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mRNA coding for oxytocin is present in axons of the hypothalamo–neurohypophysial tract

(oxytocin mRNA/*in situ* hybridization/axonal varicosities/electron microscopy/Herring bodies)

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ABSTRACT **Neuronal mRNA is thought to be restricted to perikaryal and dendritic compartments containing rough endoplasmic reticulum. We have used both *in situ* hybridization and DNA polymerase chain reaction methods to determine the precise intracellular distribution of oxytocin mRNA. Using light- and electron-microscopic detection of *in situ* hybridization with 5′-bromo-2′-deoxyuridine-labeled oligonucleotide probes, we found oxytocin mRNA in axons and Herring bodies in the lateral and ventral hypothalamus, the median eminence, and the posterior lobe of the pituitary in postpartum lactating rats. Southern blot analysis of the amplification products confirmed the presence of oxytocin mRNA in all three tissue samples. The present findings indicate that oxytocin mRNA can be transported axonally. Such transport could reflect an adventitious compartmentalization or a functional storage in Herring bodies for subsequent secretion.**

Neurons containing presumptive peptide neurotransmitters have been extensively studied by antibodies for the peptides and their prohormone precursors (see ref. 1) and by *in situ* hybridization for the mRNAs encoding these precursors (see ref. 2). A basic premise of mammalian neuronal biology is that the mRNA is restricted to the perikaryal and dendritic compartments containing rough endoplasmic reticulum (see ref. 1). However, there are multiple reports that mRNAs can be detected in peripheral axons in invertebrate systems (3–7). In the mammalian brain, specific intradendritic mRNAs are seen consistently (1, 8, 9). On the other hand, Guitteny and Bloch (10), using autoradiographic detection of vasopressin mRNA hybridization by both light and electron microscopy, reported that hybridization was restricted to perinuclear cytoplasm. Nevertheless, the micrographs presented in this study do not exclude the possibility that vasopressin mRNA is present in more distal neuronal processes. McCabe *et al.* (11) reported the presence of vasopressin mRNA in extracts of the posterior lobe by using solution hybridization. Although they did not directly determine the cellular origin of this mRNA, they suggested that it might arise from pituicytes (11), the major cell type in the posterior pituitary other than the vascular elements.

We recently developed an immunocytochemical method for *in situ* hybridization with BrdUrd-labeled oligonucleotide probes (12). This sensitive nonradioactive method also provides resolution of hybridization sites by electron microscopy. In a recent light microscopy study, we found that oxytocin mRNA hybridization was present in neuronal processes at some distance from neuronal perikarya (13). Therefore, we initiated these studies to apply this method to an evaluation of possible intraaxonal localization of the mRNA for oxytocin in the posterior pituitary. The neurohypophysial magnocellular system is ideal for analysis of the intraneuronal compartmentalization of neuropeptide mRNAs as its cell bodies are concentrated within the hypothalamus, while the axons of these neurons are concentrated in neuron-free sites in the median eminence and neurophypophysis. Early lactating animals were chosen for these experiments to take advantage of their high rates of oxytocin synthesis (14) accompanied by high levels of oxytocin mRNA (15). Our results indicate that in this physiological state, the oxytocin mRNA can indeed be detected in the axons of oxytocin-containing neurons in the median eminence and the posterior pituitary by both light microscopy and ultrastructural *in situ* hybridization as well as in RNA samples from the posterior pituitary after amplification by the polymerase chain reaction (PCR; ref. 16).

MATERIALS AND METHODS

Oligonucleotides. Three overlapping oligonucleotides were used in this study, Ox5′, Ox3′A, and Ox3′B (Fig. 1). Ox5′ is a 34-mer sense oligonucleotide directed at a distal 5′ segment of the open reading frame in exon 1. Ox3′A and Ox3′B are antisense oligonucleotides of 32 and 34 bases, respectively. The former is complementary to a sequence found in exon 2, and the latter, to a sequence lying partially in exon 2 and partially in exon 3. Ox3′B and Ox5′ were used for *in situ* hybridization and for PCR amplification. Ox3′A was used in the Southern blot analysis of the PCR products. A fourth oligonucleotide (a 20-mer complementary to a segment of exon 1, with the sequence GAACTGCCCCCTGGGCGGCA) was also used in some preliminary *in situ* hybridization experiments.

***In Situ* Hybridization.** Four female rats (Wistar), 6 days postpartum and actively lactating, and two intact male Wistar rats were killed by cardiac perfusion with 4% paraformaldehyde under chloral hydrate anesthesia. Brains were removed, and small tissue blocks were dissected from the retrochiasmatic hypothalamus, the median eminence, and the posterior lobe. Tissue samples were rinsed in sterile phosphate-buffered saline (PBS) and embedded in TAAB resin (Polysciences) through a graded ethanol series. After polymerization, semithin sections (0.5 μm thick) and ultrathin sections (85 nm thick) were cut on a Reichert Ultracut microtome. Semithin sections were mounted onto precleaned slides, and ultrathin sections were affixed onto nickel grids. *In situ* hybridization of semithin and ultrathin sections was carried out as described in detail (18). Briefly, the antisense oligonucleotide Ox3′B and the sense oligonucleotide Ox5′ were labeled with BrdUrd by the terminal deoxynucleotidyltransferase (Boehringer Mannheim) reaction (19, 20) as described (12, 21). Epoxy resin was removed with sodium methoxide (10% for 3 min for the semithin sections; 1% for 15 sec for the ultrathin sections). Sections were prehybridized thereafter for 1 hr at 37°C and then were hybridized for 1 hr at 37°C with

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Abbreviation: PCR, polymerase chain reaction.

FIG. 1. Open reading frame of the oxytocin gene. The sequence shown is from start to termination codons. Arrows mark the boundaries between exons. Boxes show the sequences of the oligonucleotides used for this study and the segment of the oxytocin gene to which they are homologs. For orientation, 3'–5' notations are used at the ends of each DNA sequence. Ox5' is a 34-base sense oligonucleotide aimed at the very 5' end of the open reading frame in exon 1. Ox3'A and Ox3'B are antisense oligonucleotides of 32 and 37 bases, respectively. The former is complementary to a segment of exon 2, and the latter, to a further-downstream segment of exon 2 and part of exon 3. Sequences were taken from ref. 17.

the antisense probe Ox3'B. Control sections were hybridized with the sense probe Ox5' under the same conditions. After hybridization, sections were washed under controlled stringency protocols (18) and then were immunostained for BrdUrd. Anti-BrdUrd (Progen, Heidelberg) and the streptavidin–peroxidase method (Vector Laboratories) was used to detect hybridization. The reaction products were stained with 3',3-diaminobenzidine (0.12 mg·ml^{-1}), nickel chloride (0.1 mg·ml^{-1}), and hydrogen peroxide (0.003%). Semithin sections were mounted with Permount for light microscopy. Ultrathin sections were examined in a Zeiss EM10 electron microscope.

PCR. For chemical analysis, two lactating rats, six days postpartum were perfused through the heart with sterile isotonic saline under chloral hydrate anesthesia. Brains were removed, and hypothalamus, median eminence, and posterior lobe were dissected. Total RNA was isolated from these three discrete areas by extraction with acid guanidinium isothiocyanate followed by phenol/chloroform, 1:1 (vol/vol); this method allows the recovery of total RNA virtually devoid of genomic DNA contaminants (22). Specific oxytocin cDNAs were prepared from total RNA by extending the antisense oligonucleotide Ox3'B (1 μM) with Moloney murine leukemia virus reverse transcriptase (BRL) at 37°C overnight. The reaction mixture contained 50 mM Tris·HCl (pH 8.3), 6 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, all dNTPs (150 μM each), 15 units of RNasin (Promega) per ml, and 10 units of enzyme per μl. Enzymatic amplification of the cDNAs obtained was performed in a DNA Thermal Cycler (Perkin–Elmer/Cetus) with the Ox3'B oligonucleotide and the sense oligonucleotide Ox5' (Fig. 1) as primers. The reaction mixture (100 μl) consisted of the four dNTPs (200 μM each), 2 μM concentration of each primer, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin–Elmer/Cetus). The reaction protocol was as follows: denaturation of template at 95°C for 1 min, annealing of oligonucleotide primers at 46°C for 2 min, and extension of primers at 72°C for 2 min—all repeated for 40 cycles and followed by 10 min at 72°C. The PCR products (1/10th of the amplification product from the hypothalamus cDNA and the total products of the amplified cDNAs from the median eminence and the posterior pituitary) were run on a 1.7% agarose gel in Tris borate/EDTA electrophoresis buffer (23). As a molecular-size standard, φX174 RF replicative form DNA digested with *Hae* III was used. After electrophoretic separation, the amplified DNAs were then transferred to a nylon blotting membrane (Bio-Rad) according to standard protocols (23, 24). The Southern blot was prehybridized in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 10% dextran sulfate, 100 μg of denatured salmon sperm DNA per ml, and 1% (wt/vol) sodium dodecyl sulfate (SDS) at 65°C for 1 hr. It was then probed overnight under the same conditions with the 32-mer oligonucleotide Ox3'A (10 ng/ml) (Fig. 1) labeled by tailing, as described above, with [α-^{32}P]dATP. The blot was then washed for 1 hr in 2× SSC at 65°C and exposed for 1 hr at room temperature on a Kodak RP film.

RESULTS

As expected, hybridization for oxytocin mRNA was detectable in magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus (Fig. 2*A*) as previously reported (13). *In situ* hybridization of semithin sections showed the most abundant BrdUrd immunoreactivity to be in perinuclear zones. These accumulations of perinuclear reaction product were absent in control sections incubated with the sense probe Ox5'. In addition, however, intense immunostaining for BrdUrd in the lactating animals was also present in presumptive axons in fields of the ventrolateral hypothalamus devoid of identifiable neuronal perikarya and in small rounded structures resembling axonal varicosities (the classical Herring bodies; refs. 1 and 21) in the median eminence (Fig. 2*B*) and in the posterior lobe (Fig. 2*D*). Limited evaluation of a second antisense probe taken from the sequence of Ox3'B gave similar positive results but of lower intensity (results not shown). In male rats, presumptive axonal hybridization in median eminence or posterior pituitary could be observed only occasionally (Fig. 2*C*). Electron microscopic examination of ultrathin sections confirmed that the hybridization in female hypothalami and posterior pituitary was present in axonal varicosities (Fig. 2 *E* and *F*). In all cases hybridization was confined to a fraction, ≈50%, of the large granular secretory vesicles; the small agranular vesicles and the surrounding axoplasm were consistently devoid of hybridization products (Fig. 2 *E* and *F*). BrdUrd immunostaining was also consistently absent in sections that had been incubated with the Ox5' sense probe (Fig. 2*G*).

To validate the detection of nonperikaryal oxytocin mRNA in the hypothalamo–neurohypophysial tract by *in situ* hybridization, we first attempted to detect oxytocin mRNA in extracts of total RNA from this region by conventional Northern blot analysis; none could be detected (results not shown). To provide a more sensitive detection strategy, we then enzymatically amplified a 330-base-pair (bp) DNA fragment from total RNA prepared from hypothalamus, median

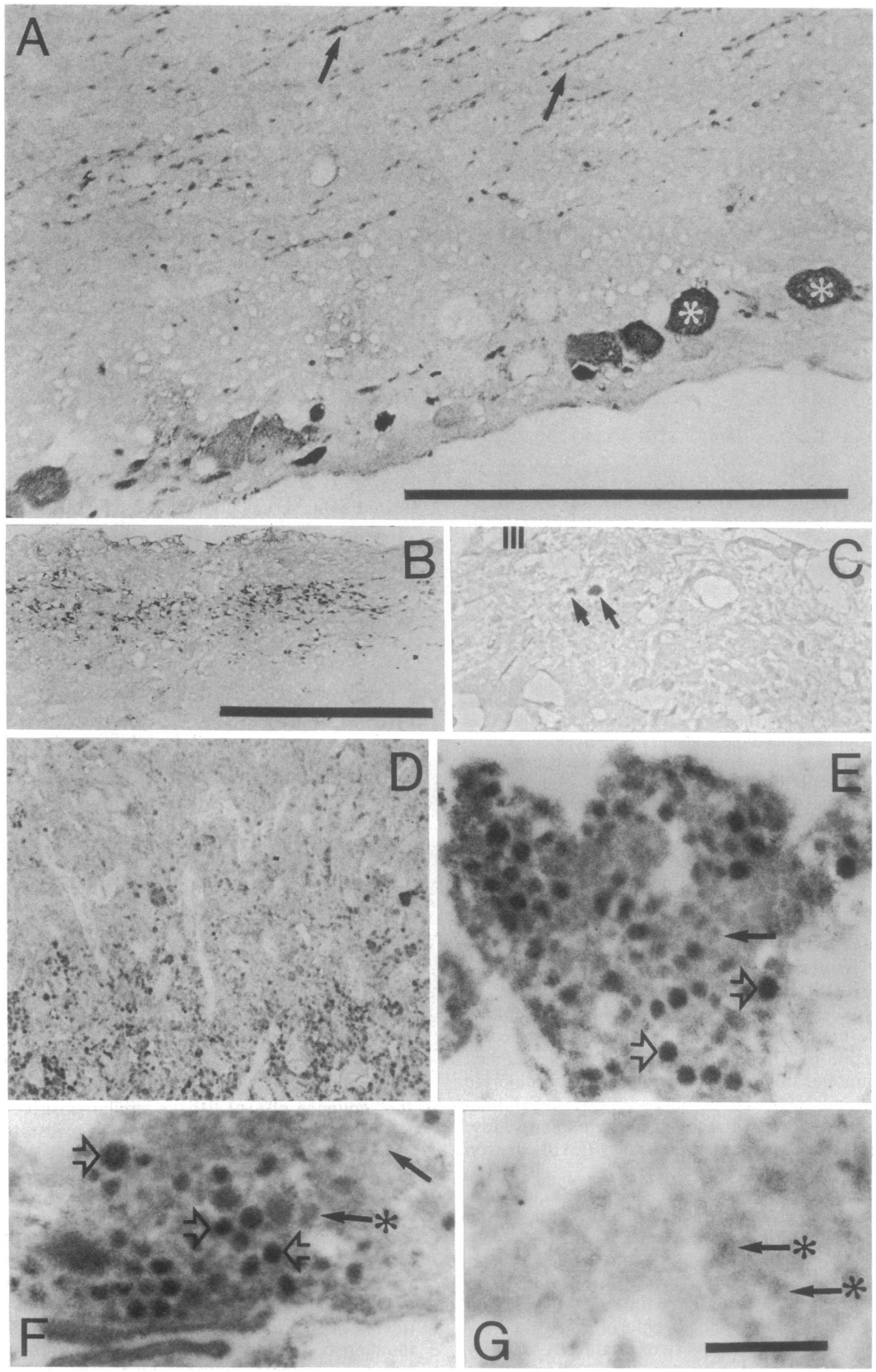

FIG. 2. (*A–D*) Semithin sections of the ventromedial hypothalamus hybridized with the BrdUrd labeled Ox3'B probe. In *A*, specific immunostaining for BrdUrd is visible in perikarya (asterisks) of the retrochiasmatic portion of the supraoptic nucleus and in axonal varicosities (arrows). (Bar = 100 μm.) In *B* and *C*, the BrdUrd immunostaining after hybridization with the Ox3'B probe is more abundant in semithin sections of the median eminence of 6-day lactating rats (*B*) than in intact male rats (*C*) (arrows). In *D*, the posterior lobe of a 6-day lactating rat contains numerous BrdUrd immunoreactive Herring bodies after hybridization with Ox3'B. (Bar = 100 μm for *B–D*.) (*E–G*) Electron micrographs of ultrathin sections from the median eminence (*E*) and the posterior lobe (*F*) of a 6-day lactating female after hybridization with Ox3'B and BrdUrd immunocytochemistry. A fraction of the large granular secretory vesicles shows specific immunoreactivity (empty arrows show examples of them), while many large granular vesicles (long arrows with asterisks) and all agranular vesicles (long arrow) do not. Sections of the posterior lobe incubated with the sense probe are devoid of BrdUrd immunostaining (*G*). (Bar = 1 μm for *E–G*.)

eminence, and the posterior lobe of the pituitary of lactating postpartum female rats by use of two oligonucleotides, Ox3′B and Ox5′, complementary to the (+)- and (−)-strand of oxytocin gene respectively (Figs. 1 and 3). The amplified fragments were detected by Southern blot hybridization (Fig. 3) with a third oligonucleotide as a detection probe. This probe, Ox3′A, is complementary to the region of the oxytocin mRNA that we have amplified (Fig. 1), the sequence of which lies in two different exons of the gene. Our PCR/Southern blot revealed a band of the expected electrophoretic mobility to be detectable in the PCR products from both the median eminence and from posterior lobe of the pituitary as well as from the amplification product derived from the hypothalamus (Fig. 3). The autoradiographic intensity of this band was stronger in the amplification product from the posterior lobe than in the sample from the median eminence, but this may reflect the smaller mass of the latter anatomical area. Because of the presence of an intron, the size of the amplified sequence differs by 220 bases from the corresponding sequence of the gene. Therefore, our results strongly support the interpretation that the amplified sequence arose from oxytocin mRNA.

DISCUSSION

The present findings provide the first direct evidence that mRNA coding for a neuropeptide can be located in axons in addition to their known localization in the perinuclear cytoplasm. Immunocytochemical studies demonstrated that neuropeptides are found throughout neuronal cytoplasm, but such studies do not reveal the site of synthesis. However, it has always been assumed that mRNA would be continued to sites where protein synthesis takes place—i.e., the perinuclear cytoplasm in which the rough endoplasmic reticulum and the Golgi apparatus are located. A few earlier studies have inferred the presence of neuropeptide mRNAs in neuronal compartments other than the perikaryon (3, 4, 6, 9). However, axons would not typically be considered to contain even small amounts of mRNA since they lack rough endoplasmic reticulum and ribosomes. The autoradiographic methods available so far have limited the precise identification of these nonperikaryal RNA hybridization sites. Our nonradioactive method for *in situ* hybridization now provides a sensitive tool for high-resolution mRNA localization at the light- and electron-microscopic level (12, 13, 21). Our finding that oxytocin mRNA is located in secretory vesicles of neurohypophysial terminals indicates that this mRNA is intraneuronal.

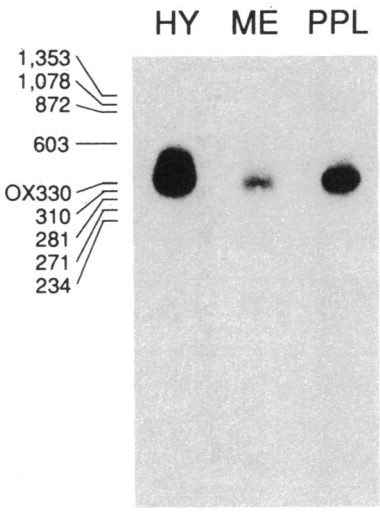

FIG. 3. Enzymatic amplification of oxytocin mRNA from hypothalamus, median eminence, and pituitary posterior lobe. Specific oxytocin cDNAs were obtained by using the antisense oligonucleotide Ox3′B as a primer, and then enzymatic amplification was performed by use of Ox3′B and the sense oligonucleotide Ox5′. The PCR products (1/10th of the reaction from the hypothalamus RNA and all the reaction from the other two areas) were then separated on a 1.7% agarose gel, Southern blotted, and probed with the oligonucleotide Ox3′A. The result of this blot is shown: HY, hypothalamus; ME, median eminence; PPL, posterior pituitary lobe. A molecular size standard (*Hae* III-cut ϕX174 RF DNA) is shown on the side.

The two oligonucleotides used for this amplification, Ox3′B and Ox5′ (Fig. 1), were designed to minimize potential artifacts arising from homology between oxytocin and vasopressin mRNA or from amplification of both oxytocin and vasopressin genes. In both cases, the 3′ nucleotide of the two oligonucleotides was a mismatch to the corresponding vasopressin sequence. Thus, vasopressin mRNA could not have been amplified with these oligonucleotides. In addition, the two primer oligonucleotides were directed at sequences that lie in two different exons (Fig. 1) separated by a 220-bp intron. This allowed us to distinguish the product of the cDNA amplification from the product of amplification of genomic DNA contaminants: the genomic possibility would have yielded a fragment of 550 bases instead of 330.

Our results suggest that during lactation, when hypothalamic oxytocinergic systems have high synthetic and secretory activity (14, 15), oxytocin mRNA can be transported axonally and be stored in varicosities and Herring bodies. The present findings indicate differences in abundance of oxytocin hybridization between axonal fields in males and lactating females. These data support the hypothesis that axonal RNA levels may reflect the functional status of the neurons. The capacity to transport RNA from the perikaryon could be an important auxiliary mechanism for control of the synthetic activity of peptidergic neurons. Neurons are known to contain virtually no RNase activity (25, 26). As peptidergic neurons can regulate their gene expression rate rapidly, intracellular segregation to the nonribosomal cytoplasm of the axon could provide an alternative to RNA degradation, as a means to reduce cytoplasmic mRNA levels. Such spatial regulation also raises the possibility of subsequent transport back to the perikaryon (and thus the site of peptide synthesis) upon certain stimuli. The existence of a mechanism of this sort might allow for rapid on and off regulation of peptide synthesis under special conditions. Transport systems allowing oligonucleotide uptake into cells have recently been described (27), suggesting that secreted RNA could have additional biological value. Further studies are needed to show whether oxytocin mRNA and oxytocin are colocalized within specific secretory vesicles and whether or not such a relationship can be detected for other neuropeptide containing neurons. Pursuit of the explanation for the intra-axonal mRNA present in these organelles may then clarify whether the mRNA could be secreted from the axon terminal or merely represents an adventitious route of excretion for an abundant message no longer needed.

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