed under UV irradiation.

RPA (RNase protection assay)

Total RNA from human atrium was analysed by RPA using the RPA III kit (Ambion, Austin, TX, U.S.A.) and an antisense RNA probe directed against the 5'-flanking sequence (–3362/–2970). Template DNA required for the synthesis of the RNA probe was obtained by PCR amplification of the BAC clone CTB-160022 (Research Genetics, Huntsville, AL, U.S.A.; GenBank® accession no. AC008627.7). The RNA probe was transcribed (Maxiscript T7 kit; Ambion) in the presence of [α -³²P]UTP (800 Ci/mmol; PerkinElmer Life Science Products, Courtaboeuf, France) and purified on a 5% polyacrylamide gel. The radiolabelled RNA probe was then hybridized with 50 μ g of total RNA from human atrium or HEK cells at 42 °C for 18 h. After digestion with RNase A and RNase T1, protected RNA fragments were separated on an 8 M urea/7% acrylamide gel. The gel was exposed to X-Omat AR film for 3 days (Eastman Kodak) at –80 °C.

Reporter construct and luciferase assay

DNA fragments were constructed by PCR using the BAC clone CTB-160022 (GenBank® accession no. AC008627.7) as a template and were cloned into the luciferase reporter vector, pGL3-Basic in either KpnI–BglII or KpnI–XhoI restriction sites (Promega). The authenticity of the nucleotide sequences was con-

firmed by DNA sequencing. For cell lines, transient transfections were performed in 24-well plates using polyethylenimine as described previously [22]. Primary culture of rat neonatal cardiomyocytes maintained for 2 days *in vitro* were transfected with Lipofectamine™ (Life Technologies) according to the manufacturer's instructions. After transfection, myocytes were cultured overnight in DMEM with 5% horse serum, then in serum-free medium. After 24 h, cells were harvested and assayed for luciferase activity. Luciferase measurements were performed using the Promega luciferase assay system according to the manufacturer's instructions in a Lumat luminometer. Results are expressed relative to the luminescence obtained with the promoterless pGL3-Basic vector. Each transfection experiment was performed in quadruplicate, repeated at least three times and was normalized per mg of cell protein.

Site-directed mutagenesis

Mutations in the Oct-1 (octamer-binding factor-1), MEF-2 (myocyte enhancer factor-2) and Nkx2.5 consensus binding sites within a region (–220/–61) were introduced using Quik Change™ site-directed mutagenesis kit (Stratagene) using the pGL3 (–220/–61) construct as a template. Sequences of the mutated oligonucleotides used were as follows (sense strand): Mut Oct-1, 5'-GGCTGTACCATACGTCAACGCGCCAAAAGCTTTTTC-CTT-3'; Mut MEF-2, 5'-GTATTGTTTATTCCTATTTCACAA-3'; Mut Nkx2.5, 5'-CAAAACAAGTCACGGTAGGAAGC-AATA-3'.

EMSA (electrophoretic mobility-shift assay) and identification of DNA-binding proteins

Nuclear extracts were prepared from IMR32 cell line as described previously [23]. Nkx2.5 protein was prepared by coupled *in vitro* transcription/translation of a T7-driven Nkx2.5 plasmid in reticulocyte lysate by using a TNT kit (Promega). The Nkx2.5 gel retardation probe was constructed by annealing complementary synthetic oligonucleotides (5'-AAACAAGTCATA-ATAGGAAGCA-3'). The oligonucleotide probe was end-labelled with [γ -³²P]ATP (3000 Ci/mmol; PerkinElmer Life Science products) and T4 polynucleotide kinase (Promega) and purified on a G-25 column. The following double-stranded oligonucleotides containing the mutated consensus sequences were used for competition assays: Nkx2.5 mut (5'-AAACAAGTCACGGTA-GGAAGCA-3'). The Ets-1 (E26 transformation-specific-1) probe has already been described in [42]. Nuclear extracts (20 μ g) or 3 μ l of programmed lysates were incubated with 0.5 ng of [³²P] double-stranded probe in 20 μ l of 20 mM Hepes (pH 7.9), 100 mM NaCl, 20 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 10% (v/v) glycerol and 1 μ g of poly(dI/dC) (Amersham Biosciences). For competition studies, before the addition of an Nkx2.5 probe, nuclear extracts were pre-incubated for 10 min at room temperature (20 °C) with a 50-fold excess of unlabelled Nkx2.5 mut and Ets-1 oligonucleotides. Reaction mixtures were incubated for 30 min at room temperature and DNA–protein complexes were resolved on a non-denaturing 6% polyacrylamide gel at 20 mA for 3 h in 0.5 $\times$ TBE (45 mM Tris/borate and 1 mM EDTA). Gels were vacuum-dried and were visualized using the Molecular Dynamics Storm PhosphorImager (Amersham Biosciences).

DNA affinity purification of nuclear proteins was performed using 300 μ g of nuclear extracts. The 5'-biotinylated oligonucleotides MEF-2-Control (AAGCTCGCTCTAAAATAACCCTGT-CCCTGGT), Mut-MEF-2-Control (AAGCTCGCTCTAAGGC-TAACCTGTCCCTGGT) and MEF-2-5-HT₄-R (TTAGTATT-GTTTATTAAATATTTCCACAAAAC) were first coupled with

streptavidin-agarose beads (Sigma) for 1 h at 4°C. Nuclear extracts were then incubated for 2 h with the precoated beads. Beads were washed three times and boiled in reducing sample buffer to elute the bound proteins. Proteins were separated on a 10 % polyacrylamide gel and electroblotted on to a PVDF membrane from Amersham Biosciences. Proteins were detected using anti-MEF-2 polyclonal antibody (Santa Cruz Biotechnology; H-300, 1:500) and visualized by the chemiluminescence system (Amersham Biosciences).

Statistical analysis

An unpaired Student's *t* test was used to evaluate differences between means; differences were considered significant when $P < 0.05$.

RESULTS

Isolation of the 5'-end of the h5-HT₄ receptor cDNA

To identify further the 5'-end of the h5-HT₄ receptor cDNA in human atria, we performed successive RT-PCR experiments on total RNA extracted from human atria using various oligonucleotide primers based on the h5-HT₄ receptor genomic DNA sequence (GenBank® accession no. AC008627.7). Since h5-HT₄ receptor mRNAs are weakly expressed in their different target tissues, especially in the atrium [15,24], we performed nested PCR to amplify the 5'-upstream sequence. To prevent any amplification of genomic DNA, sense and antisense primers were respectively selected on intron 25 and exon 27 (Figure 1A). Using the sense/antisense primers (−2340/+131) and (−2106/+40) in consecutive PCRs, a 2 kb DNA fragment was amplified (results not shown). It displayed full homology with the h5-HT₄ receptor genomic DNA sequence (GenBank® accession no. AC008627.7), indicating that the 5'-UTR of the h5-HT₄ receptor in human atria extended at least upstream of position −2340 bp from the ATG codon (results not shown). The 5'-UTR was further explored by performing nested PCR on cDNA using sense primers located upstream of position −2340 (Figure 2A). Using as templates the products of a first PCR performed with the couple of primers (−2837/−2166), a second nested PCR performed with the sense/antisense primers (−2762/−2318) led to the amplification of products of expected sizes (Figure 2B). Similarly, two PCRs performed consecutively with the couple (−2762/−2166) and the couple (−2711/−2318) amplified the predicted products (Figure 2B). In contrast, no PCR product was synthesized from reverse-transcribed RNA with the couple of primers (−3299/−2166) followed by (−2837/−2318), although an obvious fragment of the expected size was amplified from genomic DNA (Figure 2B). The specificity of the PCR products was confirmed by Southern-blot analysis using the ³²P-5'-end-labelled internal −2392 primer (Figure 2B, right panel). Altogether these results indicated that the transcription start site of the h5-HT₄ receptor gene in human atria was located within a region between −3299 and −2837 bp from the ATG codon.

Identification of the transcription start site of the h5-HT₄ receptor gene

To delineate the region containing the transcription start site, PCRs were performed using 3'-antisense primers adjacent to primer −2340 and progressively further 5'-sense primers (Figure 3). cDNA from human atria and human genomic DNA were compared. The nested PCR products obtained from this analysis are

Figure 2 Isolation of a long 5'-UTR in the 5-HT₄ receptor gene in human atria

(A) A schematic diagram representing the positions of sense (black arrows) and antisense (grey arrows) primers on intron 25 used for RT-PCR experiments. The open arrow indicates the position of the probe used for the Southern blot. (B) Results of agarose gel electrophoresis of nested PCR products are shown on the left panel. Molecular size markers are indicated in bp. Primer pairs used for the nested PCR are represented in brackets. The specificity of the PCR products was analysed by Southern blotting using the ³²P-5'-end-labelled internal −2392 primer. A 6 h exposure of the autoradiogram is shown on the right panel. gDNA, human genomic DNA.

shown in Figure 3(A). The primer pair (−3093/−2392) allowed the amplification of a DNA fragment of 701 bp from the primary PCR product amplified with the primer pair (−3134/−2318). In contrast, no PCR product was synthesized from reverse-transcribed RNA with the couple of primers (−3299/−2318) followed by (−3192/−2392). This was not due to inefficient amplification, since these primer pairs generated products of appropriate size using genomic DNA as a template (Figure 3A, right panel). Similarly, no PCR product was synthesized from reverse-transcribed RNA with the couple of primers (−3192/−2318) followed by (−3134/−2392), although an obvious fragment of the expected size was amplified from genomic DNA (Figure 3A, right panel). Altogether these results indicate that the main transcriptional start site of the h5-HT₄ receptor gene resides between −3192 and −3134 bp in human atria.

To map the transcription start site with another approach, we performed RPA analysis with total RNA isolated from human atrial tissues or HEK cells using a ³²P-labelled RNA probe encompassing the sequence −3362/−2970 of the h5-HT₄ receptor gene. As shown in Figure 3 (B, lane 1), the 392 bp probe hybridized with human atrial RNA was partially protected against degradation and gave a 215 bp fragment. The absence of any specific signal in the lane corresponding to the RNA extracted from HEK cells is in accordance with the observation that these cells do not express the h5-HT₄ receptor as assessed by RT-PCR (Figure 3B). These results demonstrated the presence of one major start site within the 5-HT₄ receptor gene in human atria around position −3185. This result is consistent with the absence of PCR product when the reaction was performed consecutively with the couple (−3192/−2318) and the couple (−3134/−2392) (Figure 3A,

Figure 3 Identification of the region of the transcription start site of the 5-HT₄ receptor gene

(A) Results of agarose gel electrophoresis of cDNA and genomic DNA (gDNA) of nested PCR products are shown on the left and right lower panels respectively. Molecular size markers indicated in bp were run with the PCR products. The positions of primers are shown in the upper panel. Sense and antisense primers are in black and grey arrows respectively. CT, PCR performed with water instead of cDNA is a negative control. (B) RNase protection analysis of the h5-HT₄ receptor gene. The labelled RNA probe (–3362, –2970) was hybridized to 60 µg of human atrium RNA (lane 1), 60 µg of HEK cell RNA (lane 2), 60 µg of tRNA (lane 3) and 60 µg of tRNA without RNase A (lane 4). The RNA molecular mass in bp is shown in lane 5. The position of the protected band and its size are indicated by arrows on the left. (C) Agarose gel electrophoresis of PCR products showing that the –3185 bp start site is unique to the heart. The PCR experiment was performed on cDNA of human placenta and atrium using primers (–2837/–2166) followed by (–2762/–2318). These primers are specific for exon 1A. Positive control was performed using human actin primers on RNA samples treated with (+) or without (–) RT. (D) Upper panel: schematic representation of the 5-HT₄ receptor gene corresponding to exons 1–26 and exon 1 bis (not to scale). The 5-HT₄ receptor gene organization is from [21] (GenBank® accession nos. AB070620 and AB070621) and has been compiled from the University of California at Santa Cruz human genome project. Each grey box corresponds to one exon. Exon 1 bis contains the 5'-UTR located on intron 25 (white box) and exon 26, and the coding region of exon 26. Two arrows indicate the possible transcription start sites. Lower panel: results of agarose gel electrophoresis of PCR products showing the presence of exons 1–4 in human atrium. Molecular size markers are indicated in bp.

left panel, lane 3). Indeed, seven bases of the forward 22-mer primer –3192 did not anneal to the template and these missing bases strongly reduced the melting-temperature (T_m) value, thus preventing PCR amplification.

Next, to check whether this transcriptional start site was unique to the heart, we performed nested RT-PCR experiments on RNA extracted from human placenta (Figure 3C). In this tissue, the major transcriptional start site of the h5-HT₄ receptor has been previously located in exon 1, which is more than 450 kb from the ATG codon [21]. As shown in Figure 3(C), no PCR product was obtained in human placenta with primers (–2837/–2166) followed by (–2762/–2318), whereas a fragment of 444 bp was amplified from human atrial cDNA. Similarly, the primer pair (–3093/–2392) allowed the amplification of a DNA fragment of 700 bp from the human heart cDNA product amplified with the primer pair (–3134/–2318) (Figure 3A). No PCR product was observed when the same experiment was performed on cDNA of human placenta (results not shown). We also demonstrated the presence in heart and placenta tissues of cDNA corresponding to the constitutively expressed β -actin gene (Figure 3C), as well as the absence of actin PCR product in a control without RT (Figure 3C). Therefore signals obtained in our study were not due to any contaminating genomic DNA, since no bands were observed when RNA was directly amplified. Altogether, these results indicate that the long 5'-UTR and the –3185 bp start site are unique to the heart. With respect to the finding of Hiroi et al. [21], we named this new long exon, which contains the 5'-UTR located on intron 25 and exon 26, exon 1 bis (Figure 3D, upper panel).

Interestingly, we found that the start site present in human placenta appeared also to be used in human atria, since we were able to detect by PCR a 220 bp product, which corresponds to a DNA fragment lying between exons 1 and 4 (Figure 3D, lower panel). This nested PCR was performed using primers (S137/S124) and (S138/S125), which have been previously shown to amplify specifically these exons [21]. These results suggest that the h5-HT₄ receptor gene may use several transcription initiation sites (Figure 3D, upper panel).

Transcriptional activity of the h5-HT₄ receptor promoter

The genomic 5'-flanking sequence lying between positions –4298 and –3100 did not contain any canonical TATA sequence nor a CAAT box (Figure 1B). Nevertheless, to test whether the region located upstream of exon 1 bis behaved as a functional promoter, three 5'-deletion fragments were generated by PCR and subcloned into the promoterless vector pGL3-Basic (Figure 4). The largest DNA fragment corresponded to sequence –4298/–3050, whereas the others corresponded to shorter proximal sequences extending from –3639 to –3050 and –3299 to –3050 (Figure 4). To date, there is no *in vitro* cardiac cellular system expressing endogenous 5-HT₄ receptors that could allow such a functional study. Therefore, the promoter activities of the luciferase constructs were evaluated by transient transfection in three human cell lines, namely IMR32, HeLa and HEK cells, as well as in monkey COS-7 cells. Among these cell lines, only human neuroblastoma IMR32 cells express 5-HT₄ receptor mRNA [8] and results not shown). After transfection in COS-7 cells, the different reporter constructs did not produce any significant increase in luciferase activity compared with the control pGL3-Basic vector (Figure 4), although a strong signal was detected on transfection of the cytomegalovirus promoter linked to the luciferase gene (results not shown). In contrast, all three constructs activate luciferase expression to approx. 1.5–7-fold above control value in transiently transfected human HeLa, HEK and IMR32

Figure 4 Transcriptional activity of the h5-HT₄ receptor promoter

Upper panel shows a diagram of the 5'-flanking region of the h5-HT₄ receptor gene containing the large exon 1 bis encoding the long 5'-UTR (–3185, +26). The schematic diagram on the lower left represents each deletion construct of the h5-HT₄ receptor promoter linked to the luciferase gene, which were transfected into different cell lines (right panel). Results are expressed as relative luciferase activity expressed as fold expression over the promoterless-pGL3-Basic vector. Values reported in the Figure represent the means $\pm$ S.E.M. for four independent transfections performed in triplicates. * P < 0.05, ** P < 0.01 and *** P < 0.001 are statistically different from the control pGL3-Basic vector.

cells (Figure 4). The highest relative luciferase expression (up to 8-fold the control value with –3299/–3050 construct) was observed in HeLa cells (Figure 4). The sequential 5'-deletions of the region from –4298 to –3299 significantly increased promoter activity in the three human cell lines, suggesting the presence of repressor elements in this promoter domain (Figure 4).

Transcriptional activity of exon 1 bis of the h5-HT₄ receptor

The h5-HT₄ receptor comprised more than 3.1 kb in its 5'-UTR located in exon 1 bis. Since transcriptional regulation by exons has been reported for several genes [25,26], we examined the promoter activity of the long 5'-UTR of the h5-HT₄ receptor. To this end, we generated by PCR a series of constructs containing serial 3'-deletions of exon 1 bis, which were subcloned into the pGL3-Basic vector (Figure 5). The constructs were then transiently transfected into HeLa, HEK, IMR32 and COS-7 cells and assayed for luciferase activity (Figure 5). As observed with previous constructs, no luciferase activity was detected in COS-7 cells with any of these new constructs (Figure 5). A modest, not significant, increase in luciferase activity was obtained with the two constructs –2340/–61 and –1200/–61 when transfected in human cell lines (Figure 5). Deletion of the region from –1200 to –845 led to a significant enhancement in transcriptional activity in human cell lines when compared with the pGL3-Basic vector (Figure 5). Most importantly, deletion from –845 to –220 produced a > 10-fold increase in luciferase activity in IMR32 and HEK cells (Figure 5). Again, the highest relative luciferase activity was observed in HeLa cells (more than 40-fold the control value) (Figure 5).

Figure 5 Functional analysis of the 5'-UTR of the h5-HT₄ receptor gene in different cell lines

5'-Deletion construct of exon 1 bis was fused to the pGL3-Basic vector and transfected into the indicated cell lines. Schematic representation of the constructs tested and the relative luciferase activity are indicated in the left and right panels respectively. Results are expressed as relative luciferase activity expressed as fold expression over the promoterless pGL3-Basic vector. Values reported in the Figure represent the means $\pm$ S.E.M. for four independent transfections. ** P < 0.01 and *** P < 0.001 are statistically different from the control pGL3-Basic vector.

An Nkx2.5-like element is required for optimal transcriptional activity of the (–220/–61) region

The prediction of potential regulatory elements within the (–220/–61) region was determined by using MatInspector [27] and the TFSearch [28]. We found that it contains putative binding sites for the transcription factors Oct-1, MEF-2 and Nkx2.5. Therefore we examined whether mutations in the binding sites of these transcription factors affected the enhancer activity of the (–220/–61) region. Mutations of the Oct-1 consensus binding site did not significantly influence the transcriptional activity of the (–220/–61) region in IMR32 cells (Figure 6A), although these mutations have been previously shown to be effective in the loss of Oct-1 binding [43]. A mutation in the MEF-2 like binding site induced a slight decrease in the transcription activity (30%) (Figure 6A). However, we were not able to show any binding of MEF-2 proteins to this DNA sequence when we performed DNA affinity purification of nuclear proteins extracted from IMR32 cells (results not shown). Mutations in the Nkx2.5-like binding site reduced the enhancer activity of the (–220/–61) region by 70% (Figure 6A). These results indicate that the Nkx2.5-like consensus sequence plays a major role in the transcriptional activity of the (–220/–61) region.

Gel mobility-shift assay was conducted to determine whether the Nkx2.5 consensus sequence was able to bind nuclear proteins. Two major complexes were formed with the radiolabelled Nkx2.5 probe and proteins extracted from IMR32 cells (Figure 6B, lane 1). All bound proteins can be displaced by a 50-fold excess of unlabelled Nkx2.5 consensus sequence (Figure 6B, lane 3). Competition with a mutant Nkx2.5-binding site failed to abolish completely the complex formation (Figure 6B, lane 2), as did an excess of unlabelled consensus Ets-1 oligonucleotide (Figure 6B, lane 4). To confirm that Nkx2.5 protein binds to the Nkx2.5 consensus sequence located in the (–220/–61) region, an EMSA was performed with *in vitro* translated Nkx2.5 protein and Nkx2.5 WT (wild-type) and mutated (Mut) probes. As shown in Figure 6(C), the Nkx2.5 protein was bound to the WT

Figure 6 Identification of an Nkx2.5-like binding site that regulated the transcriptional activity of the (-220/-61) region

(A) Effects of mutations in the potential MEF-2, Nkx2.5 and Oct-1-binding sites of the (-220/-61) region in exon 1 bis. WT and mutant constructs of the (-220/-61) region were transfected into IMR32 cells and luciferase activity was then assayed. Results are expressed as a percentage of the WT pGL3(-220/-61) luciferase activity. Values represent the means $\pm$ S.E.M. for three independent transfections. * $P < 0.05$ and *** $P < 0.001$ are statistically different from the WT pGL3(-220/-61). (B) EMSA of nuclear extracts from IMR32 cells. ³²P-labelled Nkx2.5 probe (0.5 ng) was incubated with 20 μ g of nuclear extracts in the absence of competitor (lane 1) or in the presence of a 50-fold excess of unlabelled oligonucleotides (lanes 2, 3 and 4). Lane 2, mutated (Mut) Nkx2.5 unlabelled probe; lane 3, WT Nkx2.5 probe; lane 4, Ets-1 consensus probe (lane 4). Open arrowheads indicate the two specific complexes formed. (C) WT and mutated (Mut) Nkx2.5 probes were incubated with *in vitro* translated Nkx2.5 protein. WT probe had a high binding affinity of Nkx2.5 protein, and mutations into the probe strongly reduced the binding affinity of Nkx2.5 protein. (D) Effects of Nkx2.5 on the transcriptional activity of the (-220/-61) region in primary neonatal rat ventricular myocytes. Cardiomyocytes were co-transfected with 1 μ g of control expression vector or 1 μ g of Nkx2.5 expression vector and 1 μ g of pGL3(-220/-61) or pGL3(-220/-61)-Basic for 2 days *in vitro*. Values reported in the Figure represent the means $\pm$ S.E.M. for three independent transfections. ** $P < 0.01$ is statistically different from the control expression vector.

probe and to a much lesser extent to the Mut probe. These results demonstrated that Nkx2.5 protein specifically binds to this Nkx2.5-like sequence.

Finally, to test the potential involvement of the transcription factor Nkx2.5 in the regulation of the transcriptional activity of the (-220/-61) region in cardiac cells, we performed co-transfection experiments in primary neonatal rat ventricular myocytes. Figure 6(D) shows that transfection of an expression vector encoding Nkx2.5 enhanced the transcriptional activity of the (-220/-61) region in primary neonatal cardiomyocytes. These results indicate that Nkx2.5 may participate in the regulation of 5-HT₄ receptor gene expression.

DISCUSSION

In the present study, we report the functional characterization of the 5'-flanking region of the h5-HT₄ receptor. RT-PCR experiments combined with the sequencing of the 5'-flanking region of the h5-HT₄ receptor showed that the long 5'-UTR (3.1 kb) lies between the transcription initiation site and the first ATG. Such a long 5'-UTR may reduce RNA translation and lead to low levels of expressed transcripts [29], as is the case for the h5-HT₄ receptor in the heart [24,30]. Indeed, self-complementary sequences can form stable stem-loop structures that interfere with the assembly of the preinitiation complex. These stem loops can be further stabilized by the interaction with RNA-binding proteins. Furthermore, the presence of ATG codons located upstream of the start site can inhibit translation by causing premature initiation and thereby prevent the ribosome from reaching the ATG start codon [31].

Sequence analysis of the 5'-untranslated flanking region of the h5-HT₄ receptor gene indicated that the region upstream of the putative transcriptional start site does not contain any of the typical characteristics of promoter regions, such as a TATA box, CAAT box or the GC-rich region. The TATA-less promoter is a characteristic of G-protein-coupled receptors, and the examples include promoters of the 5-HT_{2A} and 5-HT_{2C} receptors [32,45]. Interestingly, the inspection of the sequence around the transcription initiation site revealed a consensus sequence for an initiator element that consists of the sequence TTCACCTTT (Figure 1B) [33]. The initiator element is known to be functionally analogous to the TATA box, since it determines the site of transcription and its direction [33].

In the present study, we found that, in human atrium, the long 5'-UTR of the h5-HT₄ receptor mRNA is located -3185 bp upstream of the translation initiation site and is encoded by a single exon (exon 1 bis). This start site is unique to the heart and not present in placenta. However, the start site previously described in placenta was also detected in the heart (Figure 3B), suggesting that the h5-HT₄ receptor gene may use multiple transcription initiation sites. This is in accordance with previous studies showing that 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptor genes have several transcription start sites [32,44,45]. Therefore the 5'-UTR of the h5-HT₄ receptor is a result of the use of the two alternate promoters. It does not change the protein-coding region, but may affect the transcription efficiency as well as the spatial and temporal expression of the h5-HT₄ receptor. The reason for the complex arrangement of numerous start sites spread over an extended domain remains obscure. Perhaps these mRNAs are differentially regulated at the level of translation in different cell types. Alternatively, the transcription initiation sites may be regulated independently, providing a potential mechanism to control transcription of the gene differentially in different cell types and tissue regions, as has been shown for several genes [46].

Transient transfection assays with a series of h5-HT₄ receptor promoter-luciferase constructs identified an approx. 1.2 kb fragment of 5'-non-transcribed sequence that was active as a promoter in the human cell lines, IMR32, HEK and HeLa, and silent in

COS-7 cells. These results suggest that this DNA fragment is necessary to drive cell-specific expression of the 5-HT₄ promoter. Progressive deletion of the h5-HT₄ promoter resulted in an increase in luciferase activity in human cells, but not in monkey COS-7 cells. This indicates that the sequences immediately upstream of the transcription start site probably contain positive elements that are required to enhance transcription in the human cell lines tested. Surprisingly, we found that the DNA region –845/–61 of exon 1 bis increased luciferase activity on transfection in human cells. Deletion of the region located between –845 and –220 further enhanced luciferase expression, indicating the presence of strong positive elements between positions –220 and –61. Analysis of the (–220, –61) region by MatInspector and TransFac programs revealed that it contains putative binding sites for several transcription factors such as Oct-1 and Nkx2.5. Mutational analysis of either consensus sequence showed that the Nkx2.5-like binding site strongly regulates transcriptional activity of the (–220/–61) region. EMSA demonstrates specific binding of nuclear proteins to a portion of this region containing the Nkx2.5-binding site.

Nkx2.5 (also called Csx) is a member of the NK class of homeo-domain proteins required for cardiac commitment, differentiation and morphogenesis [36]. This transcription factor is continually expressed from embryonic stage to adulthood in human heart [37]. Nkx2.5 has been shown to regulate transcription of markers of cardiac hypertrophy, such as the skeletal α -actin [38] and the atrial natriuretic factor [39]. However, Nkx2.5 by itself is a weak transactivator and requires additional interactions with other transcription factors, such as serum response factor or GATA-4 [40,41]. The potential MEF-2-binding site located in the proximity of the Nkx2.5-binding site would have been a good partner, since MEF-2 response elements have been reported in several cardiac promoters and their mutations have been shown to decrease promoter activity [34,35]. However, when we performed DNA affinity purification using the MEF-2-like binding site as a probe, we were not able to detect any binding of MEF-2 proteins to this DNA sequence (results not shown). Thus the partners of Nkx2.5 have to be defined.

In summary, we have identified a second transcription start site of the h5-HT₄ receptor gene, which is under the control of a promoter region located at more than 3 kb from the first ATG codon. We also provide evidence that the long 5'-UTR isolated in this study is only coded by one exon in human atrium. The (–220/–61) region contains an Nkx2.5-like element, which regulates the transcriptional activity of the h5-HT₄ receptor gene.

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REFERENCES

- Hoyer, D., Hannon, J. P. and Martin, G. R. (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol. Biochem. Behav.* **71**, 533–554
- Dumuis, A., Bouhelal, R., Sebben, M., Cory, R. and Bockaert, J. (1988) A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol. Pharmacol.* **34**, 880–887
- Langlois, M. and Fischmeister, R. (2003) 5-HT₄ receptor ligands: applications and new prospects. *J. Med. Chem.* **46**, 319–344
- Hegde, S. S. and Eglen, R. M. (1996) Peripheral 5-HT₄ receptors. *FASEB J.* **10**, 1398–1407
- Marchetti, E., Dumuis, A., Bockaert, J., Soumireu-Mourat, B. and Roman, F. S. (2000) Differential modulation of the 5-HT(4) receptor agonists and antagonist on rat learning and memory. *Neuropharmacology* **39**, 2017–2027
- Maillet, M., Robert, S. J. and Lezoualc'h, F. (2004) New insights into 5-HT₄ receptor: a novel therapeutic target for Alzheimer's disease? *Curr. Alz. Res.* **1**, 82–86
- Lezoualc'h, F. and Robert, S. J. (2003) The serotonin 5-HT₄ receptor and the amyloid precursor protein processing. *Exp. Gerontol.* **38**, 159–166
- Robert, S. J., Zugaza, J. L., Fischmeister, R., Gardier, A. M. and Lezoualc'h, F. (2001) The human serotonin 5-HT₄ receptor regulates secretion of non-amyloidogenic precursor protein. *J. Biol. Chem.* **276**, 44881–44888
- Maillet, M., Robert, S. J., Cacquevel, M., Gastineau, M., Vivien, D., Bertoglio, J., Zugaza, J. L., Fischmeister, R. and Lezoualc'h, F. (2003) Crosstalk between Rap1 and Rac regulates secretion of sAPP α . *Nat. Cell. Biol.* **5**, 633–639
- Jahnel, U., Rupp, J., Ertl, R. and Nawrath, H. (1992) Positive inotropic response to 5-HT in human atrial but not in ventricular heart muscle. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **346**, 482–485
- Schoemaker, R. G., Du, X. Y., Bax, W. A., Bos, E. and Saxena, P. R. (1993) 5-Hydroxytryptamine stimulates human isolated atrium but not ventricle. *Eur. J. Pharmacol.* **230**, 103–105
- Kaumann, A. J. (1991) 5-HT₄-like receptors in mammalian atria. *J. Neural. Transm.* **34**, 195–201
- Quadid, H., Seguin, J., Dumuis, A., Bockaert, J. and Nargeot, J. (1992) Serotonin increases calcium current in human atrial myocytes via the newly described 5-hydroxytryptamine₄ receptors. *Mol. Pharmacol.* **41**, 346–351
- Yusuf, S., Al-Saady, N. and Camm, A. J. (2003) 5-Hydroxytryptamine and atrial fibrillation: how significant is this piece in the puzzle. *J. Cardiovasc. Electrophysiol.* **14**, 209–214
- Blondel, O., Gastineau, M., Dahmoune, Y., Langlois, M. and Fischmeister, R. (1998) Cloning, expression, and pharmacology of four human 5-hydroxytryptamine 4 receptor isoforms produced by alternative splicing in the carboxyl terminus. *J. Neurochem.* **70**, 2252–2261
- Clayeyns, S., Sebben, M., Becamel, C., Bockaert, J. and Dumuis, A. (1999) Novel brain-specific 5-HT₄ receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain. *Mol. Pharmacol.* **55**, 910–920
- Bender, E., Pindon, A., van Oers, I., Zhang, Y. B., Gommeren, W., Verhasselt, P., Jurzak, M., Leysen, J. and Luyten, W. (2000) Structure of the human serotonin 5-HT₄ receptor gene and cloning of a novel 5-HT₄ splice variant. *J. Neurochem.* **74**, 478–489
- Mialet, J., Berque-Bestel, I., Eftekhari, P., Gastineau, M., Giner, M., Dahmoune, Y., Donzeau-Gouge, P., Hoebeke, J., Langlois, M., Sicsic, S. et al. (2000) Isolation of the serotoninergic 5-HT₄(e) receptor from human heart and comparative analysis of its pharmacological profile in C6-glia and CHO cell lines. *Br. J. Pharmacol.* **129**, 771–781
- Vilaro, M. T., Domenech, T., Palacios, J. M. and Mengod, G. (2002) Cloning and characterization of a novel human 5-HT(4) receptor variant that lacks the alternatively spliced carboxy terminal exon. RT-PCR distribution in human brain and periphery of multiple 5-HT(4) receptor variants. *Neuropharmacology* **42**, 60–73
- Clayeyns, S., Faye, P., Sebben, M., Lemaire, S., Bockaert, J., Dumuis, A. and Taviaux, S. (1997) Assignment of 5-hydroxytryptamine receptor (HTR4) to human chromosome 5 bands q31-q33 by in situ hybridization. *Cytogenet. Cell Genet.* **78**, 133–140
- Hiroi, T., Hayashi-Kobayashi, N., Nagumo, S., Ino, M., Okawa, Y., Aoba, A. and Matsui, H. (2001) Identification and characterization of the human serotonin-4 receptor gene promoter. *Biochem. Biophys. Res. Commun.* **289**, 337–344
- Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B. and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7297–7301
- Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17**, 6419–6423
- Medhurst, A. D., Lezoualc'h, F., Fischmeister, R., Middlemiss, D. N. and Sanger, G. J. (2001) Quantitative mRNA analysis of five C-terminal splice variants of the human 5-HT(4) receptor in the central nervous system by TaqMan real time RT-PCR. *Mol. Brain Res.* **90**, 125–134
- Boukouvla, S., Price, N., Plant, K. E. and Sim, E. (2003) Structure and transcriptional regulation of the *Nat2* gene encoding for the drug-metabolizing enzyme arylamine N-acetyltransferase type 2 in mice. *Biochem. J.* **375**, 593–602
- Stickeler, E., Fraser, S. D., Honig, A., Chen, A. L., Berget, S. M. and Cooper, T. A. (2001) The RNA binding protein YB-1 binds A/C-rich exon enhancers and stimulates splicing of the CD44 alternative exon v4. *EMBO J.* **20**, 3821–3830
- Quandt, K., Frech, K., Karas, H., Wingender, E. and Werner, T. (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* **23**, 4878–4884
- Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A. E., Kel, O. V., Ignatieva, E. V., Ananko, E. A., Podkolodnaya, O. A., Kolpakov, F. A. et al. (1998) Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Res.* **26**, 362–370
- Meijer, H. A. and Thomas, A. A. (2002) Control of eukaryotic protein synthesis by upstream open reading frames in the 5'-untranslated region of an mRNA. *Biochem. J.* **367**, 1–11

- 30 Kaumann, A. J., Lynham, J. A. and Brown, A. M. (1996) Comparison of the densities of 5-HT₄ receptors, beta 1- and beta 2-adrenoceptors in human atrium: functional implications. *Naunyn Schmiedeberg's Arch. Pharmacol.* **353**, 592–595
- 31 Cazzola, M. and Skoda, R. C. (2000) Translational pathophysiology: a novel molecular mechanism of human disease. *Blood* **95**, 3280–3288
- 32 Zhu, Q. S., Chen, K. and Shih, J. C. (1995) Characterization of the human 5-HT_{2A} receptor gene promoter. *J. Neurosci.* **15**, 4885–4895
- 33 Smale, S. T. (1997) Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochim. Biophys. Acta* **1351**, 73–88
- 34 Akazawa, H. and Komuro, I. (2003) Roles of cardiac transcription factors in cardiac hypertrophy. *Circ. Res.* **92**, 1079–1088
- 35 Di Lisi, R., Millino, C., Calabria, E., Altruda, F., Schiaffino, S. and Ausoni, S. (1998) Combinatorial cis-acting elements control tissue-specific activation of the cardiac troponin I gene *in vitro* and *in vivo*. *J. Biol. Chem.* **273**, 25371–25380
- 36 Schwartz, R. J. and Olson, E. N. (1999) Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription. *Development* **126**, 4187–4192
- 37 Shiojima, I., Komuro, I., Mizuno, T., Aikawa, R., Akazawa, H., Oka, T., Yamazaki, T. and Yazaki, Y. (1996) Molecular cloning and characterization of human cardiac homeobox gene CSX1. *Circ. Res.* **79**, 920–929
- 38 Chen, C. Y. and Schwartz, R. J. (1995) Identification of novel DNA binding targets and regulatory domains of a murine tinman homeodomain factor, Nkx-2.5. *J. Biol. Chem.* **270**, 15628–15633
- 39 Durocher, D., Chen, C. Y., Ardati, A., Schwartz, R. J. and Nemer, M. (1996) The atrial natriuretic factor promoter is a downstream target for Nkx-2.5 in the myocardium. *Mol. Cell. Biol.* **16**, 4648–4655
- 40 Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M. and Schwartz, R. J. (1998) GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol. Cell. Biol.* **18**, 3405–3415
- 41 Sepulveda, J. L., Vlahopoulos, S., Iyer, D., Belaguli, N. and Schwartz, R. J. (2002) Combinatorial expression of GATA4, Nkx2-5, and serum response factor directs early cardiac gene activity. *J. Biol. Chem.* **277**, 25775–25782
- 42 Hadri, L., Ozog, A., Soncin, F. and Lompre, A. M. (2002) Basal transcription of the mouse sarco(endo)plasmic reticulum Ca²⁺-ATPase type 3 gene in endothelial cells is controlled by Ets-1 and Sp1. *J. Biol. Chem.* **277**, 36471–36478
- 43 Sebastian, S., White, J. A. and Wilson, J. E. (1999) Characterization of the rat type III hexokinase gene promoter. A functional octamer 1 motif is critical for basal promoter activity. *J. Biol. Chem.* **274**, 31700–31706
- 44 Parks, C. L. and Shenk, T. (1996) The serotonin 1A receptor gene contains a TATA-less promoter that responds to MAZ and Sp1. *J. Biol. Chem.* **271**, 4417–4430
- 45 Xie, E., Zhu, L., Zhao, L. and Chang, L. S. (1996) The human serotonin 5-HT_{2C}: complete cDNA, genomic structure, and alternatively spliced variant. *Genomics* **35**, 551–561
- 46 Chiu, I. M., Touhalisky, K. and Baran, C. (2001) Multiple controlling mechanisms of FGF1 gene expression through multiple tissue-specific promoters. *Prog. Nucleic Acid Res. Mol. Biol.* **70**, 155–174

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