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# Strong evolutionary conservation of neuropeptide Y: Sequences of chicken, goldfish, and *Torpedo marmorata* DNA clones

(gene/cDNA/ray/pancreatic polypeptide/peptide YY)

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ABSTRACT **Neuropeptide Y (NPY) is an abundant and widespread neuropeptide in the nervous system of mammals. NPY belongs to a family of 36-amino acid peptides that also includes pancreatic polypeptide and the endocrine gut peptide YY as well as the fish pancreatic peptide Y. To study the evolution of this peptide family, we have isolated clones encoding NPY from central nervous system cDNA libraries of chicken, goldfish, and the ray *Torpedo marmorata*, as well as from a chicken genomic library. The predicted chicken NPY amino acid sequence differs from that of rat at only one position. The goldfish sequence differs at five positions and shows that bony fishes have a true NPY peptide in addition to their pancreatic peptide Y. The *Torpedo* sequence differs from that of rat at three positions. As *Torpedo* NPY has no unique positions when compared with the other sequences, it seems to be identical to the NPY of the common ancestor of cartilaginous fishes, bony fishes, and tetrapods after 420 million years of evolution. The 30-amino acid carboxyl-terminal extension of the NPY precursor also displays considerable sequence conservation. These results show that NPY is one of the most highly conserved neuroendocrine peptides.**

Neuropeptide Y (NPY) was first purified from porcine brain (1). The name is derived from its amino- and carboxyl-terminal tyrosines (single-letter code Y). Subsequently, NPY sequences have been determined for six other mammals (2) and for a frog (3), revealing a remarkable degree of conservation. NPY occurs abundantly in the mammalian central nervous system as well as in the peripheral nervous system (4) and has been shown to have both pre- and postsynaptic actions (5).

NPY belongs to a family of peptides that includes the gut endocrine peptide YY (PYY) and the pancreatic endocrine peptides called pancreatic polypeptide (PP) in tetrapods and peptide tyrosine (PY) in fish. All of these peptides consist of 36 amino acids and have a carboxyl-terminal amide. Porcine NPY and PYY show 70% sequence identity and can act on the same receptors with similar potencies (6), whereas porcine PP is only 50% identical to NPY and PYY and appears to have distinct receptors (7–9). PP differs considerably between species; PP of pig, chicken, and bullfrog share only 44–58% identity.

Pancreatic peptides have been isolated and sequenced from four different species of fish (2). Surprisingly, these peptides were found to be more similar to mammalian NPY and PYY than to PP. Because of the unclear relationships with the mammalian peptides, the fish pancreatic peptides have been given different names by different groups of investigators. We use here the designation PY.

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Immunohistochemical studies of NPY, PYY, and PP have been interpreted to indicate that PP appeared first in evolution (10), and sequence and receptor-binding analyses have suggested that PP forms one evolutionary lineage whereas NPY and PYY form a separate lineage and diverged from each other more recently (11). To extend the structural evolutionary analyses, we decided to isolate DNA clones from species representing the major branches of the vertebrate tree. We present here NPY sequences from three distantly related vertebrates which show that NPY has remained largely unchanged during the major part of vertebrate evolution and therefore is likely to be ancestral to PP.§

## MATERIALS AND METHODS

**Screening of Chicken Genomic Libraries.** A chicken genomic library in λ phage Charon 28, kindly provided by B. Vennström and L. Frykberg (Karolinska Institute, Stockholm) (12), was screened with a $^{32}$P-labeled 287-base-pair (bp) *Xba* I–*Ava* I fragment containing exon 2 of the rat NPY gene (13). Hybridization was carried out for 16 hr at 42°C in 25% formamide/1 M NaCl/10% dextran sulfate/1% SDS containing salmon sperm DNA at 0.1 mg/ml. Filters were washed twice for 5 min at room temperature in 2× SSC/0.2% SDS (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0) and twice for 30 min at 42°C in 2× SSC/0.5% SDS. The same set of filters was screened with two "walking probes" isolated from the 3′ end of the phage insert (Fig. 1). Hybridizations and the first two washes were performed as above. The two final washes were carried out for 45 min each at 52°C in 0.2× SSC/0.5% SDS. A chicken genomic library in λFix (Stratagene), kindly provided by M. Groudine and K. Conklin (Hutchinson Cancer Research Center, Seattle), was screened with a fragment containing exon 2 of the chicken NPY gene (Fig. 1). Hybridization was carried out as above except that the formamide concentration was 50%. The two final washes were for 30 min each at 60°C in 0.2× SSC/0.5% SDS.

**Screening of Chicken (*Gallus gallus*) cDNA Library.** A chicken brain cDNA library in λgt10 (Clontech) was screened with a fragment containing exon 2 of the chicken NPY gene (Fig. 1). Hybridization was performed as described above for the Charon 28 screening. Filters were washed twice for 5 min at room temperature in 2× SSC/0.2% SDS and twice for 30 min at 60°C in 0.2× SSC/0.5% SDS.

**Screening of Goldfish (*Carassius auratus*) cDNA Library.** A cDNA library in λgt11, prepared from mRNA of regenerating retina and kindly provided by N. Schechter (State University

Abbreviations: NPY, neuropeptide Y; PYY, peptide YY; PY, peptide tyrosine; PP, pancreatic polypeptide; CPON, carboxyl-terminal peptide of NPY.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M87294–M87298).

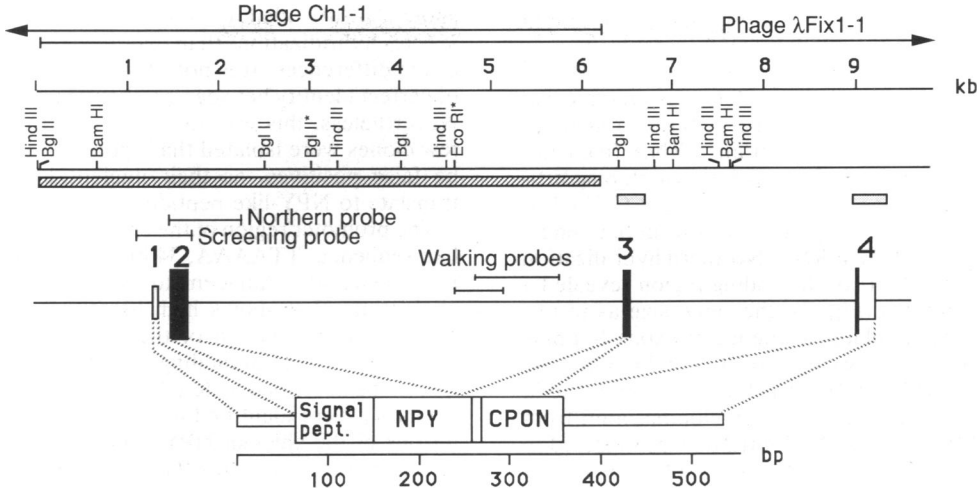


FIG. 1. Chicken NPY gene. Hatched box shows the sequenced part of the NPY gene from the Charon 28 library, and the stippled boxes show the regions sequenced in the λFIX library. The four exons are shown as boxes numbered 1–4. CPON, *c*arboxyl-terminal *p*eptide *o*f *N*PY. Stars mark two restriction sites that are absent in inserts from the λFIX library, thus reflecting allelic polymorphism (also observed for rat NPY; see ref. 12). Arrows indicate the directions in which the different phage inserts extend. kb, Kilobases.

of New York, Stony Brook), was screened using a 540-bp *Pst* I–*Hin*dIII cDNA fragment containing the entire coding region of chicken NPY. Hybridization was carried out for 16 hr at 42°C in 25% formamide/5× SSC/50 mM sodium phosphate, pH 7.0/10% dextran sulfate/0.1% SDS containing salmon sperm DNA at 0.1 mg/ml. Filters were washed twice for 5 min at room temperature and twice for 30 min at 42°C in 2× SSC/0.1% SDS. The library was rescreened with a 425-bp *Sph* I–*Nco* I goldfish NPY cDNA fragment under the same conditions as described below for *Torpedo*.

**Screening of *Torpedo marmorata* cDNA Library.** A *Torpedo* optic lobe cDNA library in λZAPII (Stratagene), kindly provided by S. Birman (14), was screened using an 850-bp *Hin*dIII fragment containing exon 2 of an NPY gene previously isolated from a shark (*Heterodontus francisci*) library (unpublished work). Hybridization was carried out for 16 hr at 42°C in 25% formamide/6× SSC/10% dextran sulfate/0.1% SDS/5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin fraction V). No salmon sperm DNA was used. Filters were washed twice for 5 min at room temperature and twice for 30 min at 42°C in 2× SSC/0.1% SDS.

**DNA Sequencing.** Sequence determinations were performed with chemical degradation (15) for most of the chicken gene and with dideoxy chain termination (16) for exons 3 and 4 of the chicken gene and for the cDNA clones. The chicken genomic sequence was partially determined on an automated fluorescent-dye DNA sequencer (Pharmacia), as were parts of the *Torpedo* sequence (Applied Biosystems).

**Northern Blots.** mRNA purification and filter preparation and hybridization were as described (12) except that washes were done at 42°C. The probe was a fragment containing exon 2 of the chicken NPY gene (Fig. 1).

## RESULTS

**Isolation of Chicken NPY Clones.** Three identical chicken genomic clones were isolated with a rat NPY exon 2 probe. A segment corresponding to rat exon 2 was identified by DNA sequencing. Additional exons were sought by hybridization with rat and human probes and by DNA sequencing to the 3′ end of the phage insert but could not be found. Walking probes (Fig. 1) were used to rescreen the library but yielded no additional clones.

To determine the chicken prepro-NPY sequence, a brain cDNA library was screened with a chicken exon 2 probe (Fig. 1). Five of six isolated clones had indistinguishable insert sizes. One of these, clone CH11-1, was sequenced and found to contain the complete coding region of prepro-NPY (Fig. 2). A segment of 34 bp immediately 5′ to the coding region in the cDNA clone was identical to a genomic segment located 134 bp 5′ to exon 2 (Fig. 1). Thus, this segment encodes exon 1, in analogy with the human and rat NPY genes.

Surprisingly, the 286 bp at the extreme 5′ end of the cDNA insert could not be found adjacent to exon 1 in the genomic sequence and did not hybridize to the genomic clone, although this clone extends 12 kb 5′ to exon 1. However, it did hybridize to chicken genomic DNA but was undetectable in brain mRNA (data not shown). Presumably, this part of the cDNA insert does not correspond to NPY mRNA but was misincorporated during the cloning procedure although it is not separated from the true NPY insert by an *Eco*RI linker.

The 3′ part of the cDNA insert contains a polyadenylylation signal followed by an additional 0.6 kb. To identify the 3′ end of the NPY gene and mRNA, another genomic library (in λFIX) was screened with a chicken NPY probe. Five phage clones were found that contained exons 3 and 4. These exons were sequenced for assignment of exon boundaries. Like the rat and human NPY genes, the chicken NPY gene consists of four exons and three introns. All three introns in the chicken gene appear at the same positions as in the rat and human genes. Introns 2 and 3 are somewhat larger in the chicken gene than in the mammalian genes, whereas intron 1 is considerably smaller in the chicken gene. The chicken gene, whose total size is ≈8 kb, is larger than the mammalian

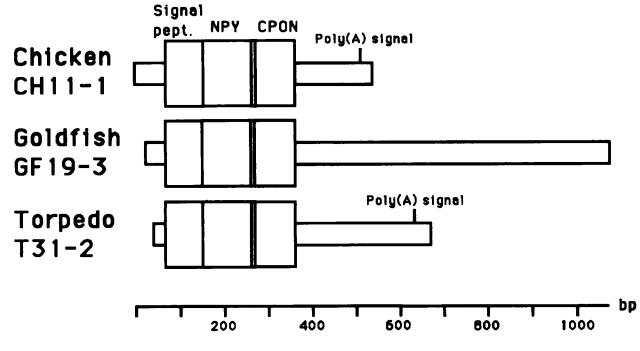


FIG. 2. cDNA clones for NPY from chicken, goldfish, and *Torpedo*.

genes. In all, 5.5 kb of the chicken gene were sequenced in addition to 1.5 kb of the 5′ flanking region (see Fig. 1).

The 0.6-kb segment 3′ to the polyadenylylation signal of the cDNA clone CH11-1 did not match the genomic sequence. Like the 5′ end, this segment did hybridize to chicken genomic DNA but not to brain mRNA (data not shown) and therefore most likely constitutes a cloning artifact. The sixth cDNA clone was dissimilar from CH11-1 at both ends and also had unidentified extensions at both ends.

**Expression of Chicken NPY mRNA.** Northern hybridization with a probe containing part of the coding region revealed a band of ≈800 nucleotides (Fig. 3), the same size as in rat, which fits with the accumulated sequence of exons 1–4 plus $(A)_{\approx 200}$ without the flanking extensions (0.3 and 0.6 kb). As expected, abundant NPY mRNA was found in cortex and in total brain and could be detected in developing brain from embryonic day 12 (Fig. 3). No NPY mRNA was detected in liver, spleen, or heart. The band at higher molecular weight visible in the autoradiogram most likely represents cross-hybridization to rRNA, as it (*i*) comigrates with 28S rRNA, (*ii*) is present in all lanes, and (*iii*) is detected also in rat mRNA although a rat NPY probe does not cross-hybridize to this band (13) (if this larger RNA were NPY-related, it should have been detected in rat mRNA also by the rat NPY probe). Further a chicken exon 4 probe detects only the 800-nucleotide band in adult chicken brain mRNA. The weak band visible at intermediate size in most lanes comigrates with 18S rRNA.

**Isolation of Goldfish NPY Clones.** Four clones were isolated with a chicken NPY probe. The clone GF19-3 was sequenced and found to contain an insert of 1065 bp encoding the entire prepro-NPY sequence. This is in rough agreement with the observed mRNA size of ≈1000 nucleotides (Chun Peng and Richard E. Peter, personal communication), although no poly(A) signal is present near the 3′ end of the insert. Rescreening with a probe from this clone yielded several additional clones, which all had insert sizes indistinguishable from that of clone GF19-3.

**Isolation of *Torpedo* NPY Clones.** Screening with a shark NPY exon 2 probe yielded three clones. The clone T31-2 was sequenced and found to contain an insert of 641 bp encoding the entire prepro-NPY sequence (Fig. 2). A poly(A) signal is present near the 3′ end of the insert. Clone T12-5 was identical to T31-2 except in the 5′ untranslated region, which was completely different. The clone T41-1 was truncated in the coding region and may correspond to either of the two previous clones.

**Nucleotide Sequence Analyses.** The nucleotide sequences encoding mature NPY of rat, chicken, goldfish, and *Torpedo* show 74–90% identity. Of greater importance for cross-species hybridizations than overall identity is the distribution of the differences. It is noteworthy that the longest segment of perfect identity between chicken and goldfish is only 17 bp. Nevertheless, this was sufficient for cross-hybridization. A few clones were isolated that upon sequencing turned out to be false positives, as their nucleotide sequences did not translate to NPY-like peptides.

The promoter region of the chicken gene contains a TATA-like sequence, TTAAAA, 34 bp 5′ to a consensus transcriptional start site. Adjacent to this segment is a block of 30 nucleotides that shows high identity to a sequence in the corresponding position of the rat and human NPY genes, including a potential binding site for the transcription factor Sp1 (to be described in detail elsewhere). No other regions of similarity were identified in the promoter regions. All three introns of the chicken NPY gene agree with the consensus sequence for splice sites (GT/AG).

**Amino Acid Sequence Analyses.** The amino acid sequences of prepro-NPY are shown in Fig. 4. The mature NPY sequences display extensive sequence identity. Also, the sequences of the 30-residue carboxyl-terminal extension of the NPY precursor, called CPON, can be aligned (Figs. 4 and 5). The signal peptides have diverged considerably but are still identical in length except for the rat sequence, which has one additional methionine codon (Fig. 4).

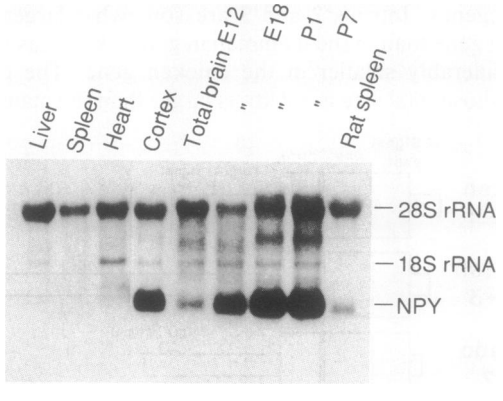


FIG. 3. Northern blot of various chicken tissues and rat spleen probed with a chicken NPY exon 2 fragment (see Fig. 1). Each lane contained 20 μg of poly(A)$^+$ RNA except for the rat spleen lane, which contained 30 μg of total RNA. E, embryonic day; P, postnatal day.

## DISCUSSION

NPY amino acid sequences have been reported for human, rat, guinea pig, rabbit, pig, cow, and sheep (2) as well as for the frog *Rana ridibunda* (3) under the name melanotropin-release-inhibiting factor (melanostatin). Only two positions vary among the mammals, and both of these involve highly conservative amino acid replacements—i.e., Glu-10 (Asp in sheep) and Met-17 (Leu in the three artiodactyls). The NPY sequences of chicken, goldfish, and *Torpedo* further emphasize this striking degree of conservation, as they differ from the rat sequence at only one, five, and three positions, respectively. The high level of identity between mammals and cartilaginous fishes, 93%, makes NPY one of the most highly conserved neuroendocrine peptides known. Over a similar evolutionary distance, glucagon shows 86% identity, vasoactive intestinal peptide, 82%; corticotropin, 72%; insulin, 71%; and relaxin, 41% (see ref. 2 for review).

The *Torpedo* NPY sequence has no unique positions as compared with the other sequences; i.e., each position is identical to at least one other NPY sequence (Fig. 4). This suggests that *Torpedo* NPY has remained unaltered since cartilaginous fishes diverged from the other vertebrates (Fig. 6). Analogously, the frog NPY sequence has probably remained constant since amphibians diverged from the tetrapod ancestor. In comparison with *Torpedo* NPY, the chicken sequence is the most divergent with its four replacements, whereas goldfish has two and the mammals have two or three replacements. This comparison also indicates that position 17 has switched between methionine and leucine on at least two occasions during vertebrate evolution.

All positions in the carboxyl-terminal part of NPY, which have been found to be important for receptor binding (18, 19), have remained perfectly conserved in all 10 NPY sequences known. Six positions in the mature NPY sequences shown in Fig. 4 display variability, and five of these are occupied by either of two very similar amino acids. The only seemingly dramatic replacement occurs at position 11, where *Torpedo* and goldfish have a polar amino acid, glycine, and all other sequences have a negatively charged amino acid, aspartate. Computer modeling with rat and pig NPY (20, 21) has shown that they are compatible with the three-dimensional structure of turkey PP determined by x-ray crystallography (22). We

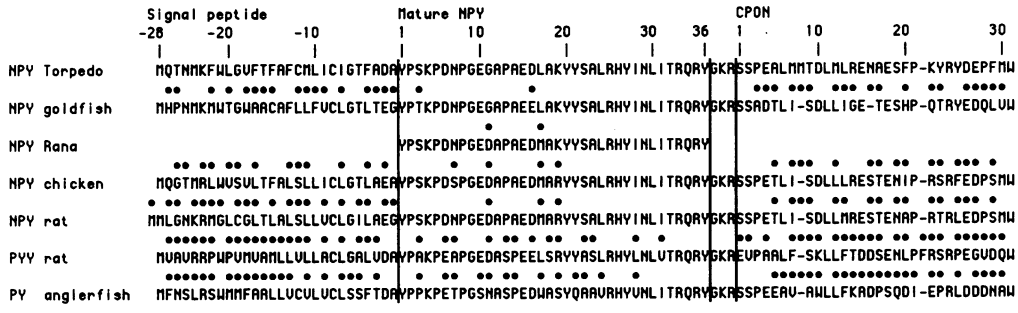


FIG. 4. Comparison of NPY, PYY, and PY prepro sequences. Dots indicate differences from the top (*Torpedo*) sequence. The NPY *Rana* sequence is the melanostatin sequence from the frog *Rana ridibunda* (3). See ref. 2 for other references.

have performed such analyses with the goldfish NPY sequence and found that it too should fit the turkey PP structure. Even the Gly-11 replacement appears compatible with a retained three-dimensional structure (alligator PP also has this replacement). However, the homodimer formation shown to occur in solution (23) may involve interactions between the Asp-11 negative side chain of one NPY subunit and the Lys-4 positive side chain of the other subunit. Thus, the replacement of Asp-11 with Gly is likely to weaken this interaction and may make NPY of goldfish and *Torpedo* less prone to form dimers. For example, the tendency of insulin to form multimers differs between species (22).

NPY-like immunoreactivity has been described in goldfish brain (24), and two NPY-like peptides have been reported to occur in goldfish retina (25). Anglerfish brain contains immunoreactivity distinct from the fish pancreatic peptide PY (26, 27) and trout brain displays immunoreactivity with an antiserum to NPY that exhibits no crossreaction with PYY (28). However, no sequence information has previously been reported for any fish central nervous system NPY-like peptide. Our goldfish sequence provides structural evidence that bony fishes have a true NPY in addition to PY. Our clone is likely to correspond to NPY rather than PY for several reasons: (*i*) it shows higher sequence identity to mammalian NPY than to fish PY (Fig. 4); (*ii*) it shows higher sequence identity to mammalian NPY than do any of the fish PY sequences (2); (*iii*) its carboxyl-terminal extension has considerably higher identity to mammalian CPON (60%) than to the anglerfish PY extension (23%) (Fig. 5); (*iv*) its carboxyl-terminal extension has higher identity to mammalian CPON than has the anglerfish PY extension (33%) (Fig. 5); and (*v*) the goldfish clone was derived from a neuronal source, retina.

The carboxyl-terminal extension of prepro-NPY (CPON) shows a surprising degree of sequence conservation. Chicken and rat CPON sequences are identical at 26 out of 30 positions (87%), and *Torpedo* and rat CPON sequences share 14 positions (47%). This degree of identity matches that of calcitonin and relaxin over similar evolutionary distances (2). The conservation of CPON may indicate that it, too, could

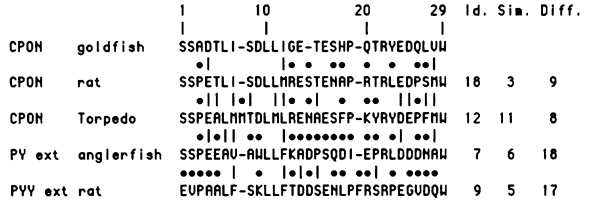


FIG. 5. Amino acid sequence comparisons of propeptide extensions. Dots indicate differences from the top (goldfish) sequence, and vertical bars indicate conservative amino acid replacements. The total numbers of identical, similar, and different amino acids are given at right. See ref. 2 for references. The sequence of the anglerfish PY extension is from Peter Hobart (personal communication).

have a function, possibly as a neurotransmitter. Indeed, preliminary binding studies with synthetic human CPON, although hampered by difficulties in radiolabeling the peptide, have indicated some degree of binding activity in rat brain extracts (C. Wahlestedt, personal communication).

Because goldfish are tetraploid (29), we investigated the number of NPY genes by Southern hybridization. Genomic DNA from three individuals of common goldfish was analyzed with a single-exon probe corresponding to the 3′ untranslated region (exon 4). This probe was expected to detect two to four bands depending on whether allelic polymorphism occurred at neither, only one, or both of the two presumed loci. Each individual was found to display two to five bands depending on the restriction enzyme used (data not shown). This is compatible with two NPY loci where both may be polymorphic. The fifth band observed for one enzyme may be due to an internal site within exon 4. It is unknown whether more than one locus is expressed and whether the polymorphism involves positions in coding or regulatory regions of the genes. All cDNA clones isolated so far show identical insert sizes. We assume that the goldfish sequence presented here encodes a functional NPY mRNA, as its predicted NPY differs from the ancestral *Torpedo* sequence at only two positions, 3 and 16, and both of these replacements are of highly conservative nature. Furthermore, Glu-16 occurs in PYY and PY sequences (2). Chicken has a single NPY gene like human and rat (2). *Torpedo* most likely has a single NPY gene, too, as several genomic clones isolated from a different cartilaginous fish, the horned shark *Heterodontus francisci*, seem to correspond to a single gene.

The finding of NPY clones in the *Torpedo* library was somewhat unexpected, as the source of the mRNA was the electric lobe, a brain region that contains primarily cholinergic neurons innervating the electric organ. Perhaps NPY

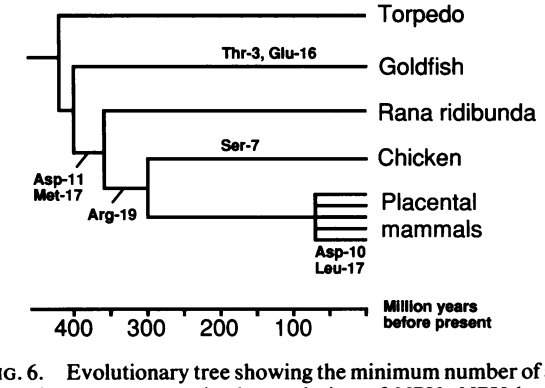


FIG. 6. Evolutionary tree showing the minimum number of amino acid replacement events in the evolution of NPY. NPY has been sequenced in five orders of mammals. The Leu-17 replacement is found in all three artiodactyls. The Asp-10 replacement is found only in sheep. Divergence times were taken from ref. 17 and M. J. Benton (personal communication).

may coexist with acetylcholine (30) or, alternatively, occur in adjacent noncholinergic neurons.

Interestingly, two distinct *Torpedo* NPY clones were found that differ only in their 5′ untranslated regions. As this region is encoded by a separate exon in the NPY genes of rat, human, and chicken (see Fig. 1), and the remainder of the two clones was identical, it is likely that both clones were derived from one and the same gene through initiation from distinct promoters. It has been reported that rat NPY mRNA has three distinct sizes (31). Although the reason for this is unknown, it remains possible that the different rat mRNAs are derived from distinct transcriptional start sites.

Studies with antisera have indicated that PP-expressing cells appeared earlier in evolution than PYY- and NPY-immunoreactive cells (10). Furthermore, comparisons of mammalian NPY, PYY, and PP sequences and receptor-binding properties led to the suggestion that this peptide family consists of two distinct evolutionary lineages, one containing PP and one containing NPY and PYY (11), as NPY and PYY peptides are clearly more similar to each other than to PP. However, our results show that NPY has remained extremely well conserved during vertebrate evolution, whereas PP has accumulated numerous replacements over a similar period of time (2). Thus, the sequence diversity of the pancreatic peptides cannot be taken as evidence for a greater evolutionary age. Rather, we suggest that the PP gene, after it was formed by duplication of the NPY or PYY gene, was allowed to accumulate mutations much faster than the NPY and PYY genes because the latter were locked in maintaining their already established functions. An NPY-like peptide from the flatworm *Moniezia expansa* has recently been sequenced, suggesting that NPY-like peptides are of great evolutionary age (32).

**Note Added in Proof.** The gap in front of exon 3 has been sequenced in phage λFIX1-1.

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1. Tatemoto, K. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5485–5489.
2. Larhammar, D., Söderberg, C. & Blomqvist, A. G. (1992) in *The Neurobiology of Neuropeptide Y and Related Peptides, Contemporary Neuroscience Series*, eds. Wahlestedt, C. & Colmers, W. F. (Humana, Clifton, NJ), in press.
3. Chartrel, N., Conlon, J. M., Danger, J.-M., Fournier, A., Tonon, N.-C. & Vaudry, H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3862–3866.
4. Lehmann, J. (1990) *Drug Dev. Res.* **19,** 329–351.
5. Wahlestedt, C., Grundemar, L., Håkanson, R., Heilig, M., Shen, G. H., Zukowska-Grojec, Z. & Reis, D. J. (1990) *Ann. N.Y. Acad. Sci.* **611,** 7–26.
6. Wahlestedt, C., Yanaihara, N. & Håkanson, R. (1986) *Regul. Peptides* **13,** 307–318.
7. Gilbert, W. R., Frank, B. H., Gavin, J. R., III, & Gingerich, R. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4745–4749.
8. Schwartz, T. W., Sheikh, S. P. & O'Hare, M. M. T. (1987) *FEBS Lett.* **225,** 209–214.
9. Jørgensen, J. C., Fuhlendorff, J. & Schwartz, T. W. (1990) *Eur. J. Pharmacol.* **186,** 105–114.
10. El-Salhy, M., Grimelius, L., Emson, P. C. & Falkmer, S. (1987) *Histochem. J.* **19,** 111–117.
11. Schwartz, T. W., Fuhlendorff, J., Langeland, N., Thøgersen, H., Jørgensen, J. C. & Sheikh, S. P. (1989) in *Neuropeptide Y*, eds. Mutt, V., Fuxe, K., Hökfelt, T. & Lundberg, J. M. (Raven, New York), pp. 143–151.
12. Ebendal, T., Larhammar, D. & Persson, H. (1986) *EMBO J.* **5,** 1483–1487.
13. Larhammar, D., Ericsson, A. & Persson, H. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2068–2072.
14. Birman, S., Meunier, F.-M., Lesbats, B., Le Caer, J.-P., Rossier, J. & Israël, M. (1990) *FEBS Lett.* **261,** 303–306.
15. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
17. Benton, M. J. (1990) *Vertebrate Palaeontology* (Hyman, London).
18. Fuhlendorff, J., Gether, U., Aakerlund, L., Johansen, N. L., Thøgersen, H., Melberg, S. G., Olsen, U. B., Thastrup, O. & Schwartz, T. W. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 182–186.
19. Krstenansky, J. L., Owen, T. J., Buck, S. H., Hagaman, K. A. & McLean, L. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4377–4381.
20. Allen, J., Novotny, J., Martin, J. & Heinrich, G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2532–2536.
21. MacKerell, A. D., Jr., Hemsén, A., Lacroix, J. S. & Lundberg, J. M. (1989) *Regul. Peptides* **25,** 295–313.
22. Blundell, T. & Wood, S. (1982) *Annu. Rev. Biochem.* **51,** 123–154.
23. Noelken, M. E., Chang, P. J. & Kimmel, J. R. (1980) *Biochemistry* **19,** 1838–1843.
24. Pontet, A., Danger, J. M., Dubourg, P., Pelletier, G., Vaudry, H., Calas, A. & Kah, O. (1989) *Cell Tissue Res.* **255,** 529–538.
25. Osborne, N. N., Patel, S., Terenghi, G., Allen, J. M., Polak, J. M. & Bloom, S. R. (1985) *Cell Tissue Res.* **241,** 651–656.
26. Milgram, S. L., Balasubramaniam, A., Andrews, P. C., McDonald, J. K. & Noe, B. D. (1989) *Peptides* **10,** 1013–1017.
27. Noe, B. D., Milgram, S. L., Balasubramaniam, A., Andrews, P. C., Calka, J. & McDonald, J. K. (1989) *Cell Tissue Res.* **257,** 303–311.
28. Danger, J.-M., Breton, B., Vallarino, M., Fournier, A., Pelletier, G. & Vaudry, H. (1991) *Endocrinology* **128,** 2360–2368.
29. Ohno, S., Wolf, U. & Atkin, N. B. (1968) *Hereditas* **59,** 169–187.
30. Leblanc, G. G., Trimmer, B. A. & Landis, S. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3511–3515.
31. Ericsson, A., Hemsén, A., Lundberg, J. M. & Persson, H. (1991) *Exp. Cell Res.* **192,** 604–611.
32. Maule, A. G., Shaw, C., Halton, D. W., Thim, L., Johnston, C. F., Fairweather, I. & Buchanan, K. D. (1991) *Parasitology* **102,** 309–316.