Vasopressin and Oxytocin mRNA Regulation in the Rat Assessed by Hybridization with Synthetic Oligonucleotides*

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Vasopressin and oxytocin are nonapeptide hormones that regulate water metabolism and lactation, respectively. To study the regulation of the expression of the vasopressin and oxytocin genes in the rat, we constructed a series of synthetic oligonucleotides, from 8 to 15 bases in length, for use in filter-blot hybridization assays (Northern blots) of hypothalamic mRNA levels and for primed synthesis of cDNAs from which we determined the nucleotide sequences of the 5' regions of the vasopressin and oxytocin mRNAs. A 20-fold increase occurred in the amounts of the two mRNAs present in the hypothalami of rats drinking 2% saline for three weeks. In addition, the sequence analyses of the cDNAs provided the complete amino acid sequences of the NH₂-terminal signal peptides of the rat vasopressin and oxytocin precursors. Thus, synthetic oligonucleotides consisting of as few as eight nucleotides can be used to prime reverse transcription of specific cDNAs from hypothalamic RNA, and pentadecanucleotide hybridization probes readily detect changes in levels of vasopressin and oxytocin mRNAs in response to osmotic stress.

Vasopressin and oxytocin are nonapeptide hormones synthesized in the magnocellular neurons of the hypothalamus in the form of two large polyprotein precursors, PreProVP-Np and PreProOT-Np, each of which consists of the nonapeptide covalently linked at its COOH terminus to a sequence-specific neurophysin (1-6). In the vasopressin precursor, the COOH terminus of neurophysin is additionally linked to a glycopeptide (2, 3, 5, 6). The two hormones, noncovalently attached to their specific neurophysins, are transported within axons to the posterior pituitary, where they are released in response to changes in the osmolality of the extracellular fluid, as well as by complex neuronal influences (2, 3). The principle actions of oxytocin and vasopressin are to stimulate lactation and renal water conservation, respectively.

We wish to study the regulation of the expression of the vasopressin and oxytocin genes in the rat. To accomplish these studies, it is useful to have available specific cDNAs with which to evaluate the levels of cellular RNAs using hybridization techniques. As such, we have chemically synthesized oligonucleotides that are complementary to the oxytocin and vasopressin mRNAs. We report that utilization of ³²P-labeled synthetic pentadecanucleotides in hybridization blots of hypothalamic RNA prepared from rats fed 2% saline in their drinking water for three weeks results in a 20-fold increase in both vasopressin and oxytocin mRNA levels. In addition, nucleotide sequence analysis of the labeled cDNAs reverse transcribed from hypothalamic mRNA using the pentadecanucleotides as hybridization primers provided the amino acid sequences of the NH₂-terminal signal peptides of both PreProVP-Np and PreProOT-Np in the rat.

EXPERIMENTAL PROCEDURES

Preparation of Polyadenylated mRNA—Adult female rats (Long-Evans, 200-225 g, body weight, were given either water or 2% saline to drink for three weeks. The rats were sacrificed by decapitation and hypothalami were immediately removed and stored in liquid nitrogen. Hypothalamic mRNA was prepared by the method of Chirgwin et al. (8). Polyadenylated mRNA was isolated from total cellular RNA by affinity chromatography on oligo(dT)-cellulose (T-3, Collaborative Research) (9).

Preparation of Synthetic Oligodeoxyribonucleotides—Four separate octanucleotides and a mixed pentadecanucleotide were synthesized by the triester method (10). The desired oligonucleotides were separated from smaller products by reverse phase high pressure liquid chromatography on C18 resin (Waters Associates) (11).

5'-End Labeling of Synthetic Oligonucleotides Using T[γ-³²P]ATP—Twenty pmol of oligonucleotide were labeled at the 5' end using a 10% molar excess of [γ-³²P]ATP (Amersham, 5000-7000 Ci/mmol) (12), to a final specific activity of 4 X 10⁶ cpm/pmol, and stored frozen at −20 °C at a final concentration of 0.5 pmol/µl.

Reverse Transcription of cDNA from Hypothalamic mRNA Using Labeled Oligonucleotide—For analytical purposes, 1 µg of mRNA, 2 pmol of ³²P-end-labeled oligonucleotide, 100 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl (pH 8.3) in a final volume of 10 µl were heated to 90 °C for 2 min and placed at 4 °C for 2 h. The reaction was adjusted to 10 mM MgCl₂, 10 mM dithiothreitol, 500 µM each of the four deoxyribonucleotides, 100 µg/ml of bovine serum albumin, 750 units/ml of reverse transcriptase (Life Sciences) in a final volume of 20 µl. The reaction was carried out successively at 4 °C for 5 min, 23 °C for 10 min, and 41 °C for 45 min, followed by extraction with phenol/chloroform, precipitation in ethanol, digestion in alkali, and denaturation as described by Noyes et al. (13). For preparative purposes, reactions were scaled up by a factor of 10.

Polyacrylamide Gel Electrophoresis and Nucleotide Sequence Analysis of cDNAs—The cDNAs of reverse transcription reactions were separated by electrophoresis on slab gels (0.04 X 40 × 15 cm) containing 10 mM Tris, 10 mM borate, 0.2 mM EDTA (pH 7.5), 7 M urea, and 5% polyacrylamide (14). Autoradiography was carried out at −70 °C using an enhancing screen (Lightning Plus, Kodak). Relevant cDNAs were eluted from the gel (15) and sequenced (16).

RNA Hybridization (Northern Blot Analysis)—Northern blot analyses were performed using the procedure of Thomas (17). Briefly, RNA was denatured in 1 M glyoxal and 50% MeSO, electrophoresed through a 1.5% agarose gel, transferred to nitrocellulose in 20 X SSC (1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) and baked (17). The filters were prehybridized for 1 h at 35 °C in 4 X SSC, 0.1% sodium dodecyl sulfate, 10 µg/ml of heat-denatured salmon sperm DNA 1 X Denhardt's solution (0.2% polyvinylpyrrolidone (M₀ = 40,000), 0.2% Ficoll (M₀ = 400,000), 0.2% bovine serum albumin), 10 mM EDTA, after which the 5'-end-labeled pentadecamer, 1 X 10⁶ cpm/ml, was added. Hybridization was for 48 h at 38 °C, followed by four 5-min washes using 5 X SSC, 0.1% sodium dodecyl sulfate at
RESULTS

Four octa-deoxyribonucleotides, $^{32}$GGGCAGTT$, were complementary to oxytocin and vasopressin (Asn-Cys-Pro) were synthesized. This tripeptide is invariant among the entire vasopressin-related family of peptides (1), and the codon degeneracy for this tripeptide is lower than that of any other region of the nonapeptides. These four octanucleotides, labeled with $^{32}$P, were used to reverse transcribe cDNA after hybridization to polyadenylated RNA isolated from hypothalami of water-fed rats (control rats) and from rats given 2% saline to drink for three weeks (salt-fed rats). With three of the four labeled octanucleotides, no differences were found in the electrophoretic patterns of the cDNAs reverse transcribed from mRNA prepared from the hypothalami of control compared to salt-fed rats (data not shown). However, one octanucleotide ($^{32}$GGGCAGTT$^3$) reverse transcribed several cDNAs from the mRNA of salt-fed rats that were not found in the comparable reverse transcription reaction of mRNA from control rats (Fig. 1).

Partial nucleotide sequences were determined from several cDNAs specific to the mRNA isolated from salt-fed rats. Although the amounts of radioactivity in the cDNAs were low (about 2000 cpm), they were sufficient to establish unambiguously that one of the cDNAs (cDNA-1 ($^{32}$GGGCAGTTCTGGAAGTAG$^3$)) was complementary to both VP-Np and OT-Np mRNA. The two pentadecamers in the mixture were separated one from the other by high pressure liquid chromatography: vasopressin-15, 5$^{5'}$GTTCTGGGAAGTAGCA$^{3'}$, complementary to VP-Np mRNA and oxytocin-15, 5$^{5'}$GTTCTGGATGTAGCA$^{3'}$, complementary to OT-Np mRNA.

Hypothalamic mRNAs prepared from control and salt-fed rats were analyzed by agarose gel electrophoresis, filter blotting, and hybridization with the mixed pentadecamers (Northern blot). These analyses revealed two hybridizable RNAs, of approximately 750 and 680 bases, detected in the mRNA from the salt-fed rats (Fig. 2a, lane 2). Although individual quantitation of each mRNA was precluded by their similar sizes, taken together, the hybrid image of these mRNAs was 20 times less intense in the mRNA from control rats (Fig. 2, a and b). This same blot was washed free of radioactivity and rehybridized consecutively to oxytocin-15 and vasopressin-15. Rehybridization showed that the larger mRNA of 750 bases represented VP-Np mRNA, whereas the mRNA of 680 bases corresponded to OT-Np mRNA (Fig. 2a, lanes 3 and 4).

The mixed pentadecamer was next used to prime the reverse transcription of polyadenylated hypothalamic RNA isolated from salt-fed rats (Fig. 2c). Three cDNAs of approximately 150 bases were reverse transcribed, each differed from the next by two bases (cDNA-1, cDNA-2, cDNA-3). These three cDNAs were isolated and sequenced. cDNA-1 and cDNA-2 were identical in sequence and corresponded to the 5$^{5'}$-end of VP-Np mRNA, while cDNA-3 was complementary to the 5$^{5'}$-end of OT-Np mRNA (Fig. 3). OT-Np mRNA contains a 5$^{5'}$-untranslated region of approximately 72 bases, followed by a nineteen amino acid signal, or leader, sequence commencing with a single methionine and containing a central region of hydrophobic amino acids characteristic of signal sequences found at the NH$_2$ terminus of proteins destined for secretion (19). The signal sequence likely terminates in alanine, and is immediately followed by cysteine, the first amino acid of oxytocin.

VP-Np mRNA consists of a 5$^{5'}$-untranslated region of approximately 78 bases, which is followed by three possible initiator methionine codons (at codon positions -22, -20, and -19), all in phase with the rest of the precursor (Fig. 3). Compared with OT-Np mRNA, the two additional sites of possible initiation of translation (at codon positions -22 and -20) are due to base substitutions resulting in the additional AUG codons in VP-Np mRNA. Otherwise, the 5$^{5'}$-ends of rat OT-Np and VP-Np mRNAs differ at 27 of 90 bases, resulting in differences of 9 amino acids. The nucleotide sequences of cDNA-1 and cDNA-2 are identical in the region sequenced.

![Fig. 1. Autoradiogram of gel electrophoresis is of cDNAs primed with the octanucleotide, $^{32}$GGGCAGTT$, using hypothalamic mRNA isolated from rats given water (cont) or 2% saline (salt) to drink for three weeks. Arrows indicate two of the most prominent cDNAs (cDNA-1 and cDNA-2) specifically primed with mRNA isolated from salt-fed rats.](http://www.jbc.org/)
FIG. 2. Northern blot analysis and reverse transcription of hypothalamic mRNAs using pentadecanucleotides. a, RNA hybridization assay (Northern blot) of hypothalamic mRNA isolated from control rats (lane 1) or salt-fed rats (lanes 2–4) hybridized with a mixed pentadecamer, VP/OT-Z5, complementary to both VP-Np and OT-Np mRNAs (lanes 1 and 2), VP-15, complementary to VP-Np mRNA (lane 3), and OT-15, complementary to OT-Np mRNA (lane 4). Unlabeled ribosomal RNAs were used as molecular weight markers. (Exposure time +48 h.) The hybrid images corresponding to the mRNA of control rats (lane 1) do not reproduce well in the photograph. b, densitometric scan of autoradiogram of Northern blot displayed in a, lane 1 (control), exposed for 96 h, and lane 2 (salt), exposed for 48 h. Number above each tracing corresponds to integrated area corrected for exposure time, in arbitrary units, under each curve. c, autoradiogram of gel electrophoresis of cDNAs primed with the mixed pentadecamer, 'GTTCTGGATGTAGCA', using hypothalamic mRNA isolated from salt-fed rats.

FIG. 3. Comparison of partial nucleotide sequence of PreProVP-Np and PreProOT-Np in rat and bovine. Deduced amino acid sequence is shown immediately adjacent to nucleotide sequence; residues in italics were obtained from the sequences of vasopressin and oxytocin. Arrows underlie portions of sequence complementary to the synthetic pentadecamer used to prime cDNA reverse transcription. Numbers indicate amino acid positions with position +1 corresponding to the first amino acid of vasopressin or oxytocin. Lines indicate identical nucleotides in rat and bovine sequences. Asterisks indicate amino acid differences between rat and bovine proteins.

Discussion

The results of these studies demonstrate that synthetic oligonucleotides as small as 8 bases can be used successfully to identify and partially characterize the primary structures of hypothalamic mRNAs encoding specific proteins. In principle, this approach could be used to characterize mRNAs encoding any protein in which only three contiguous amino acids were known. However, because of the high degree of nonspecific hybridization which occurs with such short oligonucleotides, the success of this approach depends upon the ability to compare the oligonucleotide-primed synthesis of cDNAs using mRNA from control animals with that from animals in which mRNA levels can be stimulated. Since chronic dehydration, or salt loading, is known to be a potent stimulus of vasopressin and oxytocin secretion in the rat (7), we reasoned that it might result in increased mRNA levels encoding these proteins. This in fact was the case, and allowed us to unambiguously identify cDNAs encoding PreProOT-Np and PreProVP-Np.
The relatively low efficiency of hybridization of the octanucleotides to their complementary mRNAs allowed elucidation of only a short sequence of these mRNAs, but this was sufficient to provide information for the synthesis of larger pentadecanucleotides. These larger nucleotides primed the synthesis of cDNAs at a much higher efficiency. This “primer walking” approach allowed us to obtain the sequences of most of the 5′ ends of the mRNAs encoding rat PreProVP-Np and PreProOT-Np, starting with oligonucleotides encoding less than three amino acids.

Comparison of the 5′ ends of the mRNAs encoding rat PreProVP-Np and PreProOT-Np with the bovine mRNAs reveals that the two genes are highly conserved and arose by a duplication of a common ancestral gene (20). Bovine PreProVP-Np has only one possible initiator methionine (at amino acid −19) whereas in the rat there exist three possible sites for initiation of translation (at codon positions −22, −20, and −19). It will be of interest to determine at which of these sites initiation of translation actually occurs in light of the hypothesis that the AUG codon closest to the 5′-end of an mRNA usually functions as the initiator codon (21).

The pentadecanucleotides were of sufficient length to be used as hybridization probes for the analyses of the specific mRNAs on Northern blots (22). In our preliminary hybridization analyses, we found a 20-fold increase in the amounts of hybridizable oxytocin and vasopressin mRNAs isolated from hypothalami of rats given 2% saline to drink for three weeks. This is consistent with findings that prolonged dehydration, or salt feeding, is associated with marked stimulation of blood levels of vasopressin and oxytocin (23), as well as with the depletion of posterior pituitary stores of these hormones (24). Whether the increased mRNA levels are due to increased transcription or decreased degradation of the mRNAs, or both circumstances, remains to be determined.

Each of the two pentadecamers, VP-15 and OT-15, differing in sequence by only one base, hybridized uniquely to its complementary mRNA, allowing the identification of the 750-base mRNA encoding PreProVP-Np and the smaller 680-base mRNA encoding PreProOT-Np. The sizes of these mRNAs in the rat agree well with those reported for the corresponding bovine mRNAs (3, 4). The ability to discriminate between similar mRNAs using short oligonucleotides differing by only one base has also been used successfully in analyses of closely related mutant globin genes (25, 26).

These pentadecanucleotides will be useful in more extensive and detailed analyses of the regulation of vasopressin and oxytocin gene expression. They will also be useful as hybridization probes to select full-length cloned recombinant cDNAs prepared from the rat hypothalamus, which will provide more complete nucleotide sequence of the vasopressin and oxytocin precursors in the rat.

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Addendum—After this work was submitted for publication, a partial sequence of the gene encoding rat VP-Np was published. The two sequences are in agreement (27).

REFERENCES

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