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The murine NPY-1 receptor gene

Structure and delineation of tissue-specific expression

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The murine gene for the NPY-1 receptor subtype for neuropeptide Y was characterized by DNA sequencing and expression studies. It comprises three exons with a 6,400 bp 5′-untranslated and a 80 bp internal intronic sequence. The 5′-flanking region of this gene lacks TATA or CCAAT consensus sequences in the proximity to the multiple transcription initiation sites. A 1,300 bp genomic fragment of the 5′-flanking region drives the expression of the *lacZ* reporter gene in NG108-15 cells and primary cultured neurons but not in glial and human embryonic kidney cells. In addition, it contains consensus sequences for various transcription factors including cAMP- and glucocorticoid-responsive elements.

G protein receptor; Neuropeptide Y; Neuropeptide receptor; Gene structure; Gene expression

1. INTRODUCTION

Neuropeptide Y is an amidated 36 amino acid peptide that belongs together with the pancreatic polypeptide and peptide YY to a family of structurally related peptide neurotransmitters [1]. NPY is the most abundant and widely distributed neuropeptide within the central nervous system where it participates in the control of a large number of functions, including neuroendocrine functions, stress responses, circadian rhythms, central autonomic functions, eating and drinking behavior, and sexual and motor behavior [2]. NPY is believed to exert its physiological effects by activating specific pre- and post-synaptic receptors [3]. Different orders of potency for NPY analogs in various model systems indicate that there are at least two distinct recognition sites, termed Y-1 and Y-2 [4–6]. We have recently reported the cDNA and the deduced amino acid sequences for the rat NPY-1 receptor subtype for NPY [7,8]; its primary structure shows that it belongs to the superfamily of the G protein-coupled receptors. Here we describe the molecular organization of the gene for the mouse NPY-1 receptor subtype and the functional and tissue-specific expression of the isolated promoter region of this gene.

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Abbreviations: NPY, neuropeptide Y; bp, base pair; kb, kilobases; PIPES, piperazine-*N,N′*-bis[2-ethane sulphonic acid].

2. MATERIALS AND METHODS

NPY-1 receptor genomic clones were isolated from a mouse genomic library constructed in lambda-fix [9] using a 900-bp cDNA probe covering 240 bp of the 5′-untranslated sequence of the rat NPY-1 receptor cDNA [7] (hybridization at 50% formamide, 5 × SSC, 42°C). Filters were washed at 65°C (0.5 × SSC) and exposed to Kodak XAR-5 films at −80°C. The cloned DNA was digested with *Sal*I and subcloned in the plasmid vector pBluescript SK⁻ to be analyzed by dual digestion using several restriction endonucleases. Appropriate DNA restriction fragments were then subcloned into M13mp18 and mp19 RF-DNA for DNA sequence analysis [10].

For primer extension analysis total RNA (20 μg) or polyA⁺ RNA (5 μg) from mouse cerebral cortex was hybridized for 16 h at 30°C or 35°C with 2 × 10^5 cpm of a ^{32}P-end-labelled oligonucleotide corresponding to nucleotides −57 to −73 in the 5′-flanking region of the gene (Fig. 2). The annealed RNA/primer mixture was recovered by ethanol precipitation and extended by resuspending the precipitate in 20 μl of reaction mixture containing 50 mM Tris-HCl buffer, pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml actinomycin D, 4 mM dNTP, 200 units of MMLV reverse transcriptase (GIBCO, BRL). Reactions were incubated at 37°C for 120 min. Samples were denatured and analyzed on a 6% polyacrylamide sequencing gel.

The promoterless vector pβ-Gal for *lacZ* expression was constructed by subcloning the *Hin*dIII/*Bam*HI *lacZ* fragment from pCH110 (Pharmacia LKB Biotec.) into the polylinker of pBluescript SK. A *Sal*I–*Bgl*II fragment of the murine NPY-1 receptor gene from nucleotides −1486 to −210 relative to the initiator ATG was isolated by gel electrophoresis. The *Bgl*II-digested end was filled in with the Klenow fragment of DNA polymerase I (Boehringer Mannheim) and excess of dNTPs. The resulting *Sal*I/blunt fragment was ligated into the *Sal*I/*Cla*I site of pβ-Gal, after filling the *Cla*I site of the plasmid to obtain a blunt end. Primary cultures of cortical neurons were prepared from 1-day-old rat brain following the dissociation procedure previously described [11] and used for transfection experiments after 10 days in vitro. Cells were transfected by calcium/phosphate precipitation [12] using 4 μg of plasmid DNA per dish. Forty-eight hours after transfection, cells were fixed in 1.25% glutaraldehyde and incubated overnight at 37°C with 50 mM Tris-HCl, pH 7.5, 1.25 mM

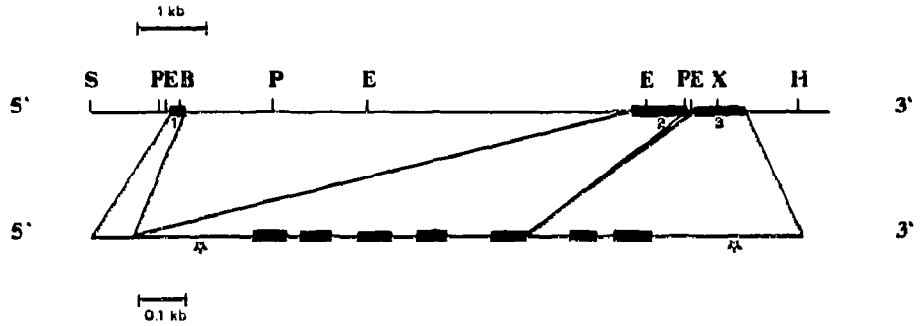

Fig. 1. Structure of the murine NPY-1 receptor gene. *Upper part*: murine NPY-1 receptor gene. *Lower part*: exon assignment to the coding region of the rat cDNA. Exons are indicated on the gene by solid boxes and numbers. Codons for putative membrane-spanning peptide segments are highlighted on the cDNA by solid boxes and initiation and stop codons are marked by stars. Restriction endonuclease sites are: B, *Bgl*II; X, *Xho*I; E, *Eco*RI; P, *Pst*I; H, *Hin*dIII.

ferrocyanide, 1.25 mM ferricyanide, 15 mM NaCl, 1 mM $MgCl_2$ and 0.5 mg/ml X-gal (Promega).

3. RESULTS AND DISCUSSION

Out of three genomic clones analyzed, one, approximately 18 kb long, was subjected to extensive characterization (Fig. 1). The DNA and the deduced amino acid sequences of the gene are shown in Fig. 2. The alignment of the mouse and rat complete amino acid sequence for the NPY-1 receptor subtype indicates an overall homology of 98%. The coding sequence of the gene is interrupted by one intron, of approximately 80 bp in length, that starts immediately after transmembrane region 5. Sequence comparison of the mouse gene and the rat cDNA 5′ to the initiator ATG indicates that they diverge upstream of base −147 (numbering from the first base of the coding sequence). The gene sequence at this point matches the consensus splice acceptor sequence, indicating the presence of an intron in the 5′-untranslated region. This intron, approximately 6.4 kb long, is flanked at the 5′ by 84 bp matching the rat cDNA sequence from bp −148 to bp −232.

Primer extension analysis indicate the presence of several closely spaced sites for transcription initiation (Fig. 3). Major transcripts seem to begin at nucleotides −167, −182, −238, −247, −263 and the initiation sites are all located close to the end of the 5′-untranslated region of the cDNA (Fig. 1). The presence of multiple transcription initiation sites is further confirmed by the analysis of the 5′-flanking sequence of the mouse NPY-1 receptor gene, indicating that no canonical promoter elements, e.g. TATA or CAAT-boxes, were found in the close proximity of the sites of initiation of transcription. However, the promoter contains the hexamer GGGGCG at position −272 which may constitute the binding site for the Sp1 transcription factor [13]. Sequence analysis of the 5′-flanking sequence of the murine gene reveals a genomic fragment by approximately 1,250 bp with several notable features. The putative promoter region of the murine NPY-1 receptor gene contains two sequences at position −849 and −478 related to the AP-1 motif, which is known to bind members of the c-*fos* and c-*jun* family [14,15]. The sequence CGTCA at position −699 was also found in the vasoactive intestinal peptide gene, where it is essential for biological activity of the cAMP-regulating enhancer [16]. Furthermore several reverse complement consensus sequences for the steroid receptor binding were found at position −1027, −858, −769, −570 and −471 [17] and, finally, at position −1,287 there is the consensus sequence GGGATTTCAC that has been shown to bind the transcription factor $NF\text{-}_KB$ [18]. To determine whether the genomic fragment 5′ to the transcription initiation sites contains promoter sequences we performed transfection experiments by using a construct in which the 5′-flanking 1.3 kb fragment of the mouse NPY-1 receptor gene was introduced immediately upstream of the reporter gene *lacZ*. In NG108-15 cells, a mouse–rat hybrid neuroblastoma glioma cell line that expresses the endogenous NPY-1 receptor gene, a significant *lacZ* expression was driven by the 1.3 kb fragment, while the promoterless vector pβGal was inactive (Fig. 4A). The murine NPY-1 receptor promoter did not drive the expression of *lacZ* in human embryonic kidney 293 cells (Fig. 4C), that lack the NPY-1 receptor mRNA, while the SV40 promoter was functional (Fig. 4D), suggesting a restricted tissue-specificity of the isolated promoter sequence. Transfection of primary cultures of cortical neurons from newborn rat brain also results in the staining for β-galactosidase of several cell bodies and neurites from cultured neurons (Fig. 4B). Moreover the expression of the NPY-1 receptor gene in primary cultures of cortical cells seems to be neuron-specific. Indeed, the NPY-1 receptor promoter region induced the *lacZ* expression only in neurons and not in glial cells, which constitute 4-6% of the total number of cultured cells [11] whereas the SV40 promoter drove the expression of the reporter gene in both neurons and glia (data not shown).

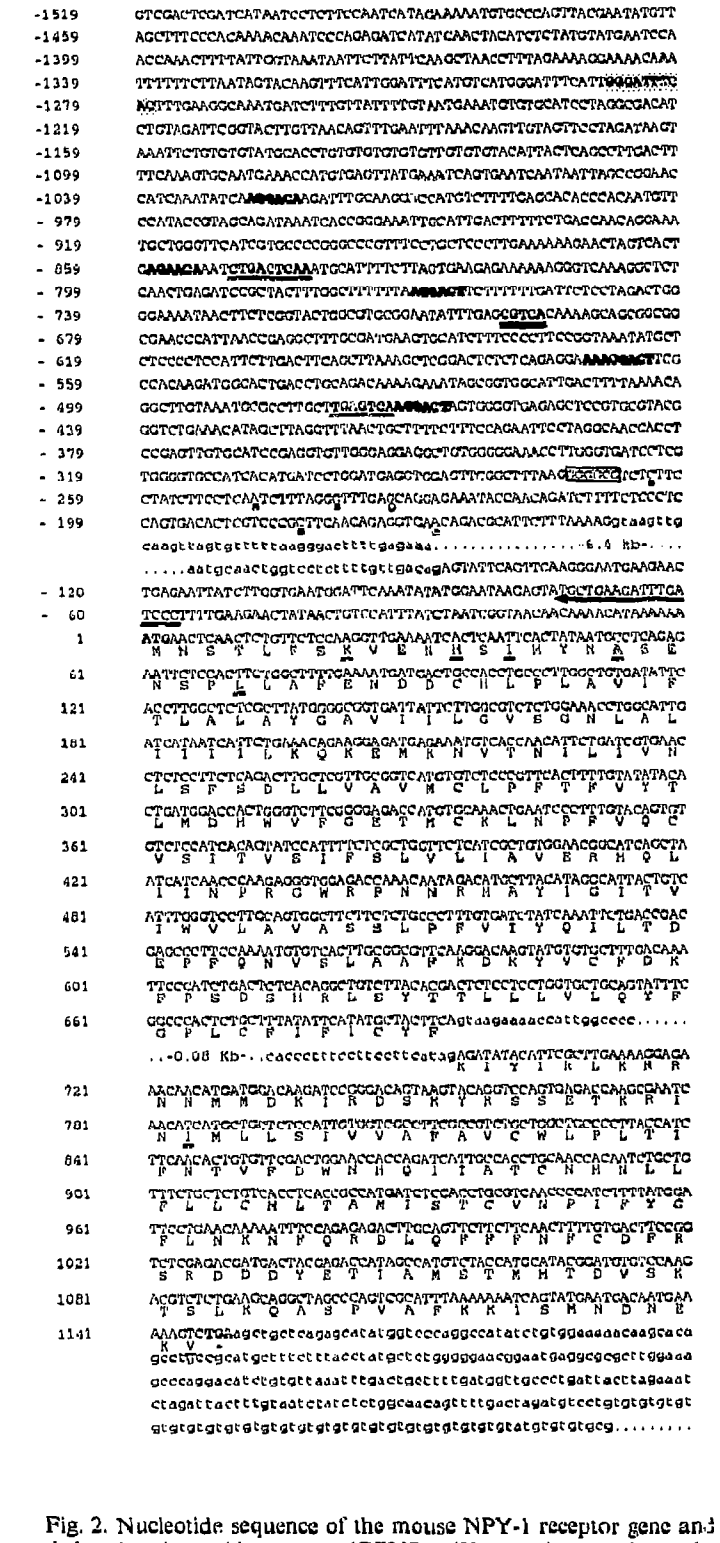

Fig. 2. Nucleotide sequence of the mouse NPY-1 receptor gene and deduced amino acid sequence (GENBANK accession numbers: (1.) NPY mouse cDNA (coding region), Z18280; (2.) NPY-1 mouse gene exon 1, Z18281; (3.) NPY-1 mouse gene exon 2, Z18282; (4.) NPY-1 mouse gene exon 3, Z18283). Amino acids that are different in the rat receptor are underlined. Potential sites for initiation of transcription are indicated by solid squares below the sequence and the 5′ end of the rat cDNA clone is indicated by an open circle. AP-1 sites are underlined, the CRE site is double underlined and the potential steroid

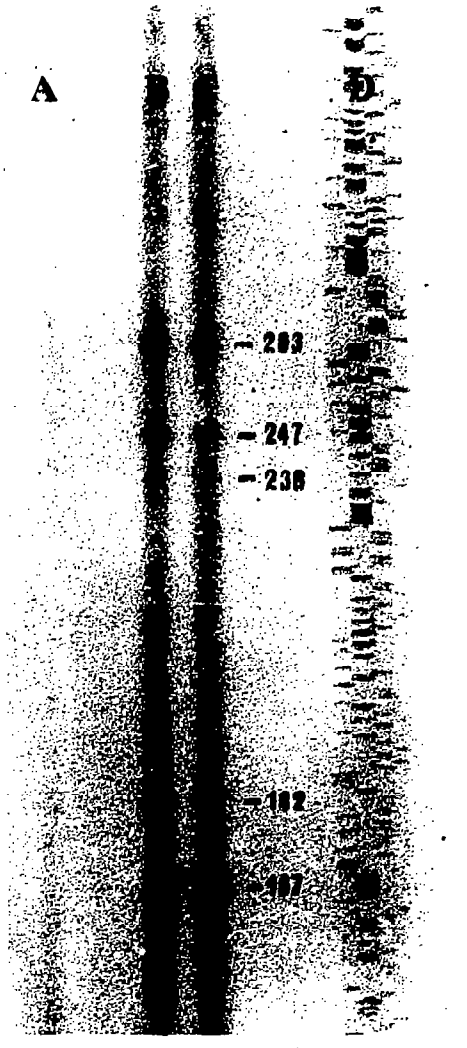

Fig. 3. Primer extension analysis. An antisense oligonucleotide corresponding to nucleotides −57 to −73 was hybridized with 20 μg of transfer RNA (A), or of total RNA at 35°C (B) and 30°C (C), then extended with M-MLV reverse transcriptase. A dideoxy sequencing ladder from the human genomic clone primed at bp −149 and extending 5′ is shown in the lanes under (D). The positions of the most prominent sites of initiation of transcription are indicated at the side.

Previous results obtained by Northern blot analysis and in situ hybridization suggest that the NPY-1 receptor mRNA is specifically expressed in brain and prominently localized in specific nuclei of the rat forebrain [7]. We now report that the NPY-1 receptor putative promoter region drives the expression of the *lacZ* reporter gene only in neurons but not in glial cells, suggesting that the expression of this gene in brain might be neuron specific.

←
receptor binding hexamers are highlighted by shadow characters. Consensus sequences for the transcription factors NF-κB and Sp1 are indicated by a stippled box and a clear box, respectively. The arrow indicates the reverse sequence of the primer used for primer extension experiments.

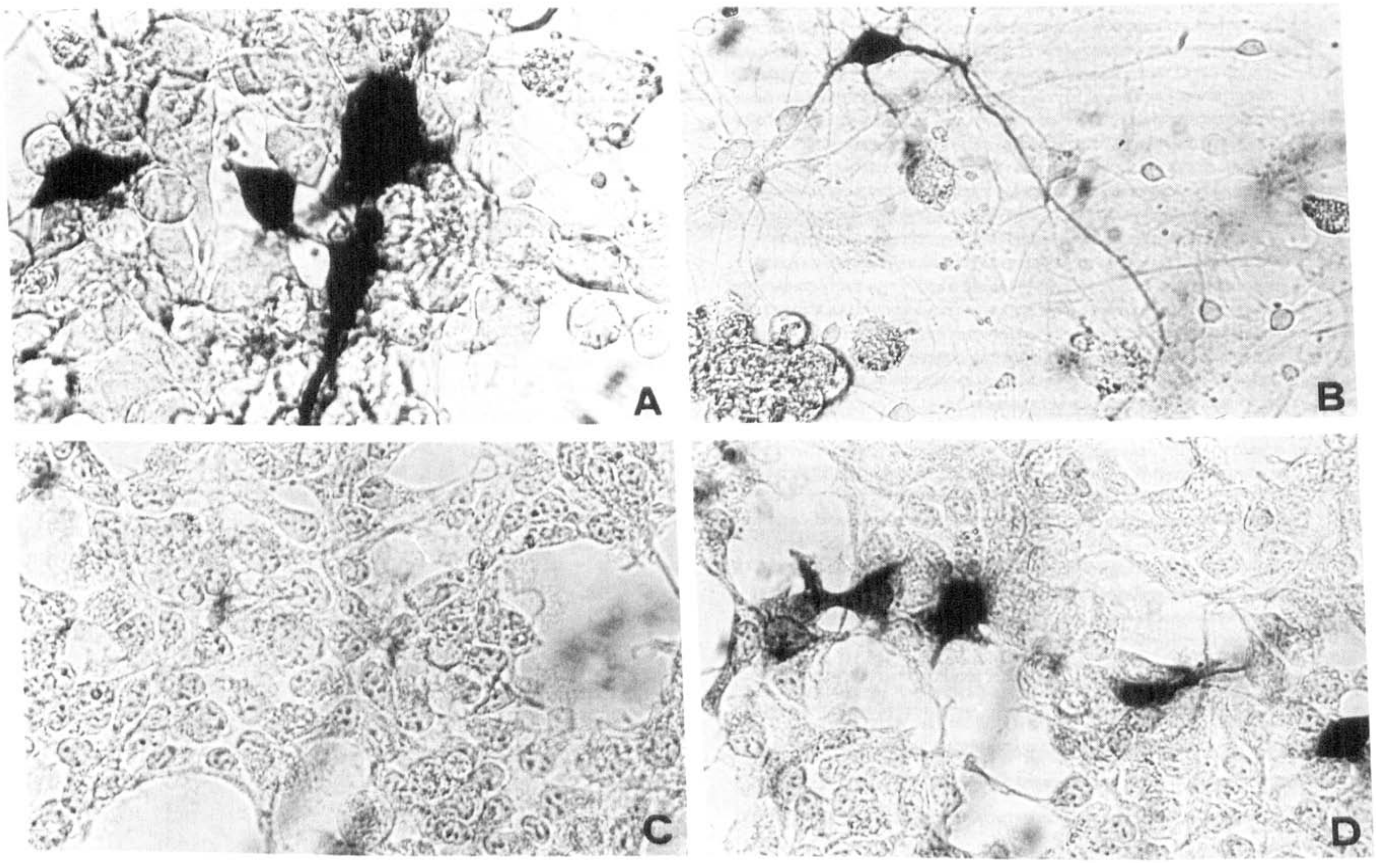

Fig. 4. Expression of NPY-1/*lacZ* in cultured cells. The mouse NPY-1 promoter region drives the expression of *lacZ* in NG108-15 cells (A) and in primary cultures of cortical neurons at the 10th day in vitro (B), but not in 293 cells (C). Transfection of 293 cells with the pCH110 plasmid vector, containing the SV40 promoter/*lacZ* fusion gene, induces a high efficiency expression of *lacZ* (D).

In conclusion, in the present study we report the molecular cloning of the murine NPY-1 receptor gene and the tissue-specific expression of its promoter region. These data provide new insights for the characterization of the structure of neuropeptide receptors and to elucidate the mechanisms controlling the expression of these genes in brain.

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