Bovine Oxytocin Transgenes in Mice

HYPOTHALAMIC EXPRESSION, PHYSIOLOGICAL REGULATION, AND INTERACTIONS WITH THE VASOPRESSIN GENE

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To gain insights into the molecular mechanisms that restrict the expression of the oxytocin gene to anatomically defined groups of neurons in the hypothalamus, we generated transgenic mice bearing bovine oxytocin genomic fragments. Appropriate neuron-specific and physiological regulation was observed in mice bearing transgene bOT3.5, which consists of the oxytocin structural gene flanked by 0.6 kilobase pair (kbp) of upstream and 1.9 kbp of downstream sequences. bOT3.5 is expressed in oxytocin magnocellular neurons in the mouse supraoptic nucleus and paraventricular nucleus, but transgene RNAs are excluded from vasopressin neurons. Replacement of the drinking diet of the transgenic mice with 2% (w/v) NaCl for 7 days significantly increased the abundance of bovine oxytocin transcripts in the supraoptic nucleus, but not in the paraventricular nucleus, in parallel with the endogenous mouse oxytocin RNA. Surprisingly, mimicry of the endogenous oxytocin gene expression pattern was lost with larger transgenes. Addition of 0.7 kbp of contiguous downstream sequences (transgene bOT) or linkage to the bovine vasopressin gene (transgene VP-bOT3.5) repressed hypothalamic expression. No mice were derived bearing transgene bOT6.4, which consists of the oxytocin structural gene flanked by 3 kbp of upstream and 2.6 kbp of downstream sequences, suggesting that the presence of this DNA is detrimental to normal embryonic development. These data suggest that while bOT3.5 contains sufficient cis-acting sequences to mediate expression to particular subsets of hypothalamic neurons, the overall regulation of the oxytocin gene is governed by multiple interacting enhancers and repressors.

Ever since pioneering studies demonstrated that the posterior pituitary contains activities that stimulate uterine contraction (1) and milk ejection (2), the oxytocin (OT) system has been a favorite model for the study of the regulation and function of an identified class of central peptidergic neurons. OT is contained in the uterus and mammary gland, OT stimulates smooth muscle contraction, leading to parturition or milk ejection. The single-copy structural gene encoding the OT prepropeptide consists of three exons and encompasses <1 kbp (5). The OT gene is highly homologous at both the structural and sequence level to the gene encoding the related neuropeptide vasopressin (VP). In mammals, the two genes are separated by a short intergenic region of 11 kbp in the rat (5) and of 3 kbp in the mouse (6) and are transcribed toward each other from opposite strands of the DNA duplex. An attractive feature of the OT system is that, within the hypothalamus, the expression of the OT gene is confined to anatomically defined groups of magnocellular neurons in the supraoptic nucleus (SON) and in the paraventricular nucleus (PVN). VP is also expressed in hypothalamic magnocellular neurons, but VP and OT are rarely found in the same cell (7–9). The OT gene is also expressed in a number of peripheral tissues (10).

Studies on the physiological regulation of OT gene expression in the hypothalamus have benefited from the exploitation of well established paradigms for the modulation of the activity of OT neurons. These experiments have revealed that OT gene expression in magnocellular cells is increased in response to functional demand. Thus, during pregnancy and lactation, when pituitary stores of OT are depleted, the abundance of the OT mRNA in the hypothalamus increases (11). Surprisingly, despite being expressed in distinct magnocellular neurons, the VP mRNA also increases in abundance during pregnancy and lactation (11). Conversely, osmotic stimuli (such as salt loading) that result in functional demand for VP and an increase in VP gene transcription (12, 13) and VP mRNA abundance (14–17) also result in similar changes in OT expression (16, 18). Liddle is known about the regulation of OT gene expression in the hypothalamus in terms of either the synaptic regulation of phenotype and physiological status or their mediation by second messengers, transcription factors, and cognate OT gene cis-acting elements. In vitro studies have described cis-elements in the OT gene promoter that are able to mediate transcriptional regulation, but the physiological relevance of these findings remains to be determined. For example, Richard and Zingg (19) have shown that the human OT promoter is able to direct the high level expression of a reporter in the mouse neuroblastoma cell line Neuro 2A. The sequences mediating this effect have been shown to consist of at least three cooperating elements located within the 50 base pairs upstream of the mRNA cap site. Both rat and human OT promoters contain...
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RAT TRANSGENES

![Diagram showing transgene orientation and expression]

BOVINE TRANSGENES

NEURONS

![Diagram showing transgene orientation and expression in neurons]

Fig. 1. Structures of bovine VP and OT transgenes. Shown are the structures of the bovine VP and OT transgenes described in this study and their relationship to the rat VP-OT locus (37) and to previously described rat (37) and bovine (30, 31) transgenes. The horizontal arrows indicate the direction of transcription. Shaded boxes represent exons. Rat transgenes are shown above the locus map, and bovine transgenes are shown below. Expression patterns for either VP (left side) or OT (right side) are described. HYP; hypothalamus; TEST, testis.

direct and inverted repeats of an AGGTCA nuclear hormone receptor-binding site. Transfections into heterologous cultured cells have shown that these sequences can mediate transcriptional up-regulation of the OT promoter in response to the ligand-dependent binding of the estrogen (20, 21), thyroid hormone (22), and retinoic acid (23) receptors. Although the presence of these receptors in oxytocinergic neurons has not been demonstrated (24), the finding that OT gene expression in the rat uterus (25) is estrogen-responsive (26, 27) may provide a physiological context within which these elements function. The bovine OT promoter is not responsive to estrogen (28).

To define broad regulatory regions required for appropriate expression, we have generated transgenic mice bearing the bovine OT gene. Here, we describe a bovine OT transgene that is expressed in a cell type-specific manner in the mouse hypothalamus; within which it is subject to physiological regulation. The hypothalamic expression of this transgene is repressed by downstream sequences and by sequences in the VP gene.

MATERIALS AND METHODS

Transgene Construction—All transgenes were derived from bovine genomic sequences isolated and kindly donated by Siegfried Ruppert and colleagues (29). Fig. 1 shows the extent of the transgenes described in this study. bOT6.4 is a 6.4-kbp transgene fragment consisting of the OT structural gene flanked by ~3 kbp of upstream and ~2.5 kbp of downstream sequences (31). bOT3.5 is a 3.5-kbp transgene consisting of the OT structural gene flanked by ~0.6 kbp of upstream (defined by an EcoRI site) and ~2.5 kbp of downstream (defined by an EcoRI site) sequences. bOT1, a 4.2-kbp EcoRI OT gene fragment, consists of the structural gene flanked by ~0.6 kbp of upstream and ~2.5 kbp of downstream sequences (31). bOT3.5 is a 3.5-kbp transgene consisting of the OT structural gene flanked by ~0.6 kbp of upstream (defined by an EcoRI site) and ~1.8 kbp of downstream (defined by an XbaI site) sequences. VP-B is a 3.45-kbp VP transgene fragment consisting of the structural gene flanked by ~1.25 kbp of upstream (defined by an Hpal site converted to a Sall site) and ~0.2 kbp of downstream (defined by an XbaI site) sequences (31). bOT3.5 and VP-B were joined at their respective downstream XbaI sites to generate transgene VP-B/bOT3.5 (see Fig. 1). The VP and OT genes in VP-B/bOT3.5 are thus arranged in a 3' to 3' transcriptional orientation. Fragments were freed from vector sequences prior to their use as transgenes.

Animals—Animals were cared for in accord with National Institutes of Health guidelines. Transgenic mice were generated by microinjecting fertilized one-cell eggs with DNA fragments derived from the bovine OT gene.

Note that numerous, independently prepared DNA preparations were used for the injection sessions using bOT6.4.

Efficiency of transgenic mouse generation following microinjection of fertilized one-cell eggs with DNA fragments derived from the bovine OT gene

Table I

| Construct | bOT3.5 | VP-B/bOT3.5 | bOT6.4 |
|-----------|--------|-------------|--------|
| Eggs injected | 454 | 242 | 1226 |
| Eggs surviving injection | 426 | 233 | 1172 |
| % of eggs injected | 93.8 | 96.3 | 95.6 |
| Injection sessions | 4 | 3 | 10 |
| Eggs transferred | 347 | 125 | 1054 |
| Surrogate mothers | 12 | 8 | 38 |
| Pups | 42 | 12 | 152 |
| % of pups | 35.7 | 33.3 | 0 |
| % of eggs transferred | 4.3 | 3.2 | 0 |

RNA using guanidinium isothiocyanate and its analysis by Northern blotting have been described (32). In one experiment (shown in Fig. 2B and Table II, Experiment 2), RNA was extracted using Trizol (Life Technologies Inc.). Northern blots were controlled for equal loading and even transfer in two ways. First, following transfer, the ethidium bromide-stained filter was viewed and photographed under ultraviolet light. Second, filters were routinely reprobed with oligonucleotides corresponding to the mRNAs encoding either glyceraldehyde-3-phosphate dehydrogenase or a-tubulin. The abundance of these RNAs does not change in the hypothalamus following physiological stimulation and thus can be used as quantitation standards. Note that a-tubulin and glyceraldehyde-3-phosphate dehydrogenase RNAs are expressed at different levels in different tissues and that the relative pattern of expression observed is consistent between animals. The oligonucleotide probes specific for the rodent and bovine OT and VP RNAs have been described (30, 31). Northern blot autoradiograms from salt loading experiments were subjected to quantitative analysis. Linear range exposures were scanned by laser densitometry (Pharmacia Biotech Ultrascan or Millipore Bio Image Visage 110), and the levels of bovine OT, mouse OT, or mouse VP RNA were determined relative to the levels of a-tubulin or glyceraldehyde-3-phosphate dehydrogenase RNAs and are expressed as a percentage of the mean of the control groups (± S.E.). Statistical
UTERUS; and PITUITARY GLAND; following tissues were examined: AP and PONS; micethatarebothlargerandsmallerthanbOT.bOT6.4isthe that are able to direct transgene expression to central and peripheral sites, we have introduced bovine OT transgenes into mice that are both larger and smaller than bOT. bOT6.4 is the same as bOT, except that it has a total of −3.0 kbp of upstream sequence of the structural gene (Fig. 1). bOT3.5 is the same as bOT, except that it lacks −0.7 kbp of distal downstream sequence (Fig. 1). We have also investigated possible regulatory interactions between the VP and OT genes by introducing transgene VP-B/bOT3.5 into mice (Fig. 1). Fertilized one-cell mouse eggs were injected with the three transgene fragments (Table I). Transgenes bOT3.5 and VP-B/bOT3.5 yielded transgenic mice at a high efficiency. However, despite injecting a large number of fertilized one-cell mouse eggs with construct bOT6.4, no transgenic animals were identified among the resulting pups (Table I).

Expression of Bovine OT Transgenes in Mice—Northern analysis demonstrated that transgene bOT3.5, like bOT, was expressed in the testis and lung (Fig. 3). Surprisingly, and unlike bOT, expression of bOT3.5 was also found in the hypothalamus (Figs. 2A and 3). Within the hypothalamus, in situ hybridization analysis revealed that transgene expression was confined to magnocellular neurons of the PVN and SON (Fig. 4). Neither the endogenous mouse OT RNA nor the transgene OT component were seen in the testis and lung (Fig. 6), but was generally excluded in the hypothalamus (Figs. 2A and 3).

Expression of the endogenous OT gene and of the bOT3.5 transgene in the murine hypothalamus is confined to the SON and PVN. Coronal brain sections from transgene bOT3.5 line 1 (BOT3.5) and wild-type (WT) mice were probed with labeled oligonucleotides corresponding to the rodent (ROT) or bovine (BOT) OT RNAs. Representative sections are shown in the vicinity of the PVN and SON. Note that the bovine OT probe does not give any signal in the wild-type mouse hypothalamus.

Physiological Regulation of Bovine OT Transgene Expression in Mice—We asked if expression of bOT3.5 in the hypothala-
mus is regulated by a stimulus that modulates the expression of the endogenous OT gene. Replacement of the drinking diet of mice with 2% (w/v) NaCl for 7 days increased the hypothalamic level of the endogenous mouse OT and VP RNAs, as assayed by Northern blotting (Fig. 2B and Table II). However, Northern blotting revealed no significant change in the level of the transgene RNA (Fig. 2B and Table II). Quantitative in situ hybridization analysis (Fig. 7) revealed that the transgene RNA level was modulated in only one group of magnocellular OT neurons; an effect was masked from detection using Northern blotting.
Thus, while salt loading had no significant effect on the levels of either the endogenous or transgene OT RNAs in the PVN (Fig. 7, B and C), both were significantly elevated in the SON (Fig. 7, A and B). Salt loading had no effect on the number of cells expressing either transgene or endogenous RNAs in either the SON or PVN (data not shown). The expression of the endogenous murine OT RNA and the transgene RNA was generally excluded from VP neurons in salt-loaded animals (Fig. 5).

**DISCUSSION**

While the molecular mechanisms underlying the cell-specific and physiological regulation of the OT gene are the subject of much interest (34), mechanistic understanding has lagged behind descriptive studies because of the lack of appropriate cell culture systems. To circumvent this obstacle (35), we have applied transgenesis to the OT system.

We have previously described the expression in mice of the 4.2-kbp transgene bOT (30, 36). bORT RNAs were detected in the testis and lung, but not in the hypothalamus. Initially, we thought that bOT must be missing elements crucial for expression in OT neurons. However, we now show that bOT contains too much sequence information, rather than too little. Surprisingly, bOT3.5, which differs from bOT only in that it lacks 0.7 kbp of distal downstream flanking sequence, is expressed consistently in the hypothalamus, within which transgene expression is confined to magnocellular cells in the PVN and SON that also express the endogenous mouse OT gene. Transgene RNAs, like the endogenous OT transcripts, are excluded from neurons expressing mouse VP RNAs. We suggest that the downstream sequences present in bOT (but absent from bOT3.5) contain one or more repressor elements that prevent the detectable expression of the former transgene in hypothalamic oxytocinergic neurons. It follows that in the normal genomic context of the OT gene, the activity of the putative repressors must be repressed or overridden.

We were unable to generate any transgenic mice bearing construct bOT6.4 (Table I). bOT6.4 differed from bOT only in that the sequence upstream of the start of transcription consisted of 3.0 kbp, rather than the 0.6 kbp found in the latter transgene. Young et al. (37) have described a similar situation with a rat OT transgene. No transgenic mice were represented in the pups resulting from the injection of fertilized one-cell mouse eggs with ROT1.63, which consisted of the entire rat OT structural gene flanked by 0.36 kbp of upstream and −0.4 kbp of downstream sequences (Fig. 1). Both rat and cattle OT genes contain sequences that are detrimental to normal development. In the bovine gene, these sequences must reside in the 2.4 kbp of distal upstream sequences, present in bOT6.4, but absent in bOT. Presumably, ectopic OT expression, mediated by these sequences at an inappropriate time, is incompatible with embryogenesis. In the context of the normal OT gene, this toxic effect must be repressed.

**TABLE II**

Effect of salt loading on the levels of the endogenous and transgene RNAs in the hypothalamus

| Control | n |Salt-loaded | n | p |
|---------|---|------------|---|---|
| Exp. 1  |   |            |   |   |
| ROT RNA | 100 ± 5.4 | 3 (4) | 149 ± 8.7 | 3 (4) | <0.005 |
| RVP RNA | 100 ± 5.6 | 3 (4) | 175 ± 22.2 | 3 (4) | <0.025 |
| BOT RNA | 100 ± 9.7 | 3 (4) | 123 ± 16.1 | 3 (4) | NS |
| Exp. 2  |   |            |   |   |
| ROT RNA | 100 ± 3.3 | 3 (6) | 143 ± 19.5 | 3 (6) | <0.05 |
| RVP RNA | 100 ± 4.2 | 3 (6) | 255 ± 31.4 | 3 (6) | <0.005 |
| BOT RNA | 100 ± 13.6 | 3 (6) | 83 ± 25.5 | 3 (6) | NS |

a n = number of independent experiments. The numbers in parentheses indicate the number of animals per group.

b ROT, rodent OT; RVP, rodent VP; BOT, bovine OT; NS, not significant.
Because of the close linkage between the VP and OT genes, it has been suggested that interactions between the two transcrip-
tion units may be important in locus control. Here, we show that the bovine VP and OT genes can affect the expres-
sion of each other. The VP-B/bOT3.5 transgene consists of the VP-B and bOT3.5 constructs linked 3' to 3' (Fig. 1). We have previously shown that VP-B, consisting of the bovine VP structural gene flanked by 1.25 kbp of upstream and 0.2 kbp of downstream sequences, is expressed in all neuronal tissues examined (31). As described above, bOT3.5 is expressed in OT neurons, testis, and lung. In mice containing the VP-B/bOT3.5 transgene, expression of the VP component could not be detected in any central or peripheral tissue, while expression of transgene OT RNA was seen only in the testis and lung. Thus, sequences within the OT component of the transgene prevented expression of the VP portion in neuronal cells, while sequences within VP-B repressed the hypothalamic expression of bOT3.5. Support for the concept of cross-talk between the VP and OT genes has also come from the experiments of Young et al. (37). These authors described mice bearing a minilocus transgene (V2) consisting of construct ROT1.63 (see above and Fig. 1) linked to sequences from the rat VP gene (transgene RVP3.55) (Fig. 1). No transgenic mice were derived from the microinjection of fertilized one-cell mouse eggs with ROT1.63 (see above). On its own, RVP3.55, consisting of the structural gene flanked by −1.4 kbp of upstream and 0.4 kbp of downstream sequences (Fig. 1), was not expressed. In the V2 minilocus transgene, the two genes were arranged in the opposite transcriptional orientation (VP 3' to OT 5') to the native genes (VP 3' to OT 3'). The VP component of the V2 minilocus was again silent. However, the OT transcription unit present in V2 was expressed in oxytocinergic magnocellular neurons (37-39). Thus, elements within the VP component must repress the deleterious effects of the OT portion, allowing transgenic mice to be generated. Thus, the close linkage of the VP and OT genes may be important for overall locus control, perhaps by coordinating responses of the two genes to developmental or regulatory cues. However, it should be noted that our data also suggest that there is redundancy in the regulatory elements contained within the two transcription units as the bOT3.5 transgene, consisting only of OT gene sequences and proximal flanks, is expressed autonomously in oxytocinergic neurons. Similarly, we have described bovine VP transgenes that are

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W. S. Young III, personal communication.
properly regulated in the hypothalamus (31).

Physiological stimuli are known to affect the pattern of OT gene expression in the hypothalamus, and we asked if these same stimuli altered the expression of the bOT3.5 transgene. We have shown that the level of bovine OT RNA increased in the hypothalamus during salt loading, in parallel with the endogenous mouse OT RNA. Thus, the sequences mediating gene expression in the hypothalamus, and we asked if these same stimuli altered the expression of the bOT3.5 transgene.

In summary, transgene bOT3.5 contains sufficient sequence information to direct expression to murine oxytocinergic magnocellular neurons, within which it is subject to physiological regulation. However, this expression pattern is not found when the same bOT3.5 sequences are linked either to the contiguous 0.7 kbp of downstream sequence (in bOT) or to the VP gene (in VP-B/bOT3.5), which is closely linked to the OT gene in mammals (5, 6). The expression patterns seen with bovine VP (31) and OT (Ref. 30 and this report) transgenes in mice suggest that the cell type-specific expression of these genes is mediated by interactions between multiple enhancers and repressors located in both transcription units.

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