



HAL
open science

Genomic organization and regulation of the human orexin (hypocretin) receptor 2 gene: identification of alternative promoters

Jing Chen, Harpal S Randeva

► **To cite this version:**

Jing Chen, Harpal S Randeva. Genomic organization and regulation of the human orexin (hypocretin) receptor 2 gene: identification of alternative promoters. *Biochemical Journal*, 2010, 427 (3), pp.377-390. 10.1042/BJ20091755 . hal-00479281

HAL Id: hal-00479281

<https://hal.science/hal-00479281>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Genomic organization and regulation of the human orexin (hypocretin) receptor 2 gene: identification of alternative promoters

Running title: Regulation of the human OX2R gene

Chen J. and Randeve H. S.

Endocrinology & Metabolism Group, Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Coventry, CV4 7AL, UK

Abstract: words 249

Word count: words 6988

Tables: 2

Figures: 7

Correspondence:

Dr Harpal S Randeve MBChB, FRCP, MD, PhD

Endocrinology & Metabolism Group

Clinical Sciences Research Institute

Warwick Medical School

University of Warwick

Coventry CV4 7AL, UK

Tel: + 44 2476 572552

Fax: + 44 2476 523701

E-mail: Harpal.Randeve@warwick.ac.uk

Disclosure statement: The authors have nothing to disclose

Keywords: Orexin (hypocretin) receptor, untranslated region, alternative splicing, promoter, Energy homeostasis

Research Grants to H.S.R. from the General Charities of the City of Coventry and British Heart Foundation

ABSTRACT

Orexins (hypocretins), acting via their receptors, are involved in the control of feeding behaviour, sleep, arousal and energy homeostasis. However, regulation of human orexin receptor 2 (hOX2R) gene remains unknown. We have identified four transcripts arising from alternative splicing from three exons. These exon 1 variants were designated as exons 1A, 1B and 1C on the basis of their 5' to 3' order. RT-PCR demonstrates the differential expression in various human tissues. The alternate 5'-UTRs possessed by these isoforms have different translational efficiencies, which regulate the level of protein expression. We demonstrate that the hOX2R gene is regulated by two promoters, the novel transcripts are regulated by the distal promoter located upstream to exon 1A. We demonstrated that the Ap-1 motif is critical for sustaining the basal activity of distal promoter. Analysis of the proximal promoter revealed the region regulating promoter activity contained putative binding elements including those for CREB, GATA-2 and Oct-1. Using the chromatin immunoprecipitation assay, we demonstrated that CRE, GATA-2 and Oct-1 transcription factors bind to these critical regulatory promoter elements. Mutational studies suggested that these motifs functioned independently but have a compound effect regulating hOX2R gene transcription. Furthermore proximal promoter activity is enhanced by both PKA and PKC pathway activation, *via* binding of CREB and GATA2 transcription factors. In conclusion, we demonstrate expression of the hOX2R is regulated by a complexity involving a proximal PKA/PKC regulated promoter and a distal promoter regulating tissue specific expression of alternate transcripts which in turn post transcriptionally regulate receptor levels.

INTRODUCTION

The orexin (hypocretin) neuropeptide gene was first cloned from rat hypothalamus (1, 2). The designation orexin (hypocretin, HCRT) was adopted to indicate that it is a hypothalamic member of the incretion family. Hypocretin is also known as orexin (from the greek word orexis which means appetite) on the basis of their expression in the hypothalamic feeding centres and role in the control of food intake. The precursor protein gives rise to two mature neuropeptides, Orexin A and Orexin B by proteolytic processing. Orexin A, a 33-amino acid peptide, and Orexin B, a 28-amino acid peptide, share 46% homology. Besides playing a role in the regulation of feeding and energy homeostasis (3, 4), intracerebroventricular administration of orexins have been reported to exert divergent actions, including modulation of physiological behaviours (5), cardiovascular regulation (6), reproductive function, regulation of the hypothalamic pituitary adrenal axis (HPA) and regulation of the sleep-wake cycle (7), with the recent demonstration of reduced cerebrospinal fluid concentrations of orexins in patients with narcolepsy (8) and involvement in drug-seeking behavior (9).

Orexin A and B orchestrate their actions by binding and activating two types of G protein-coupled receptors, orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R) which display 64% homology. The rat and human receptors display 94% and 95% homology for OX1R and OX2R, respectively, suggesting that they are highly conserved between mammalian species (1). OX1R preferentially binds Orexin A, whereas OX2R binds both Orexin A and Orexin B, with similar affinity. In recombinant systems, activation of either receptor results in a receptor-operated Ca^{2+} influx and elevation of intracellular Ca^{2+} concentrations (1, 10). Orexin A has been reported to lead to a dose-dependent increase in IP3 (11). We have recently demonstrated that hOX2R-mediated ERK1/2 and p38 MAPK activation are regulated by differential signalling pathways in HEK293 cells. Gq/PLC/PKC, Gs/AC/cAMP/PKA and Gi pathways are required for hOX2R mediated ERK1/2 activation, whereas Gs/AC/cAMP and Gi but not Gq or PKA are involved in p38 MAPK activation (12). In addition, in rat hypothalamic cultures, orexins cause protein kinase C mediated Ca^{2+} influx (13). Furthermore, there is evidence suggesting that the orexin receptors can couple to the adenylate cyclase-cAMP system (11, 14, and 15). However, the regulation by orexin of cAMP production may be tissue-dependent (15).

Orexin receptors were originally shown to be present only in the hypothalamus (1), but now their presence has been noted in peripheral tissues in both rats and humans (16), including the myenteric plexus of the enteric nervous system and the endocrine cells of the gut (17), the adrenal gland (11, 18), and the male reproductive system (19) implicating orexins and their receptors in an increasing number of physiological responses. The hOX2R gene has been mapped to chromosome 6 position 6p11-q11 and although a preliminary exon / intron arrangement of the hOX2R was reported (20), the upstream structure of the gene was not identified. As a consequence, no evidence of the mechanism and regulation of transcription for hOX2R have yet been reported.

In the present study, the structure and function of the 5'-flanking region of the hOX2R gene was investigated. We report the identification of a complex regulatory mechanism consisting of four distinct mRNA isoforms that arise via alternative splicing from novel 5' exons which are regulated by a distal promoter. The level of receptor protein expression is regulated by the translational efficiencies of these isoforms which are expressed in a tissue specific manner together with the known transcript regulated by the proximal promoter. The core regulatory regions of both distal and proximal promoters were identified by deletion reporter assays in endogenously and non-endogenously expressing cell lines. We have demonstrated that the Ap-1 motif is critical for sustaining the basal activity of distal promoter. Scanning mutational analyses and chromatin immunoprecipitation assay identified CREB, GATA-2 and Oct-1 as important regulators of hOX2R transcription. Both PKA and PKC pathway activation via CREB and GATA-2 transcription factors were demonstrated to enhance hOX2R proximal promoter activity.

EXPERIMENTAL PROCEDURES

Isolation of genomic clones by genomic walking

The 5' flanking region of the hOX2R gene was cloned by the PCR based genomic walking method. Sequences of the 5' flanking region were obtained using the Universal Genome Walker kit (BD Biosciences Clontech, Oxford, UK). The Universal Genome Walker strategy was employed together with these primers to obtain the 5' end of a hOX2R cDNA (AF041245). In the first round of PCR, the gene specific primer Exon1FR was used versus the Genome Walker adaptor primer 1 (AP1, 5'-CCATCCTAATACGACTCACTATAGGGC-3'). In the second round, the primer Exon1SR was used versus the Genome Walker Nested Adaptor primer 2 (AP2, 5'-ACTCACTATAGGGCTCGAGCGGC-3'). Reactions were held at 95°C for 60s and then cycled 40 times between 94°C for 40s, 52°C for 45s and 72°C for 3 minutes. PCR products were ligated into pGEM-T (Promega, Southampton, UK) and sequenced.

Determination of the transcription start site

The hOX2R 5' UTR cDNAs were isolated by 5' rapid amplification of cDNA ends (RACE) from adult hippocampus, hypothalamus, heart and testis cDNA libraries (BD Biosciences Clontech). One was performed by using the Marathon Ready human hippocampus, hypothalamus, heart and testis cDNA respectively, according to manufacturer's instructions. Primary PCR was carried out using a gene specific primer (Exon1FR) and the adaptor primer 1 (AP1, see above) Secondary PCR was carried out using a nested gene specific primer (Exon1SR) and the nested adaptor primer (AP2, see above) with 1.0 µl of primary PCR as template. Reactions were held at 95°C for 60s and then cycled 40 times between 94°C for 40s, 58°C for 45s and 72°C for 3 minutes. The PCR products were analyzed on a 1% agarose gel, extracted and subcloned into the pGEM T Easy (Promega) for sequencing.

PCR analysis of alternative splicing variants of the hOX2R in human tissues

These experiments were carried out with the Human Multiple Tissue cDNA Panel I kit from Clontech according to the manufacturer's recommendations. The specific hOX2R-exon-1A and exon-1C fragments were amplified by exon1A and 1C forward primers Exon1AF and Exon1CF, together with a common reverse primer Exon1FR at the start of exon 2. Thermal cycling for alternative splicing variants of the hOX2R, PCR consisted of 95 °C for 1 min followed by addition of 0.5 µl Taq DNA Polymerase; 94 °C for 45 s, 55°C for 35 s, 72 °C for 1 min (40 cycles), 72 °C for 10 min.

Cell culture, transient transfection and report gene analysis

The HEK-293 cells (human kidney epithelial; European Collection of Animal Cell Cultures, Center for Applied Microbiology and Research, Salisbury, Wiltshire, UK), Ntera/D1 cells (NT2, human neuronal-like teratocarcinoma cells; Stratagene Europe, Amsterdam, The Netherlands), and SH-SY5Y cells (human neuroblastoma; European Collection of Animal Cell Cultures, ECACC) were seeded in 6 well plates and cultured according to the ECACC recommendations, until they were approximately 60-70% confluent. Cell cultures were maintained in phenol red-free medium containing charcoal-stripped FCS, for 24 hours prior to transfection. The cells were transfected with 2 µg of plasmid DNA using Lipofectamine Plus (Invitrogen, Paisley, UK) in 1 ml of serum-free medium and were incubated for 3 hours at 37°C. All plasmid constructs were co-transfected with 0.5 µg of the pRL-TK vector (Promega) to determine the transfection efficiency. Medium was re-placed with 2 ml of fresh culture medium 3 hours after transfection and cells were grown until 90% confluent. Cells were pre-treated with either Bis and H-89 for 2 hours and 30 min prior to incubation with PMA and db-cAMP, for 12 hours. Cell extracts were prepared 24 to 48 hours after transfection and assayed using a dual-luciferase reporter assay system (Promega). Luciferase activity was measured for 10 seconds using a Luminoscan RS Luminometer (Labsystems, Helsinki, Finland). Cells transfected with the pTimer-1 reporter constructs were assayed by fluorescence microscopy.

Construction of luciferase-expression vectors and pTime-1 vectors

Flanking sequences 5' to the 5' UTR-1 (exon-1A), 5'UTR-3 (exon-1C) and exon1 of hOX2R genes were amplified by PCR from human genomic DNA (BD Biosciences), cloned into the pGL-3 basic vector (Promega) and sequence verified. Primers used for this PCR had a 5' *Xho* I or *Kpn* I restriction site in the 5' primer and a *Hind* III or *Xho* I site in the 3' primer to facilitate cloning. Constructs were designed using the primers described (supplementary table 1). The primers for analysis of the 5' flanking region of exon 1C are (1) exon 1C (-841/-821) Exon1C R primer (2) pGL3-1C-225 (-1066/-841) as same as pGL3-1-1066, (3) pGL3-1C-439 (-1280/-841) as same as pGL3-1-1280, (4) pGL3-1C-722 (-1563/-841) as same as pGL3-1-1563. Three different fragments (1A₁: -30820/-29792, 1C₂ -1280/-841 and 1₂ -638/-43) were cloned into the pTimer-1 vector (BD Biosciences Clontech), encoding the DsRed1-E5 protein, a mutant of *Discosoma* sp. Red fluorescent protein, which changes colour over time was used. Shortly after translation, the protein emits green light; its red fluorophore emerges later, hours after translation.

Cloning of splice variants into pcDNA3.1 reporter plasmid

The full length luciferase ORF derived from pGL3 basic was ligated into pcDNA 3.1 (mammalian expression vector, Invitrogen, Paisley, UK) to create pcDNA 3.1 Luc (pcDNA3.1-Luc) (21). Each of the 5'-UTR splice variants identified in human testis tissues was cloned into a pcDNA3.1-Luc vector containing the luciferase gene inserted into the *Eco*R I /*Hind* III restriction sites using the following strategy. Each of the 5' UTR splice variants identified in was cloned into the restriction sites using the following strategy. First, exon1A was amplified using primers Exon 1A SVF and Exon 1 SVR (supplementary table 1) that contains *Nhe* I /*Xho* I sites, and cloned into the *Nhe* I /*Xho* I in the (pcDNA3.1-Luc/1A). The PCR products are 172 bp The PCR products were ligated into pcDNA 3.1 Luc (pcDNA3.1-Luc). Clones with exon 1A in the correct orientation were identified by DNA sequencing.

Exon 1B was then amplified using primers Exon 1A SVF and Exon 1 SVR (supplementary table1). The PCR products are 271 bp. This fragment was then cloned in pcDNA3.1-Luc into generate a reporter gene with a 5' UTR similar to the OX2R hUTR 1B splice variant (pcDNA 3.1-Luc/ 1A+1B). Secondly, exon 1C was made by amplifying exon 1C SVF with primers Exon 1 SVRC (supplementary table 1) containing *Xba* I/*Xho* I sites which were then cloned into pcDNA3.1-Luc/1C_{short}.

Thirdly, Intron 1 was amplified using primers Intron 1 F (5'-CTAGCTACTGAGAAGGCTCTAAAGAGACC-3') and Intron1 R (5'-CCGCTCGAGTGCCTTGRAARCRAGGTGCTC-3'). that contains *Nhe* I /*Xho* I sites, and cloned into the *Nhe* I /*Xho* I in the (pcDNA3.1-Luc/intron 1 sequences, as control).The PCR products are 211 bp. This fragment was then cloned in pcDNA3.1-Luc into generate a reporter gene with a control.

All clones were sequenced to confirm the correct orientation of each exon insert. HEK-293 and SH-SY5Y cells cultured in 6 well culture plates to 80% confluence were co-transfected with 2 µg per well of pcDNA3.1-Luc/1A, pcDNA 3.1-Luc/ 1A+1B , pcDNA3.1-Luc/1C_{short} and

pcDNA3.1 -Luc/control plasmid DNA. Cotransfection with the *Renilla* luciferase vector, pRL-TK vector (Promega) at a 1:10 ratio was performed to correct for transfection efficiency. Forty-eight hours after transfection, cells were harvested and then firefly luciferase activity from the splice variant constructs and *Renilla* luciferase activity from the pRL-TK vector were measured using a dual luciferase assay kit (Promega).

Real-Time PCR

Total RNA was extracted from HEK-293 and SH-SY5Y cells that had been transfected with the pcDNA3.1-Luc splice variant and a pcDNA3.1-Luc (lacking hOX2R exons) constructs and reverse transcribed to generate cDNA as described above. For each RNA sample, a reaction lacking reverse transcriptase was performed and used in real-time analysis to ensure there was no genomic or plasmid DNA contamination. Expression levels of luciferase from

the various splice variant constructs were quantified by real-time PCR using a Roche Light Cycler™ system. PCR reactions were carried out in a reaction mixture consisting of 5 µl reaction buffer and 2.0 mM MgCl₂ (Biogene, Kimbolton, U.K), 1.0 µl of each primer (25 ng/µl), 2.5 µl of cDNA and 0.5 µl of Light Cycler DNA Master SYBR® Green I (Roche, Mannheim, Germany) as previously described (22). Protocol conditions consisted of denaturation at 95°C for 15 seconds, followed by 45 cycles of 94°C for 1 sec, 58°C for 7 sec and 72°C for 12 sec. Specific forward and reverse primers used to amplify luciferase and β-actin were used. The luciferase primers were luciferase F and R (supplementary table 1). Standard curves for luciferase and β-actin were constructed by serial dilution of the control cDNA sample and then used to quantitative expression levels. After normalization for β-actin, luciferase levels were expressed relative to the control. In order to demonstrate accuracy and reproducibility of analysis, quantification data was analysed using the Light Cycler analysis software. The RNA levels were expressed as a ratio, using the “Delta-delta method” for comparing relative expression results in real-time PCR (23). PCR products were run on 1% agarose gels for confirmation of the correct amplification size and identification of a single PCR product.

Site-Directed Mutagenesis

The pGL3-1A-300 and pGL3-1-456 fragment of hOX2R distal and proximal promoter gene were cloned into each the pGL-3 basic vector and was used as templates for the mutagenesis reaction. Mutation was introduced by a three-step PCR mutagenesis method (24). The mutation constructs were generated by PCR with *Pfu* polymerase (Stratagene) for each mutation constructed. Two internal primers bearing the desired mutated sequence and two external primers based on sequences of the pGL-3 basic vector were used. The PCR was carried out for 35 cycles with denaturation for 1 min at 95°C, annealing for 45 sec at 45°C, extension for 2 min at 72°C and a final extension for 10 min at 72°C. Two overlapping fragments gel-purified from two PCR reactions with paired external and internal primers were mixed for a second PCR reaction. Two overlapping strands were annealed and extended by the *Pfu* DNA polymerase to generate a full-length template which was then amplified by two external primers. The PCR fragments were gel-purified, digested with (*Xho* I and *Hind* III for pGL3-1A-300) and (*Kpn* I and *Xho* I for pGL3-1-456) then, ligated with the pre-digested the pGL-3 basic vector. The oligonucleotides used in site-directed mutagenesis are shown in (supplementary table 1). Mutant sequences were confirmed by DNA sequencing.

Chromatin immunoprecipitation (ChIP) Assay

SH-SY5Y cells were treated in serum-free medium in the absence or presence of 100 nM db cAMP or PMA for 6 hours and then rinsed with cold PBS and cross-linked with 1% formaldehyde at room temperature for 10 minutes. Cell lysates were sonicated on ice, 10 times for 15 sec, and separated by 45 sec. After cell lysis and sonication, the supernatant was diluted 10-fold in ChIP dilution buffer (16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, containing 1mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate, 10 µg/ml each of apportioning and leupeptin) before incubation with antibodies. ChIP was carried out by the addition of 5 µg of CREB (rabbit polyclonal, Santa Cruz Biotechnology, Inc Heidelberg, Germany), GATA-2 (goat polyclonal, Santa Cruz Biotechnology, Inc) or Oct-1 (rabbit polyclonal, Santa Cruz Biotechnology, Inc) antibody. Ten percent of the sample volume was reserved for use as input control. Cross-linking was reversed by incubation at 65°C for 4 hours. DNA was subsequently purified using Qiaquick PCR Purification kit (QIAGEN). PCR was performed using 2 µl of DNA as template for 35 cycles, using primers spanning the CREB element (-54/-165) sense CREB P2 and antisense CREB P1 (supplementary table 1) or GATA-2 (-141/-272) sense GATA-2 P3 and antisense GATA-2 P4 or Oct-1 (-297/-459) sense Oct-1 P6 and antisense Oct-1 P5 (supplementary table 1).

Statistical analysis

Non parametric unpaired *t* test was used to measure statistical significance using graph pad prism software version-4.0. Values were considered significant if $p < 0.05$.

RESULTS

5' Genomic structure of the hOX2R gene

The 5'-flanking region from the translational start site of the hOX2R gene was cloned by the PCR based genomic walking method. Upstream genomic walking was performed with commercial libraries of adaptor-ligated genomic DNA, starting with primers based on the conserved nucleotide sequence of the hOX2R cDNA. Two rounds of upstream walking resulted in overlapping PCR products of 1748 and 539 bp, altogether encompassing 1.7 kb of contiguous upstream genomic sequence (figure 1a). By aligning this nucleotide sequence with those for the hOX2R cDNA and the 5' RACE products, we were able to identify a small intron (348 bp), flanked by an upstream exon (designated exon 1C). The sequences were used to search the NCBI human genome database. The corresponding DNA sequence was located on the contig AL591718 (159.7 kb) mapped to chromosome 6. Analysis of this contig together with data obtained from the 5' RACE study described below, revealed the genomic structure of the hOX2R consists of three novel exon 1 variants and seven further exons (exon 1 to 7) contain the coding region of 444 amino acids (GenBank accession no AF041245) as determined by aligning the cDNA sequences to the genomic sequence (figure 1c).

Identification of distinct 5' UTRs transcribed from three exons in the hOX2R gene

The hOX2R 5' UTR was identified by rapid amplification of cDNA ends (RACE) from four human adult tissue cDNA libraries (hippocampus, hypothalamus, heart and testis). The 5' RACE reactions were carried out using primers specific to exon 1 of the hOX2R gene plus adaptor primers. The 5'-RACE products were subcloned into the pGEM-T vector and sequenced (figure 1b). Sequence alignment of hOX2R 5'-RACE cDNA products and the genomic DNA contig AL591718, revealed alternate 5'UTRs with multiple transcriptional initiation sites within them. These 5'UTRs are generated by alternative splicing from three novel upstream exons. These exon 1 variants were distributed over a region of 29.5 kb and designated as exons 1A, 1B and 1C on the basis of their 5' to 3' order, the translational start site containing exon was referred to as exon 1 as previously described (figure 1d). Isoform hOX2R-1A is derived from exon 1A consisting of 143 nts spliced to exon 1. Two distinct transcription start sites were identified at nt 1 and 15 of exon 1A. Isoform hOX2R-1B is derived from exon 1A splicing to exon 1B consisting of 99 nts and exon 1, exon 1B is separated from exon 1A by a 4487 bp intron. Isoform hOX2R-1C is derived from exon 1C located 24222 bp downstream of exon 1B and consisted of two variant transcripts. The first consisted of a 207 nts spliced to exon 1, separated by a 348 bp intron the second transcript contained this 348 nts intronic sequence. The short form of the hOX2R-1C isoform contained two distinct transcription starts at nt 1 and 70. The long form of the hOX2R-1C isoform contained three distinct transcription starts sites at nts 315, 339 and 347 upstream of the first base of the translation initiation codon. Table 1 summarises 5' exon size, splice site and introns.

Differential expression of hOX2R isoforms in various tissues

Tissue-specific expression of hOX2R isoforms was demonstrated by PCR in a panel of cDNAs from various human tissues. Amplification was performed using exon 1A and 1C specific sense primers and a common antisense primer located within exon 1. The amplification with the exon 1A primer resulted in two products, sequencing revealed that the long fragment corresponded to hOX2R-1B and the short fragment corresponded to hOX2R-1A. The amplification with the exon 1C primer also resulted in two products, sequencing revealed that the long fragment corresponded to hOX2R-1C_{long} and the short fragment corresponded to hOX2R-1C_{short}. Amplification with primers to exon 1 and 2 would hybridise

to all isoforms present and produced one product in all tissues (figure 1e). hOX2R-1B isoform was detected in most of the tissues examined with the exception of the kidney and liver. hOX2R-1A isoform was strongly expressed in the brain, skeletal muscle, placenta and testis, expression was weak in the kidney, lung, liver, pancreas and heart. hOX2R-1C_{short} isoform was strongly expressed in the brain, placenta, pancreas and testis and detectable in the kidney. hOX2R-1C_{long} isoform was expressed in all tissues except skeletal muscle and kidney.

Analysis of hOX2R 5'UTRs on gene expression

The 5' UTR is suggested to be involved in the regulation of translation (25-28). The alternate 5' UTRs of hOX2R mRNA transcripts could have important biological effects on the regulation of translation. Firstly, hOX2R-1A has a 5'UTR of 143 nucleotides, hOX2R-1B, hOX2R-1C_{long} and hOX2R-1C_{short} have a 5'UTR of 242, 486 and 207 nucleotides, respectively. Secondly, hOX2R-1A contains three AUG codons (uAUGs). Both hOX2R-1B, hOX2R-1C_{short} and hOX2R-1C_{long} have five, four and eight uAUGs respectively, with many partially or completely resembling a Kozak consensus sequence (A/G)CC(A/G)CCAUGG (27). No reasonable open reading frames originated from these consensus AUGs. Finally, secondary mRNA structure of the hOX2R 5' UTR sequences was analyzed using the *mfold* program (bioinfo.math.rpi.edu/~mfold/ma/forml.cgi) indicating the ability to form complex and stable structures, as indicated by the values for free energy of secondary structure. Analysis of the five different transcripts of hOX2R revealed that the secondary structure of the hOX2R-1A has estimated free energy of -32.9 kcal/mol, which is much lower than that of the hOX2R-1B, hOX2R-1C_{short}, hOX2R-1C_{long} and hOX2R-1 (Table2). All these features are regarded as important determinants of translational efficiency (29). To determine the effects of each 5' UTR on mRNA and protein expression each 5' UTR sequence was cloned into a luciferase reporter construct (30). SH-SY5Y cell line expressed all of the hOX2R transcripts (data not shown). Expression of each construct was analysed in a human neuroblastoma cell line (SH-SY5Y) which endogenously expresses hOX2R mRNA and a human kidney cell line (HEK-293) which did not, compared to activity in cells transfected with the pcDNA3.1-Luc/intron 1 (as nonsense (intron 1) sequences as control) and only a pcDNA 3.1 -Luc vector as another control, after normalising the data to the control. We have performed similar experiments using only a pcDNA 3.1 -Luc vector as another control, which has demonstrated similar results in comparison with the pcDNA3.1-Luc/intron 1 control (data not shown).

The inclusion of each 5' UTR upstream of the luciferase reporter significantly reduced luciferase activity to less than 30-45 % of the control (figure 2a), both cells lines produced comparable results. To determine whether the difference in luciferase expression was due to different amounts of mRNA, real-time PCR was employed to quantify the levels of luciferase mRNA relative to β -actin expression. 5' UTR1A showed a 3-3.5 fold ($P < 0.001$) increase in luciferase mRNA relative to the control plasmid in the two cells lines, despite having the lowest level of luciferase activity. 5' UTR1C_{short} and UTR1B also showed a higher expression compared to control of approximately 1.5 fold ($P < 0.05$), although only a slight increase was seen for 5' UTR1B in HEK-293 cells (figure 2b). The results suggest that the 5' UTRs regulate hOX2R expression at a post-transcriptional level by reducing translation efficiency to different extents.

Identification of multiple promoters in the 5' region of the hOX2R gene

The identification of 5' UTR heterogeneity associated with alternative 5' exons resulting in five isoforms suggested that multiple promoters may regulate transcription of the hOX2R gene. To determine which of the 5' proximal regions of the hOX2R gene, upstream of exon 1A, 1C and 1 can drive transcription, we generated serial deletion constructs containing different portions of the 5' flanking region of exon 1A, 1C and 1 linked to a luciferase reporter (figure 3a). The reporter constructs were used to transiently transfect SH-SY5Y and HEK-293 cells. Sequences proximal to exon 1A, constructs 1A₁ (-30820 to -29792 (1028 bp) and 1A₂ (-31578 to -29792 (1786 bp) showed high luciferase activity, although no difference was seen between constructs. Sequences proximal to exon 1C, constructs 1C₁ (-1066 to -841

(225 bp), 1C₂ (-1280 to -841 (439 bp) and 1C₃ (-1563 to -841 (722 bp) exhibited no luciferase activity above basal. Sequences proximal to exon 1, constructs 1₁ (-456 to +43 (499 bp) and 1₂ (-638 to +43 (681 bp) produced the highest luciferase activity, although no difference was seen between constructs. No difference was seen in luciferase activity between SH-SY5Y and HEK-293 cell lines. Constructs in reverse orientation (1A_{rev} and 1_{rev}) exhibited no luciferase activity above basal (figure 3b). Further confirmation of promoter activity in the 5' region of the hOX2R gene, was demonstrated by cloning constructs 1A₂, 1C₂ and 1₂ into the fluorescence-timer (pTimer-1) reporter system (31). This reporter monitors the onset of transcription of a given promoter by monitoring the appearance of green fluorescence from the translated protein, as the protein matures there is a shift to red fluorescence (31). The results demonstrated that 5' flanking regions to exons 1A and 1, but not exon 1C, had sufficient promoter activity to produce a translated product (figure 3c). The 5' flanking region to exon 1C produced no red fluorescence suggesting no translated product was produced. The green fluorescence monitored was equal to basal negative control which also produced no red fluorescence. The results from both the luciferase and fluorescence-timer reporters indicate hOX2R gene possesses two promoter regions, one 5' to exon 1 (proximal promoter) which drives expression of the known transcript and the second 5' to exon 1A (distal promoter) which regulates expression of the alternate isoforms 1A, 1B, 1C_{short} and 1C_{long}.

Analysis of distal and proximal promoter activity by luciferase reporter assay

In order to functionally characterise the distal promoter, luciferase reporter constructs of the region 5' to exon 1A were transfected into both the SH-SY5Y cell line and HEK-293 cell line. Using a dual luciferase assay, differences in transfection efficiencies were corrected for and promoter activity was measured as a ratio of the two luciferases (figure 4A). The highest levels of activity were exhibited by the 1028 and 1786 bp promoter construct ($P < 0.01$ and $P < 0.001$). A slight reduction in activity was exhibited by the 300 bp construct ($P < 0.01$), but deletion of a further 176 bp caused a significant decrease in activity by approximately 2.5 fold (124 bp construct) to a level seen in the control construct (pGL3-Basic). Therefore the basal promoter is positioned 124 bp upstream of the Exon 1A and between -124 to -300. Analysis of the 176 bp region for potential transcription factor binding site predicts that AP-1 and myeloid zinc finger gene 1(MZF1) factors bind to this region (figure 4c). We examined whether a putative AP-1 binding site is implicated in hOX2R constitutional (basal) distal promoter activity. The pGL3-1A -300 distal promoter reporter construct, which we had found to display basal promoter activity, was used as a template for site-directed mutagenesis. The pGL3-1A-300 mutant was constructed and transiently transfected into SH-SY5Y and HEK-293 cells. Mutation of the AP1 motif causes a 47% (SH-SY5Y cell) ($P < 0.001$) and a 51% (HEK-293 cell) ($P < 0.001$) reduction in promoter activity (figure 4b). It is important to note that mutation of AP-1 site significantly reduced distal promoter activity. The Ap-1 motif is critical for sustaining the basal activity of distal promoter in both cell lines.

To functionally characterise the proximal promoter region, a series of 5' deletion constructs between -2000 and +43 was generated and analyzed using a luciferase reporter assay in three cell lines (figure 5a) which included the neuronal-like teratocarcinoma (NT2) cell line. Similar luciferase activity was observed in three cell lines. Deletion of the proximal promoter sequence from -2060 to -456 had no significant effect on the level of luciferase activity. Luciferase activity was not affected until deletion after -456 nts. This resulted in a reduction in activity of approximately 2 fold. Further deletion from -241 to -123 nts resulted in luciferase activity dropping to that seen in control empty vector. The data suggests that the core promoter region lies between -456 and -123. Analysis of this 333 bp region revealed numerous potential transcription factor binding sites for CREB, GATA-2, C-Ets, and Oct-1 (figure 5b).

Mutational analysis of putative CREB, GATA-2 and Oct-1 motifs within the hOX2R proximal promoter

Sequence analysis shows the existence of three motifs. A putative CREB binding site (5'-TCACGTCA-3' located from nts -130 to -123, with 93% homology to the consensus CREB motif), a GATA-2 binding site (5'-GCCTATGTTTC-3' located from -226 to -217, with 92.5% homology to the consensus GATA-2 motif) and an Oct-1 binding site (5'-TTTCATTACTTT-3' located from nts -405 to -393, with 89.5% homology to the consensus Oct-1 motif) all in sense orientations. To determine the functional significance of these motifs in regulating hOX2R gene transcription, site-directed mutants were constructed and transiently transfected into SH-SY5Y and HEK-293 cells. Mutation of the CREB motif caused a 33% (SH-SY5Y) ($P < 0.01$) and a 35% (HEK-293) ($P < 0.01$) reduction in promoter activity. Mutation of the GATA-2 motif reduced the promoter activity by 27% (SH-SY5Y) ($P < 0.01$) and 25% (HEK-293) ($P < 0.01$). Mutation of the Oct-1 motif causes a 28% (SH-SY5Y cell) ($P < 0.01$) and a 35% (HEK-293 cell) ($P < 0.01$) reduction in promoter activity. To examine whether there is any compound effects, constructs containing double or triple mutations were generated and analyzed in the two cell lines. Constructs containing two mutations (CREB + GATA-2) had their luciferase activities reduced by 50% (SH-SY5Y) ($P < 0.001$) and 55% (HEK-293) ($P < 0.001$). Approximately 80% ($P < 0.001$) reduction in promoter activity was observed in the both cell lines, when all three motifs were mutated simultaneously (figure 6a). This suggested independent roles for each motif which have a combined effect on driving promoter activity.

Analysis of protein interactions with the putative CREB, GATA-2 and Oct-1 binding motifs

The chromatin immunoprecipitation (ChIP) assay was used to determine occupation of the endogenous hOX2R proximal promoter by CREB, GATA-2 and Oct-1 binding proteins under basal conditions. PCR amplification was conducted using primer pairs specific for the -459 to -54 bp region of the proximal promoter and for the putative CREB (-54/-165), GATA-2 (-141/-272) and Oct-1 (-297/-459) binding motifs. Immunoprecipitation with antibodies to CREB, GATA-2 and Oct-1 resulted in amplification of the proximal promoter region. The presence of the proximal promoter regions for the above genes before the ChIP assay was confirmed by PCR (Input), ChIP assays using pre-immune serum (IgG) did not detect any such amplified bands. These results demonstrate direct binding of CREB, GATA-2 and Oct-1 to the proximal promoter of hOX2R in SH-SY5Y cells (figure 6b).

Effects of protein kinase activators on promoter activity

The results obtained from reporter assays described above indicated that the CREB, GATA-2 and Oct-1 directly bind to the proximal promoter. Both CREB and GATA2 can be activated by the PKC and PKA pathways (32, 33). To investigate whether cAMP dependent protein kinase A and protein kinase C activation regulate hOX2R gene regulation, SH-SY5Y cells transfected with the proximal promoter region fused to a luciferase reporter gene, were treated with a PKA activator (100 nM db cAMP) or a PKC activator (100 nM PMA) for 12 hours. Analysis of the proximal promoter using construct pGL3-1-456 which contains the GATA2 and CREB binding sites showed significantly enhanced activity compared to the pGL3-1-123 construct which does not contain these binding sites. The db cAMP treatments of cells transfected with the pGL3-1-456 construct increased promoter activity approximately 3.0-fold, whereas PMA led to a 3.5-fold increase in activity (figure 7a). Specific inhibitors of the PKA and PKC activation were used to verify this increase in activity. Transfected SH-SY5Y cells were pre-treated with inhibitors of PKA (H-89, 10 μ M) or PKC (Bis, 1 μ M) for 30 min and 2 hour, followed by treatment with 100 nM db cAMP or 100 nM PMA for 12 hours. Both PKA inhibitor, H-89 and PKC inhibitor, Bis prevented an increase in activation ($P < 0.01$) (figure 7b). The regulation by PKA and PKC on the hOX2R promoter may be tissue specific as no increase in expression was seen in human adrenocortical tumour cells (295R) stimulated with either PKA or PKC (data not shown). To determine whether CREB and GATA-2 directly participate in the observed increase in promoter activity following PKA or PKC activation, ChIP assays were performed following stimulation with db cAMP or PMA and compared to untreated (figure 7c). The ChIP assays demonstrated that stimulation with PMA lead to an increase predominantly in GATA-2 binding with a slight increase in CREB binding.

Stimulation with db cAMP lead to an equal increase in both CREB and GATA-2 binding. Neither stimulation increased Oct-1 binding. To further illustrate that PKA and PKC promoter activation is via increased CREB and GATA-2 DNA binding, luciferase reporter constructs were repeated as before but with constructs containing site-directed mutations of the CREB and GATA-2 binding sites. The increase in luciferase activity following stimulation with db cAMP was abolished in constructs (pGL3-1-456m1 and pGL3-1-456m5) which have mutated CREB sites (figure 7d). The increase in luciferase activity following stimulation with PMA was abolished in constructs (pGL3-1-456m2 and pGL3-1-456m5) which have mutated GATA-2 sites (figure 7e).

DISCUSSION

We present the 5' structural organization of the hOX2R gene and demonstrate that it is regulated by two promoters and alternate splicing to generate transcripts with alternative 5' UTRs. Four hOX2R mRNA isoforms that differ in their 5' UTR sequences were identified, hOX2R1A, hOX2R-1B, hOX2R-1C_{long} and hOX2R-1C_{short}. Alignment of cDNA and genomic DNA sequences of the four transcript isoforms revealed that the different 5' UTRs are generated from three exons by alternate splicing. These exon 1 variants were distributed over a region of 29.5 kb and designated as exons 1A, 1B and 1C on the basis of their 5' to 3' order. Exon intron splicing junctions of the three introns found in this study, 1A and 1B followed the GT-AG rule (28), but 1C is GC-AG and thus does not match canonical GT-AG. Although the significance of introns differing from canonical GT-AG remains obscure, it has been suggested that some minor introns with AT-AC and GC-AG termini may participate in gene regulation (34-36). Expression levels of the four hOX2R transcripts were different in various human tissues analysed. The hOX2R-1B transcript shows wide distribution pattern, but is absent in the kidney and liver. The hOX2R-1A transcript was highly expressed in the brain, skeletal muscle, placenta and testis. The hOX2R-1C_{short} transcript was highly expressed in the brain, placenta, pancreas and testis, but absent in skeletal muscle, lung liver and heart. The hOX2R-1C_{long} transcript was highly expressed in the human brain, placenta, testis and pancreas, but absent in skeletal muscle and kidney. The testis and placenta expressed all of the hOX2R transcripts at high levels.

In eukaryotes, alternative promoters are known to mediate developmental or tissue-specific regulation (37, 38). Therefore, the alternate distal promoter in the hOX2R gene could provide tissue-specific regulation of hOX2R by regulating the expression of hOX2R transcripts. An important consequence of differential exon 1 usage is the generation of four hOX2R transcripts with distinct 5' UTRs, all of which encode several untranslated open reading frames (uORFs). There is increasing evidence that post-transcriptional regulation of gene expression, through modulation of mRNA stability and translation initiation, is achieved through 5' UTR encoded elements such as uORFs and stem-loop secondary structures (39, 40). An *in silico* analysis of RNA secondary structure (41) for the five different transcripts of hOX2R revealed that the secondary structure of the UTR1A has estimated free energy of -32.90 kcal/mol, which is much lower than of the other isoforms. Splice variants that alter the 5'UTR of the message, but not the protein-coding region, often show tissue-specific expression and altered translational efficiencies. Many *in vitro* and *in vivo* studies have shown that mRNAs with a high likelihood of forming a stable secondary structure in the 5' UTR tend to be inefficiently translated (37) although some secondary structure is beneficial to prevent accelerated mRNA decay. We found that in *in vitro* experiments, the presence of the hOX2R 5' UTRs upstream of the luciferase gene reduced luciferase activity compared to the control. In particular UTR1A had the lowest predicted stability and severely reduced translational efficiency of less than 18% compared to control (taking into account levels of RNA and luciferase activity). UTR1C_{short} also had low mRNA stability and a translational efficiency of approximately 33%, whereas UTR1B had the highest predicted stability of the isoforms tested and translational efficiency of 70% compared to control. Therefore the consequence of expressing different levels of hOX2R isoforms would be reflected in the levels of protein produced.

The comparison of total RNA levels between tissues or under different physiological conditions may not therefore reflect differences in protein receptor levels. For example brain expresses high levels of the hOX2R-1A transcript which is inefficiently translated *in vitro*, whereas skeletal muscle expresses high levels of hOX2R-1B transcript which is translated 3.8 times more efficiently *in vitro*. It is possible, that the hOX2R 5'UTR variants indentified inhibited protein expression and exerted cell-specific effects on mRNA expression in transfected cells. Future studies, outside the remit of the present study, will need to investigate this possibility, namely the effect of the different splice variants on translational efficiency, each 5'-UTR, along with the entire hOX2R-coding sequence and the 3'UTR.

Luciferase or fluorescence-timer reporter plasmids containing the 5' flanking region of exons 1A and 1 induced similar levels of activity, indicating the presence of two functional promoters, a proximal and an alternate distal promoter. Gene regulation by use of alternate promoters is well described (42). In the genome, approximately 18% of all genes exhibit evidence for the use of alternative promoters (43). Alternative promoters can exert different tissue specificity (44, 45), developmental activity (45, 46) and expression levels (47, 48) or regulate alternate splicing and transcriptional start sites, as is the case for the hOX2R distal promoter regulating variant 5' UTRs which affect translation efficiency (49, 50). The simplest and most common scenario involves two or more promoters that produce transcripts with identical ORFs, the protein remains unchanged, and alterations in tissue or developmental specificity are the main consequences in such cases. We have demonstrated that hOX2R gene with the alternative distal promoter is of this type. Nucleotide sequence analysis of the 5' -flanking region of the hOX2R gene revealed that the promoters lack canonical TATA or CCAT sequences which are a feature typical of housekeeping genes. In promoters which lack TATA, the mechanism of transcriptional initiation and its regulation are not uniformly established. Firstly, GC-rich promoters, found primarily in housekeeping genes, usually contain several transcription sites spread over a fairly large region and several potential binding sites for the transcription factor Sp1. Secondly, other promoters lacking TATA are not GC-rich and initiate transcription at only one or a few tightly clustered start sites (51). Due to the low GC content and the absence of a TATA box in the upstream region, the hOX2R promoters most likely belong to the latter type of promoter.

The basal distal promoter is located 124 bp upstream of the exon 1A and between -124 to -300 (relative to exon 1A). Sequence analysis of the 176 bp region for potential transcription factor binding sites, predicts that both AP-1 and MZF1 factors bind to this region. Mutation of the AP1 motif causes a 47% (SH-SY5Y cell) and a 51% (HEK-293 cell) reduction in promoter activity. Since both AP1 and MZF1 bind to this region of the promoter, it is possible that MZF1 can also contribute to the promoter activity.

It is important to note that mutation of AP-1 site significantly reduced distal promoter activity. Taken together, these results demonstrated that the Ap-1 motif is critical for sustaining the basal activity of distal promoter. The basal proximal promoter is located between -456 and -123 (relative to the translation start site) and exhibited higher promoter activities in the cell lines tested than the distal promoter. Analysis of the promoter DNA sequence between -456 and -123 nt revealed numerous potential transcription factor binding sites such as CREB, GATA-2 and Oct-1. Using the chromatin immunoprecipitation assay, we demonstrated that CREB, GATA-2 and Oct-1 transcription factors bind to this region of the hOX2R proximal promoter. Mutation of the CREB, GATA-2 or Oct-1 binding motif resulted in an approximately 30% decrease in activity in both cell lines tested. When two or all three motifs were mutated a further reduction of 50% and 80% activity was observed. These studies demonstrated that binding of CREB, GATA-2 or Oct-1 to these motifs function independently but have a compound effect regulating hOX2R gene transcription.

The db cAMP treatment of cells transfected with the +61 to -456 region of the proximal promoter increased promoter activity approximately 3 fold, whereas PMA led to an increase in activity of 3.5 fold. The PKA inhibitor H-89 and PKC inhibitor Bis prevented hOX2R proximal promoter stimulation produced by db cAMP and PMA. These results suggest the protein kinase A and protein kinase C signalling pathways regulate hOX2R proximal promoter activity. PKC activation of CREB in the SH-SY5Y neuroblastoma cell

line has previously been demonstrated (31). cAMP can also directly mediate gene expression through CRE motifs in the promoter. The upstream promoter regulatory sequences for the gene encoding tyrosine hydroxylase (52, 53) have functional CREs that are cAMP responsive and these CREs participate in PMA mediated PKC activation (54). GATA2 has also been demonstrated to activate transcription mediated by cAMP (31). We have demonstrated that PKA and PKC activation of the hOX2R proximal promoter is mediated through the transcription factors CREB and GATA2. Firstly by showing an increase in CREB and GATA-2 binding to the proximal promoter following PKA and PKC stimulation and secondly by site-directed mutagenesis of the CREB and GATA-2 sites we abolished PKA and PKC promoter activation.

In conclusion, we describe for the first time that the hOX2R gene has alternative promoters, and tissue specific splice variants. Furthermore, we have identified four novel hOX2R transcripts that differ in their 5' UTR. The four transcripts are generated by alternative splicing from three alternative 5' exons. Each transcript possesses different translational efficiencies and tissue specific expression levels. We have demonstrated that 5'-flanking regions to exon 1A and 1, but not exon 1C, drive promoter activity and the distal promoter regulates transcription of these novel transcripts. We also demonstrate that the Ap-1 motif is critical for sustaining the basal activity of distal promoter. The proximal promoter is regulated by binding of CREB, GATA-2 and Oct-1 transcription factors and by PKA and PKC pathways which increase CREB and GATA-2 activation. This increased complexity in the transcriptional and post transcriptional regulation of hOX2R transcripts has important implications in the study and understanding of tissue specific protein expression of the hOX2R under normal and pathological conditions.

REFERENCES

1. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell*. **92**, 573–585
2. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett Jr FS, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA*. **95**, 322–327
3. Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS (1999) Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr Rev*. **20**, 68–100
4. Lubkin M, Stricker-Krongrad A (1998) Independent feeding and metabolic actions of orexins in mice. *Biochem Biophys Res Commun*. **253**, 241–245
5. Ida T, Nakahara K, Katayama T, Murakami N, Nakazato M (1999) Effect of lateral cerebroventricular injection of the appetite-stimulating neuropeptide, orexin and neuropeptide Y, on the various behavioural aspects of rats. *Brain Res*. **821**, 526–529
6. Samson WK, Gosnell B, Chang J-K, Resch ZT, Murphy TC (1999) Cardiovascular regulatory actions of the hypocretins in the brain. *Brain Res*. **831**, 248–253
7. Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, Qiu X, de Jong PJ, Nishino S, Mignot E (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell*. **98**, 365–376
8. Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E (2000) Hypocretin (orexin) deficiency in human narcolepsy. *Lancet*. **355**, 39–40
9. Thomas ES and Clifford BS (2005) Orexin, drugs and motivated behaviors *Nature Neuroscience*. **8**, 1286-1288.

10. Lund PE, Shariatmadari R, Uustare A, Detheux M, Parmentier M, Kukkonen JP, Akerman KE (2000) The orexin OX1 receptor activates a novel Ca^{+2} influx pathway necessary for coupling to phospholipase C. *J Biol Chem.* **275**, 30806–30812
11. Randeve HS, Karteris E, Grammatopoulos D, Hillhouse EW (2001) Expression of orexin-A and functional orexin type 2 receptors in the human adult adrenals: implications for adrenal function and energy homeostasis. *J Clin Endocrinol Metab.* **86**, 4808–4813
12. Tang J, Chen J, Ramanjaneya M, Punn A, Conner AC, Randeve HS. (2008) The signalling profile of recombinant human orexin-2 receptor. *Cellular Signalling* **20**, 1651-61.
13. van den Pol AN, Gao XB, Obrietan K, Kilduff TS, Belousov AB (1998) Presynaptic and postsynaptic actions and modulation of neuroendocrine neurons by a new hypothalamic peptide, hypocretin/orexin. *J Neurosci.* **18**, 7962-7971
14. Karteris E, Randeve HS, Grammatopoulos DK, Jaffe RB, Hillhouse EW (2001) Expression and coupling characteristics of the CRH and orexin type 2 receptors in human fetal adrenals. *J Clin Endocrinol Metab.* **86**, 4512–4519
15. Mazzocchi G, Malendowicz LK, Gottardo L, Aragona F, Nussdorfer GG (2001) Orexin A stimulates cortisol secretion from human adrenocortical cells through activation of the adenylate cyclase-dependent signaling cascade. *J Clin Endocrinol Metab.* **86**, 778–782
16. Voisin T, Rouet-Benzineb P, Reuter N, Laburthe M (2003) Orexins and their receptors: role in peripheral tissues. *Cell Mol Life Sci.* **60**, 72–87
17. Kirchgessner AL, Liu MT (1999) Orexin synthesis and response in the gut. *Neuron.* **24**, 941–951
18. Lopez M, Senaris R, Gallego R, Garcia-Caballero T, Lago R, Seoane L (1999) Orexin receptors are expressed in the adrenal medulla of the rat. *Endocrinology.* **140**, 5991–5994
19. Karteris E, Chen J and Randeve HS. (2004) Expression of prepro-orexin and orexin receptors in the human male reproductive system: functional analysis of testicular orexin receptors. *Journal of Clinical Endocrinology and Metabolism.* **89**, 1957-1962.
20. Olafsdottir BR., Rye DB., Scammell TE., Matheson JK., Stefansson K, and Gulcher JR. (2001) Polymorphisms in hypocretin/orexin pathway genes and narcolepsy *Neurology.* **57**, 1896-1899.
21. Nikolcheva T, Pyronnet S, Chou SY, Sonenberg N, Song A, Clayberger C, Krensky AM (2002) A translational rheostat for RFLAT-1 regulates RANTES expression in T lymphocytes *J. Clin. Invest.* **110**, 119-126
22. Chen J and Randeve HS (2004) Genomic organization of mouse orexin receptors: characterization of two novel tissue-specific splice variants. *Molecular Endocrinology.* **18**, 2790-2804.
23. Pfaffi MW. (2000) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **1**, e45.
24. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* **77**, 51-59.
25. Kozak, M. (1987) AN analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15**, 8125-8148.
26. Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266**, 19867-19870.
27. Kozak, M. (1996) Interpreting cDNA sequences: some insights from studies on translation. *Mamm.Genome.* **7**, 563-574.
28. Morris, DR and Geballe, AP (2000) Upstream open reading frames as regulators of mRNA translation. *Mol. Cell. Biol.* **20**, 8635-8642.
29. Jackson RJ. and Wickens M (1997) Translational controls impinging on the 5'-untranslated region and initiation factor proteins. *Curr. Opin. Genet. Dev.* **7**, 233-241

30. Butcher NJ, Arulpragasam A, Goh HL, Davey T, Minchin RF. (2005) Genomic organization of human arylamine *N*-acetyltransferase Type I reveals alternative promoters that generate different 5'-UTR splice variants with altered translational activities *Biochem J.* **387**, 119-27.
31. Terskikh A, Fradkov A, Ermakova GA, Tan P, Kajava AV, Zhao X, Lukyanov SM, Mkim S, Weissman I and Siebert, P (2000) "Fluorescent Timer": Protein that changes color with time. *Science.* **290**, 1585-1588.
32. Rosethorne EM, Narhorski SR, Challiss RA. (2008) Regulation of cyclic AMP response-element binding-protein (CREB) by Gq/11-protein-coupled receptors in human SH-SY5Y neuroblastoma cells. *Biochem Pharmacol.* **75**, 942-955.
33. Liu W, Zhu ZQ, Wang W, Zu SY, Zhu GJ. (2007) Crucial roles of GATA-2 and SP1 in adrenomedullin-affected expression of tissue factor pathway inhibitor in human umbilical vein endothelial cells exposed to lipopolysaccharide. *Thromb Haemost.* **97**, 839-846.
34. Patel AA, Steitz JA. (2003) Splicing double: Insights from the second spliceosome. *Nat Rev Mol Cell Biol.* **4**, 960-970
35. Plant NJ, Gibson GG. (2003) Evaluation of the toxicological relevance of CYP3A4 induction. *Curr Opin Drug Discov Dev.* **6**, 50-56.
36. Farrer T, Roller AB, Kent WJ, Zahler AM: (2000) Analysis of the role of *Caenorhabditis elegans* GC-AG introns in regulated splicing. *Nucleic Acids Res.* **30**: 3360-3367.
37. Schibler U, Sierra F (1987) Alternative promoters in developmental gene expression. *Annu Rev Genet.* **21**, 237-257
38. Ayoubi TA, Van De Ven WJ (1996) Regulation of gene expression by alternative promoters. *FASEB J.* **10**, 453-460
39. Vilela C, Ramirez C, Linz B, Rodrinues-Pousada C, and McCarthy J. (1999) Post-termination ribosome interactions with the 5'UTR modulate yeast mRNA stability. *EMBO J.* **18**, 3139-3152
40. Guhaniyogi J, and Brewer G. (2001) Regulation of mRNA stability. in mammalian cells *Gene (Amst)* **256**, 11-23
41. Mathews, DH, Sabina, J, Zuker, and DH. Turner HD. (1999) Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure *J. Mol. Biol.* **288**, 911-940.
42. Landry JR., Mager DL, Wilhelm BT. (2003) Complex controls: the role of alternative promoters in mammalian genomes. *Trends Genet.* **19**, 640-648.
43. Kamat A, Hinshelwood MM, Murry BA, Mendelson CR (2002) Mechanisms in tissue-specific regulation of estrogen biosynthesis in human *Trends Endocrinol Metab.* **13**, 122-128
44. Bonham K, Ritchie SA, Dehm SM, Snyder K and Boyd FM (2000) An alternative human SRC promoter and its regulation by hepatic nuclear factor-1alpha. *J Biol. Chem.* **275**, 37604-37611
45. Saleh A, Makrigiannis AP, Hodge DL, Anderson S K (2002) Identification of a novel Ly49 promoter that is active in bone marrow and fetal thymus. *J. Immunol.* **168**, 5163-5169.
46. Landry JR Rouhi A, Medstrand P, Mager DL (2002) The Opitz syndrome gene Mid1 is transcribed from a human endogenous retroviral promoter. *Mol. Biol. Evol.* **19**, 1934-1942
47. Medstrand, P. Landry JR Mager DL (2001) long terminal repeats are used as alternative promoters for the endothelin B receptor and apolipoprotein C-I genes in humans. *J. Biol Chem.* **276**, 1896-1903.
48. Landry JR. and Mager DL. (2003) Functional analysis of the endogenous retroviral promoter of the human endothelin B receptor gene. *J. Virol.* **77**, 7459-7466
49. Phelps D, Hisao KM, Li Y, Hu N, Franklin DS, Westphal E, Lee EY, Xiong Y (1998) Coupled transcriptional and translational control of cyclin-dependent kinase inhibitor p18INK4c expression during myogenesis. *Mol. Cell. Biol.* **18**, 2334-2343.

50. Wang Y, Newton DC, Robb GB, Kay GL, Miller TL, Cheung AH, Hall AV, Van D S, Wilcox JN, Marsden P A. (1999) RNA diversity has profound effects on the translation of neuronal nitric oxide synthases. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 12150-12155.
51. Sehgal A. Patil N, Chao M. (1988) A constitutive promoter directs expression of the nerve growth factor receptor gene. *Mol Cell Bio.* **8**, 3160-3167
52. Lewis E. J, Harrington CA, and Chikaraishi DM. (1987) Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoids and cAMP. *Proc. Natl. Acad. Sci. USA.* **84**, 3553-3554
53. Piech-Dumas K M, and Tank AW (1999) CREB mediates the cAMP responsiveness of the tyrosine hydroxylase gene: use of antisense RNA strategy to produce CREB-deficient PC 12 cell lines. *Mol Brain Res.* **70**, 219-230.
54. Piech-Dumas KM., Best JA., Chen Y., Nagamoto-Combs K., Osterhout C. A., and Tank A. W. (2001) The cAMP response element and CREB partially mediate the response of the tyrosine hydroxylase gene to phorbol ester. *J. Neurochem.* **76**, 1376-1385.

Acknowledgements

We would like to thank Dr. Andrew Blanks (University of Warwick) who kindly provided us with pTimer-1 vector and advice on the confocal microscopy experiments.

Footnotes

This work was supported by Research Grants to H. S. Randeve from the General Charities of the City of Coventry and British Heart Foundation.

Abbreviations: hOX2R, human orexin (hypocretin) receptor type 2; 5'-UTR, 5'-untranslated region; RACE, rapid amplification of cDNA ends; HEK, human embryonic kidney; TSS, transcription starts sites; CREB, cAMP responsive element binding protein; PMA, phorbol-12-myristate-13-acetate; CHIP, chromatin immunoprecipitation; uAUG, upstream AUG; ORF, open reading frame; nt, nucleotide; PKA, protein kinase A; PKC, protein kinase C; db cAMP, dibutyryl-cAMP; Bis (PKC inhibitor), Bisindolylmaleimide II [2-(1-[2-(1-Methylpyrrolidino) ethyl]-1H-indol-3-yl)-3-(1H-indol-3-yl)-maleimide]; H89, (PKA inhibitor) Dihydrochloride (N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquino-linesulfonamide, 2HCl);

Appendix: Gene Accession Nos.

Homo sapiens orexin receptor 2 mRNA, partial sequence, alternatively spliced variant 1A, 1B, 1C_{short} and 1C_{long}: EF633107-EF633110

Figure legends

Figure 1 The 5' flanking region of the hOX2R gene was cloned by the PCR based genomic walking method. Two rounds: First round primer 5'-GAAGATAGGCAGCTGAGGAACTAG-3' and the adapter primer AP1. Second round primer 5'-CACTGGAGGCTGCAGCAATGACC-3' and the AP2 primer of upstream walking resulted in overlapping PCR products of 1748 bp (*EcoR* V library) and 539 bp (*Dra* I library) (A), M; marker. The hOX2R 5' UTR cDNAs were isolated by 5' rapid amplification of cDNA ends (RACE) using primers (First primer 5'-GGTCTAGAGGATCTTTGGCTC-3' and a nested primer 5'-CACATGACACTTA ACTATCGC-3') specific to exon 1 of the hOX2R gene plus adaptor primers AP1 and AP2 (B), M; marker. Alternative splicing of 5' untranslated region exons of the hOX2R gene (C). UTR 1A, UTR 1B, UTR 1C_{short} and UTR 1C_{long} transcripts are generated by alternate usage of exons 1A, 1B, and 1C. Exon 1A, 1B, 1C and 1, 5' UTRs are shown as shaded boxes (D). The translational start site coding region in exon 1 is shown as a black box. Tissue-specific expression of hOX2R transcripts in human tissues demonstrated by PCR (E). Amplification was performed using exon 1A and 1C specific sense primers and a common antisense primer located within exon 1. The amplification resulted in two DNA fragments. The nucleic acid sequence of the two fragments revealed that the long fragment corresponded to the exon 1A, 1B and exon 1 (UTR 1B), the other fragment corresponded to exon 1A and exon 1 and skipping exon 1B (UTR 1A) (i). PCR with exon 1C gene specific primers identified two different cDNA fragments. The sequence of the long fragment contained exon 1C without any splicing between the exons 1C and 1 (UTR 1C_{long}), the other fragment contained exon 1C and exon 1 (UTR 1C_{short}) (ii). PCR with exon 1 (5'-CTTGTCGCAACTGGTCATCTG-3') and exon 2 (5'-GAAGTAGTTGGTTACCGTCCTC-3') gene specific primers produced one cDNA product (245 bp) (iii). The human sequences of the sense and antisense primers used was as follows: β -actin (216 bp) 5'-AAGAGAGGCATCCTCACCT-3' and 5'-TACATGGCTGGGGTCTTGAA-3' (iv).

Figure 2 Effect of the variant 5' UTR sequences on gene expression. Variant 5' UTRs were cloned upstream of a luciferase reporter gene and then transiently transfected with a *Renilla* internal control into either HEK-293 cells or SH-SY5Y cells. Data are the mean \pm SEM. (n=6) of the firefly luciferase activity relative to the *Renilla* luciferase activity (A). Luciferase mRNA expressed by each of the variants is shown relative to expression from a control in HEK-293 cells or SH-SY5Y cells. Real-time PCR was used to quantify the amount of RNA in cells following transfection with the different variants. The results are showed as the ratio of luciferase RNA to β -actin RNA (B). *P < 0.05, **P < 0.01 and ***P < 0.001 non parametric unpaired t test.

Figure 3 Promoter activity analysis of the 5' flanking regions of the hOX2R gene alternative exon 1. Schematic representation of the pGL3 reporter constructs containing the 5' regions of the hOX2R gene. Exons and introns are indicated by boxes and solid lines, respectively. Each solid arrow represents the region inserted into the pGL3 vector. The constructs 1A₃ and 1₃ contain the same insert DNA as the constructs 1A₁ and 1₁, but in an inverse orientation (A). Promoter constructs (exon 1A: 1A₁ (-30820 to -29792 (1028 bp) and 1A₂ (-31578 to -29792 (1786 bp), exon 1C: constructs 1C₁ (-1066 to -841 (225 bp), 1C₂ (-1280 to -841 (439 bp) and 1C₃ (-1563 to -841 (722 bp) and exon 1, constructs 1₁ (-456 to +43 (499 bp) and 1₂ (-638 to +43 (681 bp) were transiently transfected into a SH-SY5Y and HEK-293 cells and measured for relative luciferase activity Data are expressed as the mean \pm SEM (n=4), ***P < 0.001 vs. pGL3-basic vector (B). Expression of 5' region hOX2R-Time-reporter in HEK-293 cells (C). Three different fragments of the 5' region of the hOX2R gene (1A₂, 1C₂ and 1₂) were cloned and studied using the fluorescence-timer (pTimer-1) reporter system. Negative control (only pTimer vector (i) 24 hours after transfection, the fluorescent image reveals that the 5' flanking

regions to the Exon 1A (ii) and 1 (iv) are red, indicating promoter activity and protein synthesis. In contrast, the 5' flanking region to Exon 1C (iii) is green, indicating no protein expression and therefore no promoter activity.

Figure 4 Transient expression analysis of the distal promoter in SH-SY5Y and HEK-293 cells. A series of 5' deletion mutants between -1786 and -124 was constructed and analyzed in HEK-293 and SH-SY5Y cells. Luciferase reporter activity is derived from the mean relative luciferase activity from four experiments ($n=4$) \pm SEM. a, $**P < 0.01$ and b, $***P < 0.001$ non parametric unpaired t test (A). Mutational analysis of the putative AP1 motif on the by a three-step PCR mutagenesis, which artificially introduced a *Xho* I and *Hind* III restriction sites in the target sequences. The hOX2R distal promoter-luciferase reporter gene constructs containing 4 bp mutation was co-transfected with the pRL-TK vector into HEK-293 and SH-SY5Y cells. The relative promoter activity is represented as the percentage of the wild-type pGL3-1A-300 whose activity was set as 100%. Data are expressed as the mean \pm SEM ($n=4$), $***P < 0.001$ vs. pGL3-1A-300 (B) distal promoter activity of pGL3-1A-300 luciferase reporter gene. AP1 motif was mutated. Genomic sequence of hOX2R distal promoter 5' UTR sequence is in bold, transcription start sites are black boxes (as identified from 5' RACE), transcription factor binding elements are open boxes (C).

Figure 5 Progressive 5' deletion analysis of the hOX2R proximal promoter in SH-SY5Y, NT2 and HEK-293 cells (A). A nested family of 5' deletion mutants was transiently transfected into the cells. The pRL-TK vector was co-transfected to normalize the transfection efficiency. Activities were normalized to the activity of the promoterless vector pGL3-Basic vector. Luciferase reporter activity is derived from the mean relative luciferase activity from four experiments ($n=4$) \pm SEM. Genomic sequence of hOX2R proximal promoter. 5' UTR sequence is in bold, transcription start sites are black boxes (as identified from 5' RACE), transcription factor binding elements are open boxes, ATG (+1) (B).

Figure 6 Mutational analysis of the putative CREB, GATA-2 and Oct-1 motif on the proximal promoter activity of pGL3-1-456 (-456 to +43) luciferase reporter gene. Each motif was mutated by a three-step PCR mutagenesis, which artificially introduced a *Kpn* I and *Xho* I restriction sites in the target sequences. A diagrammatic representation of the mutated promoter constructs is shown on the left side of the figure, crosses indicating mutations. The hOX2R proximal promoter-luciferase reporter gene constructs containing single, double or triple mutation were co-transfected with the pRL-TK vector into HEK-293 and SH-SY5Y cells. The relative promoter activity is represented as the percentage of the wild-type pGL3-1-456 whose activity was set as 100%. Data are expressed as the mean \pm SEM ($n=4$), a, $**P < 0.01$ vs. pGL3-1-456 and b, $***P < 0.001$ vs. pGL3-1-456 (A). ChIP assays were performed to investigate the transcription factor binding to the hOX2R proximal promoter, *in vivo*. Schematic at top shows the proximal promoter (-459 to +1) containing a CREB, GATA-2 and Oct-1 site. Arrows indicate the locations of primers (P1 and P2 for the CREB region, P3 and P4 for the GATA-2 region, P5 and P6 for the Oct-1 region, P1 and P6 for the proximal promoter region) used for ChIP PCR. The ChIP was performed using anti-CREB (5 μ g), anti-GATA-2 (5 μ g), anti-Oct-1 (5 μ g) and normal serum IgG (as control). Proteins recognised by IP are indicated. Purified DNA was used for PCR (35 cycles). Immunoprecipitation with antibodies to CREB, GATA-2 and Oct-1 resulted in amplification of the proximal promoter region (B).

Figure 7 The activation of the PKA and PKC signalling pathways on hOX2R proximal promoter activity was examined in SH-SY5Y cells. Cells were transfected with the wild-type pGL3-1-123 and pGL3-1-456 hOX2R proximal promoter-luciferase reporter gene constructs and 24 hours later cells were treated with 100 nM db cAMP or 100nM PMA for 12 hours. Luciferase activity was determined and expressed relative to untreated control values set to

unity. Data are expressed as the mean \pm SEM (n=4). * P < 0.001 non parametric unpaired t test (A). Effect of inhibition of the PKA and PKC pathways on db cAMP and PMA activation of the hOX2R proximal promoter. SH-SY5Y cells transfected with the wild-type pGL3-1-456 hOX2R proximal promoter-luciferase reporter gene construct were pretreated with the PKA inhibitor, H-89 (10 μ M) for 30 min or the PKC inhibitor, Bis, (1 μ M) for 2 hour, followed by 100 nM db cAMP or 100 nM PMA for 12 hours. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are expressed as the mean \pm SEM (n=4). **P < 0.01, non parametric unpaired t test (B). To investigate the transcription factors binding to the hOX2R proximal promoter *in vivo* ChIP assays were performed. SH-SY5Y cells were treated with 100 nM db cAMP or PMA for 6 hours. DNA in the CREB, GATA-2 or Oct-1 immunoprecipitated complex was used as a template for PCR with the primers to generate three fragments of hOX2R proximal promoter. Using mouse IgG immunoprecipitation serves as a negative control for specific CREB, GATA-2 or Oct-1 antibody binding. (C). Effect of PKA pathway activation on the hOX2R proximal promoter. SH-SY5Y cells transfected with the wild-type pGL3-1-456 hOX2R or pGL3-1-456 m1 (CREB mutation) or pGL3-1-456 m5 (CREB, GATA-2 double mutation) proximal promoter-luciferase reporter gene construct were pretreated with the PKA inhibitor, H-89 (10 μ M) for 30 min, followed by 100 nM db cAMP for 12 hours. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are expressed as the mean \pm SEM (n=4). **P < 0.01, ***P < 0.001 non parametric unpaired t test. $\neq \neq$ P < 0.01 vs. pGL3-1-456 (treated db cAMP), $\neq \neq \neq$ P < 0.001 vs. pGL3-1-456 (D) Effect of PKC pathway activation on the hOX2R proximal promoter. SH-SY5Y cells transfected with the wild-type pGL3-1-456 hOX2R or pGL3-1-456 m2 (GATA-2 mutation) or pGL3-1-456 m5 (CREB, GATA-2 double mutation) proximal promoter-luciferase reporter gene construct were pretreated with the PKC inhibitor, Bis (1 μ M) for 2 hours, followed by 100 nM db PMA for 12 hours. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are expressed as the mean \pm SEM (n=4). **P < 0.01, ***P < 0.001 non parametric unpaired t test. $\neq \neq$ P < 0.01 vs. pGL3-1-456 (treated PMA), $\neq \neq \neq$ P < 0.001 vs. pGL3-1-456 (E).

Table 1: Exon-Intron Organization of the Human OX2R 5 End Gene

Exon			Intron			Exon		
Number	Size(bp)	Sequence	Donor	Number	Size (bp)	Acceptor	Sequence	Number
							CCGCCAGTAC	1A
1A	143	TGCTTATCTT	gtgagctctg	1A	4487	Caccaacag	GGTTAATAC	1B
1B	99	AAAAAGTGAG	gtgagcaaat	1B	24222	Aatattcattg	AGTATATTGG	1C
1C	207	TAAAGACTCG	gcaagtacta	1C	348	Ttttctcag	CTTCAGCTCT	1
1	509	CTATCTCCAG	gtgagtctcc	1	3448	Ttcaaattag	TTTGTGTGGC	3

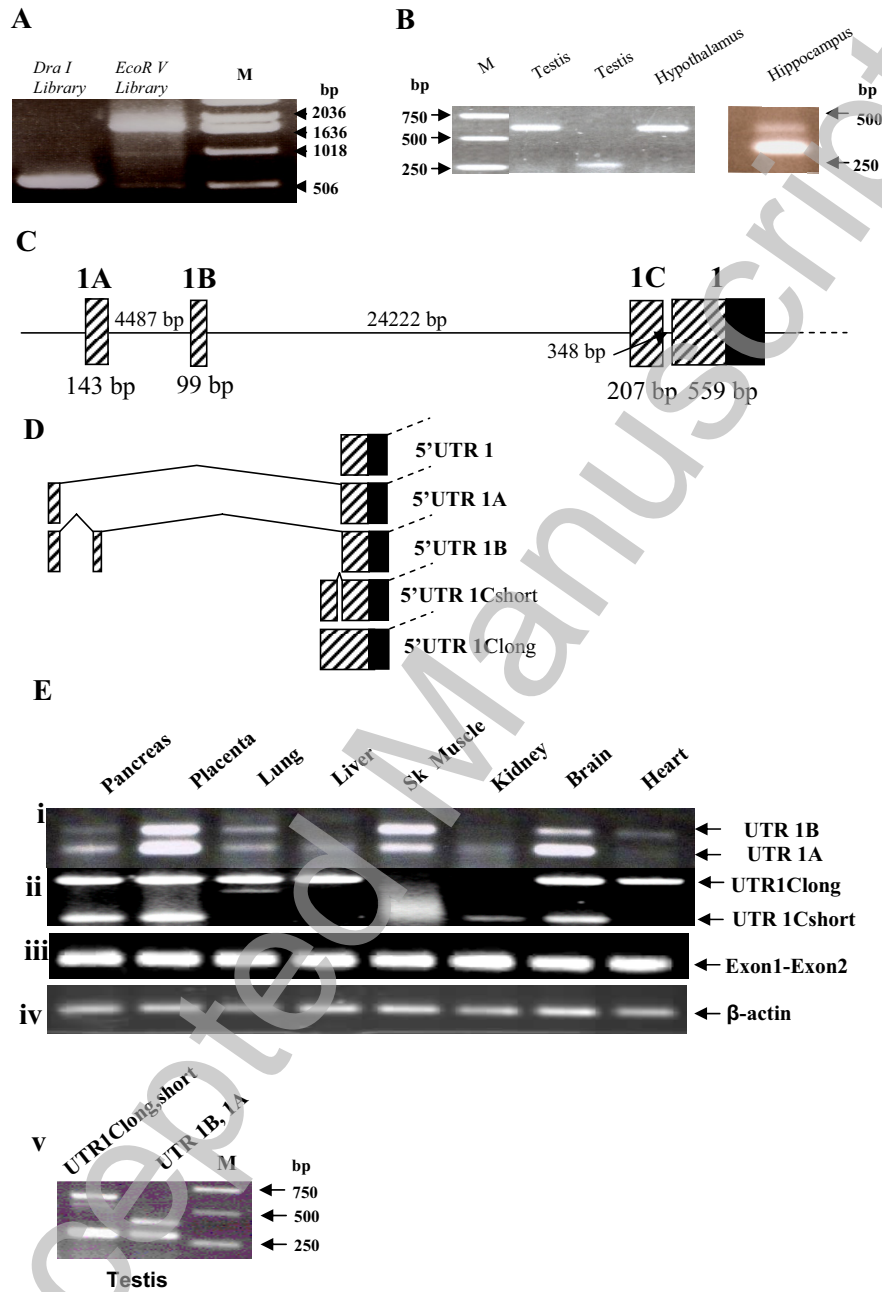
Exon and Intron sequences are shown in *upper* and *lowercase letters*, respectively.

Accepted Manuscript

Table 2. Number of AUG triplets and secondary structure free energy for hOX2R 5' UTR Sequences

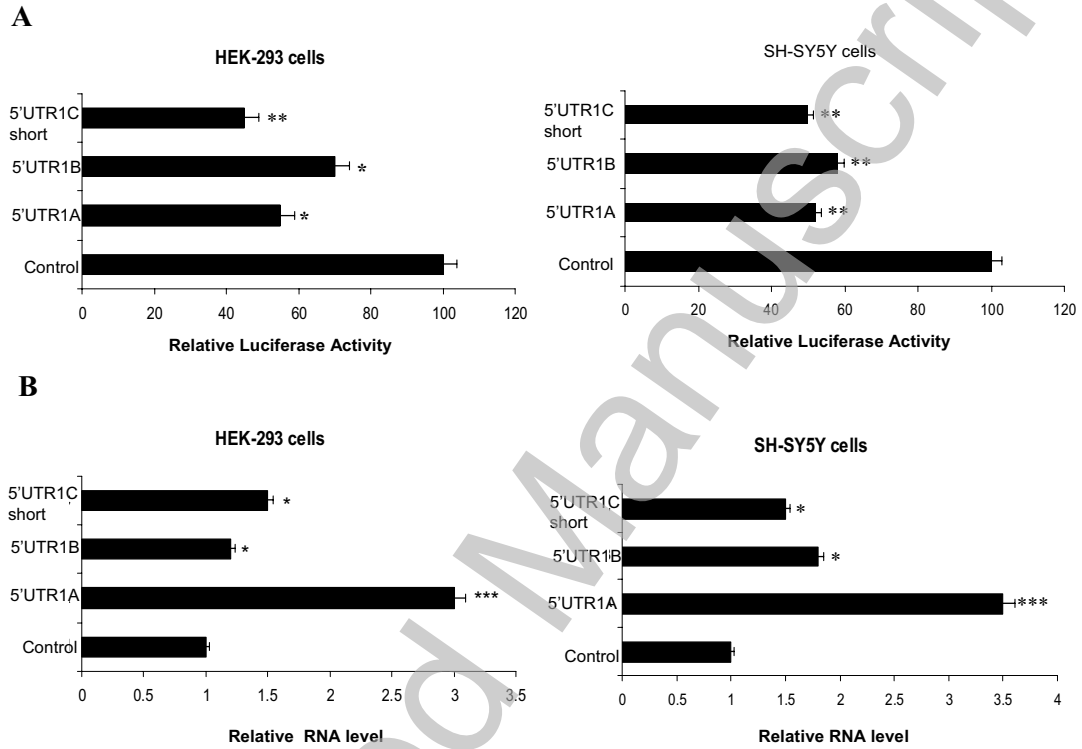
Transcripts	Size of 5'UTR (bp)	uAUG	Free Energy (kal/mol)
UTR 1A	143	4	-32.90
UTR 1B	242	5	-64.50
UTR 1C _{short}	207	4	-37.60
UTR 1C _{long}	485	8	-107.20
UTR 1	377	6	-147.70

Figure 1



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091755

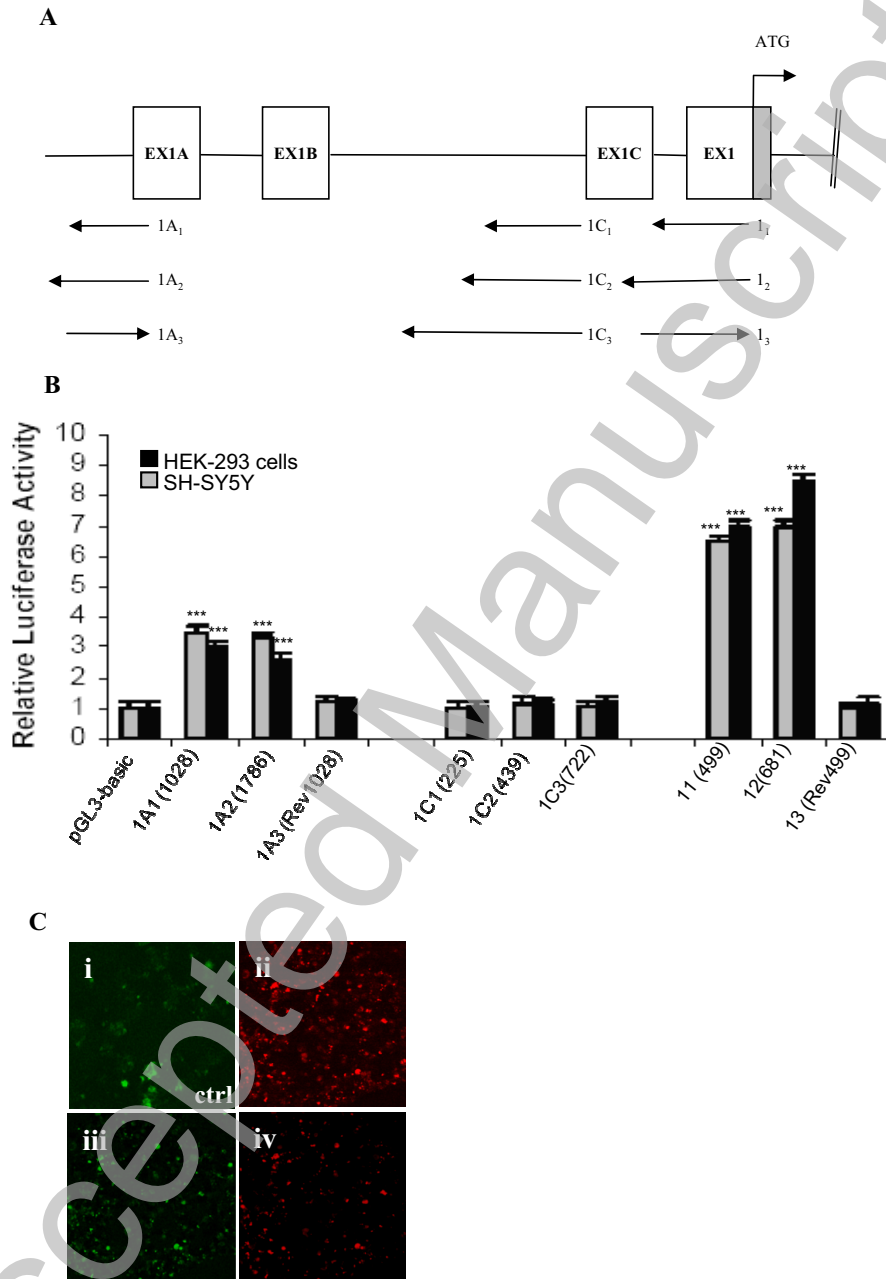
Figure 2



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091755

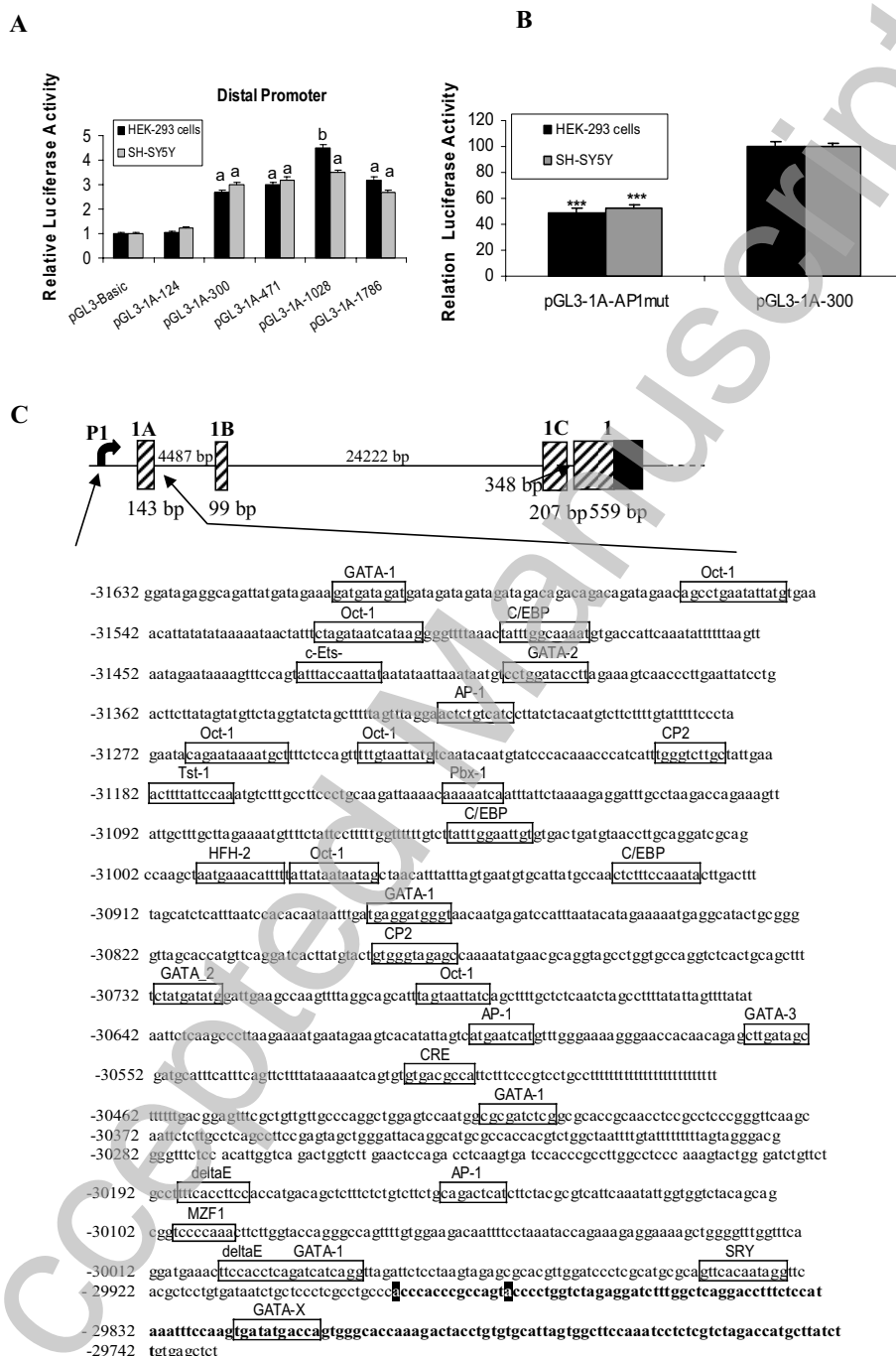
Accepted Manuscript

Figure 3



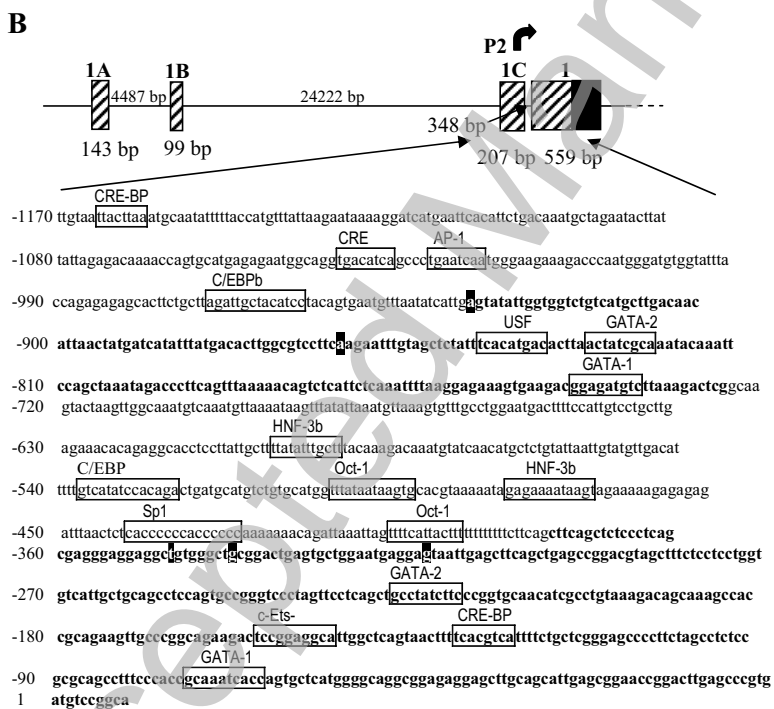
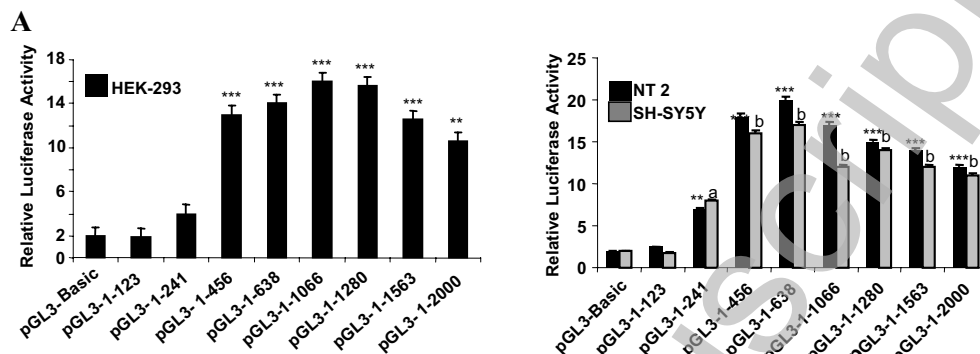
THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091755

Figure 4



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091755

Figure 5



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091755

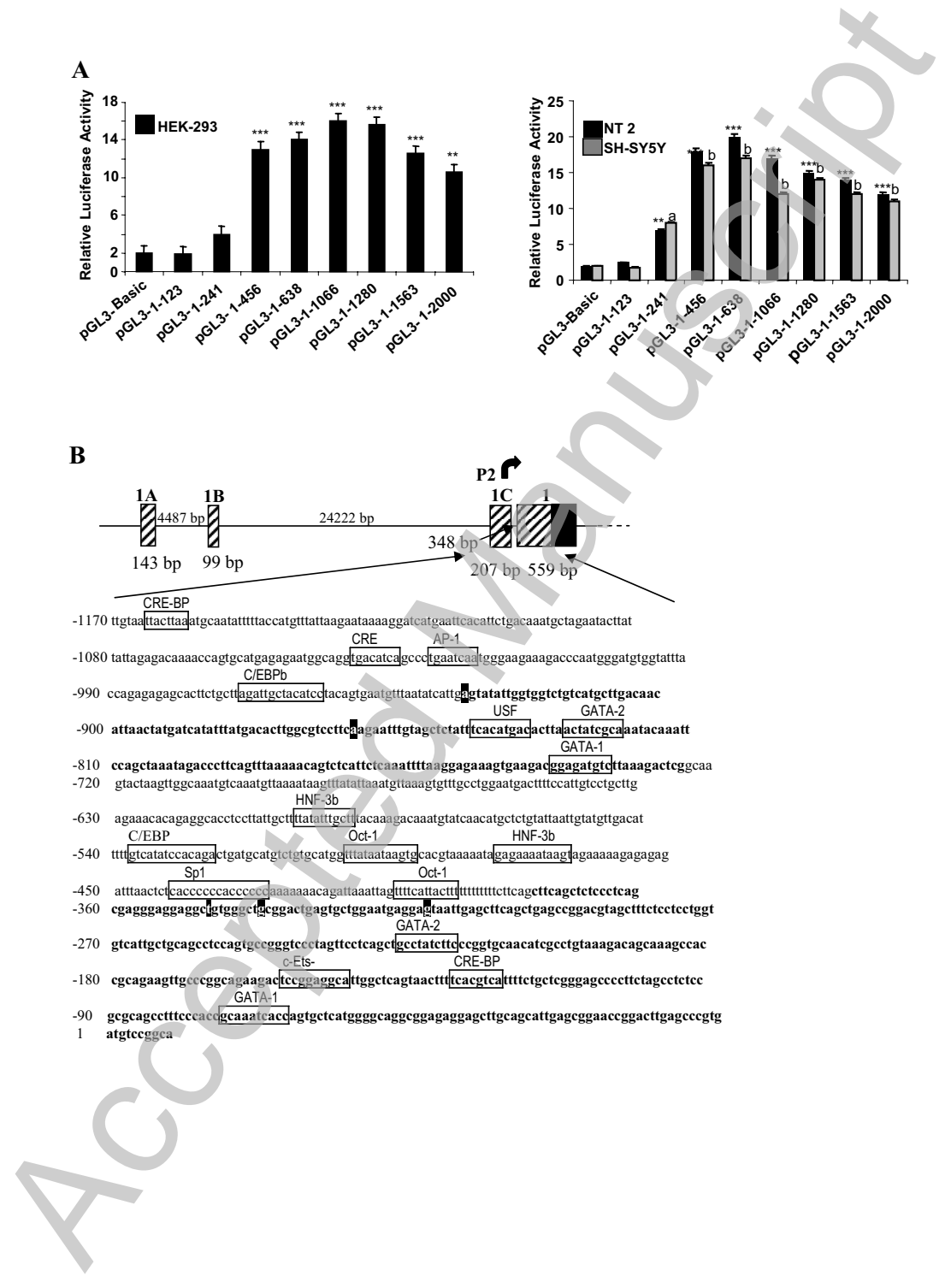
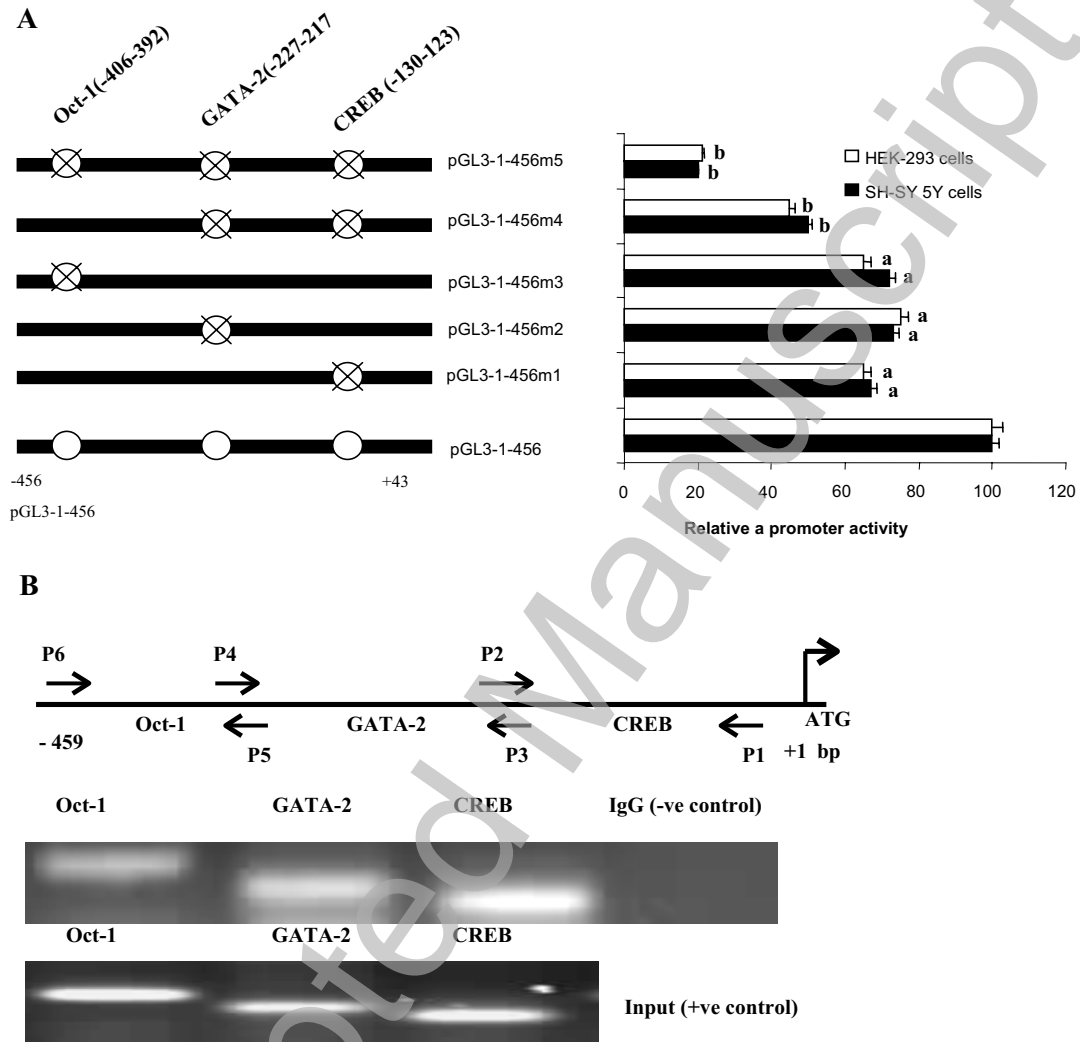
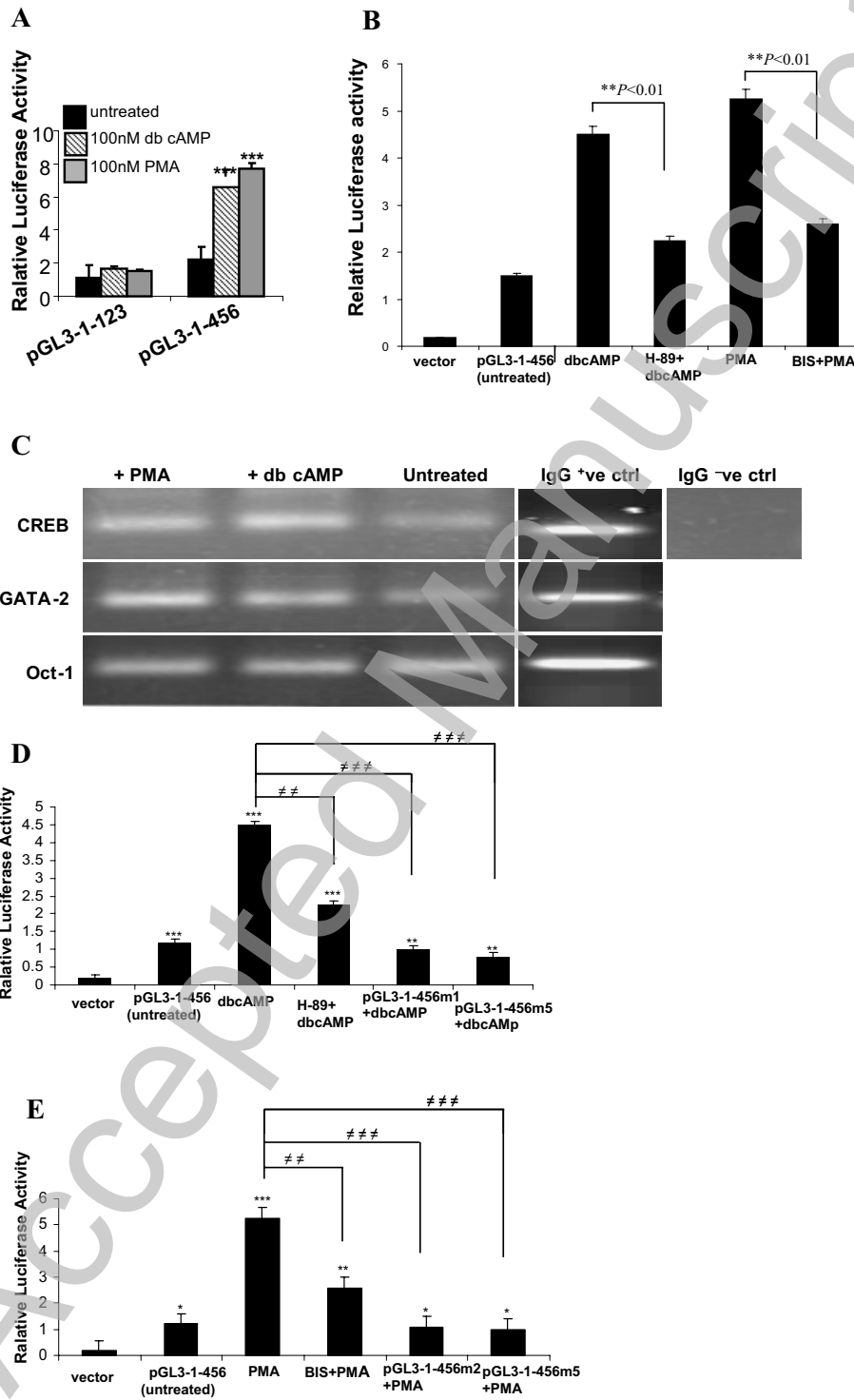


Figure 6



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091755

Figure 7



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091755