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Tissue-specific expression of the human neuropeptide Y gene in transgenic mice

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Neuropeptide Y (NPY) is the most abundant neuropeptide detected in the mammalian brain, and is found throughout the central and peripheral nervous systems. This peptide is a proposed regulator of appetite, blood pressure, and pituitary hormone release Previous experiments have demonstrated the ability of 5′ sequences within the human NPY gene to promote transcription in cultured neuronal cells. To identify sequences in this gene that regulate tissue-specific expression, a NPY/CAT fusion gene, containing approximately 850 bp of NPY sequences, was microinjected into fertilized mouse ova. Five lines of transgenic mice were derived from these ova and several tissues from mice of each line were tested for transgene expression using the CAT assay. One line demonstrated X-chromosome-linked transmission of the transgene while the other lines demonstrated autosomally-linked transmission. Three lines demonstrated transgene expression with significant levels of CAT activity detectable only in tissues which have been shown to express endogenous NPY. One autosomally-linked line did not demonstrate significant levels of transgene activity because the transgene appeared to have undergone structural alteration during genomic integration. No transgene activity was detected in either male of female mice from the X-linked line, suggesting a positional regulation of the transgene locus other than X-inactivation in this line The present research demonstrated the NPY regulatory sequences included in pCATNPYΔ796 sufficiently directed tissue-appropriate gene expression in transgenic mice

INTRODUCTION

Neuropeptide Y (NPY) is a 36 amino acid peptide first isolated from the porcine brain by Tatemoto et al.[27]. It is the most abundant neuropeptide detected in the mammalian brain. The highest levels of immunoreactive NPY have been detected in the cerebral cortex, hypothalamus, and limbic regions, while lower levels exist in the brain stem and spinal cord[1,2]. Outside the central nervous system, highest levels of NPY immunoreactivity have been detected in the adrenal medulla[28], and NPY is the major neuropeptide found in the heart[10]. Immunoreactive NPY is also found in neuronal elements of several other mammalian tissues including the eye, thyroid gland, pancreas, ovary, and kidney[9,13,26].

Due to its presence in a variety of tissues, proposed physiological roles for NPY are that of neurohormone, neuromodulator or neurotransmitter[15]. The most prominent effect of NPY is a potent stimulation of appetite when injected into the brain[7,19,22,24]. Neuropeptide Y is also involved in the regulation of blood pressure[9,13] and the release of pituitary hormones[14].

The human NPY gene, over 8 kilobases in length, includes four exons and encodes a pre-prohormone of 97 amino acids[16,17]. When fused with the gene encoding bacterial chloramphenicol acetyltransferase (CAT)[3], NPY promotor DNA can direct transcription of the CAT gene in cultured neuronal cells[18]. While these experiments are valuable for identifying *cis*-acting sequences and *trans*-acting factors which promote transcription, they are limited to specific cell-lines which are currently available.

In order to examine the regulation of the NPY gene in several tissues, 5′ non-transcribed DNA and 51 bp of exon 1 from the human NPY gene were fused upstream of the CAT gene. The construct pCATNPYΔ796, which contained sequences 796 bp upstream of the NPY transcriptional initiation site, was introduced into the mouse

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genome by pronuclear microinjection. Tissues from NPY/CAT transgenic mice were then analyzed for activity of the transgene

MATERIALS AND METHODS

Preparation of NPY/CAT construct

The plasmid pCATNPYΔ796 was constructed as previously described[18] The linearized DNA construct was isolated by agarose gel electrophoresis, purified by phenol-chloroform extraction, ethanol precipitated, and resuspended in TE buffer (10 mM Tris [pH 7 4], 1 mM EDTA) For microinjection, the DNA was diluted to 1–3 μg/ml in microinjection buffer (10 mM Tris [pH 7 4], 0 2 mM EDTA) and passed through a 0 45 μ Millipore syringe filter

Production of transgenic mice

Transgenic mice were produced by the method of pronuclear microinjection[4 12] Approximately 1–2 pl of DNA solution (1000 copies) were microinjected into one pronucleus of each (C57Bl/6J × SJL/J) F2 embryo Embryos were cultured overnight in BMOC-3 medium, and two-cell-stage embryos were transferred to the oviducts of 0 5 day post-coitus pseudopregnant ICR females mated with vasectomized ICR males Pups resulting from microinjected embryos were weaned at 19–21 days of age and analyzed for incorporation of the transgene All procedures involving husbandry, production, and analysis of transgene mice were approved by the Purdue Animal Care and Use Committee (Protocol No 87-122)

Identification of transgenic mice

Mouse genomic DNA was isolated from a tail tip biopsy by proteinase K-SDS digestion and phenol-chloroform extraction The DNA was precipitated with ethanol, resuspended in TE buffer, and stored at 4°C

To identify transgenic mice via PCR amplification, two oligonucleotide primers, 5′-AACCAGACCGTTCAGCTGG-3′ (sense strand) and 5′-ATGGAAGCCATCACAGACG-3′ (antisense strand), were designed to amplify a 501 bp sequence specific to the CAT gene. Genomic DNA (approx 1 μg) was denatured at 100°C for 5 min, then added to 50 μl of the PCR reaction mixture The mix consisted of 1× PCR reaction buffer, 200 μM of each dNTP, 1 0 μM each primer, and 2 5 units *Taq* DNA polymerase (U S Biochemical Corp., Cleveland, OH) The amplification profile included denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and extension at 72°C for 2 min (with 5 s auto-extension per cycle) for 25 cycles Fifteen μl of the reaction mixture were loaded onto a 1 0% agarose gel and visualized after staining with ethidium bromide

Alternately, transgenic mice were identified via slot blot hybridization Genomic DNA was denatured and equal volumes of the solution were vacuum-blotted through a manifold onto two nitrocellulose filters (Minifold II, Schleicher and Scheull, Inc , Keene, NH) To detect transgene incorporation, one filter was probed with a 1 6 kb *Eco*RI–*Hin*dIII fragment from pUCCAT3[18] To verify the amount of DNA used in the assay, the duplicate filter was probed with the plasmid pGem3 β-actin, which contained the avian gene encoding cytoplasmic β-actin Each filter was pre-hybridized in 5× SSPE, 5× Denhart's solution, 200 μg/ml salmon sperm DNA, and 50% formamide at 42°C for at least 4 h The DNA probes were random-prime labelled with [^{32}P]dCTP (Multiprime DNA Labelling System RPN.1600Y, Amersham Corp., Arlington Heights, IL), denatured, and added to the filter at 1–5 × 10^6 cpm per ml hybridization fluid After hybridization for a minimum of 9 h at 42°C, the filters were washed 15 min at room temperature in 1× SSC, 0 1% SDS, followed by 2 × 30 min washes at 65°C in 0 1× SSC, 1 0% SDS The filters were exposed to Kodak X-Omat AR film with an intensifying screen for 2–3 days

The integrity of incorporated DNA was analyzed using Southern analysis[23] Genomic DNA (4 μg) from NPY/CAT mice was digested with either *Eco*RI or *Nci*I and electrophoresed on a 1% agarose gel Dilutions of the *Eco*RI–*Hin*dIII CAT fragment mixed with genomic DNA from non-transgenic mice were also loaded onto the gel for use in determining the number of transgene copies per locus After capillary transfer of the DNA to nitrocellulose, the filter was processed as above The filters were rehybridized with a radiolabeled probe for rat cholecystokinin using the conditions above Densitometric analysis of the autoradiograms was used to normalize the amount of DNA loaded on the gel and to produce a standard curve for determination of copy number

RNA preparation and analysis

Total RNA was prepared by the method of Chirgwin et al [6] Twelve μg of each RNA sample was denatured, split into two samples containing 1 and 10 μg, slotted onto nytran paper, and hybridized with a radiolabelled probe for either human 28S ribosomal RNA or human NPY The filters were prehybridized, hybridized, and washed as previously described with the following modification The temperature of the final wash for the filter probed with NPY was 55°C The filters hybridized with the NPY probe were exposed for 24 h at –80°C with an intensifying screen while those hybridized with the 28S probe were exposed for 2 5 h at room temperature The amount of NPY mRNA in each sample was quantitated by densitometric analysis The signal generated by the 28S probe was quantitated either by densitometric analysis or by scintillation counting The amount of NPY mRNA present in each sample was corrected for possible loading discrepancies by division with the signal generated by its 28S counterpart

CAT assay

In order to determine transgene activity, extracts were prepared from various tissues and analyzed using a modified CAT assay[8 20] Tissue samples were dissected from mice at 8 and 16 weeks of age and placed in a 1.5 ml microfuge tube containing 100 μl 0.25 mM Tris buffer [pH 8 0] Tissues were homogenized using a Teflon pestle fitted to the microfuge tube After addition of 400 μl Tris buffer, extracts were heated at 65°C for 10 min to inactivate inhibitory proteins The extracts were centrifuged for 30 s, and the supernatant was transferred to a new microfuge tube Protein concentrations were determined via the Bio-Rad Protein Assay (Bio-Rad Labs , Richmond, CA) using bovine serum albumin as the standard Up to 100 μg protein from each tissue were incubated overnight with 5 μCi [^{14}C]chloramphenicol (Amersham Corp., Arlington Heights, IL) and 4 mM Acetyl CoA at 37°C After extraction with ethyl acetate, the mixture was spotted onto TLC plates, and the acetylated choramphenicol species were separated by thin-layer chromatography in 95% choroform 5% methanol for 45 min The TLC plates were exposed to Kodak X-Omat AR film for 2 days After autoradiography, the non-acetylated and acetylated spots were identified, excised from the plate, and quantified by scintillation counting

The data from the CAT assays were adjusted to percent conversion per hour reaction per gram tissue and subjected to Bartlett's Test of Homogeneity of Variance[25] Homogeneity of variance required a transformation of the data by $1/Y$ for the Analysis of Variance (SAS GLM procedure) Significant means were identified using the Student–Newman–Keul's test.

RESULTS

In order to determine which segments of the human NPY gene confer tissue-specific regulation, a linear NPY-CAT gene construct, containing 796 bp of NPY 5′ regulatory DNA and 51 bp of exon 1 was injected into fertilized ova to produce transgenic mice (Fig. 1). Genomic DNA from the resulting pups was initially analyzed by either PCR amplification (Fig. 2) or slot blot hybridiza-

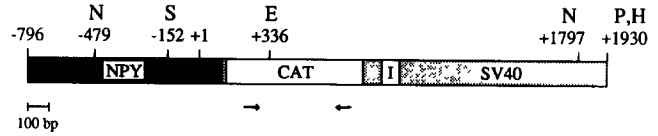

Fig 1 Schematic diagram of pCATNPYΔ796 This construct contains 796 bp of 5′ non-transcribed DNA and 51 bp of non-translated DNA from the human NPY gene (black box). These sequences are fused up-stream of the bacterial chloramphenicol acetyltransferase gene (open box) contained in the vector pUC-CAT3[18] The construct also contains the t-antigen intron and polyadenylation sites from SV40 virus (shaded box)[5] The *Eco*RI–*Hin*dIII restriction fragment was utilized as a probe for Southern and slot blot hybridizations The approximate position of the PCR primers and their direction of extension are indicated by arrows Restriction enzyme sites listed include *Nci*I (N), *Sal*I (S), *Eco*RI (E), *Pst*I (P), and *Hin*dIII (H).

tion to detect transgene incorporation. These techniques were also used to identify transgenic offspring of founder mice. Genomic DNA from non-transgenic mice did not specifically hybridize with the CAT-specific probe, nor was CAT-specific DNA amplified from these mice. Transgenic lines were established from five founder mice, and each line was designated according to the identity of the founder animal (Table I).

Southern analysis

Southern analysis of genomic DNA revealed varying integration patterns in each line (Fig. 3). The 10-1, 24-4, 24-9, and 30-2 lines all contained a 2.3 kb *Nci*I fragment which hybridized to the CAT probe (Fig. 3A). Analysis of *Eco*RI-digested DNA from these lines also revealed a 2.5 kb band that would correspond to an *Eco*RI fragment produced in a head-tail tandem array of a transgene concatamer (Fig. 3B). Thus, it appeared these four lines contained multiple copies of the intact transgene. Other bands at higher molecular weight were evident in the *Eco*RI digested DNA, and these could be due to restriction fragments produced at the junctions of the concatamer and genome, or to incomplete digestion. Only high-molecular weight restriction fragments from line 25-1 hybridized with the CAT probe.

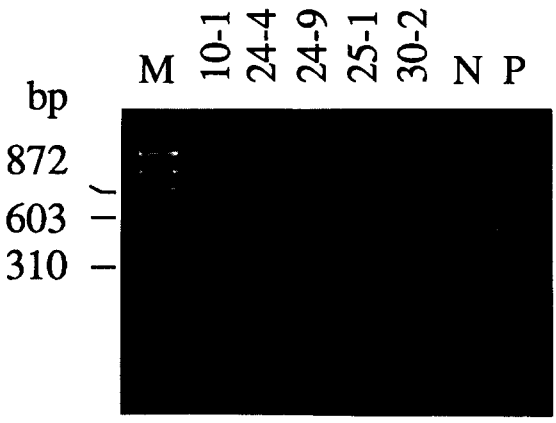

Fig. 2 PCR amplification of transgene specific DNA from mouse genomic DNA. Two 19-mer oligonucleotide primers were designed to amplify a 501 bp fragment of the bacterial CAT gene. The amplified product from each transgenic line is presented The marker lane (M) is ϕX174 DNA digested with *Hae*III The negative control template (N) is genomic DNA from a non-transgenic mouse, and the positive control template (P) is the plasmid pUCCAT3

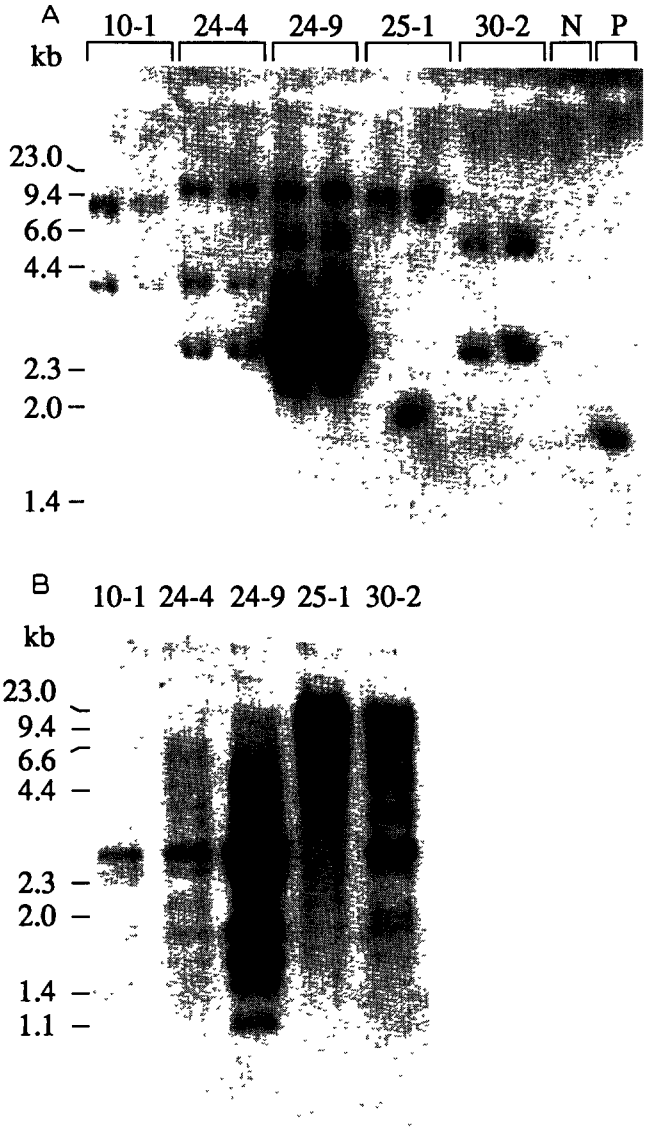

Fig. 3 A· Southern blot hybridization of genomic DNA digested with *Eco*RI. Each transgenic line is represented by genomic DNA from the founder and an offspring A non-transgenic mouse is represented in lane N For a positive control, the CAT sequences of pUCCAT3 were excised as a 1 8 kb *Sal*I fragment, and mixed with genomic DNA of non-transgenic mouse (lane P) B Southern blot hybridization of transgenic mouse genomic DNA digested with *Nci*I

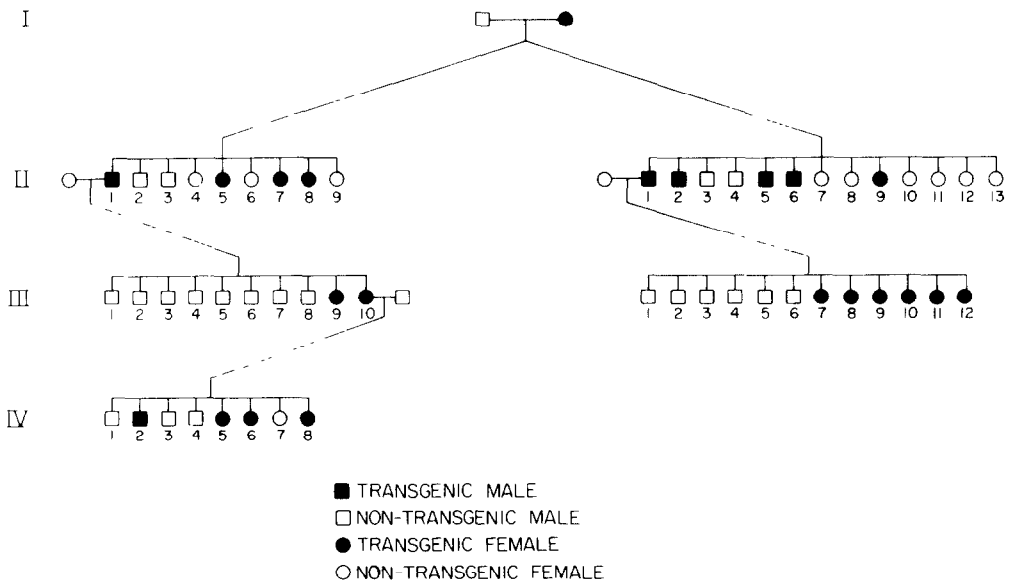

Fig 4 Partial pedigree of transgenic line 10-1 The founder female is indicated by the black circle in generation I Her first two litters are depicted in generation II, and offspring of a male from each litter are depicted in generation III Offspring of a generation III female is depicted in generation IV. The pattern of transgene inheritance suggests X-chromosome-linked transmission

Germ-line transmission of NPY/CAT genes

Transgenic lines were established for each of the five founder mice. The transgene in line 10-1 appeared to be integrated within the X-chromosome, since males carrying this transgene transmitted the gene only to their daughters (Fig. 4) Transmission followed an autosomal inheritance pattern in the other four transgenic lines

RNA analysis

NPY mRNA production in C57Bl/6J × SJL/J mice corresponded to that previously published for the rat[11]. The highest levels were present in the adrenal glands, spinal cord, and cerebral cortex followed by the limbic region, olfactory bulb and spleen Moderate to low amounts were found in the spleen, brain stem, heart and eyes, while basal levels were apparent in the cerebellum, lung, and pancreas (Fig. 5).

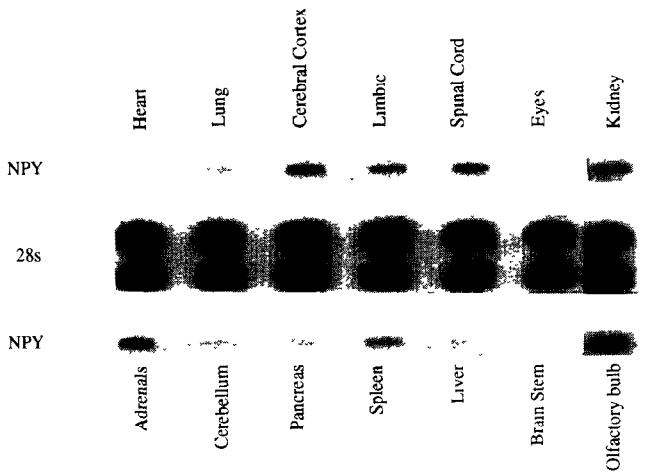

Fig 5 Slot blot analysis of mouse tissue total RNA The hybridization probe is denoted in the left margin while the source of the RNA is listed above and below the samples In each case, 10 μg of the total RNA was probed with NPY sequences while 1 μg was probed with the 28S probe

CAT activity in transgenic mice

Statistical analyses revealed significant CAT activity only in the tissues from three transgenic lines. Comparative levels of CAT activity in each line of mice are demonstrated in Fig. 6. Mice from lines 24-4, 24-9, and 30-2 exhibited significant levels of transgene activity in the olfactory bulb, cerebral cortex, and limbic region ($P < 0.01$). Significant levels of CAT activity were also detected in the adrenal glands from line 24-4 ($P < 0.05$), and in the brain stem, spinal cord, and eyes from line 30-2 ($P < 0.01$). Mice from lines 10-1 and 25-1 did not exhibit significant levels of CAT activity in any of the tissues tested ($P < 0.05$).

Due to the low levels of CAT activity and the instability of the CAT mRNA, we were not able to determine the transcriptional start site of the inserted transgene. However, accurate transcription of the fusion plasmid CATNPYΔ796 has been established in several cell lines and in an in vitro transcription system (data not shown)

DISCUSSION

In order to identify regions of the human neuropeptide Y gene that regulate tissue-specific transcription, a

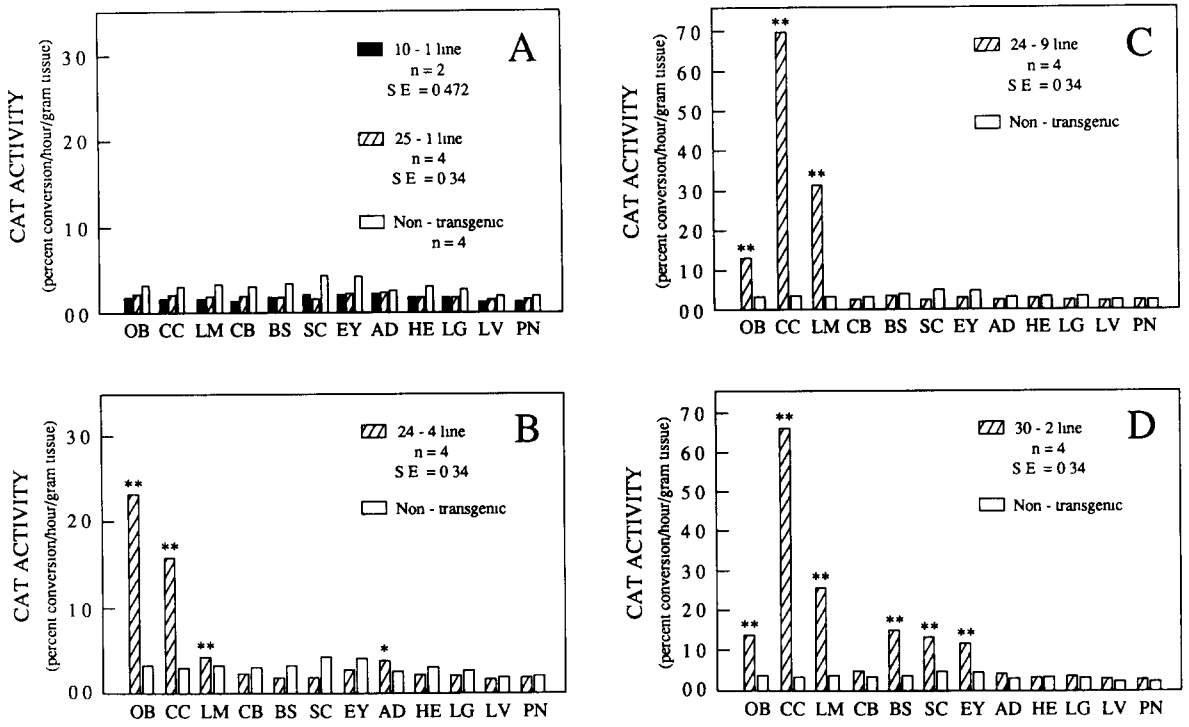

Fig. 6. Analysis of CAT enzyme activity in transgenic mice. CAT activity in tissues of lines 10-1 and 25-1 are displayed in A, 24-4 in B, 24-9 in C, and 30-2 in D. Levels of CAT activity within each tissue were compared between transgenic and non-transgenic mice. The reactions were performed with extracts from olfactory bulb (OB), cerebral cortex (CC), limbic region (LM), cerebellum (CB), brain stem (BS), spinal cord (SC), eyes (EY), adrenals (AD), heart (HE), lung (LG), liver (LV), and pancreas (PN) Significant differences ($*P < 0.05$, $**P < 0.01$) were detected using the Student–Newman–Keul's test.

NPY/CAT fusion gene was constructed and inserted into the mouse genome. This fusion gene contained 796 bp of 5′ non-transcribed DNA and 51 bp of exon 1 from the human NPY gene. Five founder transgenic mice were produced via pronuclear microinjection into fertilized ova and transgenic lines were established for each founder.

Southern analyses revealed differing patterns of transgene integration between each line. Four lines, 10-1, 24-4, 24-9, and 30-2 appeared to contain intact NPY/CAT constructs, as evident by the 2.3 kb *Nci*I fragment. Since these four lines contained multiple copies of the transgene (Table I), and multiple copy transgenes often integrate in a tandem array[21], the 2.5 kb *Eco*RI restriction fragment observed in the Southern blot would be predicted for a head-tail transgene concatamer. Hybridization of the CAT probe to only high-molecular weight *Eco*RI fragments in the 25-1 line could be explained by a single-copy transgene insertion, and our analysis determined this line contained 1-2 copies. Also, the 501 bp CAT fragment was amplified from this line. However, only high-molecular weight *Nci*I fragments from the 25-1 line hybridized with CAT, so this transgene was altered in the genome, and may have lost part or all of the NPY promoter DNA

TABLE I

Characterization of transgenic lines for pCATNPYΔ796

Founder	Sex	Number of copies	Transmission
10-1	F	7–10	X-linked
24-4	M	3–6	Autosomal
24-9	F	40–45	Autosomal
25-1	M	1–2	Autosomal
30-2	M	11–13	Autosomal

Transcriptional activity of the transgene, as determined by the CAT assay, was detected in tissues which normally express NPY, and was not detected in tissues which do not express this neuropeptide. The mice tested in the present experiment displayed CAT activity only in olfactory bulb, cerebral cortex, limbic region, brain stem, spinal cord, eye, and adrenal glands. The amount of CAT activity, however, did not always correlate with the amount of steady state NPY mRNA. This was particularly true for the adrenal gland and spinal cord which had the largest amounts of NPY mRNA but displayed very little CAT activity. There are several plausible reasons for these differences. First, the CAT assay measures transcriptional activity while the slot blot measures steady state mRNA levels. Furthermore, if message stability played an important role in the amount of NPY mRNA

present in these tissues, the instability of the CAT message would exacerbate these differences Secondly, information not present in the Δ796 construct might be necessary for efficient expression in these tissues. Finally, the genomic structure at the site of integration may influence the expression of the transgene to varying degrees in different tissues

The amount of CAT activity and NPY mRNA correlated well for the limbic region and olfactory bulb with moderate levels present in each case The 30-2 line also showed CAT activity in the brain stem and eyes Although the levels of NPY mRNA detected in these tissues was low, it was above the background level observed in the pancreas, lung, and cerebellum. Of the tissues from the central nervous system that were tested, only the cerebellum failed to show transgene activity, consistent with the data that this tissue contains very little NPY mRNA and immunoreactivity.

Two lines, 10-1 and 25-1, did not exhibit significant levels of CAT activity in any tissue. Lack of activity in the 25-1 line was probably due to structural rearrangement of the integrated transgene, resulting in loss of NPY regulatory DNA from the construct However, the apparently intact transgene in the 10-1 line was inserted within the X-chromosome Lack of activity was probably not due to X-inactivation, since CAT activity was not observed in males or in homozygous transgenic females (data not shown) The 10-1 line may represent a special case of positional regulation on the X-chromosome.

Levels of transgene expression varied between each line which expressed the transgene. Levels of CAT activity in the cerebral cortex and limbic regions of the 24-9 and 30-2 lines were twice that of the 24-4 line, whereas activity in the olfactory bulb of the 24-4 line was twice that of the other two lines Furthermore, only the 30-2 line exhibited transgene activity in the brain stem, spinal cord, and eye One would expect high levels of transgene activity in the adrenal glands, but only the 24-4 line displayed a small, but statistically significant, level of CAT activity in this tissue. In experiments which involve different gene constructs, we have detected high levels of CAT activity in the adrenals of transgenic mice. Therefore, the difference in location and level of transgene activity in the present experiment was probably due to missing regulatory elements in the construct and/or inhibitory factors at the site of genomic integration

The NPY regulatory sequences included in the present construct are sufficient to direct tissue-appropriate gene activity in transgenic mice. Perhaps *cis*-regulatory elements in non-transcribed DNA further upstream or downstream of the NPY coding sequence, or regulatory elements within introns, will be necessary for position-independent transgene expression in all tissues which endogenously produce NPY Transgenic experiments will be useful to further characterize the *cis*-regulatory regions of the human neuropeptide Y gene.

Acknowledgements This article is dedicated to the memory of Dr C Larry Chrisman This is journal paper 13054 from the Purdue University Agricultural Experimentation Station

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