Multiple Promoters Regulate Tissue-specific Expression of the Human NPY-Y1 Receptor Gene*

(Received for publication, August 8, 1995, and in revised form, September 1, 1995)

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Several cDNA clones encoding the human neuropeptide Y-Y1 receptor have been isolated that contain differing sequences at their 5'-ends. The divergence occurs at a splice junction in the 5'-untranslated region, suggesting that at least three forms of the neuropeptide Y-Y1 receptor transcript are generated by alternative splicing at this site. Genomic clones have been isolated that encompass the alternatively spliced 5'-exons. The exons are found 6.4, 18.4, and 23.9 kilobases upstream of exon 2. In the corresponding promoter regions of the various exons, possible response elements for the glucocorticoid receptor, as well as potential binding sites for the AP-1, AP-2, and NF-κB transcription factors are found. Analysis of NPY-Y1 transcripts in various cell types demonstrates the tissue-specific activation of the three promoters.

Neuropeptide Y (NPY) is an abundant neuropeptide that mediates a wide range of biological actions including the regulation of appetite, cardiovascular activity, and endocrine secretion. NPY receptors belong to the family of G protein-coupled receptors, and several receptor subtypes have been classified on the basis of their pharmacology (reviewed in Ref. 1). The NPY-Y1 and NPY-Y2 receptor subtypes can be distinguished by their ability to bind various analogs and fragments of NPY. The NPY-Y1 receptor selectively binds an analog of NPY, [Leu31, Pro34]NPY, while the NPY-Y2 receptor has higher affinity for the NPY (13–36) fragment (2, 3).

The NPY-Y1 receptor is expressed in many tissues including the brain, heart, placenta, spleen, small intestine, kidney, testis, and aortic smooth muscle (4, 5). In the cardiovascular system, the NPY-Y1 receptor mediates both direct vasoconstriction and the potentiation of other pressor agents such as noradrenaline (6). Administration of Y1 selective agonists affects appetite and the secretion of luteinizing hormone, suggesting involvement of the NPY-Y1 receptor (7, 8). NPY is considered to be the most potent endogenous orexigenic agent known with infusions of NPY into the paraventricular nucleus of rats inducing a sizable and long lasting increase in food intake (9).

DNA sequences encoding the NPY-Y1 receptor have been cloned from several species including human, rat, mouse, and Xenopus (10–13). The human cDNA encodes a protein 384 amino acids in length that is preceded by approximately 200 base pairs (bp) of 5'-untranslated region (5'-UTR) sequence. Analysis of the genomic structure has determined the presence of a 6.4-kb intron within the 5'-UTR as well as a small intron within the coding region. The gene for the receptor has been localized to chromosome 4q31.3–32 (14).

During the analysis of NPY-Y1 receptor cDNAs from a testis cDNA library, a cDNA clone was identified that differed from the previously determined NPY-Y1 receptor sequence at the 5'-end of the done. The two sequences diverged at the point where exon 1 splices onto exon 2. This suggests that two exons are alternatively spliced at this site. Although this has no effect on the protein sequence of the receptor, it does have consequences for the regulation of the gene, as transcription may be under the control of multiple promoters.

To determine whether alternative promoters are used in the transcription of the NPY-Y1 receptor gene, cDNA clones were isolated from a range of different cDNA libraries, and the 5'-sequences of NPY-Y1 mRNA were examined by 5'-rapid amplification of cDNA ends (RACE). Alternate 5'-exons were identified in the structure of the NPY-Y1 receptor gene, and the selective activation of the different promoters for the NPY-Y1 receptor was investigated by measuring the expression of the various NPY-Y1 transcripts in different cell types.

MATERIALS AND METHODS

cDNA Library Screening—Human cDNA libraries (testis, intestine, kidney (Clintech), lung (Stratagene)) were screened with a 32P-labeled 0.6-kb fragment (nucleotides 817-1437) of the human NPY-Y1 cDNA. Plasmid or bacteriophage DNA was transferred to nylon membranes (DuPont NEN) and hybridized with the probe in a solution of 5× SSPE, 5× Denhardt’s, and 0.5% SDS at 65 °C for 16 h. Membranes were washed twice in 2× SSPE, 0.1% SDS at room temperature for 15 min followed by a wash for 15 min at 65 °C in 1× SSPE, 0.1% SDS. The filters were then exposed to x-ray film (Kodak, X-Omat) at ~70 °C for 16 h using an intensifying screen. Positive colonies were purified, and DNA was isolated using a standard mini-prep or bacteriophage lysate method (15).

Genomic Library Screening—A human peripheral blood genomic library (Promega) was screened with a 32P-labeled probes derived from the 5'-end of the human NPY-Y1 published genomic sequence as well as the 5'-exon sequences found in the testis cDNA clone and 5'-RACE done. Hybridization conditions were the same as described above. DNA from the purified plaques was prepared by the bacteriophage lysate method (15). λ DNA was digested with BamHI, EcoRI, HindIII, or SacI to allow subcloning of the fragments into the pUC119 vector (Pharmacia Biotech Inc.).

Nucleotide Sequence Determination—Supercoiled plasmid DNA was alkaline-denatured and sequenced by the dideoxy chain termination method using Sequenase 2.0 (U. S. Biochemical Corp.). Oligonucleotide primers were from the vector sequence and from the genomic sequence obtained.

Southern Blotting—Human genomic DNA (μg) or a genomic clones were digested with restriction enzymes and run on a 0.7% agarose gel. The DNA was capillary-transferred to nylon membranes (Hybond N+),
Amersham) in 0.4 M NaOH and hybridized as described above.

Preparation of RNA—RNA was extracted from tissues and cell lines with guanidium thiocyanate and precipitated by centrifugation through a cesium chloride cushion (15). Poly(A)^+ RNA was prepared using a commercially available kit (Poly(A) tract system, Promega).

**5'-RACE—cDNA was synthesized from 500 ng of poly(A)^+ RNA using Superscript reverse transcriptase (Life Technologies, Inc.) and the primer Y1#1 5'-CTCTCCCTCCCGTGGGGAG-3'. The cDNA was tailed with adenosine residues using terminal deoxynucleotidyl transferase (Boehringer Mannheim) and was amplified using an anchor primer 5'-GAATTTCTTTTTTTTTTTTTTTT-3' and the Y1#1 primer. The DNA was reamplified with the same anchor primer and a nested Y1-specific primer Y1#2 5'-ATGGACAGTATGTTTCTTCA-3'.

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**Reverse Transcriptase-PCR—cDNA was synthesized using reverse transcriptase in a reaction containing 10 μg of total RNA from human kidney, artery, and the SK-N-MC cell line (16). The cDNA was synthesized as described above and reaction products were separated on a 6% acrylamide gel, dried, and visualized by autoradiography. A DNA sequencing reaction was run alongside the reaction products to enable the determination of product lengths. Each primer was designed to anneal specifically to one of the three 5'-exon sequences: Y1#3 5'-CTGAACAATCCTCTTTGGAA-3', Y1#4 (5'-AAGTGGAAAATAATGTTG-3'), and Y1#5 5'-AAGTGGAAAATAATGTTG-3'.

**Analysis of NPY-Y1 Transcripts**—The screening of cDNA libraries, from kidney, intestine, and lung, resulted in the isolation of several different NPY-Y1 receptor cDNA clones. Some of these clones extended past the splice site in the 5'-UTR, and all were found to be identical to the published NPY-Y1 receptor sequence, obtained from a hippocampus cDNA library (10). A divergent cDNA clone encoding the human NPY-Y1 receptor was isolated from a testis cDNA library. The coding region of the cDNA and the 151-bp preceding the start codon were identical to the published cDNA sequence. However, the very 5'-end of the testis clone contains a 75-bp sequence that bears no homology to the previously determined cDNA or genomic sequence (14). The divergence occurs at a splice junction within the 5'-UTR (14), suggesting the two different forms of the NPY-Y1 mRNA are the result of alternative RNA splicing at this site. To investigate the possibility of alternative 5'-exons in the NPY-Y1 gene, the 5'-RACE method was used to analyze the 5'-ends of NPY-Y1 transcripts expressed in the SK-N-MC cell line. This cell line is derived from a human neuroblastoma and endogenously expresses the NPY-Y1 receptor (16). 64 of the 85 clones, obtained using the 5'-RACE method, have an identical sequence to that previously found in hippocampal cDNA (10). None contain the sequence found in the testis cDNA clone. However, the remainder (21 clones) contain a third form of the NPY-Y1 transcript, which diverges at the same point as the other two cDNAs, indicating that at least three exons are alternatively spliced at this site. We nominated the 5'-sequence found in the published hippocampal cDNA clone as exon 1A. The sequence present in the testis clone was termed exon 1B, and the sequence obtained from the 5'-RACE clones was identified as exon 1C.

**RESULTS**

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**Structure of the Human NPY-Y1 Receptor Gene**—Chromosome walking and screening with the different 5'-exon sequences led to the isolation of several overlapping genomic clones spanning over 40 kb of the human NPY-Y1 receptor gene. A complete restriction map of the clones was prepared for the restriction endonucleases BamHI, EcoRI, and HindIII (Fig. 1). This map was confirmed using Southern blots of genomic DNA. Exon 1A is located 6.4 kb upstream of exon 2 (14). Exon 1B was found a further 12 kb upstream of exon 1A, and exon 1C another 5.5 kb upstream of exon 1B. Consensus splice donor and acceptor sequences are found at all splice donor and acceptor sites in the NPY-Y1 receptor gene (Fig. 2).
FIG. 3. 5'-Exon and flanking sequences of the human NPY-Y1 gene. The 5'-flanking sequences of exon 1A, 1B, and 1C are shown along with the exon sequences. The exons are underlined, and numbering begins at the major transcription start site for each of the exons. Minor transcription start sites are marked with asterisks. The potential recognition sequences for various transcription factors are boxed.
verse transcriptase (are negative controls that had either reverse transcriptase (lanes 1, 4, and 7) or RNA (lanes 2, 5, and 8) omitted from the reaction. A DNA sequencing reaction was run next to the reactions to enable the size of the products to be determined.

Analysis of 5'-Flanking Sequences—To aid in the identification and characterization of the different promoters, we determined the nucleotide sequence of the 5'-flanking regions of each of the 5'-exons (Fig. 3). The transcription start sites were determined by primer extension using primers specific for each exon sequence (Fig. 4). The size of exon 1A was determined to be 80 bp, and the size of exon 1B was 106 bp. Transcription of exon 1C begins at a number of positions, with the major start site being 109 bp before the splice junction. The transcription start sites are in accordance with those found in the 5'-RACE clones. The sequences of the 5'-exons contained in the 5'-RACE clones are identical to the genomic sequence, indicating that no further splicing occurs within these exons.

Promoter A lacks a typical TATA and CCAAT box. However, it contains a potential cyclic AMP response element, 5'-CCACGCTA-3', at position -92 and an AP-1 binding site, 5'-TGAATCT-3', at position -763 (17, 18). Also present is a putative binding site for octamer-binding proteins (Oct-1), 5'-ATTTCAT-3', at position -889 (19).

Promoter B also lacks the canonical TATA and CCAAT motifs and has a high G+C content (approximately 70%) in the 200 bp preceding the transcription start site. Two possible binding sites, 5'-CCGGCCGGG-3', for the transcription factor AP-2 are found at positions -49 and -110 (20). A glucocorticoid response element, 5'-TGTCTT-3', is present at position -585 (21) and a putative AP-1 binding site, 5'-TGATAAA-3', at position -744 (18). A recognition site for NF-κB is present at position -914 (22).

The only obvious transcription factor binding site in promoter C is a potential response element for the glucocorticoid receptor at position -357 (21). The promoter also lacks a TATA and/or CCAAT box.

Tissue-specific Expression of 5'-Exons—Expression of the different 5'-exons was analyzed by reverse transcriptase-PCR in the SK-N-MC cell line and several tissues including kidney and artery (Fig. 5). A Y1-specific primer from exon 2 was used to reverse transcribe NPY-Y1 mRNA. The cDNA was then amplified using the exon 2 primer and one primer specific for exon 1A, 1B, or 1C. PCR products were detected by hybridization to an internal oligonucleotide (Y1#2). Lanes 13, 14, and 15 show part of the NPY-Y1 coding region amplified from the three cell types, demonstrating that approximately equal levels of NPY-Y1 transcript are present. Lanes 4, 8, and 12 are negative controls containing no template.

have been found to contain introns within the 5'-UTR. Receptor genes displaying alternative splicing within the 5'-UTR include the porcine muscarinic acetylcholine receptor, the rat endothelial ET receptor, the human interleukin 8 (IL8RB) receptor, and the human thromboxane receptor (23-26).

Analysis of NPY-Y1 transcripts expressed in the SK-N-MC neuroblastoma cell line shows that at least three exons are alternatively spliced onto the 5'-end of the NPY-Y1 receptor mRNA. The murine NPY-Y1 receptor gene has also been cloned and, like the human gene, has a 6-kb intron situated approximately 150 bp upstream of the start codon (12). Primer extension of the murine NPY-Y1 mRNA, to determine the transcription-initiation site, revealed the presence of five transcripts of various sizes. This was interpreted to be the result of multiple transcription-initiation sites within the same exon sequence. A limitation of this method is that the sequences of the transcripts are not known, and it cannot be distinguished whether the different-sized transcripts are a continuation of the same exon sequence or the result of a splicing event. In the light of this work on the human NPY-Y1 gene, it is very likely that at least some of the multiple transcription-initiation sites found in the mouse gene are also the result of different 5'-exons being spliced onto the splice acceptor site within the 5'-UTR.

Alternative splicing of sequences within the 5'-UTR has been demonstrated to regulate receptor expression at the translational level (27). For example, the presence of open reading frames within the 5'-UTR can result in the inefficient initiation of translation and suppression of protein expression (28). The human NPY-Y1 receptor gene contains a short open reading frame in the 5'-UTR situated on exon 2. In addition, exon 1C

DISCUSSION

The genes encoding G protein-coupled receptors comprise a very large gene family. Although many members of this family have been found to lack introns, many others have a non-contiguous structure, including a growing number of genes that

![Fig. 4. Transcription start sites of the 5'-exons determined by primer extension. cDNA was reverse transcribed from SK-N-MC mRNA using radioactively labeled primers, each designed to anneal to a different 5'-exon sequence (lanes 3, 6, and 9). Also shown are negative controls that had either reverse transcriptase (lanes 1, 4, and 7) or RNA (lanes 2, 5, and 8) omitted from the reaction. A DNA sequencing reaction was run next to the reactions to enable the size of the products to be determined.](image1)

![Fig. 5. Tissue-specific expression of the 5'-exons. RNA from artery (lanes 1, 5, and 9) and kidney (lanes 2, 6, and 10) tissue and the SK-N-MC cell line (lanes 3, 7, and 11) was reverse transcribed and then amplified using one 5'-exon-specific primer and one primer for exon 2. Lanes 1–4 used a primer specific for exon 1A, lanes 5–8 have the exon 1B-specific primer, and lanes 9–12 contain the exon 1C-specific primer. PCR products were detected by hybridization to an internal oligonucleotide (Y1#2). Lanes 13, 14, and 15 show part of the NPY-Y1 coding region amplified from the three cell types, demonstrating that approximately equal levels of NPY-Y1 transcript are present. Lanes 4, 8, and 12 are negative controls containing no template.](image2)
also contains a short open reading frame; however, no start codons are present in either exon 1A or 1B. The differential use of these 5′-exons may be a mechanism for translational regulation of the NPY-Y1 receptor protein.

The selective activation of the promoters for the NPY-Y1 receptor was investigated by analyzing the relative abundance of the three different forms of Y1 transcript in various tissues. Reverse transcriptase-PCR demonstrated that exon 1C was expressed in the SK-N-MC cell line, to a lesser extent in kidney, and not at all in artery, indicating that promoter C is activated in a tissue-specific manner. The majority of 5′-RACE and cDNA clones isolated have contained the exon 1A sequence at their 5′-ends. This suggests that the transcript containing exon 1A is the most abundant NPY-Y1 transcript in the tissues analyzed. It remains possible that other 5′-exons represent the major transcripts in other tissues. For example, when the testis cDNA library was screened, a cDNA clone containing exon 1B was isolated, although no clones containing exon 1A or 1C were obtained.

The sequences upstream of the 5′-exons were analyzed for possible response elements for transcription factors. The transcription start sites were first determined by primer extension. Although it has previously been reported that a major transcription start site occurs 208 bp upstream of the start codon (14), this did not correspond with any of the transcription start sites we observed using primer extension and 5′-RACE on kidney and SK-N-MC RNA. The 208-bp start site was determined by primer extension using a primer annealing to exon 2 of the gene and did not identify which 5′-exon was involved. It is possible that the transcription start site represents an additional 5′-exon sequence and/or the utilization of a different transcription start site that was undetected in this study.

None of the three promoter regions for the human NPY-Y1 gene had typical TATA or CCAAT boxes close to the transcription start sites. Many other G protein-coupled receptor genes also lack these motifs in their promoters, including the genes for the rat bradykinin B2 receptor, human adenosine A1 receptor, as well as numerous examples from the adrenergic, dopaminergic, and serotonin receptor families (29–31). More than one transcription start site, as seen with exon 1C, is often a feature of these genes. The promoter preceding exon 1B has a very high G+C content that, along with the absence of TATA and CCAAT boxes, is characteristic of promoters for “housekeeping” genes.

Little is known about the transcriptional regulation of the NPY-Y1 receptor gene. Administration of glucocorticoids to rats causes an up-regulation of NPY-Y1 receptor expression in the arcuate nucleus, although it is not known if this is a direct effect of the glucocorticoids acting on the NPY-Y1 receptor gene promoters (32). However, in the human gene, there are potential binding sites for the glucocorticoid receptor in promoters B and C. An increase in NPY-Y1 mRNA levels in the rat spinal cord is observed in response to peripheral inflammation (33). The transcription factor NF-κB is an important regulator of genes activated during the immune response, and potential binding sites for this transcription factor exist in promoter A of the human gene as well as in the promoter for the murine NPY-Y1 receptor gene.

The studies reported here demonstrate the existence of multiple transcripts of the human NPY-Y1 receptor mRNA, which are generated by the alternative splicing of three different 5′-exons onto a splice acceptor site within the 5′-UTR of the gene. The transcription of this receptor gene is thus under the control of at least three promoters that are activated in a tissue-specific manner and that may play an important role in the regulation of expression of the NPY-Y1 receptor and its function in a wide variety of physiological responses.

Acknowledgment—We gratefully acknowledge the assistance of M. Liu with DNA sequencing.

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