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# **Functional studies of the 5'-untranslated region of the human 5-HT<sub>4</sub> receptor mRNA**

Marjorie Maillet, Monique Gastineau, Pascal Bochet, Marie-Liesse Asselin-Labat<sup>§</sup>, Eric  
Morel, Jean-Noël Laverrière<sup>#</sup>, Anne-Marie Lompré, Rodolphe Fischmeister & Frank

Lezoualc'h<sup>\*</sup>

Cardiologie Cellulaire et Moléculaire, INSERM U-446, INSERM U-461<sup>§</sup>, IRF-75, Université  
Paris-Sud, Faculté de Pharmacie, F-92296 Châtenay-Malabry;

<sup>#</sup>CNRS UMR 7079, Université Pierre et Marie Curie, Paris; France

\*Corresponding author. INSERM U-446, Faculté de Pharmacie, 5, Rue J.-B. Clément, F-  
92296 Châtenay-Malabry Cedex France, Fax 33-1-46 83 54 75  
E-mail: Frank.Lezoualc'h@cep.u-psud.fr

## **SYNOPSIS**

The serotonin 5-HT<sub>4</sub> receptor 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 this study, we isolated and characterized the 5'-flanking region of the human 5-HT<sub>4</sub> receptor (h5-HT<sub>4</sub>). We demonstrate the existence of a novel exon which corresponds to the 5' untranslated region (UTR) of the h5-HT<sub>4</sub> receptor gene. RNase protection analysis and RT-PCR experiments performed on human atrial RNA demonstrated that the major transcription start site of the h5-HT<sub>4</sub> 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-HT<sub>4</sub> receptor was characterised. 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 revealed 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'-UTR. Mutational analysis, gel shift and transfection assays identified a Nkx2.5 like binding site as a regulatory sequence of this enhancer. Our results suggest a complex regulation of the h5-HT<sub>4</sub> receptor gene expression involving distinct promoters and non-coding exons.

**Key Words: Serotonin – Promoter - Transcriptional regulation – Arrhythmia - Nkx2.5 - cyclic AMP**

**Abbreviations used:** 5-HT, 5-hydroxytryptamine; HEK, human embryonic kidney; GPCR, G protein-coupled receptors, h5-HT<sub>4</sub> receptor, human 5-HT<sub>4</sub> receptor; MEF-2, myocyte enhancer factor-2; PCR, polymerase chain reaction; RPA, RNase protection assay; RT, reverse transcriptase, UTR, untranslated region.

## INTRODUCTION

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

In the heart, 5-HT<sub>4</sub> receptors exert strong positive chronotropic, inotropic and lusitropic effects in human and pig atria. However, unlike  $\beta$ -adrenergic receptors, the 5-HT<sub>4</sub> 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-HT<sub>4</sub> 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-HT<sub>4</sub> 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-HT<sub>4</sub> 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 human 5-HT<sub>4</sub> (h5-HT<sub>4</sub>) receptor. Eight C-terminal isoforms and one internal splice variant have been currently identified and named h5-HT<sub>4(a)</sub>, h5-HT<sub>4(b)</sub>, h5-HT<sub>4(c)</sub>, h5-HT<sub>4(d)</sub>, h5-HT<sub>4(e)</sub>, h5-HT<sub>4(f)</sub>, h5-HT<sub>4(g)</sub>, h5-HT<sub>4(n)</sub> and h5-HT<sub>4(hb)</sub> [15-19]. These receptors are heptahelical transmembrane receptors that are positively coupled to adenylyl cyclase. The h5-HT<sub>4</sub> receptor gene is located on chromosome 5, bands 5q31-q33 [20] and contains 5 exons encoding the common transmembrane domains of the h5-HT<sub>4</sub> receptor as well as eight alternatively spliced cassettes which code for the internal and C-terminal splice variants [17]. The 5'-untranslated region (5'-UTR) of the h5-HT<sub>4</sub> receptor gene has recently been characterised 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 this study the entire h5-HT<sub>4</sub> receptor gene spans more than 700 kb and is divided in 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-HT<sub>4</sub> receptor expression. Therefore, to further understand the regulation of the h5-HT<sub>4</sub> receptor gene and its potential implication in cardiac disorders such as atrial arrhythmia, we characterised its 5'-UTR in human atrial RNA. RT-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 a Nkx2.5 like binding site as a regulatory sequence of this enhancer.

## **MATERIALS AND METHODS**

### **Surgery**

All protocols for obtaining human tissue were approved by the ethics committee of GREBB institute (Hôpital de Bicêtre, 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, human embryonic kidney (HEK) and human cervix epitheloid carcinoma (HeLa) cells were purchased from ATCC (Rockville, USA) and were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 1% non essential amino acids and antibiotics in humidified 5% CO<sub>2</sub>. COS-7 cells were cultured as previously described [15]. Primary cultures of neonatal rat ventricular myocytes were prepared from hearts of 2- to 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 x 10<sup>6</sup> cells per 35-mm dish and cultured overnight in DMEM containing 5% FCS and 10% horse serum. All media, media supplements and sera were obtained from Life Technologies Inc. PCR Reactions 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-HT<sub>4</sub> receptor cDNA (GenBank accession no Y12507) and the BAC clone CTB-160O22 (GenBank accession no AC008627.7): Sense primers: -2106 (position -2106/-2084); -2340 (position -2340/-2318); -2711 (position -2711/-2689); -2762 (position -2762/-2740); -2837 (position -2837/-2815); -2994 (position -2994/-2972); -3093 (position -3093/-3071); -3134 (position -3134/-3112); -3192 (position -3192/-3170); -3299 (position -3299/-3277); antisense primers: +40 (position +40/+20); +131 (position +131/+113); -2166 (position -2166/-2188); -2318 (position -2318/-2341); -2392 (position -2392/-2415); -2970 (position -2970/-2947). The sequence of S124, S125, S137, S138 and  $\beta$ -actin primers have been previously described [15, 21].

### **Reverse Transcription – Polymerase Chain Reaction (RT-PCR)**

Total RNA was prepared from human atrial tissues and cell lines using the Trizol RNA purification system (Life Technologies Inc.). After purification, RNA was treated with DNase I (Life Technologies Inc.) and five µg of total RNA were then hybridized with oligo(dT) primer and reverse transcribed using Superscript reverse transcriptase II (Life Technologies Inc.). The resulting single strand cDNAs were used as templates in two successive PCR reactions using the different primers indicated in the text. Both PCR reactions 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 sec 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 U.V. irradiation.

### **RNase protection assay (RPA)**

Total RNA from human atrium was analysed by RPA using the RPA III kit (Ambion, Austin, TX, USA) 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-160O22 (Research Genetics, Huntsville, USA; GenBank accession no AC008627.7). The RNA probe was transcribed (Maxiscript T7 kit; Ambion) in presence of [ $\alpha$ -<sup>32</sup>P]UTP 800 Ci/mmol (Perkin Elmer Life Science products, France) and purified on a 5% polyacrylamide gel. The radiolabeled RNA probe was then hybridised with 50 µg 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-160O22 (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 confirmed by DNA sequencing. For cell lines, transient transfections were carried out in 24 well plates using polyethylenimine as previously described [22]. Primary culture of rat neonatal cardiomyocytes maintained 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 carried out using the Promega luciferase assay system according to manufacturer instructions in a Lumat luminometer. Results are expressed relative to the luminescence obtained with the promoter-less pGL3 basic vector. Each transfection experiment was done in quadruplicate, repeated at least 3 times and was normalized per mg of cell protein.

### Site directed mutagenesis

Mutations in the Oct-1, MEF-2 and Nkx2.5 consensus binding sites within region [-220/-61] were introduced using QuickChange™ site-directed mutagenesis kit (Statagene) using the pGL3[-220/-61] construct as a template. The sequences of the mutated oligonucleotides used were as follows (sense strand): Mut Oct-1 5'-GGCTGTACCATACGT CAACGCGCCAAAAGCTTTTGGCTT-3', Mut MEF-2 5'-GTA TTGTTTATTCCCTATTTC CAAAA-3', Mut Nkx2.5 5'-CAAAACAAG TCACGGTAGGAAGCAATA-3'.

### Electrophoretic mobility shift assay and identification of DNA-binding Proteins

Nuclear extracts were prepared from IMR32 cell line as previously described [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'-AAACAAGTCATAATAGGAAGCA-3'). The oligonucleotide probe was end-labelled with  $\gamma$ -[<sup>32</sup>P]ATP (3000 Ci/mmol) (Perkin Elmer Life Science products, France) 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'-AAACAAGTCACGGTAGGAAGCA-3'). Ets-1 probe has already been described [42]. Nuclear extracts (20  $\mu$ g) or 3  $\mu$ l of programmed lysate were incubated with 0.5 ng of [<sup>32</sup>P] double-stranded probe in 20  $\mu$ 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 phenylmethylsulfonyl fluoride, 10 % glycerol and 1  $\mu$ g of poly(dI-dC) (Amersham Biosciences). For competition studies, before the addition of Nkx2.5 probe, nuclear extracts were preincubated for 10 min at room temperature with a 50-fold excess of unlabeled Nkx2.5 mut and Ets-1 oligonucleotides. Reactions mixtures were incubated for 30 min at room

temperature and DNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel at 20 mA for 3 hr in 0.5× 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 were performed using 300 µg of nuclear extracts. The 5'-biotinylated oligonucleotides MEF2-Control (AAGCTCGCTCTAAAAATAA-CCCTGTCCTGGT), Mut-MEF2-Control<sub>1</sub> (AAGCTCGCTCTAAGGCTAACCCTGTCCC-TGGT) and MEF2-5-HT<sub>4</sub>-R (TTAGTATTGTTTATTAAATATTTCCACAAAAC), were first coupled to streptavidine agarose beads (Sigma) for 1 hr at 4°C. Nuclear extracts were then incubated for 2 hrs 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 onto Amersham Pharmacia Biotech PVDF membrane. Proteins were detected using anti-MEF-2 polyclonal antibody (Santa Cruz, 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-HT<sub>4</sub> receptor cDNA

To further identify the 5' end of the h5-HT<sub>4</sub> receptor cDNA in human atria, we carried out successive RT-PCR experiments on total RNA extracted from human atria using various oligonucleotide primers based on the h5-HT<sub>4</sub> receptor genomic DNA sequence (Genbank accession no AC008627.7). Because h5-HT<sub>4</sub> receptor mRNA 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 PCR reactions, a 2 kb DNA fragment was amplified (data not shown). It displayed full homology with the h5-HT<sub>4</sub> receptor genomic DNA sequence (Genbank accession no AC008627.7) indicating that the 5' UTR of the h5-HT<sub>4</sub> receptor in human atria extended at least upstream of position -2340 bp from the ATG codon (data not shown). The 5' UTR was further explored by carrying out nested PCR on cDNA using sense primers located upstream 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 PCR reactions performed consecutively with couple [-2762/-2166] and couple [-2711/-2318] amplified the predicted products (Figure 2B). In contrast, no PCR product was synthesized from reverse transcribed RNA with 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 using the <sup>32</sup>P-5'-end-labeled internal -2392 primer (Figure 2B, right panel). Altogether these results indicated that the transcription start site of the h5-HT<sub>4</sub> receptor gene in human atria was located within a region between -3299 bp and -2837 bp from the ATG codon.

### Identification of the transcription start site of the h5-HT<sub>4</sub> 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 shown in Figure 3A. 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 observed with primers [–3192/–2392] and [–3134/–2392]. This was not due to inefficient amplification since all primer pairs generated products of appropriate size using human genomic DNA as a template (Figure 3A, right panel). 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 <sup>32</sup>P-labelled RNA probe encompassing the sequence –3362/–2970 of the h5-HT<sub>4</sub> receptor gene. As shown in Figure 3B (lane 1), the 392 bp probe hybridised with human atrial RNA was partially protected against degradation and gave a 200 bp fragment. This result demonstrated the presence of one major start site within the 5-HT<sub>4</sub> receptor gene in human atria around position –3185. 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-HT<sub>4</sub> receptor as assessed by RT-PCR (Figure 3B). Altogether these results indicate that the main transcriptional start site of the h5-HT<sub>4</sub> receptor gene resides between –3192 and –3134 bp in human atria.

Next, to check whether this transcriptional start site was unique to the heart, we carried out nested RT-PCR experiments on RNA extracted from human placenta (Figure 3C). In this tissue the major transcriptional start site of the h5-HT<sub>4</sub> receptor has been previously located in exon 1 which is at more than 450 kb from the ATG codon [21]. As shown in Figure 3C, 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 (data not shown). We also demonstrated the presence in heart and placenta tissues of cDNA corresponding to the constitutively expressed  $\beta$ -actin gene (Figure 3C), as well as the absence of actin PCR product in a control without reverse transcriptase (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 data 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 exon 1 and exon 4 (Figure 3D, lower panel). This nested

PCR was performed using primers [S137/S124] and [S138/S125] which have been previously shown to specifically amplify these exons [21]. These results suggest that the h5-HT<sub>4</sub> receptor gene may use several transcription initiation sites (Figure 3D, upper panel).

### **Transcriptional activity of the h5-HT<sub>4</sub> receptor promoter**

The genomic 5' flanking sequence lying between position -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 promoter-less vector pGL3-Basic (Figure 4). The largest DNA fragment corresponded to sequence -4298/-3050 while 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-HT<sub>4</sub> receptors which could allow such a functional study. Therefore, the promoter activities of the luciferase constructs were evaluated by transient transfection in three human cell lines, IMR32, HeLa and HEK cells as well as in monkey COS-7 cells. Among these cell lines, only human neuroblastoma IMR32 cells express 5-HT<sub>4</sub> receptor mRNA ([8], and data not shown). After transfection in COS-7 cells, the different reporter constructs did not produce any significant increase in luciferase activity compared to the control pGL3-basic vector (Figure 4) although a strong signal was detected upon transfection of the cytomegalovirus (CMV) promoter linked to the luciferase gene (data not shown). In contrast, all three constructs activate luciferase expression to approximately 1.5- to 7-fold above control value in transiently transfected human HeLa, HEK and IMR32 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-HT<sub>4</sub> receptor**

The h5-HT<sub>4</sub> 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-HT<sub>4</sub> receptor. To this end, we generated by PCR a series of constructs containing serial 3' deletions of exon 1 bis which were sub-cloned 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 -1200 to -845 led to a significant enhancement in transcriptional activity in human cell lines as compared with the pGL3-basic vector (Figure 5). Most importantly, deletion from -845 to -220 produced a greater than 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).

### **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 (Fig 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 of the transcription activity (30%) (Fig 6A). However, we were unable 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 (data not shown). Mutations in the Nkx2.5 like binding site reduced the enhancer activity of the [-220/-61] region by 70% (Fig 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 radio-labelled Nkx2.5 probe and proteins extracted from IMR32 cells (Figure 6B, lane 1). All bound proteins can be displaced by 50-fold excess unlabeled Nkx2.5 consensus sequence (Figure 6B, lane 3). Competition with a mutant Nkx2.5 binding site failed to completely abolish 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 electrophoretic mobility shift assay

was performed with *in vitro* translated Nkx2.5 protein and Nkx2.5 wild type (WT) and mutated (Mut) probes. As shown in Figure 6C, Nkx2.5 protein was bound to the WT 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 carried out co-transfection experiments in primary neonatal rat ventricular myocytes. Figure 6D shows that transfection of an expression vector encoding Nkx2.5 enhanced the transcriptional activity of the [-220/-61] region in primary neonatal cardiomyocytes. These data indicate that Nkx2.5 may participate in the regulation of 5-HT<sub>4</sub> receptor gene expression.

## **DISCUSSION**

In the present study we report the functional characterization of the 5'-flanking region of the h5-HT<sub>4</sub> receptor. RT-PCR experiments combined with the sequencing of the 5'-flanking region of the h5-HT<sub>4</sub> receptor showed that the long 5'-UTR (3.1 kb) lays between the transcription initiation site and the first ATG. Such long 5'-UTR may reduce RNA translation and lead to low levels of expressed transcripts [29] as it is the case for the h5-HT<sub>4</sub> 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 preventing the ribosome from reaching the ATG start codon [31].

Sequence analysis of the 5'-untranslated flanking region of the h5-HT<sub>4</sub> 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 characteristic of GPCRs and examples include promoters of the serotonin 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors [32,45]. Interestingly, inspection of the sequence around the transcription initiation site revealed a consensus sequence for an initiator element (INR) which consists of the sequence TTCACCTT (Figure 1B) [33]. The INR is known to be functionally analogous to the TATA box since it determines the site of transcription and its direction [33].

In our study, we found that, in human atrium, the long 5'-UTR of the h5-HT<sub>4</sub> 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-HT<sub>4</sub> receptor gene may use multiple transcription initiation sites. This is in accordance with previous studies showing that 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptor genes have several transcription start sites [32,44,45]. Therefore, the 5'-UTR of the h5-HT<sub>4</sub> 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-HT<sub>4</sub> 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 differentially control transcription of the gene in different cell types and tissues regions as it has been shown for several genes [46].

Transient transfection assays with a series of the h5-HT<sub>4</sub> receptor promoter-luciferase constructs identified a ~1.2 kb fragment of 5'-nontranscribed sequence that was active as a promoter in the human cell lines, IMR32, HEK and HeLa and silent in COS-7 cells. These data suggest that this DNA fragment is necessary to drive cell specific expression of the 5-HT<sub>4</sub> promoter. Progressive deletion of the h5-HT<sub>4</sub> 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 upon 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 position -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 sequences 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 homeodomain 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  $\alpha$ -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 (data 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-HT<sub>4</sub> 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 our study is only coded by one exon in human atrium. The [-220/-61] region contains a Nkx2.5 like element which regulates the transcriptional activity of the h5-HT<sub>4</sub> receptor gene.

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**FIGURE LEGENDS**

**Figure 1.** **A)** 5' gene organization of the h5-HT<sub>4</sub> 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-HT<sub>4</sub> receptor gene showing the position of various putative transcription factor binding sites (underlined). Bases relative to the ATG translation initiation codon (in boldface, designated +1) are numbered on the left and on the right. The intron sequence is shown in small letters and the coding sequence in bold letters.

**Figure 2. Isolation of a long 5' UTR in the 5-HT<sub>4</sub> receptor gene in human atria.** **A)** Upper Panel. A schematic diagram representing the positions of sense (dark 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 into brackets. The specificity of the PCR products were analysed by Southern blotting using the <sup>32</sup>P-5'-end-labeled internal -2392 primer. A 6 h exposure of the autoradiogram is shown on the *right panel*. gDNA; human genomic DNA.

**Figure 3. Identification of the region of the transcription start site of the 5-HT<sub>4</sub> 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 dark and grey arrows respectively. CT, PCR performed with water instead of cDNA is a negative control. **B)** RNase protection analysis of the h5-HT<sub>4</sub> receptor gene. The labelled RNA probe [-3362, -2970] was hybridised to 60 µg of human atrium RNA (lanes 1), 60 µg of HEK cells RNA (lanes 2), 60 µg of tRNA (lanes 3), 60 µg of tRNA without RNase A (lanes 4). The RNA molecular weight in bp is shown in lane 5. The position of a major protected band and its size is indicated by an arrows on the left. Asterisks show non-specific background. **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 of Exon 1A. Positive control was

performed using human actin primers on RNA samples treated with (+RT) or without (-RT) reverse transcriptase. **D)** The upper panel shows the schematic representation of the 5-HT<sub>4</sub> receptor gene corresponding to exons 1-26 and exon 1 bis (not to scale). 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. The 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.

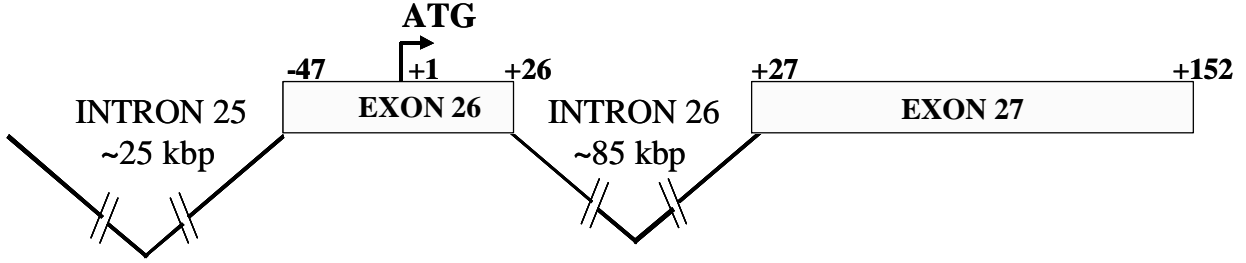
**Figure 4. Transcriptional activity of the h5-HT<sub>4</sub> receptor promoter.** The upper panel shows a diagram of the 5' flanking region of the h5-HT<sub>4</sub> receptor gene containing the large exon 1 bis encoding the long 5' UTR (-3185, +26). The schematic diagram on the lower left represents each deletion construct of the h5-HT<sub>4</sub> 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 promoter-less pGL3 Basic vector. Values reported in the figure represent the mean  $\pm$  S.E.M of four independent transfections performed in triplicates. Bars bearing “\*” (p<0.05), “\*\*\*” (p<0.01), “\*\*\*\*”(p<0.001) are statistically different from the control pGL3 Basic vector.

**Figure 5. Functional analysis of the 5' UTR of the h5-HT<sub>4</sub> receptor gene in different cell lines.** 5' deletion construct of exon 1 bis were 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 on the left and right panel, respectively. Results are expressed as relative luciferase activity expressed as fold expression over the promoter-less pGL3 Basic vector. Values reported in the figure represent the mean  $\pm$  S.E.M of four independent transfections. Bars bearing “\*\*\*” (p<0.01), “\*\*\*\*”(p<0.001) are statistically different from the control pGL3 Basic vector.

**Figure 6. Identification of a 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. Wild type (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 wild type pGL3[-220/-61] luciferase activity. Values represent the mean  $\pm$  S.E.M of three independent transfections.

Bars bearing “\*” (p<0.05), “\*\*\*”(p<0.001) are statistically different from the WT pGL3[-220/-61]. **B)** Electrophoretic mobility shift assay of nuclear extracts from IMR32 cells. 0.5 ng of [<sup>32</sup>P] labelled Nkx2.5 probe were incubated with 20 µg of nuclear extracts in the absence of competitor (lane 1) or in the presence of a 50 fold excess of unlabeled oligonucleotides (lanes 2, 3 and 4). Lane 2, mutated (Mut) Nkx2.5 cold probe; lane 3, wild type Nkx2.5 probe; lane 4, Ets-1 consensus probe (lane 4). Opened arrows indicate the two specific complexes formed. **C)** Wild type (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 µg of control expression vector or 1 µg of Nkx2.5 expression vector and 1 µg of pGL3[-220/-61] or pGL3[-220/-61] basic at two days *in vitro*. Values reported in the figure represent the mean ± S.E.M of three independent transfections. The bar bearing “\*\*\*” (p<0.01) is statistically different from the control expression vector.

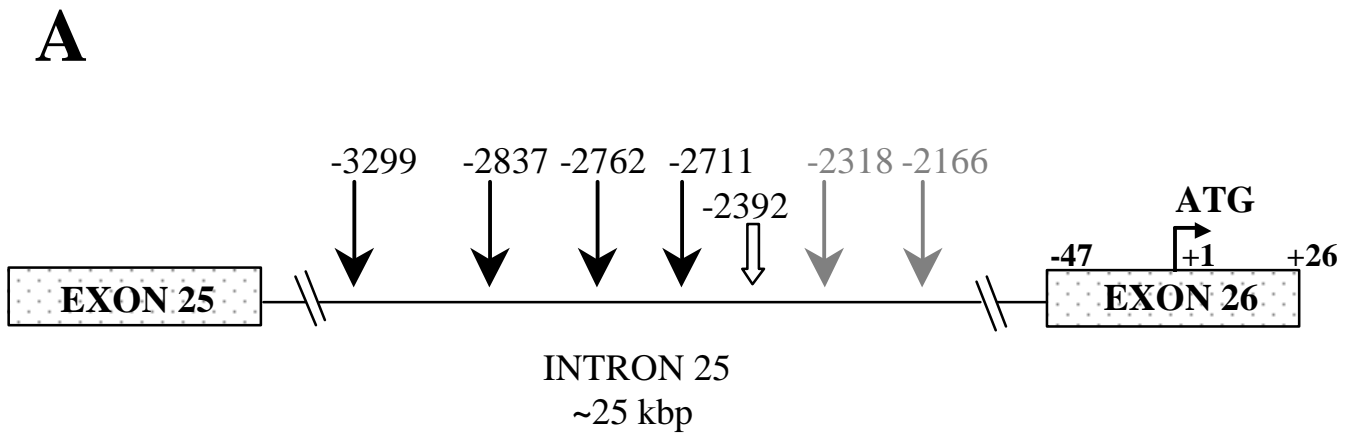
**A**



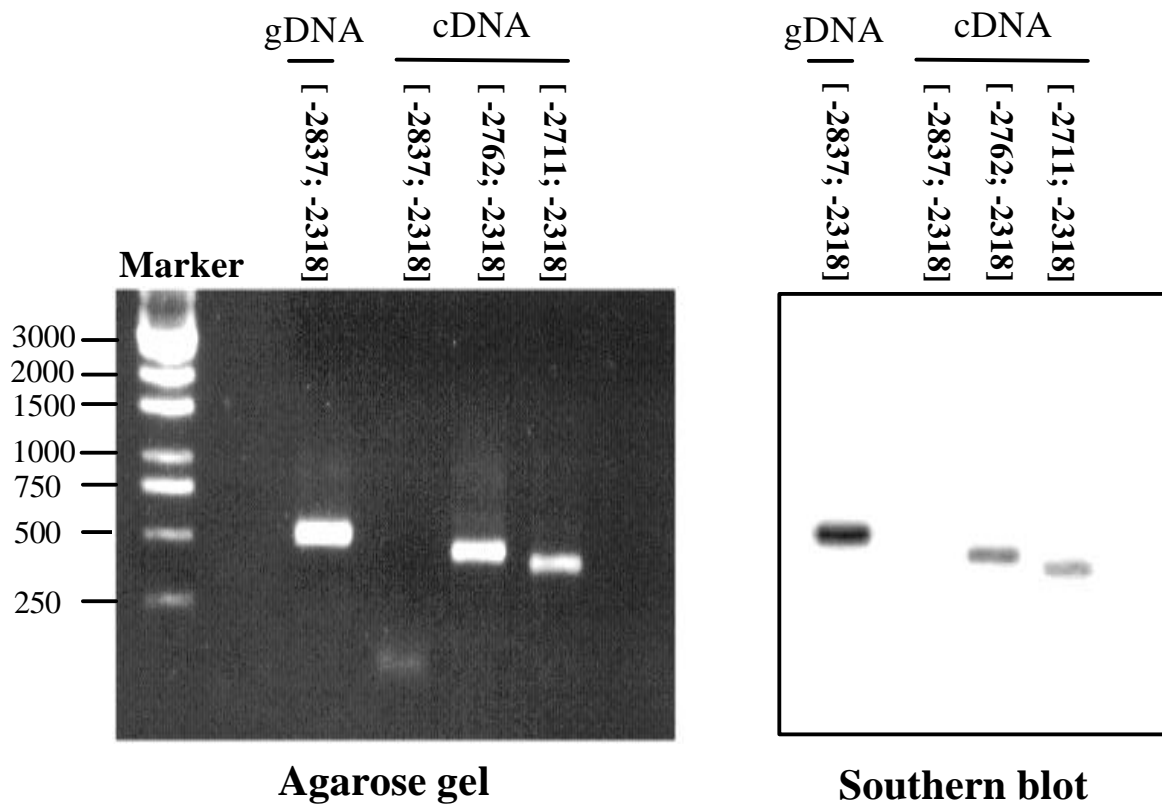
**B**

-4299	CATACACACC	AGGTCTTAAT	GGATTGGAAT	TGGCAGAATT	TTTAGAAGTG	ACACAACCTG	TACCTGAGCA	TAGGAGATGC	TCTGAGTTGA	TTGTGTTTTT	-4200
-4199	AATTAGCTAG	GCAGTGTFFF	GTGAAGAAGC	CAGAAATATA	<u>AGTATTC</u> AG	ATAAAAGCAC	TGGTTCCCTC	CACATCCCCC	TCACCCTATG	CAATCTTCCC	-4100
					<u>Oct-1</u>						
-4099	AGCAAGAGAT	GTTCCTATAA	TCATGGCTCT	GATTAGTTCT	CCCTGGTGAG	TCCTGCAGAA	AATAGTCAGG	CATAATCAAT	TAATCAAATT	AGGATAGGTA	-4000
-3999	CTAATCATCA	ACTCCTCCTT	CCTCTGTTC	TTTCTCTCTG	<u>ATAAAA</u> TGCA	TGCTATCTCA	<u>CTGGTTTGT</u> T	<u>GAAAG</u> CAAT	CATTTTAAAT	TTGCTCATTG	-3900
					<u>GATA-1</u>		<u>C/EBP</u>				
-3899	GGTTTCAGTG	CAAAAAATGT	AACAATCTAT	ATGCTATGAA	<u>TATACA</u> AGAT	TTTCCTTGTG	TTGTAACATT	TTCACATGCA	GTTTAAATGA	TACAAACTTT	-3800
					<u>Oct-1</u>						
-3799	AGGTTTTAAT	GGCTTTTAAA	GCTGAAACTG	CCCAGCTTTT	CTTCCTTTTA	ATCTGCAAAA	CAATACAATA	TTGATTTTAT	TGTATATTAC	TCAGCATTCA	-3700
-3699	TTTGCTTGCT	GACTGGAACA	CAACTCAAAC	TGGCTTAAGG	AGGCAAAAAG	AATTTGTTGG	CTCTAGTAAT	TAGGACATTC	<u>AGAGT</u> TAAAA	<u>TTAT</u> CTTAAG	-3600
									<u>MEF2</u>		
-3599	ACACTGCTGG	ATCTTAAGCC	TCAAATTATG	TAGTCAGGAC	<u>ACTGGC</u> ATTC	<u>CCTGT</u> GTTGT	GGCTCTGCCT	TCCTTTGGGT	TAGTTTTCTT	CTCAGACAAG	-3500
					<u>NF-kB</u>						
-3499	CTCCTCCCAT	GGGGTGGCAA	TGATGGCCAC	TAGCAGCTTC	AAACTGTCTG	ACAACACCAG	<u>AGGAA</u> TACA	GAGTCTTTCT	AGCAAAAATT	CTAGGACTGG	-3400
						<u>NFAT</u>					
-3399	CTTTCCTGG	TCCAGCCAGT	AAGCTAAGG	ATTCCTTGTG	CCAGGAATGG	ACTATTCCTG	TCGACCAGCC	CTGCTTATGT	GTTTATCACT	GGAGCTAGGG	-3300
-3299	ATTGGAGAAG	GGAAGAGGTG	GTTCCAGTAA	<u>GGAAA</u> TCCA	GACATTATTC	TCAGAGAAAAG	<u>ATGAA</u> TAGA	TGGATGGACA	GCAGAAAATT	ATAGTGGATG	-3200
				<u>NFAT</u>		<u>GATA-3</u>					
-3199	<u>TTCACTTT</u> CA	TCAGGATACT	CTGTGCTCC	AGTGGGAGGA	CGTATAATAA	GATGACAAGA	ATGATGTAAG	CTAAAGCTCG	AGTTTTGGAA	TTCATAGCCG	-3100
	<u>INR</u>										
-3099	TGAATCTAT	TCCCAGATTG	GTCAAGTGCT	GCTGTGTATG	ACTTTGGCAA	TTCAATTACC	CTCTCTGACC	CTCAGTCTCT	TCATCTATAG	GATGGAGGTT	-3000
-2999	GGACTCGGAG	GTTGGACTCA	AAGGTTGGAG	GAGGAGAAAA	AGGGCAATGT	CTGGTACTTA	TAAACACGTA	GTAAGGGGCA	ACTAATTAAG	ATATAATACT	-2900
-2899	TTTGCTATT	TTTAAATTAC	AATATCTAT	TCTGAGATGG	TCCCTTCCTT	CCCAGTGTGA	AAGTCAAAAA	CTCCTAAGCC	TCAGTTTTCT	CATTTGCAAA	-2800
-2799	ACTAATGAAC	TACTTAGTTG	ACTCAATAAA	TATCTATGAT	GAGGACACAA	TATACCAGGC	ATTTCCTGAG	GGCAGTGAAG	ATGTACAGT	GATCAAGACA	-2700
-2699	GTGCTCTACG	CCCTCTTGAA	ATTTATATTC	TAGTGAGCAA	GACAGATCAC	AAATAAAGAA	GCAATTGACT	ATATATATAT	ATATATATAT	ATATATACTG	-2600
-2599	GGAAGAAAA	AATAGAACAG	AGAAAGAGGA	TAGACATTTT	TAGAAAATGG	GGTGTAAGAT	TGTAGTTAGG	TGTTGAGAGA	AGACCTGTCT	GAGGAAGTGA	-2500
-2499	TATTTGAGCG	GCACCCTAA	GGAAGTGAGA	GAGTAATCTA	CATGGTCACA	TTCCAGGGAA	AAGAGACAAA	GTGAAAAGCC	CCTGAGATGG	GAACCTACGG	-2400
-2399	TTACGGCCCA	GGAAGCAGC	AAAACCAAGT	AGCTGGTGGG	TGGGAAAGGG	GAGGGGCATG	GCTACAGTTG	AGTTTGGAGA	AGAGTGGGGG	CCAGATCATG	-2300
-2299	TAAGGTCGG	TTAGCTATGA	TAAGGAATTT	GGATTGTATG	CTAAGTGTGA	TAGGAAGCCA	CTAGAATTTT	ATGCAAGCAA	GAAATGACCT	GACTTACATT	-2200
-2199	TAAAAAATAT	CATCTGGCA	GGAGACAGAT	TGGGGTGTG	GGTGAGTAAT	TCAGAGAAAAG	AAATGGGTGG	CTTGGACCCA	GATGCTGGGT	CCAGAGGTGA	-2100
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-1499	GGGAGTCTAC	AGTGAATAGT	ATTGTAATTT	TACTGTATTT	TCAGCCACCA	GATGGATGTG	ATCCTCTCAG	GAAAAATTTT	AGATAGACAA	GGAAGAGGA	-1400
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-1299	CTACTTTCCT	TCAGATTAGG	GGAGGGGGCT	CAGAGTCTTG	CAGGTGGAGC	CTTCCCATG	CCTGCCCCAG	GCCAGAAAT	GCCCATGGGA	GCTCCTGTTA	-1200
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-1099	CTGTTTAGCT	CACCCCTGGCC	AAGGTGTCCA	AGGGCCAGG	CTGAGTTTCA	TGGTTTTTTT	ATGTAGATAA	CAGATTTCCA	GGTTCTTCTT	GGTAAGATT	-1000
-999	AACTAGAAAA	CTAGCAAAAC	GAACATAGA	ATATCAGAGC	TAGGAGTGAA	CTCAGAGATT	ATTTTTTTC	AATCTCTTCT	CTTTACTGAG	AAGTGAATGA	-900
-899	TGATGTTTCA	AAGGAAAAGG	GACTTGCTCA	TGTTGACCCT	AGAAGAGACA	GCCCGACCCT	GCCCATACAC	TTACTGATTC	ATCATGCTGT	GGAACAGATG	-800
-799	TGATGAAGAT	ATAAAAATGC	AAAAATGGAA	CTGAACAATT	AGTAAAGAGG	TTTTGATTTT	TGGAGATCAG	TGAAATGCCA	TCATTAAGAG	AGTGTGAGGT	-700
-699	CTTCTGAGGG	TCTGTGATAG	AGGAACTTTG	<u>GTAATTT</u> GAA	GTTAACTCTC	CTTCGATTGG	GAATTCCTAG	CCTGGGGTTC	CTGGATGGGC	CTGGGATTCC	-600
				<u>C/EBP</u>							
-599	TGGATTCTCTG	AGTTCAAAGG	ATCATGGACT	CTCTGGAAAAG	TGTGCATGTG	TGTGCATGCG	TATCTATTTT	<u>TTTCTGGG</u> CA	<u>GACAGT</u> TCTC	AAGAGTTTAC	-500
								<u>GRE</u>			
-499	TAGATTCTCA	AAAGAATCTG	TGATTTCTTA	CCCTTAAAAT	GATTTAAACA	TTTTTTTAAA	ATCCTCATAG	GAACAAAATG	GCCATTTTGG	GCAATATACT	-400
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										<u>Oct-1</u>	
-199	<u>GCCAAA</u> AGCT	TTTTGCTTTA	GATATTGTTT	<u>TTAAA</u> TATTT	<u>CCACAAA</u> ACA	AGT <u>CATA</u> ATA	<u>GGAAG</u> CAATA	GTTATTTGAG	TTTTTTTAAA	CAAGGACTTG	-100
				<u>MEF2</u>		<u>Nkx2.5</u>					
-99	TATTTTTCAA	TGCTGCTTTC	TTTTATAACA	TCTTTTTTACT	TCCCCATT	TAGGACCCTG	TGGGCATTGA	AATCCAACCT	ACTCATGCTT	ATTTCTGTGA	0
+1	<u>ATG</u> GACAAAC	<u>TTG</u> ATGCTAA	<u>TGTG</u> AGgtat	gttacctttc	tgtacatatt	gtgttgtaaa	ctgagaacat	tctctgtatg	aagactctaa	aaatctgttg	100
+101	ttgtagagtt	ataaagttaa	tggagtgtgga	ttttcttttga	tgtttcatat	ataatcatgt	tcacagtaet	gtttgattat	tagtgttttg	tcatttctgt	200

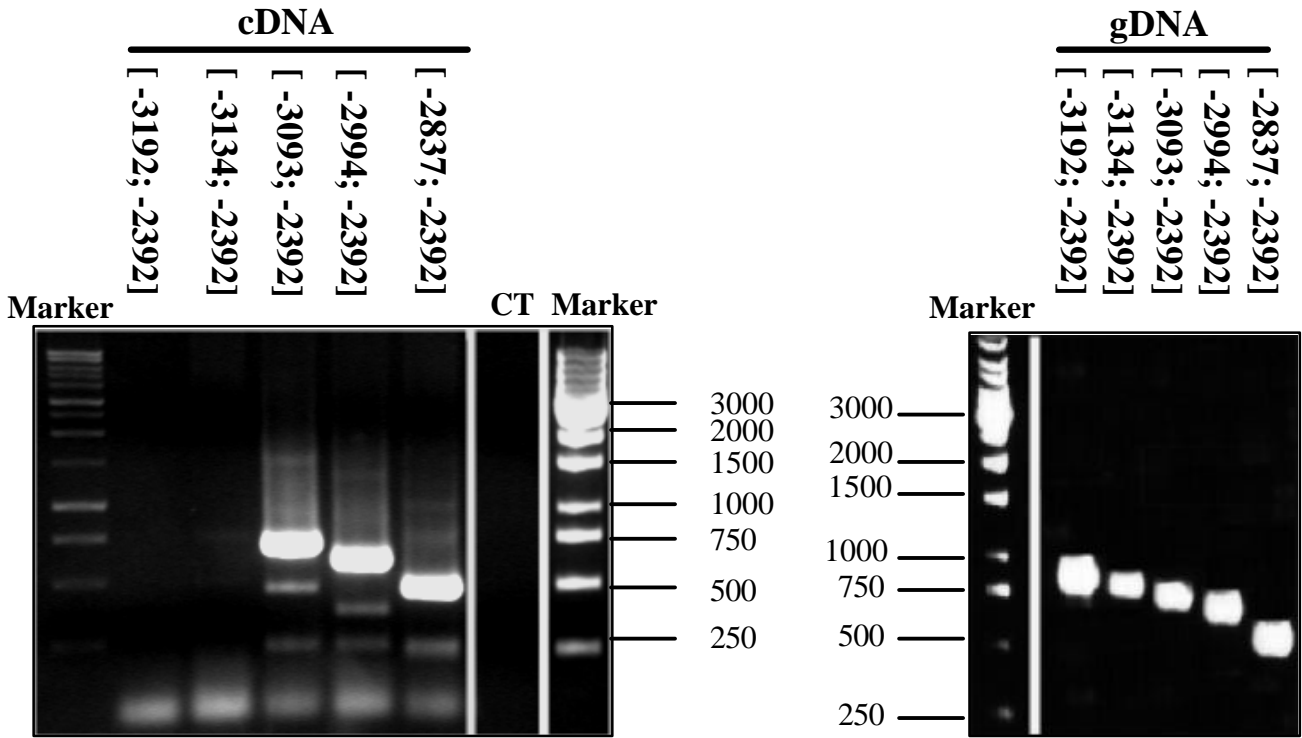
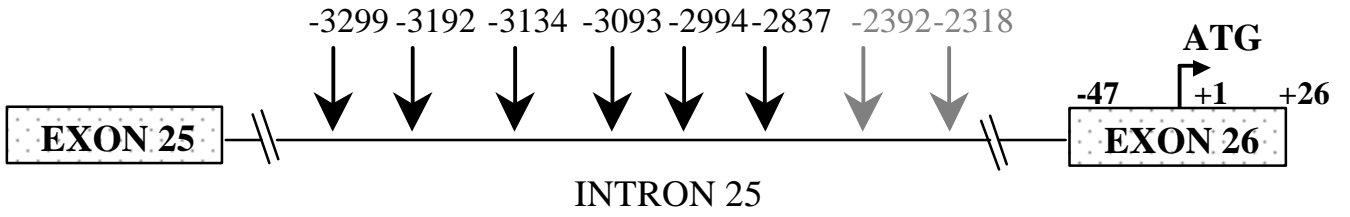
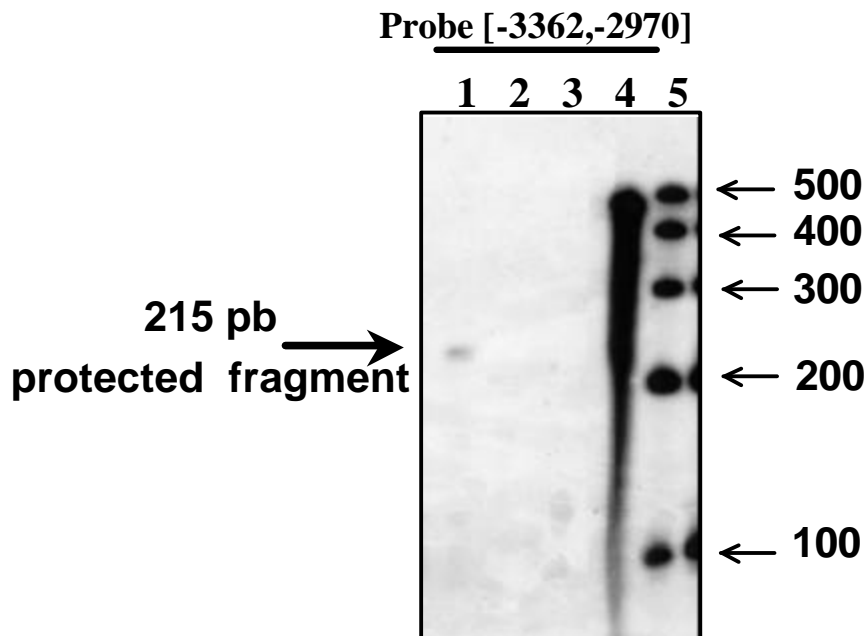
Figure 1

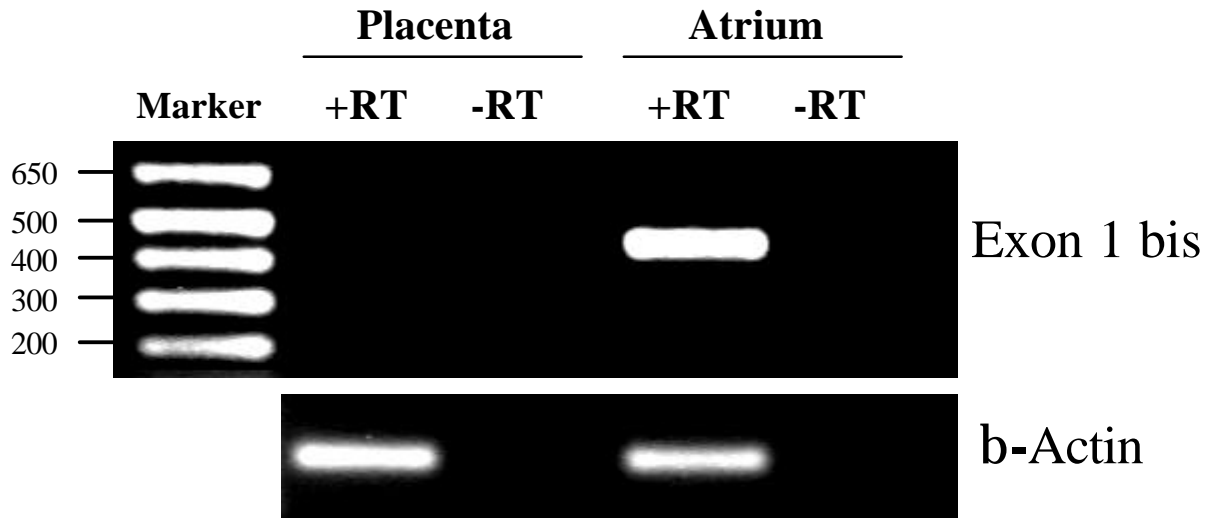
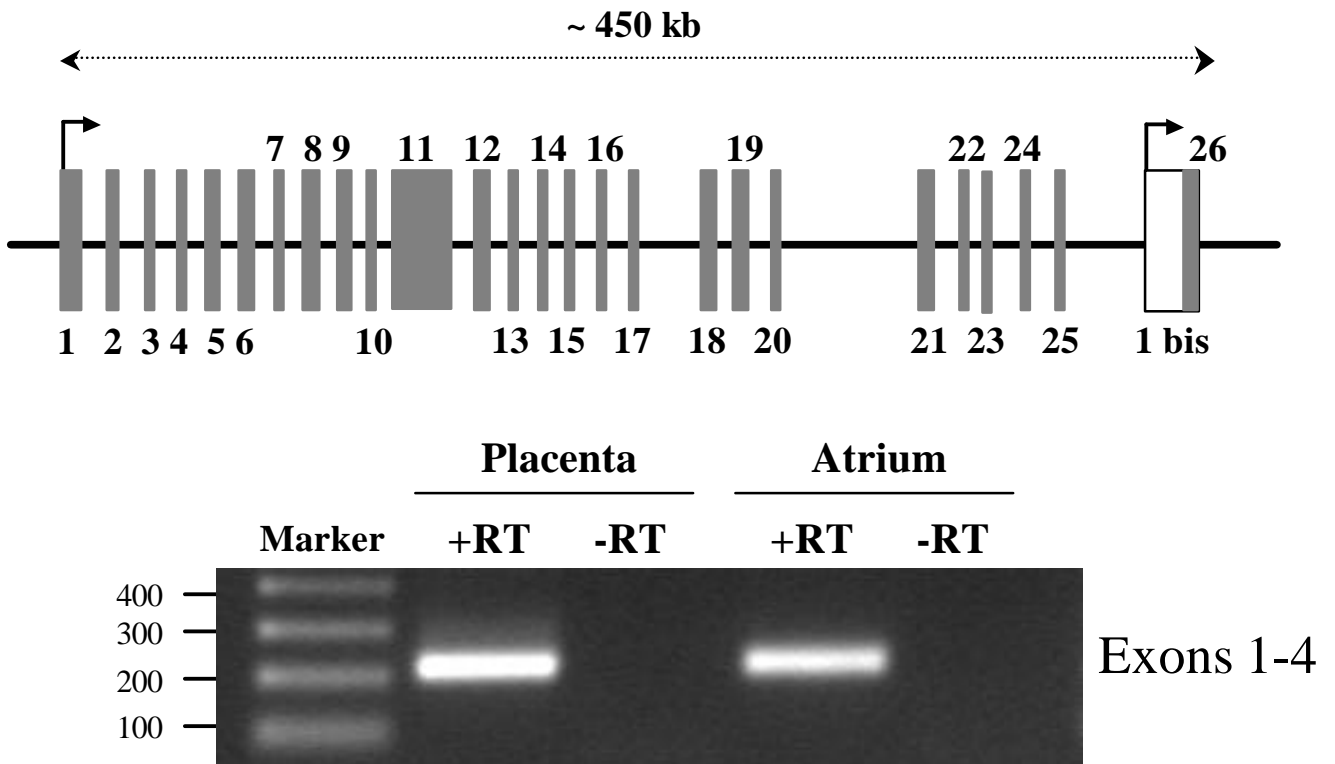


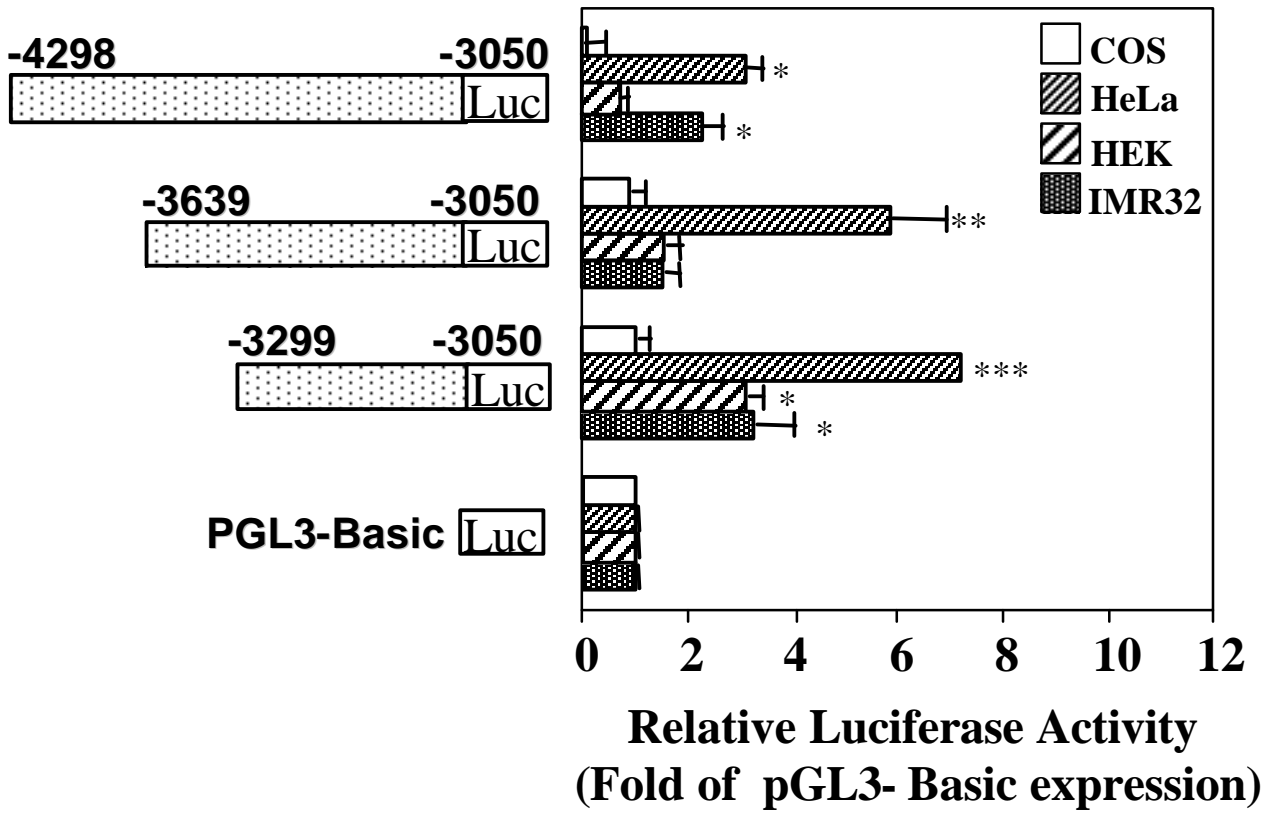
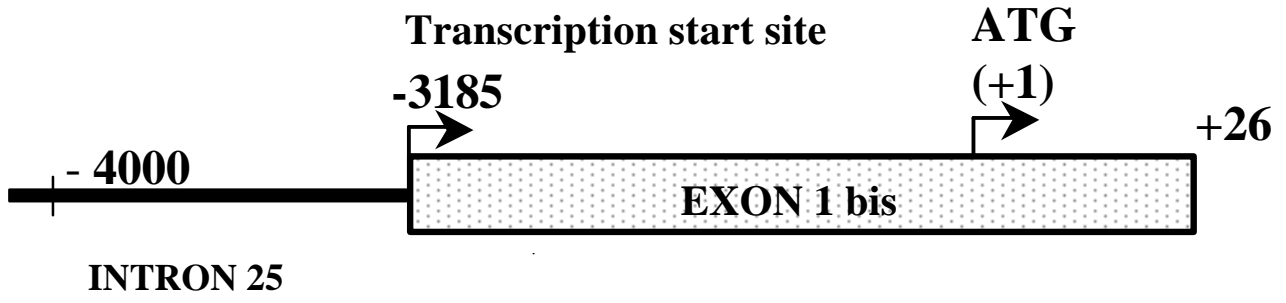
**B**



**Figure 2**

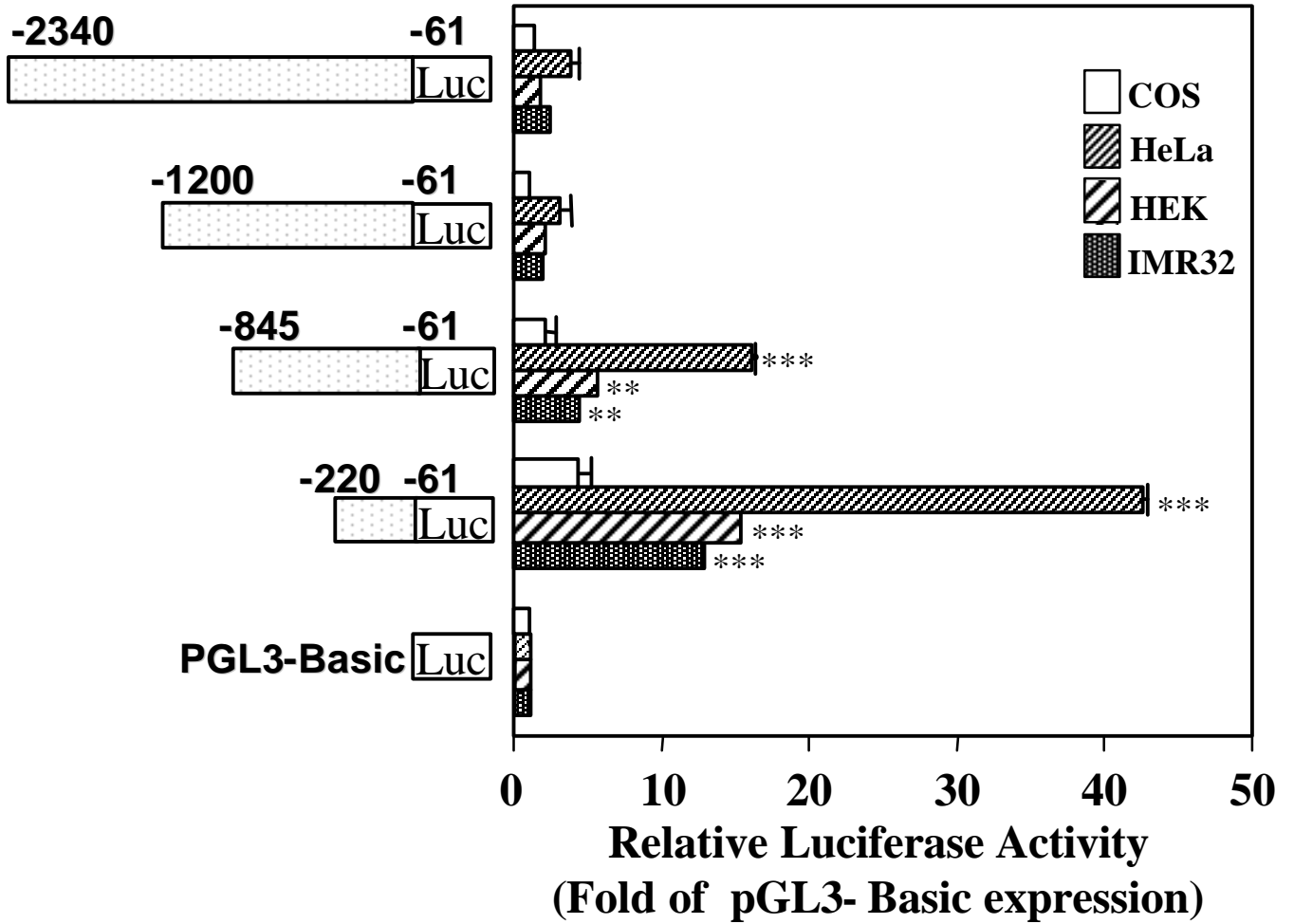
**A****B****Figure 3**

**C****D****Figure 3**

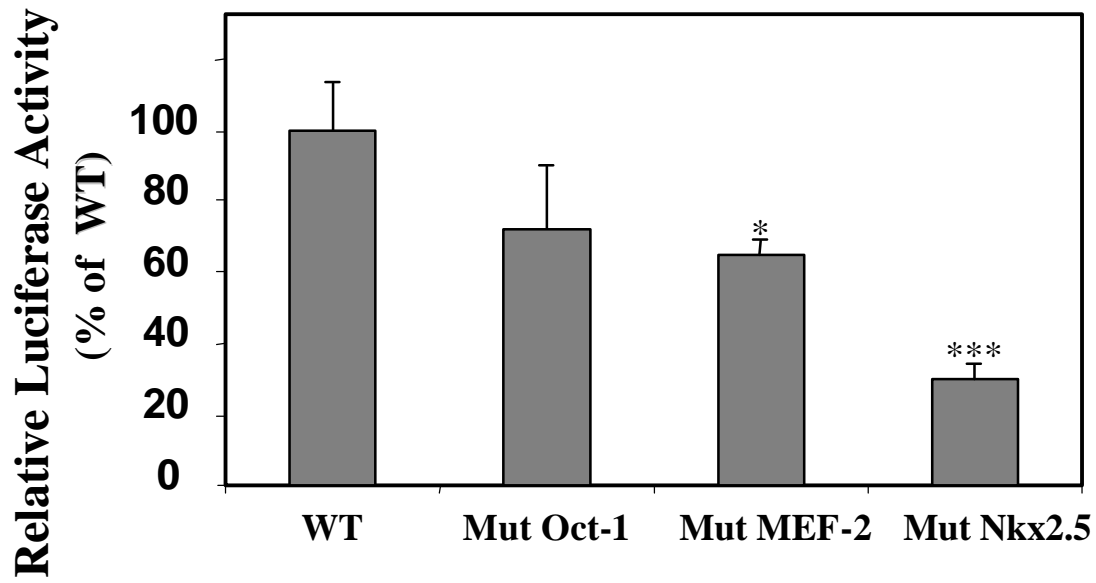
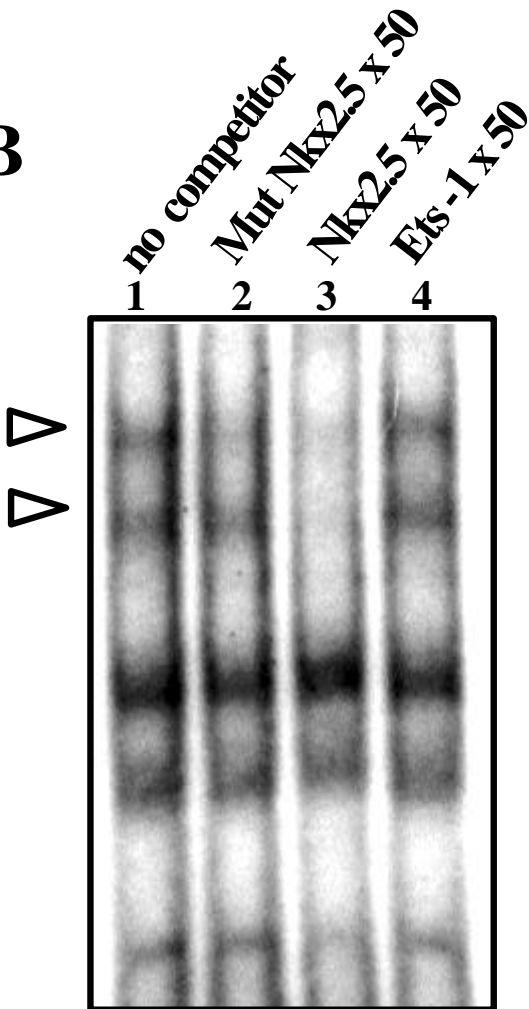
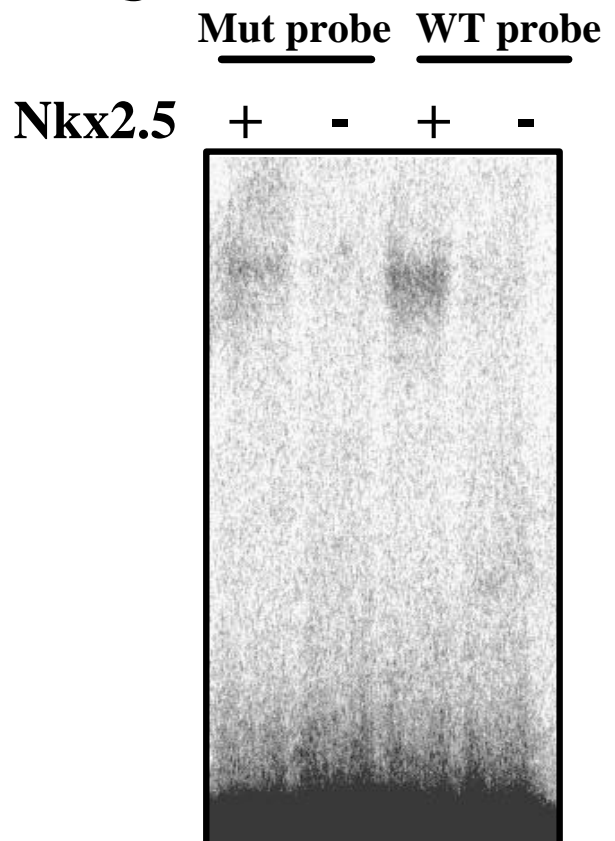


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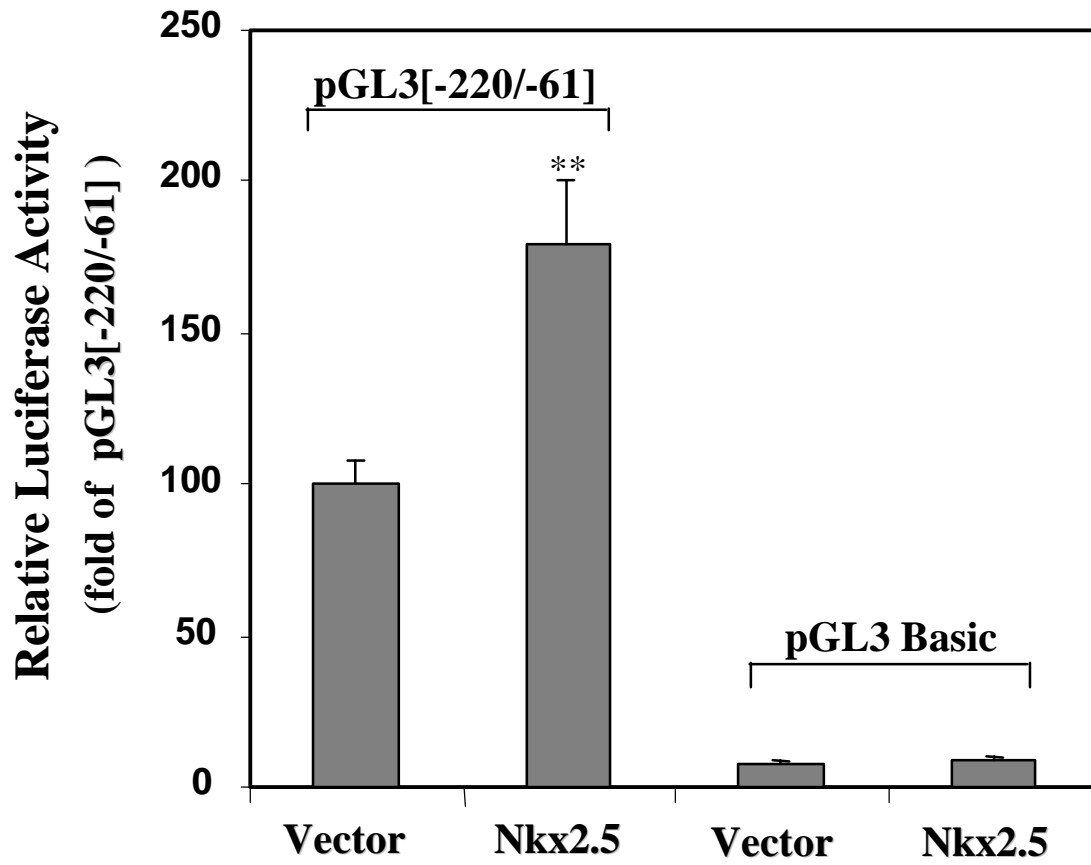




**Figure 5**

**A****Figure 6****B****C**

**D**



**Figure 6**