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Transgenic Targeting of Neuroendocrine Peptide Genes in the Hypothalamic-Pituitary Axis

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Abstract

A large number of neuroendocrine peptide genes have been tested for their ability to target expression to the hypothalamus and pituitary in transgenic mice. This has resulted in a number of powerful applications, for example, ablation or immortalization of specific cell types, and analysis of transcription regulatory sequences. The greatest amount of success in targeting cells of the neuroendocrine axis has been in the pituitary and has utilized regulatory sequences of genes that are normally expressed in pituitary. Greater difficulties have been encountered in directing expression to specific neurons in the hypothalamus. A primary goal of this review is to consider collectively the data obtained by a number of laboratories in order to draw conclusions about the general sequence requirements for achieving cell-specific expression. The data suggest that the mechanisms controlling cell-specific expression of neuropeptide genes in the hypothalamus are complex and involve multiple regulatory elements that may reside within the gene or many kilobases away from the promoter. These elements act positively and negatively in different cells to enhance or restrict expression, and may include sequences that shield a transgene from regulatory influences of other genes near the point of chromosomal insertion.

Index Entries: Growth hormone; somatostatin; prolactin; gonadotrophin-releasing hormone (GnRH); follicle-stimulating hormone (FSH); luteinizing hormone (LH); proopiomelanocortin (POMC); thyroid hormone-releasing hormone (TRH); glycoprotein hormone alpha subunit; growth hormone-releasing factor (GRF); vasopressin; oxytocin; vasoactive intestinal peptide (VIP); neuropeptide Y; enkephalin.

Introduction

Many investigators have sought the ability to direct the expression of an exogenous gene to a specified tissue in an intact organism. Targeting the expression of a gene provides the opportunity to explore mechanisms regulating the expression of the gene, to study the biological activity of the gene product in its natural environment, or to dissect an intracellular process within a specific cell type. In addition, the approach can generate valuable experimental

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reagents, such as immortalized cell lines. Some of the applications of transgenic targeting are listed in Table 1.

The major question to be addressed here is: What gene sequences are needed to specifically target expression to a particular cell type? This review focuses on the use of regulatory sequences of genes that are normally expressed in the cell type to be targeted. Thus, although some coincidental targeting of desired cell types has been accomplished with viral or other strong promoters, this particular strategy will not be considered here.

A large number of neuroendocrine peptide genes have now been explored with respect to their ability to direct expression to specified cell-types in transgenic mice. In particular, many investigators have successfully targeted specific cell types in the pituitary. This is discussed first because it illustrates some of the general mechanisms by which cell-specific gene expression is achieved, and demonstrates a number of useful applications of the technology. However, it is apparent that accomplishing cell specific expression in the hypothalamus is more problematic. A special section discusses important considerations in the interpretation of such data. Results obtained by a number of laboratories using hypothalamic neuroendocrine peptide genes are then considered collectively. The data demonstrate that cell-specific expression of neuropeptide genes in the hypothalamus is complex and requires multiple spatially distinct regulatory elements on the genes. These elements may act positively or negatively to enhance or restrict expression.

Targeting of Transgenes to Specified Phenotypes in the Anterior and Intermediate Lobes of the Pituitary

The pituitary represents a model system to study molecular mechanisms that specify cell phenotype, because individual cell types in this tissue are well-defined, and because its overall development is relatively well understood. The anterior pituitary is considered to contain five major endocrine cell types. These are the somatotrophs, lactotrophs, corticotrophs, and thyrotrophs (which produce growth hormone, prolactin, adrenocorticotropic hormone [ACTH], and thyroid-stimulating hormone [TSH], respectively) and the gonadotrophs, which produce both follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

It has been shown that only 310 bp of the rat growth hormone promoter are necessary to target expression to the pituitary, predominantly to somatotrophs (Lira et al., 1988). This allowed investigators to ablate somatotrophs by fusing promoter sequences to either the diphtheria toxin (Behringer et al., 1988) or the Herpes Simplex thymidine kinase (Borrelli et al., 1989) gene, and thereby produce a dwarf phenotype. Similarly, a *trans*-dominant mutant cAMP-responsive element binding (CREB) transcription factor was targeted to somatotrophs with the same promoter (Struthers et al., 1991). This strategy, designed to interfere with the growth hormone-releasing factor (GRF) signal transduction pathway in somatotrophs (cAMP induction) also produced a dwarf phenotype. Conversely, a giant phenotype was produced with a transgene consisting of the same promoter fused to cholera toxin, which constitutively activates the cAMP pathway (Burton et al., 1991).

The described studies show how a cell-specific promoter can be used to study the biology of a specified cell type. However, the strategy can also be used to study transcription control elements if a transgene is expressed at sufficiently high levels. Although regulatory elements are generally localized by transfection analysis in tissue culture models and/or by analysis of binding of nuclear proteins to DNA sequences, it is desirable to confirm that a regulatory element has activity in vivo. Further, in many cases, appropriate tissue-culture models to localize these elements do not exist. The growth hormone and prolactin genes have been subject to a systematic deletion/mutation analysis in transgenic mice, and the biological

Table 1
Possible Applications of Transgene Targeting to the Hypothalamus

Identification or confirmation of gene regulatory elements
Ablation of specific cell populations
Immortalization of a specific cell type
Blockade or enhancement of gene expression
Interference of an intracellular process within a specific cell
Assessment of gene sequence requirements for gene therapy

significance of several putative regulatory elements identified on these genes has been confirmed (Crenshaw et al., 1989; Lira et al., 1993). Moreover, an analysis of the rat growth hormone gene in transgenic animals demonstrated the existence of previously unknown enhancer-like sequences located farther upstream on the gene. The evidence for the latter is that transgene expression in the pituitary was approx 10-fold higher in animals containing 1.7 kb of the growth hormone gene promoter vs a shorter (320 bp) promoter (Lira et al., 1993).

It has recently been shown that transgene expression in pituitary gonadotrophs can be achieved with the FSH beta subunit (FSHβ) gene promoter, if sufficient genomic sequences are used. Transgenic mice made with 2.3 kb of the bovine FSH beta subunit (FSHβ) promoter fused to the herpes simplex virus type 1 thymidine kinase gene had very low expression in the pituitary and consistently high (and presumably ectopic) expression specifically in the testes and ovaries across pedigrees (Markkula et al., 1993), suggesting that this transgene lacked elements required for pituitary expression as well as elements that might repress expression in the gonads. On the other hand, expression specifically in gonadotrophs was accomplished with a 10 kb FSHβ, gene fragment containing the intact human gene and approx 4.0 kb 5' and 2.0 kb 3' flanking sequence (Kumar et al., 1993). Expression of this FSH transgene was shown to be regulated in a manner similar to the endogenous mouse FSHβ in response to castration and gonadal hormone supplementation (Kumar and Low, 1993). Thus, it seems likely that gonadotroph-specific FSH-β gene expression requires elements that enhance expression, as well as elements that restrict expression to the gonadotroph. In contrast to the potentially large sequence requirements for FSH-β expression, gonadotroph-specific expression was recently accomplished with a transgene consisting of 1.9 kb of the ovine LH beta subunit (LH) gene promoter fused to the bacterial reporter chloramphenicol acetyltransferase (CAT) (Brown et al., 1993).

The gene encoding the glycoprotein hormone α subunit (the common subunit shared by FSH, LH, and TSH) has also been used to target expression to the pituitary. Constructs containing either 313 bp of bovine promoter or 1.6 kb of human promoter fused to CAT resulted in high expression in the pituitary (Bokar et al., 1989). However, low (possibly ectopic) expression in the brain was observed with both transgenes utilizing this sensitive reporter. Ectopic expression was much more evident with a construct consisting of only 168 bp of the human promoter fused to sequences encoding the SV40 virus transforming T-antigen (Horn et al., 1992). This resulted in tumors primarily in the brain and pancreas. In contrast, when 1.8 kb of the promoter were used, only tumors of the pituitary occurred, indicating that some sequences restricting expression to the pituitary reside on the latter construct (Horn et al., 1992).

The larger glycoprotein hormone α subunit gene fragments used in the described studies contain sequences necessary for targeting expression to the gonadotrophs, but appear not to have the sequences necessary for thyrotroph expression. This was directly determined in

studies in which the 1.6 kb human promoter was fused to the β-galactosidase (lacZ) reporter (Hamernik et al., 1992). Histochemical assays indicated that expression occurred exclusively in the gonadotrophs. Further evidence that the sequences specifically target the gonadotroph is that the pituitary tumors induced by the 1.8 kb of the human promoter fused to the SV40 virus T-antigen (discussed earlier) were only of the gonadotrophic lineage (Horn et al., 1992), and a construct consisting of 313 bp of the bovine promoter fused to diphtheria toxin A chain resulted in mice that specifically lacked gonadotrophs (Kendall et al., 1991). Although sequences that target expression to the thyrotroph in vivo have not yet been identified, the general principle can be inferred that elements targeting expression to different tissues may reside on completely different parts of the gene. The existence of sequences that target expression of the α subunit gene to the thyrotroph might become apparent with a larger transgene. A large transgene was utilized by Fox and Solter, consisting of a 17 kb human genomic fragment containing the intact gene, including 5.7 kb of 5' and 1.9 kb of 3' flanking sequence (Fox and Solter, 1988). Transgene expression, analyzed under conditions in which probes were specific for the human form of the gene, was confined to the pituitary. Identical populations of cells were labeled with probes for rodent and human α subunits. This suggests that both gonadotrophs and thyrotrophs expressed the transgene, although direct evidence for this was not provided.

The studies on transgene expression in the pituitary strongly implicate three general principles that appear to govern cell-specific expression. These are that:

1. Multiple positive regulatory elements are required to achieve a full level of expression;
2. Expression in different populations of cells may require spatially-distinct regulatory elements; and
3. Cell-specific expression requires regulatory elements that enhance expression as well as those that repress or restrict expression.

Data on transgene expression in the hypothalamus discussed in the following indicate that the same principles hold true but that the regulatory order increases in complexity.

Cell-Specific Targeting in the Hypothalamus

General Considerations

It will be seen herein that a major problem investigators have faced in targeting the neurons of the hypothalamus is that the level of transgene expression is in many cases variable and far below that of the endogenous gene. In some applications, the low level of expression has been compensated by the use of very sensitive reporters. The more difficult problem of variability in levels of expression is believed to be owing to the fact that the injected gene typically incorporates randomly into the genome. This results in transgene expression that is subject to the positive and negative regulatory effects of other genes near the point of insertion. The problem is amplified in cases where the inherent level of transgene expression is low because of the lack of gene sequences that might be necessary for optimal expression, for example, enhancers, or sequences that might promote mRNA transport out of the nucleus (Maquat, 1991), mRNA stability (Theil, 1993), or translation (Peltz and Jacobson, 1992).

One general approach that has been used in transgenic research to circumvent this problem has been to use large genomic fragments containing intact genes, including all exon, introns, and as much 5' and 3' flanking sequence as possible. This approach in many cases has resulted in transgene expression that is not only position independent and tissue-appropriate, but also at levels that are comparable or even higher than that of the endogenous gene. It is still a matter of debate, however, if this is owing to the inclusion of:

1. Tissue-specific enhancer elements located on exons, introns, or flanking sequences;
2. Sequences, possibly introns, which simply stabilize the expression of the transgene; or

3. Other sequences that do not necessarily act as enhancers, but that may interact with chromatin structure and influence transcriptional activity.

Examples of the later sequences include locus control regions (LCR)s (which are defined as developmental stable DNAse hypersensitive sites that confer expression that is copy-number dependent and integration-site independent [Felsenfeld, 1992]), and chromosomal matrix attachment sites (MAR)s (which bind chromatin tightly in vitro, and may shield a gene from the regulatory influences of nearby genes [Bonifer et al., 1991]). In any case, it is difficult to predict the location of important regulatory sequences *a priori.* Tissue-specific enhancers, for example, can reside on 5' flanking sequence as much or more than 18 kb upstream from the transcription start site (Goldhamer et al., 1992). Some genes, for example those in the β-globin family, are organized in clusters and are coordinately controlled by elements (LCRs) that act over part, or all of the gene cluster (Behringer et al., 1990; Felsenfeld, 1992). All of these considerations argue for the use of the largest transgene possible. However, the practical limitations of using large transgenes are that the initial approach and subsequent application may be technically difficult because a large segment of DNA is typically difficult to obtain and manipulate in vitro. Nonetheless, the several potential causes for poor or variable transgene expression should be taken into account when interpreting existing data on transgene expression in the hypothalamus.

One might also consider the effect of using gene sequences from a different species to make transgenic mice. It seems reasonable to expect that transgene expression will appropriately occur in tissues in which the endogenous gene is expressed by both species if the necessary regulatory sequences are present on the transgene. This is probably so because it seems unlikely that transcription factors and their cognate binding sequences would both change in parallel during evolution. However, if a true species difference in expression exists in a particular tissue, then transgene expression may reflect what occurs in the mouse, or what occurs in the species from which the transgene is derived. This is because the differences in expression may have arisen during evolution from a change in gene regulatory sequences or a change in transcription factors present in the cell.

Vasopressin

The vasopressin gene has probably undergone the most extensive analysis to determine the sequences necessary for cell-specific expression in the hypothalamus. The mRNA encoding this peptide is normally most easily detected in the magnocellular neurons of the supraoptic and paraventricular hypothalamic nuclei (SON and PVN, respectively), but is also expressed in the parvicellular neurons of the PVN and the dorsomedial neurons of the suprachiasmatic nucleus (SCN). The emerging picture from transgenic studies is that tissue-appropriate expression requires sequences that enhance expression, as well as sequences that restrict expression to specific cell types. These elements appear to be located within the gene as well as on the 5' flanking sequences, extending to a region located 1.25–9.0 kb upstream of the transcription start site.

The evidence for gene elements that restrict expression comes from transgene constructs consisting of short portions of vasopressin promoter fused to various reporters. These have resulted in significant and sometimes widespread ectopic expression. For example, a transgene consisting of 1.5 kb of the rat arginine vasopressin (AVP) gene promoter fused to an intact human growth hormone gene reporter (construct A, Fig. 1), exhibited very high levels of ectopic expression in many areas of the brain (Russo et al., 1988). Similar conclusions can be reached from experiments in which mice were generated with a transgene containing 1.25 kb of the bovine vasopressin promoter fused to the bacterial CAT gene (construct B) (Ang et al., 1993), or to sequences encoding the SV40 virus transforming large T-antigen (Tag) (construct C) (Murphy et al.,

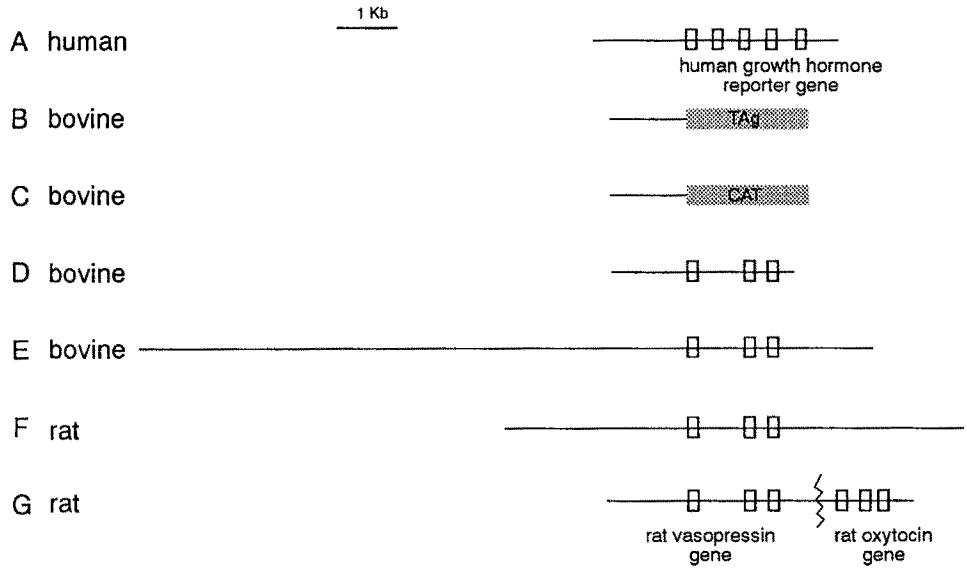

Fig. 1. Transgenes used in attempts to target expression to vasopressin-expressing neurons. Constructs A, B, and C, utilized as reporters, respectively, the intact human growth hormone gene, sequences encoding the SV40 virus T-antigen (TAg) and the *E. coli* chloramphenicol acetyltransferase (CAT) gene. The other transgenes utilized the intact vasopressin genes obtained from other species. Construct G is a fusion of the intact rat AVP and oxytocin genes. *See references* in text for a more complete description of transgenes.

1987). The former resulted in reporter expression in all tissues analyzed (including liver, spleen, and heart), whereas the latter produced tumors in the pancreas and anterior pituitary. Because the ectopic expression was consistently seen in different pedigrees containing the same transgene, and with the same promoter sequences fused to a variety of reporters, it seems reasonable to conclude that tissue-specific expression of this gene, in its normal context, is regulated in part by gene elements that restrict its expression to specific cells.

Data obtained with larger vasopressin transgenes also support the contention that gene expression is controlled in part by elements that restrict expression. For example, when transgenic mice were made with the intact bovine vasopressin gene (consisting of 1.25 kb promoter, all exons and introns, and 0.2 kb 3' flanking sequence [construct D]), transgene expression (measured with a bovine-specific probe) was much more confined to the nervous system, although high levels of expression were observed at some CNS sites expressing little or no AVP (Ang et al., 1993). This varied among pedigrees, presumably because of the aforementioned chromosomal positional effects. One pedigree exhibited transgene expression in the hypothalamus, confined to those nuclei expressing the endogenous gene, suggesting that the sequences necessary for restricted expression within the hypothalamus were in fact present on the transgene. The occurrence of cell-specific expression in the hypothalamus in this pedigree might have been owing to fortuitous integration of the transgene into a genomic site that allowed the cell-restriction effects of these transgene sequences to be exerted. However, the fact that ectopic expression was observed in extrahypothalamic CNS sites, and that other pedigrees exhibited inappropriate expression in the hypothalamus and other sites (or no expression in the hypothalamus), suggests that this transgene still lacked other important regulatory elements, or was highly subject to the regulatory influences of genes near the points of chromosomal integration.

Transgenic mice made with a larger bovine vasopressin transgene (construct E) exhibited a much more restricted and appropriate pattern of expression (Ang et al., 1993). Ectopic expression was only occasionally observed, and of four pedigrees analyzed, three exhibited expression in the hypothalamus; in two of these, expression in the brain was confined to this structure. Two pedigrees were examined by *in situ* hybridization and found to have transgene expression in appropriate hypothalamic nuclei. A comparison of expression patterns between animals made with constructs D and E, suggests that construct E might contain elements not present on D, particularly of the type that restricts expression or shields the gene from the regulatory influences of nearby genes. An unresolved point, however, is that construct E, but not D, resulted in consistent transgene expression in the ovary, suggesting that transgene E contains sequences responsible for this. The contention that construct E contains a biologically relevant ovarian specifier assumes, of course, that vasopressin is expressed in the ovary. There is some evidence that this is the case (Ho and Lee, 1992).

Another group used an intact rat AVP gene that included approx 3 kb each of 5' and 3' flanking sequence (construct F). Expression, analyzed by a very sensitive RT-PCR assay, was reported for one pedigree (Grant et al., 1993). Highest expression was observed in the hypothalamus. Lower transgene expression was detected in CNS sites that also tested positive for endogenous gene expression by the same assay, and also at a few ectopic sites. It would be interesting to compare these results with those using construct E, but this is difficult because of the different assay procedures used, and because cell-specific localization studies were not reported in studies with construct F.

Young et al. tested the hypothesis that vasopressin expression is regulated in part by sequences on the oxytocin gene because these genes are located close to each other on the same chromosome (approx 11 kb apart in the rat) and might require coordinate regulation. Thus, a set of transgenic mice were made with an intact rat gene (construct G) that was similar to bovine construct D, except that it was fused to an intact rat oxytocin gene (Young et al., 1990). These mice did not express the rat AVP gene, but expressed the rat oxytocin gene specifically in the magnocellular neurons that expressed the mouse oxytocin gene (*see the following*). The lack of any detectable rat vasopressin expression in appropriate hypothalamic sites in the four lines of mice made with the latter construct suggests that elements that enhance expression are missing from the transgene, or that expression was extinguished by suppressive effects at the sites of chromosomal integration.

The relative success in targeting the expression of constructs D and E to appropriate hypothalamic nuclei in selected pedigrees allowed an examination of the ability of the transgenes to respond to an osmotic stimulus like the endogenous gene. Expression from both constructs responded appropriately to an osmotic challenge, indicating that the elements responsible for this are contained within the first 1.5 kb of 5' flanking sequence, within the gene, or in the 3' flanking sequence contained in these constructs (Ang et al., 1993). Consistent with this finding is the fact that the expression of construct F was also appropriately regulated in the hypothalamus by osmotic challenge (Grant et al., 1993).

Very recently, transgenic rats were made with an intact rat vasopressin gene tagged in the third exon with the CAT gene. The transgene contained 5 kb of 5' and 3 kb of 3' flanking sequence. Expression was appropriately targeted in two out of two pedigrees to the vasopressin magnocellular neurons of the paraventricular and supraoptic nuclei, and to at least a few cells of the suprachiasmatic nuclei. Expression in the magnocellular neurons was appropriately regulated in response to osmotic stress. However, high ectopic expression in the heart and thymus was observed in both pedigrees. Considering again the lack of ectopic expression with construct E, the conclusion stands that elements restricting expression to the hypothalamus reside far

upstream from the transcription start site (Zeng et al., 1994).

Oxytocin

An intact bovine oxytocin gene containing 600 bp of promoter and 100–150 bp 3' flanking sequence was used to make three sets of transgenic mice (Ang et al., 1991). Northern analysis showed no expression in the hypothalamus or any other tissue except the testes. The expression in the testes should not necessarily be considered ectopic, because oxytocin is expressed at this site in cattle. Notwithstanding, the data suggest that the construct used lacked the minimal elements necessary for expression in the SON and PVN magnocellular neurons, where the endogenous gene is expressed at high levels. In contrast, the vasopressin/oxytocin construct discussed earlier (Fig. 1, construct G), resulted in expression of rat oxytocin mRNA confined to the oxytocin-producing SON and PVN cells, except in one pedigree that exhibited ectopic expression elsewhere in the brain (Young et al., 1990). Rat oxytocin mRNA was also appropriately upregulated in the hypothalamus by lactation. Assuming the oxytocin gene sequences on rat and bovine transgenes were similar, the original hypothesis is supported that the mechanisms regulating vasopressin and oxytocin gene expression are interdependent.

Gonadotrophin-Releasing Hormone (GnRH)

Two groups fused the GnRH promoter to the gene encoding the SV40 virus T-antigen. Mellon et al. (1990) used 2.0 kb of the rat promoter, whereas Radovick et al. (1991) used 1.1 kb of the human promoter. Transgenic mice made with each construction developed tumors that expressed GnRH. Cell lines expressing the GnRH gene have been developed from these tumors, and are now being used to study mechanisms controlling GnRH synthesis and release. The fact that these promoters cause tumors specifically in GnRH cells shows that the promoters used contain sequences necessary to target expression to these cells. It would be of interest to know if these transgenes also contained the sequences necessary for active regulation of the GnRH gene. In this regard, reproductive function was restored to the hpg mouse germ line (which lacks a functional GnRH gene) with a transgene consisting of an intact mouse GnRH gene along with 5.0 and 3.5 kb flanking sequence on the 5' and 3' end (Mason et al., 1986).

Proenkephalin A

The proenkephalin A gene is expressed in several regions of the brain, including a number of hypothalamic nuclei. A transgene consisting of a short portion of the enkephalin promoter and part of the first exon (–193 to +210) fused to the CAT gene followed by 1 kb of 3' enkephalin gene flanking sequence resulted in CAT activity in extracts from several areas of the brain (Donovan et al., 1992). CAT activity was upregulated in the striatum and lumbar spinal cord in a manner similar to the endogenous gene in two experimental models of transsynaptic gene induction (Takemura et al., 1991; Donovan et al., 1992), indicating that elements conferring this were present in the construct. It should be taken into account, however, that expression in the brain was at least two orders of magnitude lower than in the testes, whereas the expression of the endogenous gene is normally about equal at these two sites. The studies indicate that elements that direct high levels of expression to the testes and some expression in the CNS are present on the transgene, whereas elements required for high levels of CNS expression may be located elsewhere.

Another group utilized a transgene that included 3 kb of 5' flanking sequences of the human enkephalin gene fused to the β-galactosidase reporter. Downstream from this reporter was attached 1.2 kb of 3' flanking sequence from the same gene. Transgene expression was observed in all major enkephalin-expressing regions in the hypothalamus in one pedigree, and in a subset of these regions in other pedigrees (Comb et al., 1992;

Borsook et al., 1994). Expression in the paraventricular and supraoptic nuclei was appropriately regulated by osmotic stress. The level of basal expression in a given cell was comparable to that in the testes (M. Comb, personal communication), suggesting that regulatory elements for expression in the testes and these CNS neurons were present on the transgene. However, no expression was observed in the striatum or olfactory bulb that expresses high levels of the endogenous gene, suggesting that gene elements required for this were missing. Finally, no ectopic expression in the brain was observed, suggesting that restriction elements may be present on this transgene.

Vasoactive Intestinal Peptide (VIP)

VIP is expressed at high levels in the SCN, and at lower levels in other hypothalamic nuclei and in the pituitary, and is regulated in some of these sites by various endocrinological manipulations. We and others have made transgenic mice with several different constructions (Fig. 2). A transgene consisting of 5.9 kb of the human VIP gene fused to CAT (construct A) resulted in no expression in the brain being seen in four pedigrees, with the exception of one pedigree exhibiting ectopic CAT activity in the cerebellum (Agoston et al., 1990). Transgene expression was observed in the intestine, however, in all four pedigrees. Limited studies were performed with constructs B and C. No expression in the brain or intestine was observed in the two pedigrees produced with construct B (Agoston and Waschek, unpublished data), and construct C resulted in no detectable brain expression in two pedigrees (O'Hara and Waschek, unpublished data). One pedigree containing construct C appeared to express a high-mol-wt transgene mRNA in the intestine. The larger size may have occurred because that construct lacked a preferred VIP gene polyadenylation signal.

The results with these human gene constructs contrast with those obtained with the shorter 2.5 kb of the rat VIP promoter (construct D). This transgene was variably expressed in certain areas of the brain that express VIP, although some of the expression was ectopic, and no expression was observed in the SCN of any of the mouse lines (Low, unpublished work). This transgene did, however, show some expression in the intestinal neurons that express VIP.

Our most recent attempts to target the VIP-expressing cells of the hypothalamus have used a very large mouse VIP/lacZ fusion gene (construct E). The preliminary results so far are that no expression in the SCN or other hypothalamic nuclei or brain regions has been detected in three lines of mice by the standard histochemical assay. Thus, the construct appears to lack elements required for expression in the CNS. The fact that constructs A, B, C, and E resulted in little or no ectopic CNS expression suggests that sequences on these transgenes might restrict expression in the CNS. In addition, although an intestinal enhancer appears to reside on the human and rat VIP gene constructs, the elements that confer consistent and high levels of cell-specific expression in the CNS may occur on sequences not on any of these transgenes.

Pro-Opiomelanocortin (POMC)

The polypeptide precursor POMC, which gives rise to ACTH, β-endorphin, melanocyte-stimulating hormone (MSH), and other peptides, is expressed at highest levels in the arcuate nucleus and in the intermediate and anterior lobes of the pituitary. Transgenes containing 323, 480, and 706 bp of the rat POMC gene promoter directed reporter gene expression to the intermediate pituitary lobe melanotrophs and anterior lobe corticotrophs, but not to a level of detection in hypothalamic cells that normally express the gene (Tremblay et al., 1988; Hammer et al., 1990; Liu et al., 1992). Expression from the transgenes containing the 323 and 706 bp of promoter was tested and found to be appropriately regulated in pituitary corticotrophs by glucocorticoids. Interestingly, in transgenic mice containing 706 bp of the POMC promoter fused to the SV40 large T-antigen (Low et al., 1993), tumors formed in

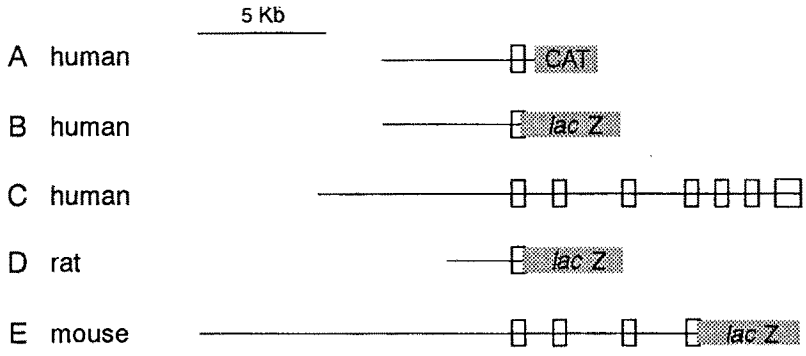

Fig. 2. Transgenes used in attempts to target expression to VIP-expressing neurons. Construct A contained 5.2 kb of the human VIP gene promoter, the first exon, part of the first intron, and a synthetic splice sequence fused to the gene encoding *E. coli* chloramphenicol acetyltransferase (CAT) Constructs B and D contained the indicated 5' portions of the human and rat VIP genes fused in their first exons to the *E. coli* beta-galactosidase *(lacZ)* gene. Construct E contains 16.5 kb of the mouse VIP gene (including 12.0 kb of 5' flanking sequences) fused in exon four in frame to *lacZ*. *See references* in text for a more complete description of transgenes.

the intermediate, but not anterior pituitary, possibly because of higher expression, or because the melanotroph is more susceptible than the corticotroph to the transforming effects of the T-antigen.

A recent study utilized two larger constructions, one containing 4.0 kb of the rat POMC promoter fused to *lacZ*, and the other, an intact mouse POMC gene containing 2.0 kb of 5' and 2.0 kb of 3' flanking sequence (Rubenstein et al., 1993). The latter was "tagged" by the insertion of a short DNA sequence into exon 3 so that its expression could be distinguished from that of the endogenous gene. In approximately half of the mouse lines containing the 4 kb of promoter fused to *lacZ*, expression was targeted to the melanotrophs and to a portion of the corticotrophs, but significant ectopic expression was observed in several areas of the brain. The construct containing the intact gene, on the other hand, was more efficiently targeted to pituitary corticotrophs. Ectopic expression in the brain was observed in only two of six pedigrees analyzed and was confined to the hippocampus dentate gyrus. Neither of these constructions correctly targeted expression to the arcuate nucleus. The results suggest that all transgenes used contain some of the elements necessary for expression in the melanotrophs and for glucocorticoid hormone-regulated expression in corticotrophs, but not for expression in the arcuate nucleus. The largest construct, containing the intact gene, contained additional elements at confer high and efficient constitutive expression in corticotrophs, and elements that restrict expression in the brain.

Neuropeptide Y

Neuropeptide Y mRNA is expressed in several hypothalamic nuclei. A transgene consisting of approx 800 bp of the human neuropeptide Y promoter fused to CAT resulted in reporter activity in the limbic region and other neural sites where the peptide is expressed but not in sites where little or no expression occurs (Waldbieser et al., 1992). It is of interest to know if the sequences on this transgene target expression to the appropriate hypothalamic nuclei, especially in light of the studies on vasopressin discussed earlier, which indicate that targeted expression to a particular brain region does not necessarily mean cell-specific expression.

Somatostatin

An intact mouse somatostatin gene containing 12 kb of 5' and 2 kb of 3' flanking sequence (tagged with a short foreign DNA sequence

inserted into the 5' untranslated region) was used to make transgenic mice (Rubenstein et al., 1992). These animals consistently expressed the transgene in specific hypothalamic nuclei that in the adult do not express somatostatin, and they did not express the transgene in periventricular nucleus of the hypothalamus, which normally expresses a high level of somatostatin. The data suggest that elements necessary for expression in the adult rodent brain were missing, and because ectopic expression in certain cell types was consistently observed, the transgene must have been missing a repressor element that normally silences the gene in these cells.

The biological significance of a silencer on the somatostatin gene has been proposed by Oster-Granite (1993), who reported that the endogenous somatostatin gene is transiently expressed in mice during development in those hypothalamic nuclei in which ectopic expression occurred (Bendoth et al., 1990; Oster-Granite, 1993). Thus, the transgene may have contained sequences that specify expression in these nuclei during development, but lacked the elements necessary to extinguish expression in the adult.

Shorter portions of the rat somatostatin gene 5' flanking sequence (4.0 or 0.4 kb) ligated to either the β-galactosidase or a nontransforming mutant form of the T-antigen (K1Tag) reporter produced a variable pattern of brain expression. Interestingly, in several pedigrees, substantially correct expression was produced in cortical neurons, amacrine cells of the retina, and the hilus of the dentate gyrus of the hippocampus (Low, personal communication), suggesting that some elements necessary for expression at these sites were present on the transgene, but perhaps variably suppressed by positional effects.

Growth Hormone-Releasing Factor (GRF)

Several constructs containing different portions of the GRF gene fused to SV40 virus T-antigen sequences were tested in transgenic mice. These consisted of either 4.2 kb of the human GRF gene promoter, or 1.6 kb of the same promoter plus the first intron and part of the second exon fused to T-antigen-encoding sequences (Botteri et al., 1987). None of these constructs resulted in transgene expression in the hypothalamus, and all resulted in thymic hyperplasia, presumably because of ectopic expression. Similarly, a transgene consisting of 872 bp of the mouse GRF gene promoter fused to TAg sequences resulted in no expression in the hypothalamus, and tumors in the adrenal medulla, again presumably because of ectopic expression (Giradli et al., 1994).

Thyrotropin-Releasing Hormone (TRH)

No full reports have appeared in the literature in which the TRH gene has been examined in a transgenic model. However, transgenic mice have been made with approx 5 kb of rat TRH gene 5' flanking sequence fused to β-galactosidase (Low, personal communication). These animals displayed inconsistent expression of the transgene, with expression most commonly seen in septal nuclei, the dorsomedial nucleus of the hypothalamus, olfactory bulb, and brain stem. No expression was observed in the hypothalamic PVN.

Conclusions

Much evidence has accumulated now suggesting that targeting cells in the hypothalamic-pituitary axis requires that multiple gene elements be present on the transgene. These elements act positively to increase expression or negatively to restrict expression to specific cell types. The minimal sequences necessary for high and tissue-specific expression are close to the promoter in only a limited number of cases. Better targeting of hypothalamus-expressed genes has been observed when intragenic sequences (exons and introns) were included on transgenes, but the reason for this is uncertain. Higher, more consistent and appropriate expression has also been observed with increased amounts of gene flanking sequences. The additional sequences may con-

tain enhancers, silencers, or elements that shield the promoter from the regulatory effects of other genes near the point of genomic insertion, or in some other way allow the gene to function as an independent transcriptional unit.

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