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Isolation and characterization of the *Xenopus laevis* cDNA and genomic homologs of neuropeptide Y

David Griffin [a], Carolyn D. Minth [b], William L. Taylor [a]

[a] *Department of Molecular Physiology and Biophysics, School of Medicine, Vanderbilt University, 737 Light Hall, Nashville, TN 3723-0615, USA;*
[b] *Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109, USA*

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Abstract

We have isolated *Xenopus laevis* cDNA and genomic clones encoding the neuropeptide Y (NPY) mRNA and gene using a probe from the human NPY gene. The longest open reading frame in the cDNA encodes a peptide 76% identical to human prepro-NPY and 73% identical to rat prepro-NPY. The putative mature *Xenopus* NPY (XNPY) peptide is 94% identical to both human and rat peptides. A genomic clone containing 422 base pairs of 5′-flanking sequences and the 5′-end of the mRNA was also isolated. Primer extension analysis was used to map the transcription initiation site of the *Xenopus* NPY gene. Comparison of the 5′-flanking sequences of the *Xenopus laevis,* human, and rat NPY genes resulted in areas of high conservation, including the TATA box and the CT box previously shown to interact with Spl-like proteins. Distribution of the *Xenopus* NPY message was analyzed by Northern analysis and RNAse protection. XNPY transcripts were not detected in whole developing embryo RNA, but were detected in adult frog brain RNA. We have also conducted preliminary studies of the XNPY promoter, utilizing an XNPY/chloramphenicol acetyl-transferase fusion construct. This study has demonstrated that *Xenopus* NPY shares a high degree of identity to its human and rat counterparts and that this homology extends to the gene, which contains similar *cis*-elements positioned near the transcription start site.

Key words: cDNA isolation; Promoter isolation; Neuropeptide Y; Regulation; Expression; (*Xenopus laevis*)

1. Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide which is involved in many processes occurring in both the central and peripheral nervous systems. The initial isolation of the NPY peptide was from porcine brain (Tatemoto, 1982). Subsequently, homologs have been isolated from a large number species at both the peptide and cDNA level. NPY is widely distributed within the mammalian nervous system, being one of the most abundant neuropeptides known (Mione et al., 1990). It is often co-localized with other neurotransmitters, including catecholamines or somatostatin (Mione et al., 1990; O'Donohue et al., 1985). The actions of NPY are diverse, involving complex behavioral patterns and biochemical activities, along with its fundamental activity as a neurotransmitter (McDonald, 1987). In this study we have begun an investigation of neuropeptide Y in *Xenopus laevis*, isolating and characterizing the NPY gene and determining the tissue distribution of NPY mRNA.

NPY has been implicated or shown to function in several diverse and important systems in mammals, including the regulation of feeding and carbohydrate metabolism (Stanley et al., 1989; McGregor et al., 1990; Chua et al., 1991; Brady et al., 1990), LHRH release (O'Donohue et al., 1985), and of of several aspects of the circulatory system (Mione et al., 1990; Kawamura et al., 1989; Waeber et al., 1990).

The ability of NPY to elicit hormone effects has also been extended to non-mammalian systems. Synthetic NPY inhibits α-MSH release from the neurointermediate lobes of *Xenopus laevis* (Verburg-Van Kemenade et al., 1987), and binds a specific cell type in the *Xenopus laevis* pituitary (De Rijk et al., 1991). Recently, McKay et al. (1992) isolated and sequenced the

* Corresponding author. Tel.: 615-322-7980; Fax: 615-322-7236.

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NPY peptide homolog from *R. temporaria*. This peptide only had one amino acid substitution when compared to human NPY, further validating the results obtained with synthetic NPY in *Xenopus laevis*.

Several groups have studied the expression and regulation of NPY mRNA in mammalian systems. Expression of NPY is highest in the frontal cortex and the striatum of the human brain, along with the adrenal gland (Higuchi et al., 1988). The mRNA has also been detected in a non-neuronal cell type, rat megakaryocytes, but not in human megakaryocytes (Ericsson et al., 1987; unpublished results). Several studies have documented various ways in which NPY mRNA transcription is regulated. Induction of seizures have been shown to increase the concentration of NPY mRNA in certain areas of the brain (Bellman et al., 1991). Insulin can effect mRNA levels either positively or negatively (Fischer-Colbrie et al., 1988). Studies using rat pheochromocytoma (PC12) or neuroblastoma (NG18TG-2) cell lines indicated that NPY mRNA can be positively regulated by the actions of glucocorticoids, cAMP elevation, and protein kinase C activation, either alone or when acting synergistically (Higuchi et al., 1988). Finally, in Minth et al. (1986) and Minth and Dixon (1990), the isolation of the human gene and a study of its promoter region are reported.

The lack of a detailed and coherent picture of NPY action along with its wide distribution make further study of this peptide and its gene important. In this study we report the isolation of a cDNA homolog of NPY in *Xenopus laevis*. We then isolated genomic clones containing the 5′-flanking sequences, mapped the transcription start, and studied the distribution of the NPY transcript in *Xenopus*. We have also begun preliminary investigations of the *Xenopus* NPY promoter. These studies will provide an important basis for elucidating the regulatory mechanisms governing neuronal gene expression conserved throughout evolution.

2. Materials and methods

Molecular cloning

Xenopus laevis NPY clones were isolated from a *Xenopus laevis* brain cDNA library constructed in phage λgt11 (obtained from Drs. K. Richter and I. Dawid). The initial NPY probe was a 418 bp *Sca*I/*Sph*I fragment from the human NPY cDNA which was labeled by the random primer method (Prime-It, Stratagene; La Jolla, CA, USA). The random primer probe was then used for low stringency hybridization of the above library (Minth et al., 1986). Plaque lifts on nitrocellulose were prehybridized 4 h at 42°C in hybridization buffer (43% formamide, 5 × SSPE (750 mM NaCl, 50 mM NaH_2PO_4, 5 mM ethylenediamineteraacetic acid [EDTA]), 5 × Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 1 mg/ml bovine serum albumin [BSA]), 10 μg/ml tRNA, 0.1% sodium lauryl sulfate [SDS]) followed by addition of the labeled probe at 500 000 cpm/ml for 48 h at 42°C. Filters were washed at 42°C twice for 1 h in 2 × SSPE, 0.1% SDS and then twice for 1 h in 0.5 × SSPE, 0.1% SDS.

Genomic clones were subsequently isolated from a genomic library prepared from homozygous diploid *Xenopus laevis* genomic DNA partially digested with *Sau*IIIA and ligated into the phage vector EMBL 4 (obtained from Z. Jonas and I. Dawid). The genomic library was screened using a *Xenopus* NPY probe prepared by labeling the XNPY insert from the plasmid pXNPY-1 by the random primer method as described. Screening was performed as described for the phage λgt11 library. Several positive plaques from the genomic library were analyzed and all represented an identical clone. All clones from both cDNA and genomic phage screens were removed from the vector with *Eco*RI and subcloned into the *Eco*RI site of plasmid pGEM-7 Zf + (Promega; Madison, WI, USA).

Plasmid pPCRXGNPY-.9 was prepared using genomic clone pXGNPY-.9 and the polymerase chain reaction (PCR) essentially as described by the manufacturer (Perkin-Elmer-Cetus; Norwalk, CT, USA). The first PCR primer corresponded to the 15 nucleotides immediately 5′ to the first exon-intron boundary (position 107 to 126; 5′-ATCCCGGGTACTTTGGGTCTGTCGAAGATA) with a *Sma*I containing linker. The second primer was complementary to pGEM-7 Zf + (positions 154 to 169 in the sequence from Promega, 5′-AACAGCTATGACCATG). The PCR amplified fragment of pXGNPY-.9 was digested with *Xho*I and *Sma*I, isolated, and subcloned into the *Xho*I/*Sma*I sites of pGEM-7 Zf +.

Plasmid pEUCATXGNPY-.9 was constructed by isolating the insert from pPCRXGNPY-.9 by restriction enzyme digestion with *Sal*I/*Hin*dIII. This fragment was then ligated into similarly digested pEUCAT. Plasmid pEUCAT is a derivative of pEMBL8 CAT with a strong terminator of transcription (Upstream Mouse Sequence, UMS) upstream of the polylinker (Piaggio and DeSimone, 1990).

Sequencing

Sequencing of double stranded cDNA and genomic subclones was carried out using the dideoxy sequencing method and the Sequenase kit (USB; Cleveland, OH, USA). Overlapping fragments on both strands of clones pXNPY-1, pXNPY-8, pXGNPY-.9 and pPCRXGNPY-.9 were sequenced completely. Alignments were performed utilizing Intelligetics software, release 5.35 (IntelliGenetics; Mountain View, CA, USA) and MacVector software, release 4.0 (Eastman Kodak; Rochester, NY, USA).

Northern analysis

The total RNA was prepared for Northern analysis and RNAse protection following the procedure of Chomczynski and Sacchi (1987). The total RNA samples were isolated from adult tissues and embryos reared in the laboratory and staged according to Nieuwkoop and Faber (1967). Gels (1.5% agarose, 1 × TAE (40 mM Tris-acetate, 2 mM EDTA), 3% formaldehyde) were loaded with 10 μg of total RNA per lane. The RNA was then transferred to Nytran membranes (Schleicher and Schuell; Keene, NH, USA) and hybridized with random primed probes prepared from the cDNA clone pXNPY-1 as described. The Nytran membranes on which the RNA was immobilized were pre-hybridized in 20% formamide, 5 × SSPE, 5% SDS, 500 μg/μl yeast RNA, and 1 × Denhardt's for 1 h at 65°C. These blots were then hybridized with the prehybridization buffer containing 10% dextran sulfate and 3×10^6 cpm/ml [α-^{32}P]ATP-labeled probe for 12–24 h at 65°C. Subsequently, two washes were done in 2 × SSPE, 1% SDS for 30 min at 65°C followed by two additional washes in 0.2 × SSPE, 1% SDS for 30 min at 65°C.

Oocyte injections

Ovarian tissue was isolated from adult *X. laevis* and defolliculated in 100 mM $NaPO_4$ (pH 7.0), 0.4 mg/ml collagenase (Sigma; St. Louis, MO, USA, type 1) for 2–3 h at room temperature. The defolliculated oocytes were washed extensively with OR-2 [82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM Hepes (pH 7.8)], and stored in OR-2 at 18°C. Stage VI oocytes (Dumont, 1972) were selected for injection, which was carried out as described in Pfaff et al. (1989) and Pfaff and Taylor (1992). The DNA samples were injected into germinal vesicles in a volume of 4 nl controlled by a picospritzer II (General Valve; Fairfield, NJ, USA). The NPY/CAT chimeric gene construct pEUCATXGNPY-.9 or the reporter construct pEUCAT dissolved in water was injected at 0.4 mg/ml. After a 24 h incubation at 18°C, pools of 20 oocytes were selected for primer extension analysis of RNA.

Primer extension analysis

The pooled, injected oocytes were homogenized by vigorous vortexing in 600 μl of 1 × SET (0.15 M NaCl, 5 mM EDTA, 50 mM Tris, pH 7.8, 2% SDS and 0.5 mg/ml proteinase K). The oocyte mixture was incubated for 40 min at room temperature. The mixture was then extracted twice with phenol, twice with phenol-chloroform (1:1) and once with chloroform. The RNA was recovered by ethanol precipitation.

The poly (A)+ *Xenopus* brain RNA for primer extension analysis was prepared as follows. Total brain RNA was prepared from approxiamatly 80 young *Xenopus laevis* frogs according to the method of Chomczynski and Sacchi (1987). The amount of total RNA recovered was 165 μg. An mRNA spin column kit (5 Prime-3 Prime; Boulder, CO, USA) was then used to select for poly (A)+ RNA, utilizing an oligo(dT)-cellulose spin column as described by the manufacturer. All of the total brain RNA was applied to the column.

The oligonucleotide utilized in the primer extension analysis was B915 (5′-CTTTGGGTCTGTCGAAGATA), which corresponds to the twenty nucleotides of the non-sense strand immediately 5′ to the first exon/intron boundary. This oligonucleotide was 5′ end-labeled utilizing [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled oligonucleotide was purified from unincorporated ATP by spin column chromatography with Bio-Gel P6 (Bio-Rad; Richmond, CA, USA).

The hybridization of the labeled oligonucleotide and the oocyte and brain RNA was carried out according to Ausebel et al. (1989), with some modifications. Each RNA sample was ethanol precipitated, then resuspended in 15 μl hybridization buffer (0.15 M KCl, 10 mM Tris, pH 8.5, 1 mM EDTA) containing 500000 cpm of oligonucleotide B915. The tubes were then incubated for 90 min in a 65°C water bath. The tubes were next removed and allowed to cool to room temperature. After cooling, 80 μl of the reverse transcriptase cocktail [10 mM $MgCl_2$, 20 mM Tris, pH 8.5, 10 mM DTT, 10 mM each dATP, dTTP, dCTP, dGTP and 2.5 units avian myoblastosis virus reverse transcriptase (Promega; Madison, WI, USA)] was added. The tubes were then incubated 60 min in a 37°C water bath. Next, the reaction mixture was then extracted once with phenol, once with phenol-chloroform (1:1) and once with chloroform. The samples were then ethanol precipitated (without additional salt). The pellets were resuspended in 1:1 water, sequencing buffer (90% deionized formamide, 20 mM EDTA, 0.03% xylene cyanol and bromophenol blue). Finally, the samples were run on a 5% sequencing gel.

A sequencing ladder was run alongside the primer extension products to accurately determine the transcription start site. This sequencing ladder was made using the same labeled B915 oligonucleotide used as a primer in the primer extension reactions, with pPCRXGNPY-.9 as the template. The sequencing reactions were carried out using the CircumVent thermal cycle dideoxy DNA sequencing kit (New England Biolabs; Beverly, MA, USA) as described by the manufacturer.

3. Results

Isolation of cDNA clones encoding Xenopus NPY

A cDNA library from adult *Xenopus* brain mRNA was screened for the presence of *Xenopus laevis* NPY clones. Several positive cDNAs were isolated and two

were extensively analyzed. pXNPY-1 is 600 nucleotides long, the first nucleotide being 13 base pairs 3′ to the transcription start site. pXNPY-8 is 862 nucleotides in length, beginning at +29 and ending with a tract of 15 adenosines (Fig. 1A,B). A polyadenylation signal sequence (AATAAA) is located at +854, and nucleotide +876 corresponds to the polyadenylation site. The open reading frame encoding the putative XNPY begins with a methionine codon at +127 and ends with a stop codon at +418 (TGA). Other cDNA clones were

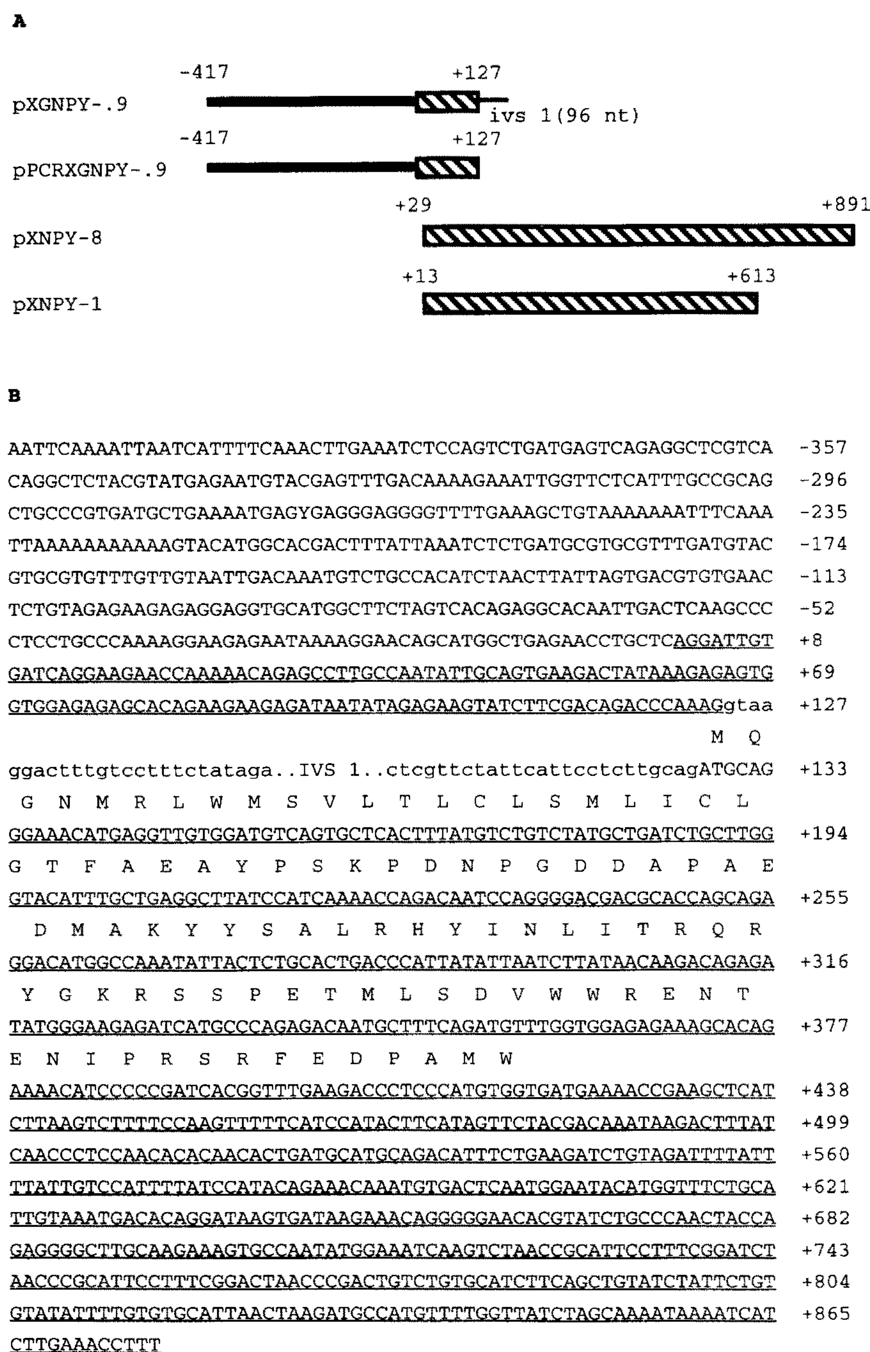

Fig. 1. (A) Schematic representation of the *Xenopus* NPY sequences used in this study. pXGNPY-.9 was isolated from a genomic library using the pXNPY-1 cDNA clone as a hybridization probe (ivs 1 indicates a portion of intron 1). pPCRXGNPY-.9 is a derivative of pXGNPY-.9 produced by the polymerase chain reaction (Materials and methods). pXNPY-8 and pXNPY-1 were isolated from a *Xenopus laevis* brain cDNA library using probes derived from pXNPY-1 and a human NPY cDNA fragment respectively. The clones were sequenced on both strands. The nucleotide defined as the mRNA initiation site (+1) was determined using a primer extension assay (Fig. 2). (B) DNA and peptide sequences of *X. laevis* NPY, combining pXGNPY-.9 and pXNPY-8 (underlined). Sequence corresponding to prepro-XNPY peptide is shown in single letter amino acid code above corresponding DNA sequence.

partially sequenced and all were contained within pXNPY-8.

Isolation of Xenopus NPY gene and determination of the transcription start site

pXNPY-1 was used as a probe to screen a *Xenopus* genomic DNA library. One clone was isolated and sequenced. Comparison of the genomic clone (pXGNPY-.9) to the sequence of pXNPY-1 showed that it contained 417 nucleotides 5′ to the transcription start, the first exon, first intron, and second exon of the XNPY gene (Fig. 1A,B).

The transcription start site of the chimeric and native XNPY transcripts was determined by primer extension analysis. The major start site for the chimeric pEUCAT/XNPY promoter construct in oocytes was three nucleotides 5′ to the start site detected in *Xenopus* brain RNA (Fig. 2). No product was detected in oocytes injected with the pEUCAT vector alone. The start site detected in the *Xenopus* brain RNA sample was defined as +1.

Comparison of Xenopus NPY to the human and rat homologs

The sequence of the *Xenopus* NPY cDNA was found to be highly similar to that of human (Minth et al., 1986) and rat (Larhammar et al., 1987) NPY, except for several large insertions in the 3′ untranslated region (Fig. 3). The greatest homology in sequence was found in the region which codes for the prepro-hormone (underlined sequence). Disregarding the insertions in the XNPY cDNA, the maximal alignment for these three cDNA's results in 61% identity. This increases to 74% if only the prepro-hormone coding sequences are aligned.

The genomic sequences also contained regions of similarity, especially in the region immediately upstream of the NPY gene's transcription start site (Fig. 4). Similarity of the *Xenopus* upstream sequences to the human (Minth et al., 1986; Minth and Dixon, 1990) and rat (Larhammar et al., 1987) sequences was found in clusters, the most striking corresponding to a non-consensus TATA box (*Xenopus* −32, human −30) and an Sp1-like *cis*-element (*Xenopus* −56, human −57), which were previously mapped in the human gene (Minth and Dixon, 1990). Other *cis*-elements identified in the *Xenopus* NPY gene upstream sequences include an AP1-like element (*Xenopus* −65, human −70) and a CAAT box (*Xenopus* −69, human −76) (for review see Mitchell and Tjian, 1989). The exon/intron boundaries analyzed in the *Xenopus* clone all occurred in exactly the same relative position as in the mammalian NPY gene. The first of these exon/intron boundaries occurs directly before the first AUG (human +85), such that the first exon is entirely within the 5′ untranslated sequence in the mRNA.

Further comparisons between *Xenopus* NPY and human NPY were made by aligning the precursor peptide encoded by the XNPY open reading frame with the 97 amino acid NPY precursor from human and rat. Comparison of these peptides revealed that the putative mature hormone from *Xenopus* is 94% identical to both human and rat, and the putative *Xenopus* prepro peptide is 75% and 73% identical

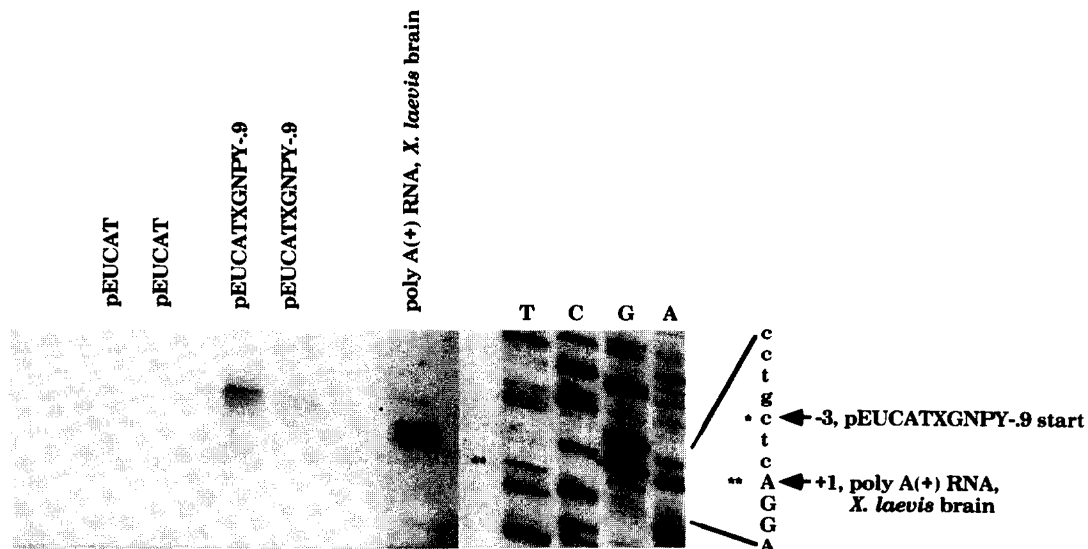

Fig. 2. Primer extension analysis of *Xenopus laevis* poly-A (+) RNA and oocyte total RNA injected with pEUCAT and pEUCATXGNPY-.9. An α-^{32}P end-labeled oligonucleotide immediately 5′ to the first exon/intron boundary was utilized as the primer. The autoradiogram was exposed 2 weeks to visualize the primer extension product in the *X. laevis* brain poly A(+) RNA lane. The remainder of the autoradiogram was exposed two days. The sequencing ladder was prepared by thermal cycle sequencing as described (see Materials and methods). The sequence as read from the ladder corresponds to the antisense strand; the sequence written to the right is that corresponding to the opposite (sense) strand.

when compared to human and rat respectively (Fig. 5). Additionally, the mature *Xenopus* NPY peptide sequence has one conservative amino acid change at position 10, glu to asp, from the recently published *R. temporaria* NPY peptide (McKay et al., 1992).

XNPY mRNA distribution

Northern blot analysis and RNase protection were employed to study the distribution of *Xenopus* NPY mRNA during development and among various tissues of adult *Xenopus laevis*. The XNPY message is 940 nucleotides according to Northern analysis (Fig. 6). This corresponds to the size predicted from cDNA clones and the 5′ and 3′ boundaries of the transcript, along with the addition of 50–100 adenine residues. Two higher molecular weight species are also detected intermittently (3 kb and 6 kb). XNPY message was detected in adult *Xenopus* brain but was not detected in any of the other adult tissues tested (heart, spleen, lung) (Fig. 6). Furthermore, XNPY message was not detected in total RNA from *Xenopus* embryos of various stages or the *Xenopus* cell line A110I. The results of RNase protection experiments essentially parallel the Northern analysis, with XNPY RNA detected only in adult brain tissue (data not shown). However, the possibility exists that XNPY is expressed at a level below the limits of detection, especially in *Xenopus* embryos, considering that only a small fraction of the whole embryos, which were used to prepare the RNA used in these experiments, consists of neural tissue.

XNPY-CAT chimera expression

Primer extension analysis was performed on RNA isolated from *Xenopus* oocytes injected with a fusion construct containing the *E. coli* chloramphenicol acetyl-transferase gene the expression of which is driven

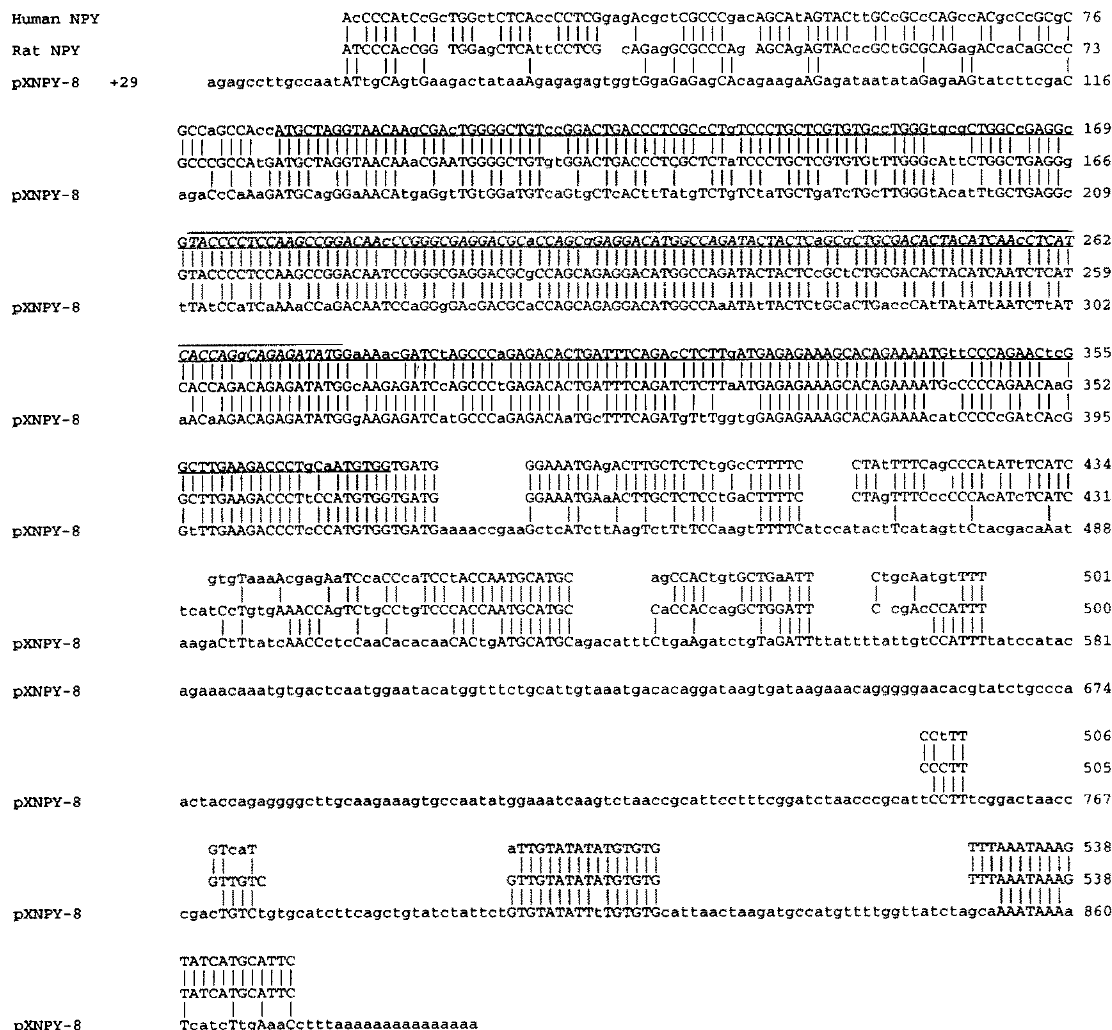

Fig. 3. *Xenopus laevis* NPY cDNA sequence, aligned to human and rat cDNA sequences. The portion of the sequence corresponding to prepro-NPY in the human cDNA is underlined (87–377). The sequence corresponding to the mature NPY peptide in the human cDNA is underlined and overlined (171–278). Capital and small case letters indicate matching and non-matching bases respectively. Gaps were introduced to increase the number of aligned bases.

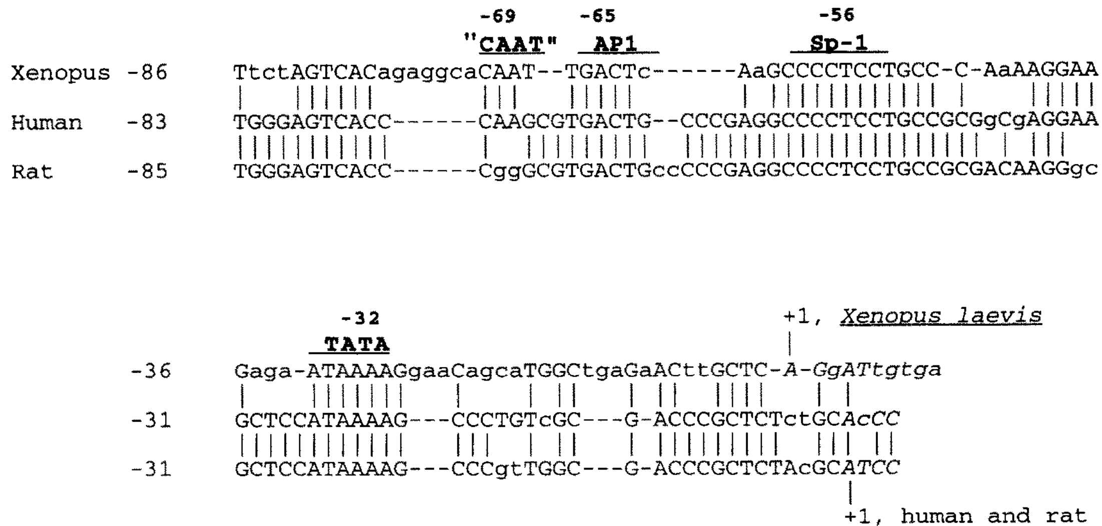

Fig. 4. Comparison of the upstream regions of NPY genes. +1 is indicated for the *Xenopus* gene as determined by RNase protection. The sequences of the human, rat, and *Xenopus* genes are compared. The presence of the consensus binding sequences for various *trans*-acting factors (Sp1, AP1, CAAT and TATA) are indicated for the *Xenopus* upstream sequence. Capital and small case letters indicate matching and non-matching bases respectively. Gaps were introduced to increase the number of aligned bases.

by NPY upstream sequences (−417 to +126). This chimeric construct demonstrated a low level of expression (Fig. 2) in *Xenopus* oocytes. As previously stated, the major primer extension product of the XNPY promoter construct injected into oocytes is three nucleotides larger that the primer extension product of *Xenopus laevis* brain RNA.

4. Discussion

This study has established that an NPY homolog exists in *Xenopus laevis*. The extensive similarity between the NPY sequence of an amphibian versus that found in mammals suggests a basic, conserved function for the protein. Amphibians and mammals diverged several hundred million years ago (Creighton, 1984). Since that time, proteins such as cytochrome *c* and hemoglobin-β have diverged 17% and 46% respectively (Creighton, 1984). The divergence in NPY is only 5.5% for the mature peptide and 27% for the prepro-peptide. This evidence suggests that along with the conservation of sequence, common properties relating to function may also be conserved.

Several studies have ascertained the existence of an NPY-like molecule and have used synthetic NPY to elicit responses in amphibians. One such study carried out by Danger et al. (1985) demonstrated that NPY elicited α-MSH release from the pituitary. Subsequent studies using *Xenopus* further suggested the possibility of an NPY-like molecule. Verburg-Van Kemenade et al. (1987) showed that NPY immunoreactivity is pre-

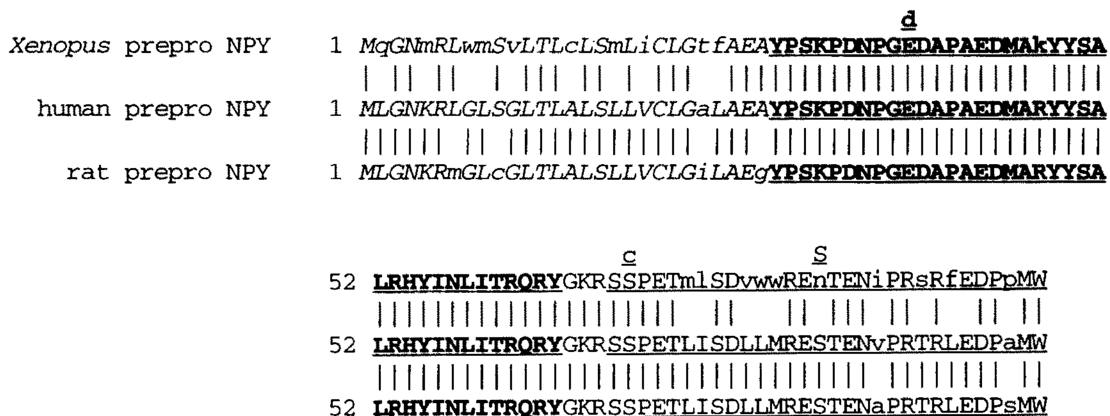

Fig. 5. Comparison of the *Xenopus*, human and rat prepro-NPY peptides with an alignment of the amino acid sequences. Portions of the sequences are identified as follows: signal sequence (*italics*), mature NPY (bold and underlined), C-terminal peptide (underlined). The deduced amino acid sequence for the Xenopus cDNA clones pXNPY-8 (top) and pXNPY-1 (bottom) are shown.

sent in the hypothalamus and pituitary of *Xenopus laevis*. They also showed that perfusion of isolated neurointermediate lobe tissue with NPY inhibited the release of α-MSH and other pro-opiomelanocortin derived peptides, apparently by blocking their secretion. De Rijk et al. (1991) subsequently have shown that labeled NPY specifically binds to a subset of cells in the neurointermediate lobe of *Xenopus* (folliculo-stellate cells), and that these cells may be a required intermediate for NPY's action on α-MSH release. The results of our study, along with those of McKay et al. (1992), further affirms the validity of the previous NPY studies in amphibians, and demonstrates that the messenger RNA for an NPY-like peptide exists in *Xenopus laevis*.

The distribution of NPY in *Xenopus* may be even more restricted than that seen in rats. The only detectable message in *Xenopus* was found in adult brain, while rat studies have also detected it in varying amounts in heart, ovary, spleen, thymus, kidney, lung, bone marrow and adrenal gland, in addition to the brain (Ericsson et al., 1987; Higuchi et al., 1988). Notably, the only definite non-neuronal expression of NPY has been shown in rat megakaryocytes (Ericsson et al., 1987). The inability to detect the NPY message in non-brain samples in *Xenopus* may be due to lower expression of NPY in peripheral locations and/or a more restricted expression in neural subtypes. Developmentally, the message for NPY was not detected in whole embryos at stages 20 and 45 by Northern blot analysis (Fig. 6), or by RNase protection in embryos at stages 26, 32, and 46 (data not shown). Neural development in *Xenopus* is well underway in stage 20 embryos. Neural folds are in the process of fusing, certain brain subdivisions are becoming apparent, and the eyes are beginning to protrude (Nieuwkoop and Faber, 1967). By stage 45, circulation of blood to the gills is beginning, sense organ structures are becoming organized, and motor activity, which begins at stage 24, is well established (Nieuwkoop and Faber, 1967). This pattern of developmental regulation of NPY is similar to that found in rat. In rat, the mRNA for NPY was shown to be detectable only in late embryogenesis (Larhammar et al., 1987).

In Northern blots of RNA isolated from *Xenopus* tissues, larger molecular weight RNA's hybridized to the NPY cDNA sequences. The detection of larger forms of NPY mRNA is also consistent with results from rat (Larhammar et al., 1987). Analysis of these anomalous bands in rat indicated that they were not due to incomplete processing of the message (Larhammar et al., 1987). Thus, it is currently not apparent what these larger RNA species represent.

The sequencing of the upstream region of the *Xenopus* NPY gene allows comparison with previously characterized *cis*-acting elements present in the human and

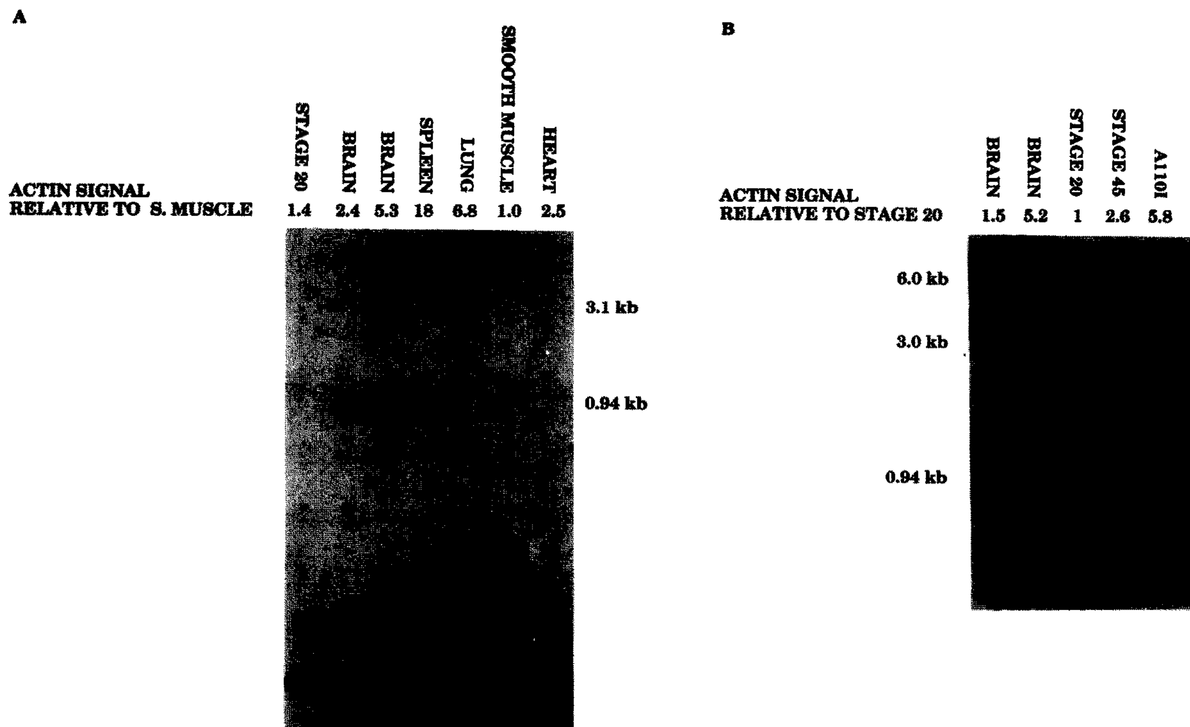

Fig. 6. Northern analysis of the *Xenopus* NPY transcript. Both blots were hybridized with the pXNPY-1 sequence. Blots were subsequently stripped by boiling and reprobed with a *Xenopus* actin probe and the actin hybridization was quantified by densitometry. (A) Northern blot using 10 μg (5 and 10 μg from brain) of total *Xenopus laevis* RNA from various tissues. (B) Northern blot using 10 μg (5 and 10 μg from brain) of total *Xenopus laevis* RNA from brain, stage 20, and stage 45 *Xenopus* embryos (Nieuwkoop and Faber, 1967) and a *Xenopus laevis* cell line (A110I).

rat 5′-flanking sequences. Four well characterized *cis*-elements can be found in the *Xenopus* NPY promoter region reported here. The TATA box (−32) is divergent from the consensus sequence, TATAA, but matches the human and rat NPY promoter sequences exactly. Previous work has shown that *Xenopus* cellular extracts contain proteins which can specifically bind an Sp1-like element (Ben-Hattar et al., 1989). The proximal Sp1-like region of the *Xenopus* NPY promoter (CCCCTCC, −50 to −56 with respect to the transcription initiation site) also is completely conserved in both human (−51 to −57) and rat (−51 to −57) NPY genes. Studies of the human NPY promoter indicate that the proximal Sp1-like element (−51) may be important for transcription, and that an Sp1-like protein binds to the −51 element in the human gene (Minth and Dixon, 1990). Lastly, the *Xenopus* NPY gene upstream region contains two other recognizable *cis*-acting elements, a CAAT box (−69) and an AP1-like element (TGACTCA) (−65). Proteins which bind CAAT-like elements have been detected in *Xenopus* by several groups (Tafuri and Wolffe, 1990; Ben-Hattar et al., 1989). Studies have also identified a fos homologue in *Xenopus* which binds an AP1 element and activates transcription when heterodimerized with murine jun (Kindy and Verma, 1990). These two elements have also been identified in the human upstream sequences. The presence of consensus sequences for common transcription factors in the *Xenopus* NPY gene upstream sequences, along with the similarities existing between these sequences and those for the human NPY gene, indicate regions which may be important for efficient transcriptional regulation of the *Xenopus* NPY gene.

This study has identified the *Xenopus* homolog of NPY. These results further validate the applicability of previous studies in amphibians, where NPY is seemingly present and active. Analysis of the peptide encoded further illustrates the conservation of NPY over hundreds of millions of years of evolutionary time. This conservation indicates that amphibian experimental systems may provide a means to further elucidate NPY regulation and function.

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