MOLECULAR AND CELLULAR NEUROSCIENCES 5, 505–514 (1994)

Expression of Corticotropin-Releasing Hormone Transgenes in Neurons of Adult and Developing Mice

CATHERINE E. KEEGAN,*,† I. JILL KAROLYI,* LAUREN T. KNAPP,* FRANCIS J. BOURBONAIS,‡ SALLY A. CAMPER,* AND AUDREY F. SEASHOLTZ‡,[1]

*Department of Human Genetics, †Graduate Program in Cellular and Molecular Biology, and ‡Department of Biological Chemistry and Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan 48109-0720

Received for publication May 10, 1994

The DNA sequences important for cell-specific expression and developmental regulation of corticotropin-releasing hormone (CRH) were analyzed in transgenic mice. A construct containing 0.5 kb of CRH 5′ flanking DNA linked to the chloramphenicol acetyltransferase reporter gene was expressed in many brain regions and in several ectopic peripheral sites, suggesting that this portion of the CRH gene contains basal promoter activity but lacks DNA elements necessary for appropriate tissue specificity. Cell specificity of transgene expression was examined with a CRH-β-galactosidase reporter construct containing the same 0.5-kb CRH promoter fragment, but also including the CRH structural gene and 2 kb of CRH 3′ flanking DNA. Transgene expression was observed in inappropriate regions of the brain, but no expression was detected in peripheral tissues, suggesting that these additional CRH sequences suppress inappropriately high levels of peripheral expression. Cell-specific expression improved significantly with the inclusion of 8.7 kb of CRH 5′ flanking DNA. Individual transgenic lines exhibited expression in a number of the major CRH neuronal groups including the paraventricular nucleus, medial geniculate nucleus, inferior olivary nucleus, and Barrington's nucleus. Transgene expression was properly activated in Barrington's nucleus during development. This study demonstrates that the regulatory control of cell-specific and developmentally appropriate CRH expression is complex, utilizing multiple DNA sequence elements located upstream and downstream of the CRH transcription start site. © 1994 Academic Press, Inc.

INTRODUCTION

The endocrine response to stress is mediated by the hypothalamic–pituitary–adrenal (HPA) axis. The 41 amino acid neuropeptide corticotropin-releasing hormone (CRH) is a key regulator of this axis. In response to stress, CRH is released from the median eminence into the hypophyseal portal blood stream. It binds to CRH receptors on anterior pituitary corticotrope cells, causing increased synthesis and secretion of adrenocorticotropin hormone (ACTH). ACTH stimulates steroidogenesis in the adrenal cortex, resulting in the production of glucocorticoids which mediate the body's adaptive responses to stress. Glucocorticoids inhibit ACTH and CRH synthesis and secretion at the level of the pituitary and hypothalamus, respectively, thus completing the feedback regulatory loop (1).

Appropriate HPA axis regulation is important for maintenance of homeostasis. Aberrant regulation of the HPA axis can lead to elevated CRH levels, which have been associated with several psychiatric disorders including depression and anorexia nervosa (reviewed in 1). In addition, a primary deficiency of hypothalamic CRH is thought to cause increased susceptibility to streptococcal-cell-wall-induced arthritis in Lewis rats (2). A critical step in understanding the regulation of the stress response is to identify cis-acting DNA regulatory sequences that control cell-specific expression and hormonal and developmental regulation of the CRH gene.

CRH, like many neuroendocrine genes, has a characteristic pattern of cell-specific expression which includes both major and minor sites. One major site of CRH production is the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) (3–5). CRH is expressed at similar levels in many other extrahypothalamic regions of the brain, including the inferior olivary nucleus, central nucleus of the amygdala, medial geniculate nucleus, and Barrington's nucleus, where CRH is thought to act as a neurotransmitter or neuromodulator (3–12). Lower levels of CRH mRNA have been detected by *in situ* hybridization histochemistry in many other neuronal nuclei, such as substantia innominata, anterior and medial amygdala, lateral reticular nucleus, lateral parabrachial nucleus, lateral dorsal tegmental nucleus, and external cuneate

[1] To whom correspondence should be addressed at Department of Biological Chemistry and Mental Health Research Institute, 205 Zina Pitcher Place, Ann Arbor, MI 48109-0720. Fax: (313) 747-4130. e-mail: audrey.seasholtz@med.umich.edu.

1044-7431/94 $6.00
Copyright © 1994 by Academic Press, Inc.
All rights of reproduction in any form reserved.

nucleus (12). These data are consistent with mRNA levels determined by Northern blotting analysis of dissected brain regions in adult rat (13). CRH immunoreactivity has been detected in several peripheral sites (14), and the presence of CRH mRNA has been confirmed in testis, adrenal, spinal cord, and pituitary by Northern blotting analysis (13). However, the levels of CRH mRNA detected in these tissues are 10- to 20-fold lower than those in the brain stem, and cell specificity of CRH expression has not been determined. Trace amounts of CRH mRNA have also been detected in spleen and thymus by reverse transcriptase–polymerase chain reaction assays in combination with Southern blotting, while no transcripts were detected in liver or kidney (15). Furthermore, high levels of CRH mRNA are present transiently during development in peripheral sites such as lung and celiac ganglion (12). However, a role for CRH in many of these peripheral tissues has not been established.

Very little is known at this time about the mechanisms by which the CRH gene maintains its precise pattern of cell-specific expression and regulation. CRH transcription has been studied by transfection in multiple cell lines derived from a wide variety of tissue types including rat adrenal pheochromocytoma (PC12) cells, human choriocarcinoma (JAR) cells, human neuroblastoma (SK-N-SH) cells, mouse pituitary corticotropes (AtT20), transformed fetal hypothalamic cells , and monkey kidney cells (CV-1) (16–20, Seasholtz, unpublished observations). Promoter deletion analyses in several of these cell lines demonstrated that 0.5 kb of CRH 5′ flanking DNA is sufficient for basal expression and cAMP and glucocorticoid regulation of the CRH gene. With the exception of fetal hypothalamic cells, none of the transfected cell lines express the endogenous CRH gene, emphasizing the importance of taking an *in vivo* approach to identify the CRH cis-acting regulatory sequences by using transgenic mice. This approach makes it possible to examine the onset of gene expression during development and rigorously test the cell specificity of expression in many regions of the adult brain that normally produce CRH. The data presented here demonstrate that 0.5 kb of CRH 5′ flanking DNA is not sufficient for cell-specific expression of the CRH gene *in vivo*. Sequences within the CRH structural gene or 3′ flanking DNA may repress expression in peripheral tissues, and sequences between 8.7 and 0.5 kb 5′ of the transcription start site are important for transgene expression in appropriate CRH neurons.

MATERIALS AND METHODS

Construction of 0.5 CAT Transgene

The 0.5 CAT construct is identical to the previously described plasmid CRHCAT-497 (16). Briefly, it contains CRH genomic sequences extending from the *Bgl*II site at −497 to the *Pst*I site at +94 bp fused to the chloramphenicol acetyltransferase (CAT) gene in the pOCAT vector (21). The 3′ polyadenylation sequences are from the herpes simplex virus thymidine kinase gene.

Construction of CRH-β-galactosidase Transgenes

CRH genomic clones were isolated from a Sprague–Dawley rat genomic library as previously described (13). The 0.5 CRH β-gal was generated as follows. A 4.4-kb *Bgl*II/*Sac*I CRH genomic DNA fragment was isolated and subcloned into pUC18 vector DNA digested with *Bam*HI and *Sac*I to create rCRH4.4*Bgl*II/*Sac*I. This clone includes CRH genomic DNA from the *Bgl*II site at −497 bp to the *Sac*I restriction endonuclease site approximately 3900 bp 3′ to the transcription initiation site. In total, this construct contains 497 bp of CRH 5′ flanking DNA, exon I, intron I, exon II, and approximately 2.0 kb of CRH 3′ flanking DNA. rCRH4.4*Bgl*II/*Sac*I was digested with *Kpn*I restriction endonuclease which cleaves at +117 bp in the untranslated first exon. The *Kpn*I site was blunted with T4 DNA polymerase and a 3.1-kb DNA fragment containing nucleotides 1309 to 4373 of the *Escherichia coli* β-galactosidase gene (GenBank Accession No. J01636) was ligated into this vector to create 0.5CRH β-gal. This β-galactosidase sequence includes the translation start and stop codons. Polyadenylation signal sequences are provided by the CRH gene.

To produce 8.7 CRH β-gal 1, the plasmid pGem9Zr-CRH12.6*Hin*dIII/*Sac*I was used. This plasmid includes 8.7 kb of CRH 5′ flanking DNA (from the *Hin*dIII site approximately 8700 bp 5′ to the transcription initiation site) and 3900 bp of DNA 3′ to the transcription initiation site including exon I, intron I, exon II, and approximately 2.0 kb of CRH 3′ flanking DNA in *Hin*dIII/*Sac*I-digested pGEM9Z vector DNA (Promega, Madison, WI). A 3.6-kb *Kpn*I/*Hin*dIII DNA fragment from pnlacF (kindly provided by Jacques Peschon and Richard Palmiter) was converted to a 3.6-kb nlacF *Kpn*I fragment using *Kpn*I linkers. This fragment contains a nuclear localization signal at the amino terminus of the β-galactosidase coding sequence and mouse protamine 3′ untranslated sequences including an intron and polyadenylation signal (22). The 3.6-kb nlacF *Kpn*I fragment was ligated with *Kpn*I-digested pGEM9ZrCRH12.6*Hin*dII/*Sac*I to create 8.7 CRHβ-gal 1 which contains the nuclear β-galactosidase gene in the *Kpn*I site of CRH untranslated exon I.

The 8.7 CRH β-gal 2 was generated as follows. A 3.6-kb *Nco*I/*Pst*I DNA fragment was isolated from pnlacF and ligated to oligonucleotides to create Rsr II restriction endonuclease cohesive ends. The modified β-galactosidase fragment was inserted in frame into the CRH protein coding sequence at the Rsr II site (+1290 bp) immediately preceding the CRH peptide sequence in the pGEM9zrCRH12.6*Hin*dIII/*Sac*I DNA clone.

In all cases, restriction endonuclease digests and DNA sequence analysis (Sequenase Version 2.0, United States Biochemicals, Cleveland, OH) were utilized to confirm

the orientation and integrity of the β-galactosidase DNA insert.

Generation of Transgenic Mice

A 3.2-kb insert containing 0.5 CAT was isolated from the plasmid vector by restriction endonuclease digestion with *Bgl*II and *Sac*II. The 7.4-kb insert containing 0.5 CRH β-gal was released from the pUC18 vector by digestion with *Sac*I and *Xba*I restriction endonucleases. The 8.7 CRH β-gal 1 and 8.7 CRH β-gal 2 inserts (16.2 kb each) were isolated from the plasmid vector by digestion with *Hin*dIII and *Sfi*I restriction endonucleases. The *Sfi*I site is in the pGEM9Z vector just 3′ to the *Sac*I site present in rat genomic DNA. All fragments were separated by agarose gel electrophoresis, electroeluted and purified by CsCl centrifugation (23) or Nucleobond AX column chromatography (The Nest Group, Southborough, MA). The purified DNA fragments were microinjected into F2 hybrid zygotes from C57BL/6J × SJL/J parents at a concentration of approximately 2 ng/μl (23). After overnight incubation, the eggs which survived to the two-cell stage were transferred to Day 0.5 postcoitum pseudopregnant CD1 females. Approximately 66% of the zygotes survived injection, 9% of the implanted embryos resulted in live mice, and 14% of the mice were transgenic.

C57BL/6J and SJL/J mice (The Jackson Laboratory, Bar Harbor, ME) and CD1 mice (Charles River, Wilmington, MA) were bred at the University of Michigan. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accord with the principles and procedures outlined in the NIH "Guide for the Care and Use of Laboratory Animals."

Identification and Breeding of Transgenic Mice

Genomic DNA prepared from tail biopsies was screened for the presence of the CAT or β-gal reporter gene using polymerase chain reaction (PCR) (24, 25). Amplification of a 364-bp β-gal fragment was accomplished with a 30-bp sense oligo (5′-TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA -3′) and a 30-bp antisense oligo (5′- ATC TGA GCG AGT AAC AAC CCG TCG GAT TCT- 3′). Oligonucleotides specific for the CAT gene amplified a 244-bp fragment (26). The PCR reaction mix, consisting of genomic DNA (100–200 ng), primers (0.5 pmol/μl), and 2.5 m*M* $MgCl_2$ (25), was subjected to 29 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1.5 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Coamplification of a 454-bp fragment of the endogenous β-globin gene was used as a positive control for the quality of the genomic DNA sample (27).

After identification of transgenic mice by PCR, Southern blots were performed to verify the integrity of the transgenes and to estimate copy number. Copy number standards were generated by mixing the appropriate amount of plasmid with genomic DNA from a nontransgenic mouse (28). Mouse tail genomic DNA (10 μg) was digested for 20 h in the presence of 4 m*M* spermidine. Southern blotting and hybridization conditions have previously been described (27). Hybridization probes were selected for detection of the 5′ or 3′ ends of the transgene tandem array, permitting the detection of multiple insertion sites. Founder mice were bred to C57BL6/J to produce lines.

Timed Pregnancies

$B6SJLF_1$/J females were superovulated and mated with male transgenic mice (23). The presence of a vaginal plug on the morning following mating was set as Day 0.5 of gestation (e0.5), and postnatal day 1 (p1) was designated as the day of birth. Gestation period in the mouse is 19–20 days.

CAT Assays

Brain regions (cerebellum, hypothalamus, medulla/pons, midbrain, thalamus, hippocampus, striatum, and cortex) and peripheral tissues (liver, spleen, testis, heart, kidney, thymus, lung, and adrenal) were dissected from transgenic mice and homogenized in 0.25 *M* Tris–HCl, pH 7.5. Total protein content in the homogenate was determined using Bradford reagent (BioRad, Hercules, CA). An aliquot (60 μl) of the tissue homogenate was heat inactivated at 65°C for 10 min and centrifuged and the supernatant was assayed for CAT activity overnight at 37°C with acetyl coenzyme A and [^{14}C]chloramphenicol as previously described (16). ^{14}C-acetylated products were separated by thin-layer chromatography and quantitated by liquid scintillation counting. A nontransgenic mouse liver was used as a negative control for background CAT activity. Expression greater than twofold over background was considered significant.

Tissue Preparation for Histochemical Analyses

Brains from adult and p7 transgenic mice were removed, frozen in 2-methylbutane at −25 to −35°C, and stored at −80°C. Whole e16.5 fetuses and heads from p1 pups were frozen by the same procedure. Peripheral tissues, liver, kidney, spleen, thymus, adrenal, and gonads were embedded in OCT medium, frozen on dry ice, and stored at −80°C. Frozen sections (20 μm) were cut on a cryostat microtome (Bright Instrument Company, Huntingdon, England), thaw mounted onto poly-L-lysine (Sigma, St. Louis, MO)-coated slides, and stored at −80°C until use.

Evaluation of CRH β-gal Transgene Expression

CRH β-gal transgene expression was evaluated by *in situ* hybridization histochemistry with a β-gal riboprobe,

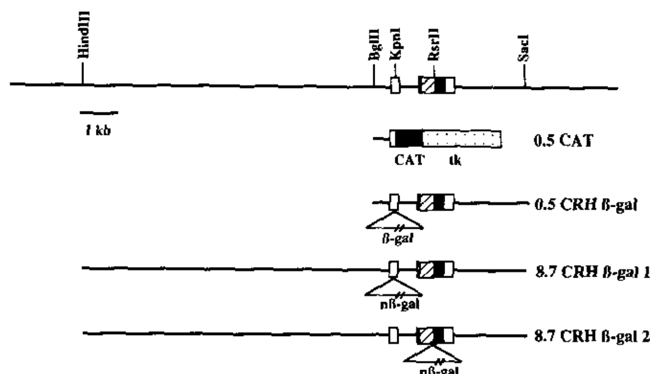


FIG. 1. Endogenous CRH gene and CRH transgenes. The CRH gene contains two exons (boxes, top line). The CRH propeptide sequence (hatched box) is encoded by exon 2 and processed to the mature CRH peptide (black box). The 0.5 CAT construct contains rat CRH gene sequences from the *Bgl*II site at −497 to the *Pst*I site at +94 fused to the chloramphenicol acetyltransferase (CAT) reporter gene (shaded) in the pOCAT vector (21). The 3′ sequences are from the herpes simplex virus thymidine kinase (tk) gene (stippled). The 0.5 CRH β-gal construct extends from the *Bgl*II site at −497 to the *Sac*I site at +3900 with the cytoplasmic β-gal reporter gene (β-gal) inserted into the *Kpn*I site of exon 1 (+117 bp). The 8.7 CRH β-gal 1 and 2 transgenes contain rat CRH genomic sequences from the *Hin*dIII site at −8700 to the *Sac*I site at +3900. For 8.7 CRHβ-gal 1, a nuclear localized β-galactosidase reporter gene (nβ-gal) was inserted into the same *Kpn*I site of exon I as in the 0.5 CRH β-gal construct. In the 8.7 CRH β-gal 2 construct, the nβ-gal reporter was inserted in frame into the *Rsr*II site, creating a CRH fusion protein, but terminating prior to the CRH peptide sequences. None of the constructs should produce CRH peptide. All constructs are shown to scale except the β-gal reporter genes, which are 3 kb.

by immunohistochemistry with a polyclonal antibody for β-gal (Organon Teknika Corp., Cappel Research Products, Durham, NC), and by enzymatic histochemical assay using the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). All methods were successful in detecting transgene expression. X-gal histochemistry was as sensitive as the other methods and was selected for these studies because the blue precipitate was most easily visualized.

For X-gal histochemical assays, frozen tissue sections were fixed for 5 min in either 4% paraformaldehyde or 0.5% gluteraldehyde in 0.1 *M* sodium phosphate buffer, pH 7.3, 5 m*M* EGTA, and 2 m*M* magnesium chloride. Sections were rinsed in wash buffer (0.1 *M* sodium phosphate, pH 7.3, 2 m*M* magnesium chloride, and 0.02% Nonidet P-40) and stained for 16–20 h at 37°C in wash buffer containing 1 mg/ml X-gal [stock solution 25 mg/ml X-gal (Boehringer Manneheim, Indianapolis, IN) in dimethyl formamide (Sigma, St. Louis, MO)], 5 m*M* potassium ferrocyanide (Sigma), and 5 m*M* potassium ferricyanide (Sigma). Following staining with X-gal, sections were lightly counterstained with eosin or 0.5% neutral red and dehydrated, and coverslips were mounted with Permount (Fisher, Pittsburgh, PA).

Brain and peripheral tissue sections were evaluated for β-gal expression by light microscopy. Cells expressing β-gal were noted by a blue precipitate under brightfield illumination. Under the more sensitive darkfield illumination, the β-gal expressing cells appeared pink (29). Tissues from nontransgenic C57BL/6J mice were examined for background β-gal staining. No background was ever observed in brain. Significant endogenous β-gal activity was observed in epididymis and ovary, and scattered, faintly stained cells were occasionally noted in the other tissues examined.

RESULTS

0.5 kb of CRH 5′ Flanking DNA Directs Higher Levels of Transgene Expression in Peripheral Tissues Than in Brain Regions

Four lines of transgenic mice containing 0.5 kb of CRH 5′ flanking DNA and 94 bp of CRH 5′ untranslated sequences fused to the CAT gene (Fig. 1) were examined by assaying CAT activity in dissected brain regions and peripheral tissues. Two of the four lines expressed the transgene at low levels in the brain (Fig. 2A). Expression was distributed in most brain regions, and one of the two

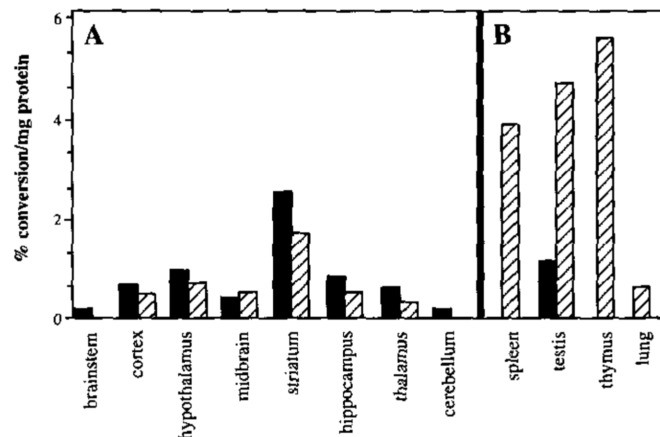


FIG. 2. The 0.5 CAT transgene is expressed in brain and peripheral tissues. Four 0.5 CAT transgenic lines were analyzed for CAT activity in dissected tissues. CAT activity is expressed as percentage conversion of [^{14}C]chloramphenicol to acetylated products per milligram protein, with background CAT activity subtracted (see Materials and Methods). Two insertion sites demonstrated significant expression in brain regions, 1142-A (shaded) and 1374 (hatched) (A). Line 1374 (hatched) did not exhibit detectable CAT activity in brain stem and cerebellum, but exhibited high levels of expression in spleen, testis, thymus, and lung (B). Founder 1142-A (shaded) exhibited expression in testis (B). Other peripheral tissues were not examined, and no line was established from this founder. No brain expression was detected in lines 1396 and 1142-B (not shown). Line 1396 exhibited expression only in liver (0.05% conversion/mg), and line 1142-B expressed the transgene only in testis (0.8% conversion/mg) (not shown). Each value represents the average of two determinations for each tissue from different animals within a line, except for 1142-A, where the founder was analyzed. No CAT activity was detected in kidney or heart in any lines examined.

lines exhibited inappropriate expression of the transgene in the cerebellum. However, the level of transgene expression within each region did not resemble the pattern expected for the endogenous CRH gene: brain stem ≫ cerebral cortex = hypothalamus > midbrain > striatum > hippocampus (13). In direct contrast to endogenous CRH expression, all four lines exhibited CAT activity in at least one peripheral tissue at levels greater than that observed in the majority of discrete brain regions (Fig. 2B). One line exhibited expression in the liver (not shown). Although CRH immunoreactivity has been reported in the liver (14), no mRNA transcripts are detectable by Northern blot or PCR analysis (13, 15). CAT activity was detected in lung in one line, yet CRH mRNA is not detectable by *in situ* hybridization analysis of adult lung (Keegan, unpublished observations). Surprisingly high levels of transgene expression were detected in spleen and thymus in one line. Testis expression, present in three of four lines, is attributable to the herpes simplex virus thymidine kinase sequences contained in the construct (30). A construct with 3.6 kb CRH 5′ flanking DNA fused to the CAT reporter gene gave similar results in transgenic mice. Two of six insertion sites expressed the transgene. One line exhibited expression in brain regions, testis, and thymus, and the other line expressed the transgene only in thymus (data not shown).

0.5 CRH β-gal Transgenes Are Expressed Only in Brains of Transgenic Mice

Because the 0.5 CAT transgene did not exhibit appropriate region-specific expression, we tested a construct with additional CRH genomic sequences, including both exons, the intron, and 2 kb of 3′ flanking sequence (Fig. 1). This construct utilized the *E. coli* β-galactosidase (β-gal) reporter gene to facilitate assessment of the cell specificity of transgene expression. Many other groups have used this reporter gene successfully in transgenic mice (22, 29, 31–34).

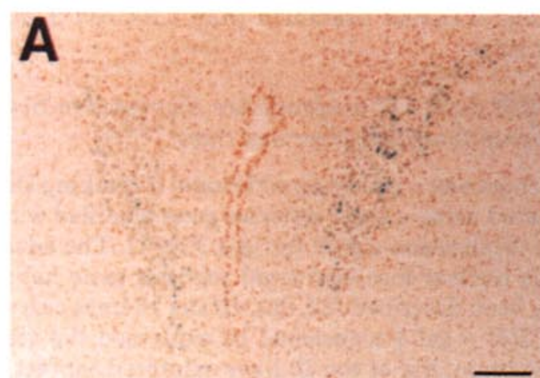
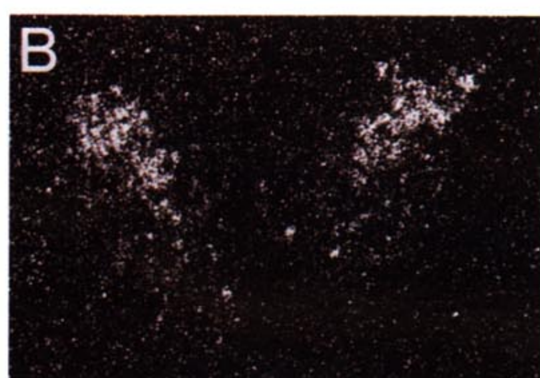
Coronal brain sections and peripheral tissue sections from seven lines containing the 0.5 CRH β-gal transgene were evaluated for expression by X-gal enzymatic histochemical assay. No endogenous β-gal activity was noted in brains from nontransgenic mice, but expression was detected in brains of two of the seven transgenic lines. Expression of 0.5 CRH β-gal was observed mainly in inappropriate brain regions. In line 4489, appropriate cortical expression (layer II) was noted, but strong inappropriate expression was detected in the hippocampus, dentate gyrus, and habenular nucleus (data not shown). Line 4312 exhibited inappropriate expression of the transgene in the piriform cortex (data not shown). None of the seven lines exhibited expression in the major regions which express the endogenous CRH gene, such as the PVN, inferior olivary nucleus, medial geniculate nucleus, and Barrington's nucleus. At least two individuals were examined from each expressing line, and expression patterns were identical between individuals within a line. No β-gal staining above background levels was detected in any peripheral tissues examined from 0.5 CRH β-gal transgenic mice.

8.7 CRH β-gal 1 Transgenes Are Expressed in Several Appropriate CRH Neuronal Sites

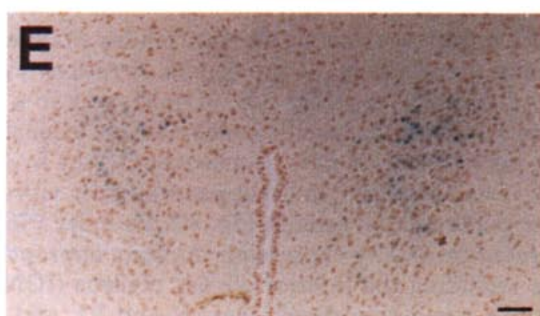
A construct containing additional 5′ flanking DNA was prepared using a β-gal reporter gene modified with a nuclear localization signal (nβ-gal, Fig. 1). The advantages of the nuclear-localized β-gal reporter gene have previously been described (22, 35). Of the 16 lines carrying the 8.7 CRH β-gal 1 transgene, 10 expressed the transgene in the brain. Six of the 10 lines exhibited expression in a subset of the appropriate CRH neurons, with 3 of those lines expressing the transgene in major CRH neuronal groups. Line 7901 correctly expressed the transgene in the medial parvocellular division of the PVN (Fig. 3A), consistent with the localization of endogenous CRH mRNA to the medial parvocellular neurons of the mouse PVN(12). Appropriate expression in line 7901 was also noted in Barrington's nucleus (BAR) (Fig. 3C), medial geniculate nucleus (GEN), and cerebral cortex (layer II). The appropriateness of transgene expression was confirmed in both the PVN and BAR by demonstrating endogenous CRH gene expression by *in situ* hybridization histochemical analysis of adjacent slides (four and five sections, respectively, in the caudal direction) as previously described (12) (Figs. 3B, 3D). Moving in a rostrocaudal direction, the Barrington's nucleus is located in a more dorsal position (36). Consequently, the *in situ* hybridization signal in Fig. 3D appears slightly more dorsal to the β-gal-stained cells in Fig. 3C. Founder 7765 demonstrated transgene expression in many appropriate regions including the inferior olivary nucleus (ION), Barrington's nucleus, cortical layers V and VI, and central nucleus of the amygdala. Line 7907 expressed the transgene in BAR, and in three additional lines transgene expression was noted in some appropriate cortical layers. In each expressing line the β-gal activity appeared to be confined to neuronal cells although some inappropriate neuronal expression was noted. Several of the 10 expressing lines demonstrated inappropriate neuronal expression in thalamic nuclei, medial habenular nucleus, and hippocampus; however, the overall pattern of ectopic expression appeared to be random. As for 0.5 CRH β-gal, no significant β-gal activity was observed in any peripheral tissues examined.

Placement of the Reporter Gene Does Not Influence Transgene Expression

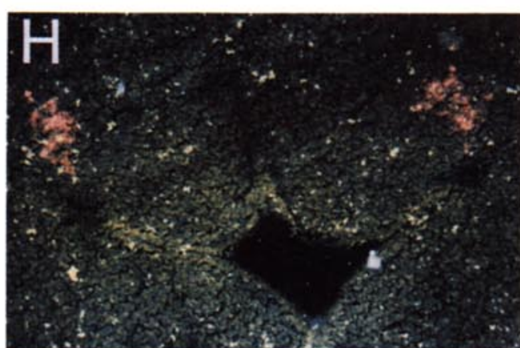
In the 8.7 CRH β-gal 1 transgene, the nβ-gal reporter gene was inserted into the 5′ untranslated region of the CRH gene. Because the 5′ untranslated region can influence the efficiency of protein synthesis and can contain


A

B

C

D

E

F

G

H

I

transcriptional control elements, the 8.7 CRH β-gal 2 construct was designed with nβ-gal inserted into the CRH protein coding region within exon 2, leaving the 5′ untranslated region intact (Fig. 1). Three of seven transgenic lines expressed the transgene in the brain. Two of the three expressing lines demonstrated transgene expression in a subset of the appropriate CRH neurons. One line (9599) expressed the transgene in the parvocellular neurons of the PVN (Figs. 3E and 3F), in appropriate cortical layers, and in BAR. In line 9600, appropriate transgene expression was detected in the medial geniculate nucleus and cerebral cortex. However, inappropriate neuronal expression was detected in all three expressing lines in several of the same neuronal regions as with the 8.7 CRH β-gal 1 transgene, providing further support for the idea that additional DNA sequences are necessary for complete restriction of transgene expression to appropriate neuronal sites. The observation of parvocellular PVN expression in line 7901 (8.7 CRH β-gal 1) and line 9599 (8.7 CRH β-gal 2) suggests that expression in these hypothalamic neurons is not simply due to an effect of the transgene integration site.

Transgene Expression Is Not Copy Number Dependent

A correspondence between transgene copy number and level of transgene expression has been attributed to locus control regions or a combinatorial interaction of multiple enhancer elements (37–41). None of the constructs tested exhibited a correlation between the level of transgene expression and transgene copy number (data not shown). For example, the most appropriate transgene expression was observed in 8.7 CRH β-gal 1 line 7901 and 8.7 CRH β-gal 2 line 9599, which had one and five copies of the transgene, respectively. 8.7 CRH β-gal 1 line 7951, which did not express the transgene at all, contained 100 copies. The copy numbers of other nonexpressing lines ranged from 1 to 25 copies.

Developmental Expression of CRH Transgenes

One of the transgenic lines exhibiting expression in several appropriate regions was examined for developmental regulation of transgene expression. Progeny of line 7901 (8.7 CRH β-gal 1) were collected at three time points, Embryonic Day 16.5 (e16.5), Postnatal Day 1 (p1), and Postnatal Day 7 (p7). In BAR, β-gal activity was noted at e16.5 (Figs. 3G and 3H). Correct identification of BAR was confirmed by demonstration of endogenous CRH expression in an adjacent slide by *in situ* hybridization histochemistry (Fig. 3I). Transgene and endogenous gene expression in BAR was also detected at P1 and P7 (data not shown). The endogenous mouse CRH gene is expressed in the PVN at e16.5 (12). However, no transgene expression was detected in the PVN at this time. Transgene expression in the PVN was barely detectable at p1, with higher levels evident by p7 (data not shown).

DISCUSSION

Transgenic animals provide an ideal system for the analysis of regulatory sequences within the context of the physiology of the animal and the opportunity to examine tissue-specific and developmental signals controlling gene expression. In the present study we have analyzed the expression of several CRH reporter constructs in transgenic mice in order to identify the cis-acting regulatory sequences controlling CRH gene expression *in vivo*.

Two of 4 lines of transgenic mice carrying the 0.5 CAT transgene exhibited low levels of CAT activity in the brain, and 4 of 4 lines exhibited significant levels of expression in peripheral tissues. In contrast, 2 of 7 0.5 CRH β-gal lines expressed the transgene in the brain, while no transgene expression was detected in peripheral tissues. Although we cannot rule out a contribution of the reporter gene, this result suggests that sequences contained within the CRH gene and 3′ flanking DNA suppress

FIG. 3. The 8.7 CRH β-gal 1 transgene is expressed in the parvocellular division of the PVN and in Barrington's nucleus. Coronal brain sections are shown depicting transgene expression in the PVN and Barrington's nucleus of an adult mouse from 8.7 CRH β-gal 1 line 7901. A and C were stained for β-gal activity by X-gal histochemistry and counterstained with 0.5% neutral red. The cells expressing the transgene can be easily visualized by the blue precipitate. B and D represent *in situ* hybridization histochemical analysis, demonstrating endogenous mouse CRH expression in the PVN and Barrington's nucleus (four and five sections, respectively, in the caudal direction). Antisense and sense CRH riboprobes were transcribed from a mouse CRH 578-bp *Pst*I exon 2 fragment (59) and hybridized as previously described (12). The antisense CRH riboprobe does not hybridize to the transgene because transcription termination occurs at the mouse protamine polyadenylation sequences of the transgene (see Materials and Methods, Fig. 1). Magnification bars shown in A and C equal 100 μm, and magnifications are constant for each nucleus. (E, F) The 8.7 CRH β-gal 2 transgene is expressed in the PVN. The photomicrographs depict X-gal histochemical staining of a 20-μm coronal brain section demonstrating transgene expression in the parvocellular division of the PVN of an animal from line 9599 carrying the 8.7 CRH β-gal 2 transgene. Expression in the PVN under brightfield is evident (E); however, β-gal expression is more apparent when the same section is viewed under darkfield illumination (F), in which the cells expressing β-gal appear pink. The section is counterstained with 0.5% neutral red. The magnification bar shown in E equals 50 μm. The same magnification was used in F. (G–I) The 8.7 CRH β-gal 1 transgene is expressed appropriately during development in Barrington's nucleus. G and H show a coronal section from an e16.5-day fetus from 8.7 CRH β-gal 1 line 7901. The section was stained for β-gal activity in the Barrington's nucleus using X-gal histochemistry, counterstained with neutral red (0.5%), and viewed under brightfield (G) and darkfield (H). Endogenous CRH expression is shown by *in situ* hybridization histochemistry of an adjacent slide (four sections away) with an antisense CRH riboprobe (I). The magnification bar shown in G equals 100 μm. The same magnification was used in H and I.

the inappropriately high levels of expression in the periphery. This is supported by analysis of a construct similar to 0.5 CRH β-gal that contained SV40 T-antigen inserted into exon 1 of the CRH gene instead of β-gal. Nineteen founders carrying this transgene were observed for 1 year. One founder developed a brain tumor. The other 18 lines did not develop tumors in the brain or in any peripheral tissues (Karolyi, unpublished observations). The presence of DNA sequences that repress expression in peripheral tissues has also been demonstrated for the vasopressin and SCG10 genes in transgenic mice (42, 43), and the existence of cis-acting DNA sequences at internal and downstream positions is not uncommon (39, 44–46).

The idea that CRH internal sequences contribute to CRH expression in transgenic mice has been previously proposed (47). However, that study used the metallothionein promoter, which can act in a combinatorial manner with other structural genes to produce novel cell specificities including hypothalamic expression in transgenic mice (48–50). The lack of PVN expression in seven transgenic lines with the 0.5 CRH β-gal construct supports the notion that the metallothionein promoter contributed significantly to the PVN expression observed in the metallothionein–CRH fusion transgene.

When transgenic mice were produced with additional CRH 5′ flanking DNA, there were multiple examples of expression in appropriate CRH neurons. For example, transgene expression was evident in the PVN, medial geniculate nucleus, Barrington's nucleus, and appropriate cortical layers in an 8.7 CRH β-gal 1 line. Similar patterns of transgene expression were demonstrated with the 8.7 CRH β-gal 2 transgenic mice, suggesting that the position of β-gal within the 5′ untranslated region in 8.7 CRH β-gal 1 did not disrupt a regulatory element. Since 20% (5/23) of transgenic lines with 8.7 kb of CRH 5′ flanking DNA exhibited transgene expression in one or more of the major CRH neuronal groups (PVN, ION, BAR, GEN) compared to none (0/7) of the 0.5 CRH β-gal mice, important regulatory information may lie between 8.7 and 0.5 kb. Although the penetrance of expression in major CRH neuronal groups is low, it is unlikely that this rate could occur by chance. Additional DNA sequences are probably required to recapitulate endogenous gene expression in every transgenic line, and further localization of regulatory elements between 0.5 and 8.7 kb of 5′ flanking DNA would be extremely difficult because of the variation in transgene expression between lines.

An interesting facet of the regulation of CRH expression is its developmental profile. CRH mRNA is detected at Day e13.5 in the PVN, BAR, and inferior olivary nucleus. Adult levels of expression are attained in these brain regions at different times during development, suggesting independent cell-specific transcriptional control during ontogeny (12). Developmental regulation of transgene expression was consistent with this hypothesis. In line 7901 (8.7 CRH β-gal 1), transgene expression was similar to the expression of the endogenous gene in fetal BAR. However, no transgene expression was detected in fetal PVN 3 days after the onset of endogenous gene expression. This is unlikely to be due to insensitivity of the β-gal assay because transgene expression in BAR was readily detected. It probably reflects a delay in activation of transgene expression relative to the endogenous gene, similar to that observed for other neuronal transgenes (33, 34, 51). However, the delay is specific to the PVN and not BAR. Our observation of appropriate expression in fetal BAR suggests that DNA regulatory sequences controlling fetal expression in BAR are present in this construct, and that additional DNA sequences are required to ensure the timely onset of CRH gene expression in the PVN.

The presence of variable ectopic neuronal expression in CRH transgenic lines suggests that an additional enhancer or locus control region is required to insulate the transgene from position effects (37). Although we cannot exclude the possibility that species differences between the rat and mouse genes contribute to the lack of appropriate expression, their nearly identical patterns of cell-specific expression and regulation suggest that the factors regulating their expression should be similar. A more likely possibility is that additional DNA regulatory sequences are required, which is supported by numerous examples of genes in which the enhancer elements lie a large distance (up to 50 kb) away from the gene (38–41, 44, 52, 53).

The requirement for cell-specific neuronal expression is a rigorous test of transgene expression. There are several examples in which appropriate organ- or region-specific transgene expression in tissue homogenates or dissected brain regions has been demonstrated (43, 54–56). In cases where cell specificity has been examined, both appropriate and inappropriate neuronal expression have been observed, similar to our observations with 8.7 CRH β-gal transgenes (22, 42, 50, 57, 58). This may suggest that neuronal specificity is particularly elusive. However, with the exception of vasopressin (42), most of these transgenes have not included substantial 5′ and 3′ flanking sequences. Another intriguing possibility is that neuronally expressed genes are more susceptible to position effects due to the large number of genes expressed in the brain relative to other tissues.

In this study we present an analysis of four different constructs containing various segments of the rat CRH gene in transgenic mice. Regulatory elements within 0.5 kb of the CRH gene appear to be sufficient for basal expression *in vivo*, but sequences within the CRH gene or 3′ flanking DNA contribute to repression of inappropriately high levels of peripheral transgene expression. Regulatory sequences between 8.7 and 0.5 kb of CRH 5′ flanking DNA are important for obtaining expression in major sites of CRH expression in the brain, such as the paraventricular nucleus, inferior olivary nucleus, medial

geniculate nucleus, and Barrington's nucleus. Additional DNA regulatory sequences are probably necessary in order for the transgene to perfectly recapitulate expression of the endogenous CRH gene. This evidence supports the hypothesis that the CRH gene has multiple interacting transcriptional regulatory elements.

ACKNOWLEDGMENTS

We thank Dr. Thom Saunders, Scott Keller, and Leslie McAndrews for their contributions to the early stages of this work, and Drs. Jim Herman and Bill Cullinan for help with neuroanatomical identification. We also wish to thank Drs. Miriam Meisler and Jeff Chamberlain for their critical reading of this manuscript. We acknowledge the University of Michigan Reproductive Sciences Center Morphology and Molecular Biology Cores (P30-HD18258), the Central Research Core Facility for the Department of Anatomy and Cell Biology, and Kaye Brabec, Wendee Wegenek, and Bruce Donohoe for their helpful assistance. This work was supported by Young Investigator Awards from the National Alliance for Research on Schizophrenia and Depression (S.A.C. and A.F.S.), and by the National Institutes of Health (R29MH49463, S.A.C.; R29DK42730, A.F.S.; and R03MH46532, S.A.C. and A.F.S.). C.E.K. was supported by NIH Training Grants T32GM07863, T32GM07315, and T32HD07274.

REFERENCES

1. Owens, M. J., and C. B. Nemeroff (1991). Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol. Rev.* **43:** 425–473.
2. Sternberg, E. M., W. S. Young, III, R. Bernardini, A. E. Calogero, G. P. Chrousos, P. W. Gold, and R. L. Wilder (1989). A central nervous system defect in biosynthesis of corticotropin releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. *Proc. Natl. Acad. Sci. USA* **86:** 4771–4775.
3. Merchenthaler, I., S. Vigh, P. Petrusz, and A. V. Schally (1982). Immunocytochemical localization of corticotropin-releasing factor (CRF) in the rat brain. *Am. J. Anat.* **165:** 385–396.
4. Cummings, S., R. Elde, J. Ells, and A. Lindall (1983). Corticotropin-releasing factor immunoreactivity is widely distributed within the central nervous system of the rat: An immunohistochemical study. *J. Neurosci.* **3:** 1355–1368.
5. Swanson, L. W., P. E. Sawchenko, J. Rivier, and W. Vale (1983). Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: An immunohistochemical study. *Neuroendocrinology* **36:** 165–186.
6. Young, W. S., III, L. C. Walker, R. E. Powers, E. B. DeSouza, and D. L. Price (1986). Corticotropin-releasing factor mRNA is expressed in the inferior olives of rodents and primates. *Brain Res.* **387:** 189–192.
7. Palkovits, M., C. Leranth, T. Gorcs, and W. S. Young, III (1987). Corticotropin-releasing factor in the olivocerebellar tract of rats: demonstration by light- and electron-microscopic immunohistochemistry and in situ hybridization histochemistry. *Proc. Natl. Acad. Sci. USA* **84:** 3911–3915.
8. Sakanaka, M., T. Shibasaki, and K. Lederis (1987). Corticotropin releasing factor-like immunoreactivity in the rat brain as revealed by a modified cobalt-glucose oxidase–diaminobenzidine method. *J. Comp. Neurol.* **260:** 256–298.
9. Ju, G., L. W. Swanson, and R. B. Simerly (1989). Studies on the cellular architecture of the bed nuclei of the stria terminalis of the rat. II. chemoarchitecture. *J. Comp. Neurol.* **280:** 603–621.
10. Imaki, J., T. Imaki, W. Vale, and P. E. Sawchenko (1991). Distribution of corticotropin-releasing factor mRNA and immunoreactivity in the central auditory system of the rat. *Brain Res.* **547:** 28–36.
11. Mezey, E., and M. Palkovits (1991). Time dependent changes in CRF and its mRNA in the neurons of the inferior olive following surgical transection of the olivocerebellar tract in the rat. *Brain Res. Mol. Brain Res.* **10:** 55–59.
12. Keegan, C. E., J. P. Herman, I. J. Karolyi, K. S. O'Shea, S. A. Camper, and A. F. Seasholtz (1994). Differential expression of corticotropin releasing hormone (CRH) in developing mouse embryos and adult brain. *Endocrinology* **134:** 2547–2555.
13. Thompson, R. C., A. F. Seasholtz, and E. Herbert (1987). Rat corticotropin-releasing hormone gene: Sequence and tissue-specific expression. *Mol. Endocrinol.* **1:** 363–370.
14. Petrusz, P., I. Merchenthaler, J. L. Maderdrut, and P. U. Hertz (1985). Central and peripheral distribution of corticotropin-releasing factor. *Fed. Proc.* **44:** 229–235.
15. Aird, F., C. V. Clevenger, M. B. Prystowsky, and E. Redei (1993). Corticotropin-releasing factor mRNA in rat thymus and spleen. *Proc. Natl. Acad. Sci. USA* **90:** 7104–7108.
16. Seasholtz, A. F., R. C. Thompson, and J. O. Douglass (1988). Identification of a cyclic adenosine monophosphate-responsive element in the rat corticotropin-releasing hormone gene. *Mol. Endocrinol.* **2:** 1311–1319.
17. Van, L. P., D. H. Spengler, and F. Holsboer (1990). Glucocorticoid repression of 3′,5′-cyclic-adenosine monophosphate-dependent human corticotropin-releasing-hormone gene promoter activity in a transfected mouse anterior pituitary cell line. *Endocrinology* **127:** 1412–1418.
18. Spengler, D., R. Rupprecht, L. P. Van, and F. Holsboer (1992). Identification and characterization of a 3′,5′-cylic adenosine monophosphate-responsive element in the human corticotropin-releasing hormone gene promoter. *Mol. Endocrinol.* **6:** 1931–1941.
19. Mugele, K., H. Kugler, and J. Spiess (1993). Immortalization of a fetal rat brain cell line that expresses corticotropin-releasing factor mRNA. *DNA Cell Biol.* **12:** 119–126.
20. Adler, G. K., C. M. Smas, and J. A. Majzoub (1988). Expression and dexamethasone regulation of the human corticotropin-releasing hormone gene in a mouse anterior pituitary cell line. *J. Biol. Chem.* **263:** 5846–5852.
21. Prost, E., and D. D. Moore (1986). CAT vectors for analysis of eukaryotic promoters and enhancers. *Gene* **45:** 107–111.
22. Mercer, E. H., G. W. Hoyle, R. P. Kapur, R. L. Brinster, and R. D. Palmiter (1991). The dopamine β-hydroxylase gene promoter directs expression of E. coli *lacZ* to sympathetic and other neurons in adult transgenic mice. *Neuron* **7:** 703–716.
23. Hogan, B., F. Costantini, and E. Lacy (1986). *Manipulating the Mouse Embryo.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
24. Miller, S. A., D. D. Dykes, and H. F. Polesky (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16:** 1215.
25. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.
26. Keller, S. A., M. P. Rosenberg, T. M. Johnson, G. Howard, and M. H. Meisler (1990). Regulation of amylase gene expression in diabetic mice is mediated by a *cis*-acting upstream element close to the pancreas-specific enhancer. *Genes Dev.* **4:** 1316–1321.
27. Kendall, S. K., T. L. Saunders, L. Jin, R. V. Lloyd, L. M. Glode, T. M. Nett, R. A. Keri, J. H. Nilson, and S. A. Camper (1991).

Targeted ablation of pituitary gonadotropes in transgenic mice. *Mol. Endocrinol.* **5:** 2025–2036.

28. Camper, S. A. (1987). Research applications of transgenic mice. *BioTechniques* **5:** 638–650.
29. Flenniken, A. M., and B. R. Williams (1990). Developmental expression of the endogenous TIMP gene and a TIMP-lacZ fusion gene in transgenic mice. *Genes Dev* **4:** 1094–1106.
30. Al-Shawi, R., J. Burke, C. T. Jones, J. P. Simons, and J. O. Bishop (1988). A *Mup* promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. *Mol. Cell. Biol.* **8:** 4821–4828.
31. Hammer, G. D., V. Fairchild-Huntress, and M. J. Low (1990). Pituitary-specific and hormonally regulated gene expression directed by the rat proopiomelanocortin promoter in transgenic mice. *Mol. Endocrinol.* **4:** 1689–1697.
32. Forss-Petter, S., P. E. Danielson, S. Catsicas, E. Battenberg, J. Price, M. Nerenberg, and J. G. Sutcliffe (1990). Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. *Neuron* **5:** 187–197.
33. Lem, J., M. L. Applebury, J. D. Falk, J. G. Flannery, and M. I. Simon (1991). Tissue-specific and developmental regulation of rod opsin chimeric genes in transgenic mice. *Neuron* **6:** 201–210.
34. Zack, D. J., J. Bennett, Y. Wang, C. Davenport, B. Klaunberg, J. Gearhart, and J. Nathans (1991). Unusual topography of bovine rhodopsin promoter-lacZ fusion gene expression in transgenic mouse retinas. *Neuron* **6:** 187–199.
35. Bonnerot, C., D. Rocancourt, P. Briand, G. Grimber, and J.-F. Nicolas (1987). A β-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl. Acad. Sci. USA* **84:** 6795–6799.
36. Paxinos, G., and C. Watson (1986). *The Rat Brain in Stereotaxic Coordinates,* 2nd ed. Academic Press, Orlando, FL.
37. Palmiter, R. D., and R. L. Brinster (1986). Germ-line transformation of mice. *Annu. Rev. Genet.* **20:** 465–499.
38. Hammer, R. E., R. Krumlauf, S. A. Camper, R. L. Brinster, and S. M. Tilghman (1987). Diversity of α-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. *Science* **235:** 53–58.
39. Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kolias (1987). Position-independent, high level expression of the human β-globin gene in transgenic mice. *Cell* **51:** 975–985.
40. Carson, S., and M. V. Wiles (1993). Far upstream regions of class II MHC Ea are necessary for position-independent, copy-dependent expression of Ea transgene. *Nucleic Acids Res.* **21:** 2065–2072.
41. Palmiter, R. D., E. P. Sandgren, D. M. Koeller, and R. L. Brinster (1993). Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice. *Mol. Cell Biol.* **13:** 5266–5275.
42. Ang, H.-L., D. A. Carter, and D. Murphy (1993). Neuron-specific expression and physiological regulation of bovine vasopressin transgenes in mice. *EMBO J.* **12:** 2397–2409.
43. Wuenschell, C. W., N. Mori, and D. J. Anderson (1990). Analysis of SCG10 gene expression in transgenic mice reveals that neural specificity is achieved through selective derepression. *Neuron* **4:** 595–602.
44. Yoo-Warren, H., V. Pachnis, R. S. Ingram, and S. M. Tilghman (1988). Two regulatory domains flank the mouse H19 gene. *Mol. Cell. Biol.* **8:** 4704–4715.
45. Liska, D. J., J. L. Slack, and P. Bornstein (1990). A highly conserved intronic sequence is involved in transcriptional regulation of the alpha I(I) collagen gene. *Cell. Regul.* **1:** 487–498.
46. Wang, J., and S. Chen (1992). Identification of a promoter and a silencer at the 3′-end of the first intron of the human aromatase gene. *Mol. Endocrinol.* **6:** 1479–1488.
47. Stenzel-Poore, M. P., V. A. Cameron, J. Vaughan, P. E. Sawchenko, and W. Vale (1992). Development of Cushing's syndrome in corticotropin releasing factor transgenic mice. *Endocrinology* **130:** 3378–3386.
48. Swanson, L. W., D. M. Simmons, J. Arriza, R. Hammer, R. Brinster, M. G. Rosenfeld, and R. M. Evans (1985). Novel developmental specificity in the nervous system of transgenic animals expressing growth hormone fusion genes. *Nature* **317:** 363–366.
49. Low, M. J., R. M. Lechan, R. E. Hammer, R. L. Brinster, J. F. Habener, G. Mandel, and R. H. Goodman (1986). Gonadotroph-specific expression of metallothionein fusion genes in pituitaries of transgenic mice. *Science* **231:** 1002–1004.
50. Murphy, D., H.-L. Ang, Q. Zeng, M.-Y. Ho, J. Funkhouser, and D. Carter (1992). Neuropeptide gene expression in transgenic animals. *Prog. Brain Res.* **92:** 77–96.
51. Julien, J. P., I. Tretjakoff, L. Beaudet, and A. Peterson (1987). Expression and assembly of a human neurofilament protein in transgenic mice provide a novel neuronal marking system. *Genes Dev* **1:** 1085–1095.
52. Pinkert, C., D. M. Ornitz, R. L. Brinster, and R. D. Palmiter (1987). An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. *Genes Dev.* **1:** 268–276.
53. Lew, D., H. Brady, K. Klausing, K. Yaginuma, L. E. Theill, C. Stauber, M. Karin, and P. L. Mellon (1993). GHF-1 promoter-targeted immortalization of a somatotropic progenitor cell results in dwarfism in transgenic mice. *Genes Dev.* **7:** 683–693.
54. Donovan, D. M., M. Takemura, B. F. O'Hara, M. T. Brannock, and G. R. Uhl (1992). Preproenkephalin promoter "cassette" confers brain expression and synaptic regulation in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89:** 2345–2349.
55. Waldbieser, G. C., C. D. Minth, C. L. Chrisman, and J. E. Dixon (1992). Tissue-specific expression of the human neuropeptide Y gene in transgenic mice. *Brain Res. Mol. Brain Res.* **14:** 87–93.
56. Grant, F. D., J. Reventos, J. W. Gordon, S. Kawabata, M. Miller, and J. A. Majzoub (1993). Expression of the rat arginine vasopressin gene in transgenic mice. *Mol. Endocrinol.* **7:** 659–667.
57. Mason, A. J., S. L. Pitts, K. Nikolics, E. Szonyi, J. N. Wilcox, P. H. Seeburg, and T. A. Stewart (1986). The hypogonadal mouse: Reproductive functions restored by gene therapy. *Science* **234:** 1372–1378.
58. Young, W. S., III, K. Reynolds, E. A. Shepard, H. Gainer, and M. Castel (1990). Cell-specific expression of the rat oxytocin gene in transgenic mice. *J. Neuroendocrinol.* **2:** 917–925.
59. Seasholtz, A., F. Bourbonais, C. Harnden, and S. Camper (1991). Nucleotide sequence and expression of the mouse corticotropin-releasing hormone gene. *Mol. Cell. Neurosci.* **2:** 266–273.