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Studies of oxytocin and vasopressin gene expression in the rat hypothalamus using exon- and intron-specific probes

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Yue, Chunmei, Noriko Mutsuga, Elka M. Scordalakes, and Harold Gainer. Studies of oxytocin and vasopressin gene expression in the rat hypothalamus using exon- and intron-specific probes. Am J Physiol Regul Integr Comp Physiol 290: R1233–R1241, 2006. First published December 15, 2005; doi:10.1152/ajpregu.00709.2005.—To develop a comprehensive approach for the study of oxytocin (OT) and vasopressin (VP) gene expression in the rat hypothalamus, we first developed an intronic riboprobe to measure OT heteronuclear RNA (hnRNA) levels by in situ hybridization histochemistry (ISHH). Using this 84-bp riboprobe, directed against intron 2 of the OT gene, we demonstrate strong and specific signals in neurons confined to the supraoptic (SON) and paraventricular (PVN) nuclei of the rat hypothalamus. We used this new intronic OT probe, together with other well-established intronic and exonic OT and VP probes, to reevaluate OT and VP gene expression in the hypothalamus under two classical physiological conditions, acute osmotic stimulation, and lactation. We found that magnocellular neurons in 7- to 8-day lactating female rats exhibit increased OT but not VP hnRNA. Since VP mRNA is increased during lactation, this suggests that decreased VP mRNA degradation during lactation may be responsible for this change. In contrast, whereas there was the expected large increase in VP hnRNA after acute salt loading, there was no change in OT hnRNA, suggesting that acute hyperosmotic stimuli produce increased VP but not OT gene transcription. Hence, the use of both exon- and intron-specific probes, which distinguish the changes in hnRNA and mRNA levels, respectively, can provide insight into the relative roles of transcription and mRNA degradation processes in changes in gene expression evoked by physiological stimuli.

heteronuclear ribonucleic acid; lactation; hyperosmotic

STUDIES OF NEUROPEPTIDE GENE expression in the central nervous system (CNS), typically performed by in situ hybridization histochemistry (ISHH) using exon-specific probes, measure the steady-state levels of mRNA, which reflect both gene transcription and mRNA degradation processes in the neuron. In contrast, measurements using intron-specific probes measure pre-mRNA or heteronuclear RNA (hnRNA) levels in the neuron, which, because of the rapid turnover of the primary transcript and intermediate forms of RNA in the cell nucleus, are believed to primarily reflect the transcription rate of the gene (16). An effective intron-specific vasopressin (VP) (19) probe has been widely used for studies of the regulation of VP gene expression in the hypothalamo-neurohypophyseal system (HNS) in vivo and in vitro (2, 23, 31).

In contrast, studies of oxytocin gene expression using intron-directed probes have been relatively sparse. The first report describing the design and use of an oxytocin (OT) intronic probe was reported by Brooks et al. (8), and this was a 201-bp fragment complementary to the 220-bp-long intron 1 in the OT gene. This OT intronic probe labeled with [3H]dNTPs successfully detected changes of OT hnRNA in the rat hypothalamus under various physiological conditions (8, 13). Another laboratory using a 211-bp intronic probe from OT intron 1, which was labeled with [35S]UTP, did not detect OT hnRNA in the rat hypothalamus (38). In preliminary experiments, we designed a 35S-labeled probe directed at intron 1 of the OT gene, and using this probe, obtained very variable signals in magnocellular neurons (MCNs) in the rat hypothalamus. Hence, here our objective was to develop a reliable intron-specific probe for the study of OT hnRNA using 35S-labeling and ISHH procedures comparable to that previously used in studies of VP hnRNA (19). To our surprise, we found that 35S-labeled probes directed at the smaller, 84-bp intron 2 were much more reliable for detecting OT hnRNA in hypothalamic neurons. In this paper, we describe an OT intron-specific riboprobe and use it in a study that compares OT and VP gene regulation in the hypothalamus under various physiological conditions.

MATERIALS AND METHODS

Animals. Adult male (Taconic, Germantown, NY) and female (Charles River Laboratories, Wilmington, MA) Sprague-Dawley rats were used. Animals were individually housed under temperature (21–23°C)- and light (12:12-h light-dark cycle)-controlled facilities and given food and water ad libitum. All procedures were carried out in accordance with the guidelines set forth by the National Institutes of Health Animal Care and Use Committee. For the acute osmotic stimulation experiment, male rats (n = 6 per group) received either an isotonic (0.9% normal saline) or hyperosmotic (1.5 M NaCl) intraperitoneal injection (1 ml solution/100 g body wt) and were killed by decapitation after 2 h. Lactating females (n = 6) were killed on day 7 or 8 of lactation. Diestrus females (n = 5) were killed at the time of lights off (±1 h). After decapitation, all brains were frozen on...
powdered dry ice and stored at −80°C until sectioning. To determine the stage of the estrous cycle, daily vaginal smears were conducted. After two estrus cycles, females were killed on the day of the cycle when the cell morphology corresponded to a diestrus smear.

**Design and preparation of intron-specific probes for OT.** DNA sequences in intron 1 and intron 2 of the OT gene were used to design the PCR primers for the OT intronic probes 1 and 2 (In1 and In2), respectively, using DNASIS-Mac v3.5 software (Hitachi Software Engineering, Tokyo, Japan). The BLAST results showed that these primers are specific for the OT gene. The PCR primers used to generate OT In1 are: 5′-ATGAAAGTCTTGTTTGCGGAAGGCTTAAAG-3′ (sense) and 5′-ATCTGCGACAAAATCATTGCGGACTG-3′ (antisense). Using these primers, we were able to obtain a 162-bp fragment of OT intron 1 after PCR (see Fig. 1). The primers used to obtain OT In2 are: 5′-ATGAAAGCTTGGAGCAGAGGGCCCT A-3′ (sense) and 5′-ATGCTGCAGCTGCAAGAGAATAATGG-3′ (antisense). These produced an 84-bp fragment of In2 (see Fig. 1). PCR was carried out in a 50-μl reaction volume, containing 0.8-μg rat genomic DNA, 1 × PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 200 μM each of dNTPs, 200 nM each of the primers and 2.5 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). PCR was performed on a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA) and consisted of an initial 2 min, 94°C denaturation, followed by 30 cycles of denaturing (94°C, 30 s), annealing (60°C, 30 s) and extension (72°C, 45 s), followed by a final extension of 7 min at 72°C. The PCR products were loaded on the 1.5% agarose gel and purified by the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Both PCR fragments were subcloned into pBlueScript II SK (+) (Stratagene, La Jolla, CA). Modified T7 (GGCGCCATAATACGACTCATAAGGG) and T3 (GGCGGATTAACCCCTACTCATAAAGG) primers were used to PCR amplify the fragments, and the resulting PCR products were used as templates for the synthesis of riboprobes. T7 and T3 RNA polymerases were used to obtain both sense- and antisense-labeled probes, respectively. Hybridization of sense probes were performed as negative controls.

The rat VP intronic riboprobe (kindly provided by Dr. Thomas Sherman, Georgetown University, Washington, D.C.) was a 155-bp fragment of intron 1 of rat VP gene subcloned into pGEM-3 vector (Promega, Madison, WI) (19). The rat VP exonic probe was a 229-bp fragment of the rat VP gene, and the rat OT exonic probe was a 487-bp fragment obtained from Dr. W. Scott Young (National Institute of Mental Health). Both were subcloned into pGEM-3 vectors (34). Modified T7 and SP6 (CATACG ACTTATAGGG) primers were used to PCR amplify all of these three fragments to make templates that were used to synthesize the riboprobes. The T7 RNA polymerase was used to synthesize the antisense of hnVP and exonic VP probes and the SP6 the antisense of the OT exonic riboprobe.

**Labeling of the VP intronic, VP exonic, and OT exonic riboprobes.** The VP intronic riboprobe was synthesized and purified by polycrylamide gel electrophoresis (Sigma, St. Louis, MO). A BLAST search of the nucleotide sequences in GenBank failed to reveal any nonspecific matches for these probes. We used a labeling hybridization protocol for the oligonucleotide probes, which is described in detail on the following web site: http://intramural.nimh.nih.gov/lcmr/snge/. Briefly, in this method, 5 pmol of oligonucleotide is labeled with 57.5 μCi of [α-35S]-deoxyadenosine 5′-triphosphate (Perkin Elmer Life Sciences, Boston, MA) using terminal deoxynucleotidyltransferase (Invitrogen) with an incubation of half an hour at 37°C, followed by precipitation by 100% ethanol.

**ISHH.** In this study, we used a quantitative ISHH protocol, described elsewhere (34, 47) with minor variations. Briefly, serial 10-μm brain sections were cut on a cryostat and placed onto poly-L-lysine-coated slides (Fisher Scientific, Newark, DE), dried on a slide warmer for 10–30 min at 37°C, and then stored at −80°C. Before hybridization with riboprobes, the sections were fixed in 4% formaldehyde for 10 min at room temperature, rinsed once, and washed twice for 5 min in 1× PBS, put into 0.1 triethanolamine-HCl (pH 8.0), containing 0.25% acetic anhydride for 10 min at room temperature, rinsed with 2× SSC buffer, and transferred through graded ethanol (75–100%), and then air-dried. Hybridization was carried out in 80 μl of hybridization solution (20 mM Tris·Cl pH 7.4, 1 mM EDTA pH 8.0, 300 mM NaCl 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 100 μg/ml salmon sperm DNA, 250 μg/ml yeast total RNA, 250 μCi/ml yeast tRNA, 0.0625% SDS, 0.0625% sodium thiosulfate) containing 105 cpm denatured [35S]-labeled riboprobe. After overnight hybridization at 55°C, the sections were washed four times in 4× SSC, incubated with TNE buffer (10 mM Tris·Cl pH 8.0, 0.5 M NaCl, 0.25 mM EDTA, pH 8.0) containing 20 μg/ml ribonuclease A for 30 min at 37°C, and then washed twice in 2× SSC, once in 1× and 0.5× SSC at room temperature, and twice in 1× SSC at 65°C. The sections were rinsed in graded ethanol solutions, and then air-dried. Finally, the sections were apposed to a low-energy storage phosphor screen (Amersham Biosciences, Piscataway, NJ) for 1–10 days, and developed using a phosphor imager (Storm 860, Amersham Biosciences).

For double-labeled ISHH, digoxigenin (DIG)-labeled VP or OT exonic and intronic riboprobes and the [35S]-labeled OT In2 antisense riboprobe were cohybridized to identify the specificity of the OT In2 riboprobe we designed. DIG labeling was done per the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN) using a PCR amplified VP (30 ng) and OT (100 ng) sequences as a template as described above. Overnight hybridization at 55°C was done using 106 cpm [35S]-labeled antisense riboprobe and 1 μl of the 50 μl purified DIG-labeled antisense riboprobe per slide in a total of 80 μl hybridization solution (same as the hybridization solution used in a single ISHH hybridization). Then, the slides were washed as in the single-probe hybridization method, processed for the DIG labeling probe by incubating in 1:2,000 of antidigoxigenin-AP (Roche Diagnostics) made in 1× TBS (0.1 M Tris·Cl, pH 7.5, 150 mM NaCl), 5% normal goat serum (Vector Laboratories, Burlingame, CA) at 37°C for 5 h, and developed with 0.33 mg/ml nitro blue tetrazolium chloride/0.165 mg/ml 5-bromo-4-chloro-3-indolyphosphate (BCIP/NBT combo from Invitrogen) and Levamisole (Vector Laboratories) in 0.1 M Tris·Cl, pH 9.5, 0.1 M NaCl, 50 mM MgCl2 for 30 min to 1 h at room temperature. The slides were coated with Ifford K.5D nuclear emulsion (Poly Sciences, Warrington, PA) and developed using D-19 (Kodak, Rochester, NY) after 14–28 days of exposure when the grains were visible. We used the Ifford K.5D nuclear emulsion for the double-labeled ISHH, because, unlike the NTB nuclear emulsion (Kodak), the DIG reaction products do not interfere with the development of the photographic grains on the Ifford K.5D nuclear emulsion. For single-labeled ISHH, the slides hybridized with [35S]-labeled OT In2 antisense riboprobe were coated with the more sensitive NTB nuclear emulsion (Kodak) and exposed for 10 days. The slides incubated with [35S]-labeled OT exonic antisense riboprobe were exposed at −80°C.
were coated with the less sensitive Ilford K.5D nuclear emulsion and exposed for 3 days. The slides were mounted with Cytoseal 60 mount medium and coverslipped.

For ISHH using oligonucleotide probes, the preparation of the serial brain sections, prehybridization, and hybridization are as similar as described above, except the hybridization temperature is 37°C. After the hybridization for 20–24 h, sections were washed four times in 1× SSPE/1 mM DTT at 55°C for 15 min each time, followed by 5 min at room temperature twice in 1× SSPE/1 mM DTT. The sections were rinsed in 75% ethanol for a few seconds and dried by a hair dryer. Finally, the sections were exposed to a low-energy storage phosphor screen (Amersham Biosciences) for 1–14 days and developed using a phosphor imager (Storm 860, Amersham Biosciences).

Quantitative analysis of ISHH. To evaluate the levels of hnRNA or mRNA in the SONs, PVNs, and suprachiasmatic nuclei (SCNs), the average densities and unit areas from two representative sections in the central region of the nuclei recorded on the phosphor screen were measured using the Image Quant software version 5.2 (Amersham Biosciences). Statistical significance of differences between groups was calculated by an unpaired t-test using the Statview 5.0 (SAS Institute, Cary, NC) program. Differences between groups were considered statistically significant when P < 0.05. Results are expressed as a percentage of controls (means ± SE).

RESULTS

Design and characterization of the rat OT intronic probes

The sequences of the OT intronic riboprobes and oligoprobes used in this study are shown in Fig. 1. A 162-bp fragment localized entirely within intron 1 of the OT gene and a 84-bp fragment complementary to the entire 84-bp In2 sequence were used to generate the labeled riboprobes (see MATERIALS AND METHODS). Both of these riboprobes are short sequence were used to generate the labeled riboprobes (see MATERIALS AND METHODS). Both of these riboprobes are short sequence and a 84-bp fragment complementary to the entire 84-bp In2 fragment localized entirely within intron 1 of the rat OT gene probes used in this study are shown in Fig. 1. A 162-bp Design and characterization of the rat OT intronic probes

A 162-bp fragment of In1, and the In2 sequences, respectively. The In1 probe is complementary to a 162-bp fragment of In1, and the In2 probe is complementary to the entire 84-bp In2 sequence. The oligonucleotide probes (O1–3) are directed at sequence in In1 of the OT hnRNA, while O4 is directed against In2 (Fig. 1). All oligonucleotide probes are 40 bp in length.

Comparisons of the signals produced by these OT-intron-directed oligonucleotide probes and riboprobes are shown in Fig. 2. The O1 probe failed to produce a signal for OT hnRNA even after 4 wk of exposure (Fig. 2A). Similar results were obtained with the other two intron 1-directed probes, O2 and O3 (data not shown). In contrast, the O4 probe directed against intron 2 produced a strong signal in the SON (Fig. 2B). Consistent with these oligonucleotide probe data, the hybridization signal produced by the OT In1 riboprobe (Fig. 2C) is considerably weaker than the intron 2-directed riboprobe (Fig. 2D). The In2 riboprobe produced the strongest signal-to-noise ratio compared with the results with the In1 and O4 hnRNA probes. On the basis of these data, we decided to use the In2 probe to study OT gene expression in the hypothalamus.

ISHH of the OT In2 probe to neurons in the SON is visualized by nuclear emulsion autoradiography as shown in Fig. 3. The signal of the OT In2 antisense probe is clearly over SON neurons (Fig. 3A), and there is no detectable signal over cells from the sections that were incubated with the sense-strand control probes (Fig. 3B). Preincubations of the sections with RNase also yielded no cell-specific signals after incubation with OT In2 antisense probes (Fig. 3C), thereby demonstrating that the OT In2 probe is specifically detecting OT RNA and not genomic DNA. In comparison, the ISHH signals obtained using the labeled OT exonic probe (Fig. 3D) had much higher intensity than that seen with the OT In2 probe, consistent with the expectation that OT mRNA will far exceed OT hnRNA abundance in the cell. In fact, we estimate from measurements of autoradiographic grain densities in the SON that the mRNA:hnRNA ratio in the OT MCN is about 160:1 (data not shown). Figure 4 illustrates the cell-specificity of the OT signals detected by the OT In2 probe in the SON (Fig. 4, A and B) and PVN (Fig. 4, C and D) exposed to photographic nuclear emulsion for autoradiography. Each section was hybridized with a 35S-labeled In2 probe and either a DIG-labeled OT exonic probe (Fig. 4, A and C) or a DIG-labeled VP exonic probe (Fig. 4, B and D). Note that in Fig. 4, A and C, the densely packed red (pseudocolored) grains reflecting the OT hnRNA from the In2 probe hybridization are clearly localized in the OT neurons as visualized by the exonic OT DIG-labeled probe both in SON and PVN (dark color). In contrast, the VP cells in the SON (B) and PVN (D) do not overlap with OT

Fig. 1. Schematic diagram of the rat oxytocin gene and descriptions of the intron-specific probes used in this paper. The oxytocin gene contains three exons A, B and C, which are separated by a long intron 1 (In1) and a short intron 2 (In2). The sequences of the two riboprobes and four oligonucleotide (O) probes, which we used to detect oxytocin (OT) hnRNA, are shown in bold type and as underlined sequences, respectively. The In1 probe is complementary to a 162-bp fragment of In1, and the In2 probe is complementary to the entire 84-bp In2 sequence. The oligonucleotide probes (O1–3) are directed at sequence in In1 of the OT hnRNA, while O4 is directed against In2. All oligonucleotide probes are 40 bp in length.
hnRNA. These data illustrate that the OT In2 probe selectively hybridized to RNA in OT neurons.

Physiological influences on OT and VP gene expression in the rat SON and PVN. Given the above reliable riboprobe for the detection and measurement of OT hnRNA levels, we next examined OT and VP gene expression in two physiological states, lactation and acute hyperosmotic stimulation, in a comprehensive fashion, using both intron- and exon-specific probes directed against both OT and VP hnRNAs and mRNAs.

The effects of lactation on OT hnRNA, OT mRNA, VP hnRNA, and VP mRNA levels in SON are shown in Fig. 5. Lactation resulted in a significant increase in OT hnRNA ($P < 0.0001$) and OT mRNA (Fig. 5A, $P < 0.01$), and in VP mRNA (Fig. 5B, $P < 0.01$), but no changes in VP hnRNA (Fig. 5B).

The significant increase in VP mRNA in the absence of a comparable increase in VP hnRNA, suggests a decreased degradation of the VP mRNA under this physiological condition (see DISCUSSION).

Figure 6 shows the changes in OT and VP hnRNA and mRNA in the PVN. Lactation significantly increased OT hnRNA expression (Fig. 6A, $P < 0.05$), whereas OT mRNA (Fig. 6A) and VP hnRNA (Fig. 6B) were not significantly
changed. The apparent increase in VP mRNA in lactating rats in the PVN, although not statistically significant \((P = 0.2)\), is similar to that seen in the SON (compare VP mRNA changes in Figs. 5B and 6B).

The VP hnRNA signals were significantly increased in the SON (Fig. 7B) and the PVN (Fig. 8B) of rats receiving an acute salt load \((P < 0.01\) for each). However, the expression of OT hnRNA, OT mRNA, and VP mRNA was unaltered under these conditions (Figs. 7 and 8). Thus acute hyperosmotic stimulation significantly increases only VP hnRNA expression in the HNS, indicating that only VP but not OT gene expression is increased under this physiological condition.

**DISCUSSION**

The regulation of OT and VP gene expression in the HNS has been extensively studied under a wide variety of physiological conditions (9, 41). Most of these studies have measured mRNA levels, which are the products of both de novo transcription and mRNA degradation processes. About 20 years ago, Roberts and coworkers (15, 16) introduced the approach of using intron sequence-specific probes and in situ hybridization procedures to detect the pro-opiomelanocortin gene primary transcript in individual neurons of the hypothalamus. The view that changes in pre-mRNA levels as measured by intron-specific probes reflect changes in gene transcription rates is predicated on the common observation that excision of the intron from the primary transcript and the subsequent degradation of the intron is very rapid, typically with half-lives \(<30–60\) min (12, 17, 36). The above view was supported by several studies, which compared the changes in transcription determined by the ISHH method to results obtained using nuclear “run-on” assays (15, 19). Consequently, assays of gene transcription changes using intron-specific probes and quantitative ISHH have been used in studies of a wide variety of genes in the CNS (2, 10, 14, 18–20, 24, 29, 30, 32, 35, 39).

The development of intron-specific probes to study neurohypophysial peptide gene expression in the HNS was first accomplished for VP, and this study provided evidence that the intron 1-directed riboprobe that was used was indeed detecting changes in hnRNA with much more rapid kinetics than exonic probes did for changes in mRNA (19). These authors then concluded that this approach provided a “relatively reliable method for detecting rapid changes in gene transcription” in neurons in the CNS. This riboprobe has since been successfully used to measure rapid changes in VP hnRNA in HNS neurons in MCNs in response to acute osmotic stimulation (19, 23), in parvocellular (CRF) neurons in the PVN in response to various forms of stress (24, 28–30, 37), after direct stimulation by forskolin in vitro (2), and in the SCN in vivo (46) and in vitro (3).

An effort to develop an intron-specific probe to detect and measure OT hnRNA was also made (8), but use of this probe that was directed against intron 1 of the OT gene has received much less use. Two reports used a \(^{3}S\)-labeled cDNA probe and 50 days of exposure to nuclear emulsion to successfully detect changes in OT hnRNA (13, 22). A third report used a similar \(^{3}S\)-labeled intronic OT riboprobe and 4-wk exposure to nuclear emulsion but failed to detect OT hnRNA in hypothalamic sections (38). In preliminary experiments, we used a \(^{3}S\)-riboprobe directed against intron 1 of the OT gene (Fig. 1) and obtained very variable results usually of low intensity (a typical result is shown in Fig. 2C). We then attempted to adopt a multiple oligonucleotide approach that had been described in the literature as being a highly effective probe strategy for quantitative ISHH (6). Figure 1 describes the four oligonucleotide probes that we used, and to our surprise, all three oligonucleotide probes directed against intron 1 \((O1–3)\) failed to detect OT hnRNA at the exposure times used. However, the single probe against intron 2 \((O4)\) did provide robust and reliable signals (Fig. 2B). Consequently, we constructed a
small 84-bp riboprobe directed to intron 2, and this too provided relatively reliable and robust detection of OT hnRNA (Fig. 2D and Fig. 4). We are not certain why the intron 2 sequence provides a better probe for OT hnRNA than intron 1. Structural analyses of the secondary structures of the two intronic sequences in the OT hnRNA, as well as the secondary structures in the two intronic probes revealed no significant differences (data not shown). We presume that the most likely explanation for the difference is that intron 1 is excised from the primary transcript much more rapidly than intron 2.

If this is the case, then our intron 2 probe would be detecting and measuring the sum of the primary transcript and the intron 1-less OT hnRNA intermediate. Previous investigators have cautioned that the presence of stable intermediates in multiple-intron genes could confound interpretations of the relationship of hnRNA to transcription vs. to varying pre-mRNA processing rates (18, 25). Evidence for persistent hnRNA intermediates, whose rates appear to be regulated, has been reported for specific biological systems (11, 21, 25, 45). We do not know whether the pre-mRNA processing rates of either the OT or VP primary transcripts undergo changes under the experimental conditions that we have studied nor is there evidence to suggest that this is occurring for either peptide gene. Therefore, following the precedent set by previous reports of changes in VP hnRNA (19, 23), we interpret these changes as due to transcription, with the awareness that future studies could find that the changes are due to changes in pre-mRNA processing, or some combination of pre-mRNA processing and transcription. Given the availability of the above reliable intronic riboprobe for OT hnRNA detection, we then used it together with the more established OT exonic, VP intronic, and VP riboprobes, for a comprehensive analysis of two previously well-studied physiological conditions: acute osmotic stimulation and lactation. Although much of the data presented and discussed here confirms the literature, these data together with the novel measurements reported in this paper provide new insights with respect to the regulation of OT and VP gene expression under these physiological conditions.

It is well known that acute hyperosmotic stimulation in the rat produces intense activation of both OT and VP MCNs in the hypothalamus of rats, as well as substantial secretion of both OT and VP peptides into the general circulation (5, 43). Indeed, one study suggests that even more oxytocin is secreted than VP after acute hyperosmotic stimulation (43). Murphy and Carter (33) used nuclear run-on assays to demonstrate that an acute osmotic stimulation results in rapid and relatively
large increase in VP gene transcription that do not correlate with increases in the VP mRNA level. Various ISHH studies of VP gene expression under these conditions, have demonstrated a robust burst (up to 3 h) of VP transcription, but no increase in VP mRNA in response to acute hyperosmotic stimulation (4, 19, 23, 26, 27; see also Figs. 7B and 8B in this paper). In addition, it has been shown previously using oligonucleotide probes that OT mRNA does not change in response to an acute hyperosmotic stimulus (26), and it is confirmed here through riboprobe analysis (Figs. 7A and 8A). To our knowledge, our paper contains the only study of VP hnRNA during lactation, and surprisingly, we found no change in VP hnRNA in the HNS after 7 days of lactation when VP mRNA is already at increased levels (Figs. 5B and 6B). Our interpretation of these data is that during sustained lactation, the OT mRNA increases are maintained by increased transcriptional mechanisms, whereas the increase in VP mRNA does not involve increased transcription but rather depends mainly on a decrease in VP mRNA degradation. These data do not discount the

Although the above studies on the effects of acute hyperosmotic stimulation showed differential responses in the MCNs where VP hnRNA is increased and OT hnRNA is unchanged, we found the opposite during lactation. Substantial literature exists that shows that both OT and VP mRNA levels increase significantly in the HNS after 7 days of lactation (1, 7, 27, 42, 44, 48). We also confirm these findings for the HNS in rats after 7 days of lactation (Figs. 5 and 6). Only one study has been done that measured OT hnRNA levels, but this was performed after 2 days of lactation when no increased OT mRNA was found (13). Interestingly, these authors found a 187% increase in OT hnRNA 2 days after lactation began, thereby indicating an increase in OT gene expression at that time, presumably to increase the OT mRNA stores for enhanced translation of OT prohormone. In this regard, we also found a similar increase of OT mRNA in the HNS, especially in the SON after 7 days of lactation (Fig. 5A). To our knowledge, our paper contains the only study of VP hnRNA during lactation, and surprisingly, we found no change in VP hnRNA in the HNS after 7 days of lactation when VP mRNA is already at increased levels (Figs. 5B and 6B). Our interpretation of these data is that during sustained lactation, the OT mRNA increases are maintained by increased transcriptional mechanisms, whereas the increase in VP mRNA does not involve increased transcription but rather depends mainly on a decrease in VP mRNA degradation. These data do not discount the

Fig. 7. Comparisons of OT and VP hnRNA and mRNA levels in SONs of acutely hyperosmotically stimulated male rats vs. SONs in isotonic controls. ISHH of SONs in brain sections from males that had received an acute hyperosmotic injection of 1.5M NaCl (n = 5), were compared with ISHH of SONs from control males that had received an isotonic saline injection of equal volume (n = 6). ISHH was used to analyze changes in OT hnRNA and OT mRNA (A) and VP hnRNA and VP mRNA (B). The acute osmotic stimulus produced significant increases only in VP hnRNA (P < 0.01), but it does not effect the expression of OT hnRNA, OT mRNA, or VP mRNA levels. Data are presented as percentages of isotonic control. *Significantly different from male isotonic control, P < 0.05.

Fig. 8. Comparisons of OT and VP hnRNA and mRNA levels in PVNs of acutely hyperosmotically stimulated male rats vs. PVNs in isotonic controls. As was found in the SON (see Fig. 7), acute hyperosmotic stimulation significantly increases only VP hnRNA expression in PVN. *Significantly different from male isotonic controls, P < 0.05.
possibility that the OT mRNA is also degraded more slowly but focuses us on the differential transcriptional responses of these two genes during the state of lactation.

In summary, we have developed an intron-specific riboprobe that allows us to reliably detect OT hnRNA. We demonstrate here that by using this probe in combination with those already available, that is, for detecting VP hnRNA, VP mRNA, as well as OT mRNA, we can obtain new insights regarding the regulation of OT and VP gene expression. We have applied this comprehensive gene expression analysis to the study of two well-known stimuli, acute hyperosmotic stimulation and lactation, and provide evidence for new and unexpected, differential responses of the OT and VP MCNs to these conditions. Many questions remain to be answered. For example, are the differential effects of osmotic stimulation and lactation on OT and VP gene expression due to different neurotransmitter inputs and receptor activations or to different signal-transduction mechanisms in the OT and VP MCNs? What is the situation for OT transcription in chronic dehydration, where VP hnRNA and both OT and VP mRNAs are increased? Our preliminary studies using chronic salt loading with 2.1% saline for 7 days produce robust increase in both OT and VP hnRNAs (unpublished data, not shown), consistent with expectations from the literature (9, 19, 26, 27). This would suggest that there is a kinetic difference between the gene expression changes in the OT and VP MCNs in response to osmotic perturbations, in which acute osmotic stimuli selectively affect VP gene expression, in contrast to chronic stimuli, which affects the expression of both genes. Further experiments that address these questions are currently being done in our laboratory. Finally, we note that in a recent review, that after carefully assessing the conflicting literature on OT mRNA abundance during pregnancy, Russell et al. (40) write that there is still no consensus on whether OT gene expression changes during pregnancy. We suggest that use of the comprehensive gene expression analyses described in this paper, might help to resolve the status of OT (and VP) gene expression in pregnancy, and other physiological states.

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GRANTS

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