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Reduction of exogenous vasopressin RNA poly(A) tail length increases its effectiveness in transiently correcting diabetes insipidus in the Brattleboro rat

(intrahypothalamic injections of RNA/accumulation and axonal transport of RNA)

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ABSTRACT **Magnocellular hypothalamic neurons in Brattleboro rats can accumulate, transport, and translate exogenous [Arg^8]vasopressin (AVP) mRNA after injection in the hypothalamo–hypophysial tract in amounts sufficient to reverse transiently the animals' characteristic diabetes insipidus. In the present study, different preparations of hypothalamic RNA extracted from normal rats or synthetic AVP RNA were injected into the lateral hypothalamus of Brattleboro rats. Poly(A)$^-$ RNA and poly(A)$^+$ RNA from which tails were removed by RNase H digestion were much more effective than poly(A)$^+$ RNA in expressing AVP in the magnocellular hypothalamic neurons and in raising urine osmolarity. Synthetic AVP RNA lacking a poly(A) tail also produced a very potent dose-dependent diabetes insipidus reversal. Our results suggest that a short or absent poly(A) tail may facilitate the accumulation, transport, or expression of exogenous AVP mRNA by magnocellular neurons.**

We previously reported that oxytocin mRNA can be found associated with the secretory vesicles within the axonal compartment of rat hypothalamo–neurohypophysial system neurons (1). [Arg^8]Vasopressin (AVP) mRNA has also been found in the axonal compartment by several investigators (2–7). Previous studies indicate that the length of total hypothalamic AVP mRNA poly(A) tail is increased from 250 to 400 nucleotides following a hyperosmotic stimulus (8). Structural changes in the AVP mRNA have also been found to be associated with its translocation; using osmotically challenged rats, Mohr *et al.* (9) have shown that the poly(A) tail length of AVP mRNA is progressively shortened during axonal transport in the hypothalamo–neurohypophysial tract and is virtually nonexistent by the time it reaches the posterior lobe.

More recently, we have studied the functional significance of the intraaxonal mRNA by experiments with the Brattleboro rat. This naturally occurring mutant rat, with a single base deletion in exon B of the propressophysin gene (10), is unable to translate functional AVP and has chronic diabetes insipidus, a condition characterized by the inability to concentrate urine. When total RNA extracted from the hypothalami of osmotically challenged normal rats was microinjected into the hypothalamo–neurohypophysial tract of homozygous Brattleboro rats, AVP mRNA was accumulated by magnocellular neurons, transported, and translated into immunoreactive vasopressin (11). Exogenous mRNA from normal rats and synthetic AVP RNA were both capable of causing a temporary correction of diabetes insipidus as well as production of significant plasma levels of immunoreactive AVP. Control injections with total RNA extracted from the cortex or hypothalamic RNA digested with RNase, as well as injections with the sense RNA probe into the lateral ventricle or into the cerebral cortex, were all ineffective in changing urine osmolarity or vasopressin levels.

In an effort to increase the concentration of specific AVP mRNA in the RNA extract, we initially sought to enrich the polyadenylylated fraction by passage over oligo(dT)-cellulose, since in general poly(A)$^+$ mRNAs have been considered the main biologically active form (12). In preliminary experiments (13), we made stereotaxic injections of the poly(A)$^-$ fraction into the locale of hypothalamic neurohypophysial axons in homozygous Brattleboro rats. Although this poly(A)$^-$ fraction contained very little AVP mRNA, these injections caused a much greater increase (2.5-fold) in urine osmolarity than injection of the poly(A)$^+$ fraction (1.3-fold). This unexpected finding suggested that the length of the poly(A) tail might be functionally important for accumulation, transport, or translation of the exogenous RNA. To evaluate this hypothesis, we compared the effectiveness of poly(A)$^-$ and poly(A)$^+$ fractions of hypothalamic RNA extracts as well as poly(A)$^+$ extracts from which the poly(A) tail had been removed by treatment with RNase H to determine whether the lack of effect was due to the tail length or potentially to the presence of an unknown inhibitor in the poly(A)$^+$ population.

MATERIALS AND METHODS

Animals. Two different sources of Brattleboro rats were used: one group of animals was obtained from Harlan–Sprague–Dawley, and the second group was a gift from the National Institutes of Health. Both strains of rats were bred in The Scripps Research Institute vivarium. Homozygous animals were selected according to their water consumption and urine osmolarity (14). During the experiment, the rats were singly housed in metabolic cages. Water consumption, urine output, and urine osmolarity (osmometer; Advanced Instruments) were measured daily.

RNA Isolation and Analysis. Total RNA was isolated from hypothalami of 16 Lewis rats (average weight, 300 g) that had been osmotically challenged by intraperitoneal injections of 1.5 M saline 2 h prior to decapitation. Cytoplasmic RNA was prepared according to Schibler *et al.* (15) by tissue homogenization in Tris buffer followed by digestion with proteinase K in the presence of SDS and phenol/chloroform/isoamyl alcohol extraction. The RNA was then separated by a single passage over oligo(dT)-cellulose (type 3) (16). The poly(A)$^+$ fraction was further digested with RNase H. Aliquots of 1 μg of poly(A)$^+$ RNA in 30 μl of Hepes·KOH buffer were mixed

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Abbreviations: AVP, [Arg^8]vasopressin; PVN, paraventricular nucleus.

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with 500 μg of oligo(dT) (20-mer). After a 65°C incubation for 3 min and a 37°C incubation for 30 min, the DNA·RNA hybrid was digested for 1 h at 37°C with 1 unit of RNase H following the manufacturer's (Boehringer Mannheim) protocol. The RNA concentration of each preparation was determined by measuring absorbance at 260 nm. The different RNA fractions were separated by electrophoresis on agarose gels in the presence of 1 M formaldehyde and were then blotted onto nitrocellulose (17). Blots were hybridized with 6×10^7 cpm of ^{32}P-labeled AVP cDNA [200-bp fragment, complementary to the glycoprotein part of the AVP precursor (gift from H. Schmale, University of Hamburg, Hamburg, F.R.G.), subcloned in Bluescript vector (Stratagene), and labeled by random priming (Boehringer Mannheim kit)], washed under stringent conditions, and exposed for a week.

RNA Synthesis. A synthetic AVP mRNA was generated by *in vitro* SP6 (Promega) transcription of the full-length AVP cDNA clone [donated by D. Richter, Eppendorf Institute, University of Hamburg, Hamburg, F.R.G. (18)] cut as indicated with *Dra* II to eliminate the poly(A)$^+$ tail. Precise quantitation was done by solution hybridization as further described.

Solution Hybridization. Various amounts of the different RNA fractions were hybridized overnight at 62°C to ^{32}P-labeled AVP antisense RNA (25,000 cpm) with T7 polymerase and then digested with RNase A (40 mg/ml) and RNase T1 (2 mg/ml) for 1 h at 37°C as described by Ludwig *et al.* (19). After precipitation with trichloroacetic acid and filtration on a nitrocellulose filter, radioactivity was counted and the amount of specific AVP was calculated by using a standard curve obtained with known amounts of unlabeled sense RNA generated from the same clone with SP6 polymerase. Estimated doses for injections calculated from these standard curves have not been corrected for efficiency of gel extraction or possible quenching effects.

RNA Injections. Each rat was stereotaxically injected unilaterally into the medial portion of the hypothalamo–neurohypophysial tract [coordinates: *x*, 2.2 mm right; *y*, +2 mm; *z*, +2.2 mm from Bregma, according to Paxinos and Watson (20)], while under halothane anesthesia, with 5 μl of isotonic saline containing different RNA fractions coded to allow for blind observations of urinary osmolarities after injection. An injection aid (21) rather than a stereotaxic apparatus was used to perform all injections.

Vasopressin Immunostaining. Two hours after poly(A)$^+$ or poly(A)$^-$ RNA unilateral intrahypothalamic injection, rats were killed by cardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at 4°C. Hypothalami were removed and sectioned on a Vibratome (Oxford) into serial frontal sections (50 μm thick). Sections were washed in PBS and immunostained with a rabbit anti-AVP antiserum (Chemicon) that had been preabsorbed with synthetic oxytocin (Sigma) and by the peroxidase-antiperoxidase method. Immunoprecipitates were visualized with diaminobenzidine and hydrogen peroxide. For control purposes, some sections were stained with antisera to adrenocorticotropic hormone and to β-endorphin by the method described above (both antisera obtained from Chemicon).

RIA. Tissue preparation and RIAs were carried out as double antibody RIAs, according to Häussler *et al.* (22), by Paul Plotsky (Emory University, School of Medicine).

RESULTS

Effect of Different Cytoplasmic RNA Preparations on Urine Osmolarity of Brattleboro Rats. Total RNA was extracted from the hypothalami of osmotically challenged Lewis rats, yielding 0.6 μg of RNA per mg of tissue, and fractionated over oligo(dT)-cellulose, yielding a poly(A)$^+$-enriched fraction (5.08%) and a poly(A)$^+$-depleted fraction (94.92%) referred to here as poly(A)$^-$. The amount of specific AVP mRNA present in those fractions was determined by Northern blot analysis (Fig. 1*A*). To detect a specific signal in the poly(A)$^-$ fraction, 40 μg of RNA was loaded on the gel. Such a large amount affected migration and resulted in the AVP band residing higher on the gel than expected. A third sample, poly(dA)$^+$, was prepared from the poly(A)$^+$ fraction by digesting the poly(A) tail with RNase H. With this treatment, only a part of AVP mRNA had its tail shortened by ≈400 bp (Fig. 1*B*). A quantitative analysis of specific mRNA coding for vasopressin in these fractions was done by solution hybridization (Table 1). The poly(A)$^+$ fraction contained 237 times more AVP mRNA per μg than the poly(A)$^-$ fraction.

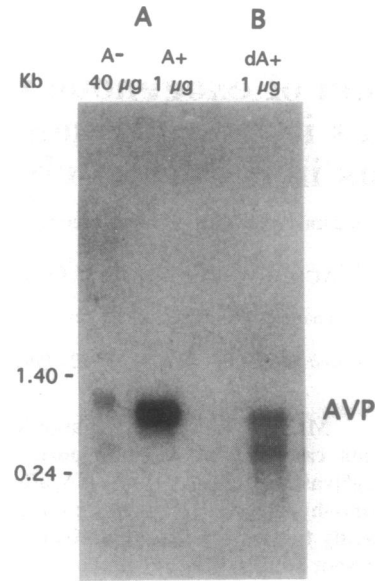

FIG. 1. Northern blot analysis: 40 μg of poly(A)$^-$, 1 μg of poly(A)$^+$, and 1 μg of poly(A)$^+$ RNA from which tails were partially removed by RNase H were separated by electrophoresis, blotted onto nitrocellulose, and hybridized with 6×10^7 cpm of ^{32}P-labeled AVP cDNA.

Pairs of homozygous Brattleboro rats (National Institutes of Health) were injected unilaterally into the hypothalamo–neurohypophysial tract with 5 μl of isotonic saline containing 1 μg of poly(A)$^+$ RNA, 1 μg of poly(A)$^-$ RNA, 80 ng of digested poly(A)$^+$ RNA, or saline only. Urine osmolarity was measured before and 2 h after injection and then daily for 6 days.

The urine osmolarity of control homozygous Brattleboro rats (injected intrahypothalamically with saline) had an overall approximate value of 200 mosM throughout the time observed. Animals treated with 1 μg of poly(A)$^-$ RNA [≈4 amol (i.e., 4×10^{-18} mol) of AVP; see *Materials and Methods*] showed a 2.5-fold increase in urine osmolarity within 2 h after the injection, which returned to control levels over the course of the next 5 days (Fig. 2). Animals treated

Table 1. Amount of specific AVP mRNA present in total, poly(A)$^-$, poly(A)$^+$, and digested poly(A)$^+$ RNA fractions

RNA fraction	AVP mRNA	
	pg per μg of RNA	amol per μg of RNA
Total RNA	8.34 ± 0.42	34.1 ± 1.7
Poly(A)$^+$	225.00 ± 12.0	919.7 ± 49.0
Poly(A)$^-$	0.95 ± 0.18	3.9 ± 0.7
Digested poly(A)$^+$	102.60 ± 5	421.2 ± 20

Data are expressed as pg or amol (10^{-18} mol) of standard AVP sense RNA as measured by solution hybridization.

with 1 μg of poly(A)$^+$ RNA (≈920 amol of AVP) showed only a 1.35-fold increase in urine osmolarity 2 h after the injection when compared to the control, but these differences were not significant. However, animals injected with 80 ng of poly(A)$^+$ RNA digested with RNase H (≈34 amol of AVP, the amount found in 1 μg of total RNA) showed as much increase in their urine osmolarity as animals injected with 1 μg of poly(A)$^-$ RNA, both treatments causing a 2.5-fold increase in urine osmolarity (Fig. 2). To control for a possible effect of RNase H, a pair of animals was injected with 1 μg of poly(A)$^-$ RNA digested with RNase H; this caused results identical to those from injections with 1 μg of poly(A)$^-$ RNA.

Dose–Response Curve. To detect the minimal dose of AVP RNA capable of producing changes in urine osmolarity, pairs of homozygous Brattleboro rats (Harlan–Sprague–Dawley) were injected with serial dilutions of synthetic poly(A)$^-$ AVP RNA (Fig. 3). Within this group, uninjected control animals and animals injected with saline had an approximate urine osmolarity value of 260 ± 42 mosM. As little as 1 fg of AVP RNA (4×10^{-21} mol; see *Materials and Methods*) gave a significant ($P < 0.05$) increase in urine osmolarity after 2 h. With this group of rats having higher basal urine osmolarity levels, the effect of the injection was briefer, with a return to basal levels after 3 days. Although some increases in urine osmolarity were observed with injections of 1 fg and above for 1–2 days after injection, the differences were not significant at longer time points.

To compare the minimal dose effect of synthetic RNA and hypothalamic poly(A)$^-$ RNA, four animals were injected

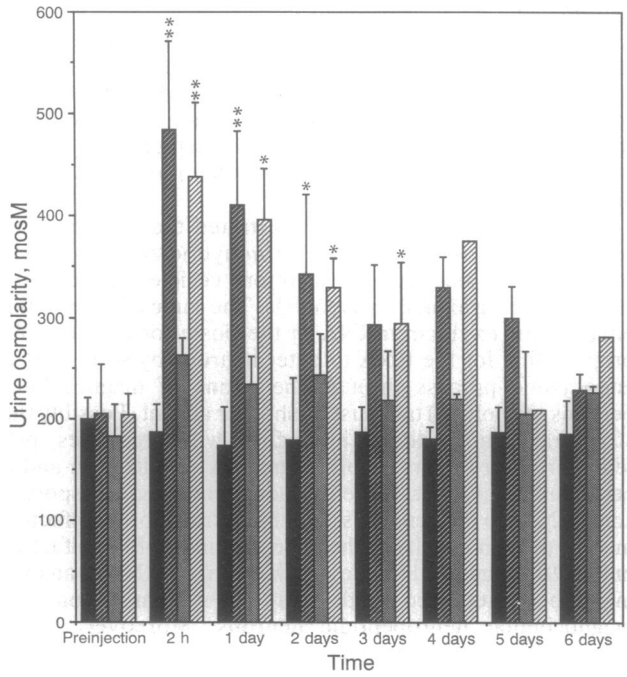

FIG. 2. Urine osmolarity of Brattleboro rats after unilateral intrahypothalamic injections with RNA fractions. Pairs of rats were stereotaxically injected unilaterally into the medial portion of the hypothalamo–neurohypophysial tract with 5 μl of isotonic saline containing (left to right) nothing, 1 μg of poly(A)$^-$, 1 μg of poly(A)$^+$, or 80 ng of detailed poly(A)$^+$. Urine osmolarity was measured before and 2 h, 1, 2, 3, 4, 5, and 6 days after injection. Results are shown as means ± SD of five different experiments with n animals. For preinjection, 2 h, and 1 day, $n = 5$ saline, 9 poly(A)$^-$, 6 poly(A)$^+$, and 6 poly(dA)$^+$. For 2 and 3 days, $n = 2$ saline, 6 poly(A)$^-$, 4 poly(A)$^+$, and 3 poly(dA)$^+$. For 4 and 6 days, $n = 4$ saline, 5 poly(A)$^-$, 2 poly(A)$^+$, and 1 poly(dA)$^+$. For 5 days, $n = 5$ saline, 7 poly(A)$^-$, 4 poly(A)$^+$, and 1 poly(dA)$^+$. Statistical significance of data was evaluated by repeated measures of analysis of variance and a Scheffe F post hoc test. **, $P < 0.005$; *, $P < 0.05$.

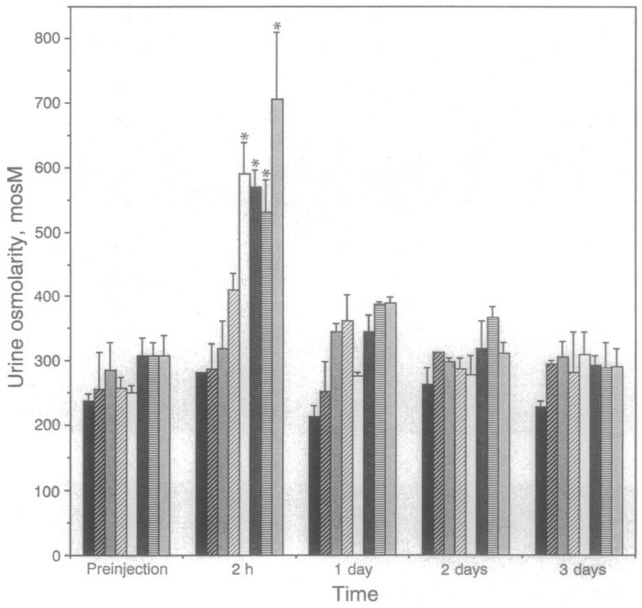

FIG. 3. Pairs of Brattleboro rats (Harlan–Sprague–Dawley) were each stereotaxically injected unilaterally into the medial portion of the hypothalamo–neurohypophysial tract with 5 μl of isotonic saline containing increasing amounts of synthetic AVP RNA [left to right: control (no injection), saline, 0.1 fg, 1 fg, 10 fg, 100 fg, 1 pg, and 1 ng of AVP RNA]. Urine osmolarity was measured before and 2 h, 1, 2, and 3 days after injection. Results are expressed as means ± SD of two measurements from each pair of animals. **, $P < 0.005$; *, $P < 0.05$.

with 1 ng of hypothalamic poly(A)$^-$ RNA (containing ≈1 fg AVP). As seen with synthetic RNA, this small dose increased urine osmolarity to 565 ± 140 mosM. However, experiments with injections of as much as 5 μg of hypothalamic poly(A)$^+$ RNA as well as 10, 40, or 100 ng all yielded the same maximal 1.4-fold increase in urine osmolarity after 2 h (data not shown).

AVP Expression: Immunohistochemistry and RIAs. As in our earlier studies (11), we confirmed the presence of immunoreactive AVP in magnocellular perikarya of the paraventricular nucleus (PVN) and, to a lesser extent, in the supraoptic nucleus 2 h after injection of poly(A)$^-$ RNA (Fig. 4*a*). Immunoreactive AVP was also present in neuronal processes in the PVN as well as in axons of the median eminence and the posterior lobe. However, the magnocellular nuclei and the posterior lobe of Brattleboro rats injected with the poly(A)$^+$ fraction were generally devoid of AVP immunoreactivity (Fig. 4*b*).

RIAs of plasma samples showed significant increases in AVP levels after intrahypothalamic injections of 1 μg of poly(A)$^-$ RNA fraction (Table 2), which were particularly pronounced after 18 h. Intrahypothalamic injections with 1 μg of poly(A)$^+$ also caused a significant but much smaller increase in AVP plasma levels.

DISCUSSION

The data presented here, including the increase of urine osmolarity, the elevation of AVP plasma levels, and the immunocytochemical and RIA findings, suggest that injected AVP poly(A)$^-$ RNA as well as detailed poly(A)$^+$ RNA were preferentially accumulated by neurons of the hypothalamo–neurohypophysial system and translated. Apparently the reduction of the poly(A) tail length can modulate the efficiency of the overall process of RNA accumulation, transport, and expression.

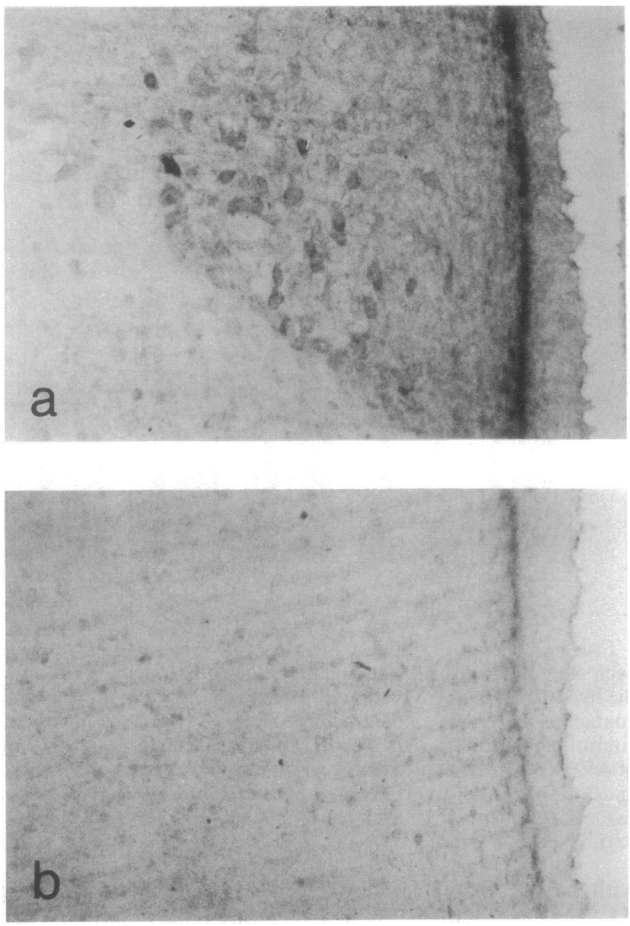

FIG. 4. Vasopressin immunostaining of the PVN of Brattleboro rats 2 h after unilateral injection of poly(A)$^-$ or poly(A)$^+$ hypothalamic RNA. PVN contains numerous AVP immunoreactive magnocellular perikarya in poly(A)$^-$ injected animals while in animals injected with poly(A)$^+$ RNA, PVN perikarya were mostly devoid of immunostaining (*b*).

It has been demonstrated (23, 24) that due to the lack of an existing osmotic medullary gradient in the Brattleboro rat's renal papilla, the AVP-treated animal is initially unable to concentrate its urine above 600 mosM, even with a very high concentration of AVP, far below the 1200 mosM lowest value of a normal rat. An explanation of the discrepancy between AVP levels and urine osmolarity could be that, with the increase in intracellular salt concentration in the tubules, the animal becomes more responsive to circulating AVP. Furthermore, circulating AVP is necessary for expression of functional V2 receptor (25).

Table 2. Plasma vasopressin levels in Brattleboro rats after intrahypothalamic injections of poly(A)$^-$ and poly(A)$^+$ RNA extracted from osmotically challenged Lewis rats

	Pre-injection	2 h	18 h	2 days	4 days	6 days
Poly(A)$^-$ RNA	<1.7	333.8*	1048*	90.3*	8.1	6.9
		(33)	(142)	(7.7)	(1.2)	(2.4)
Poly(A)$^+$ RNA	<2	92.0	103	45.3	4.5	5.7
		(22)	(28.5)	(7.4)	(2.2)	(2.4)

RIA for AVP measured on plasma samples (fg per ml of plasma) taken from Brattleboro rats before and after injections with 1 μg of poly(A)$^+$ or poly(A)$^-$ fraction of total hypothalamic RNA extracts. Results are means ± SD (parentheses) from two animals. Statistical significance of data was evaluated by *t* test.
*$P < 0.05$ relative to the respective poly(A)$^+$ value.

The poly(A)$^-$ fraction of total RNA hypothalamic extracts proved to be more efficient in reversing diabetes insipidus than the total RNA fraction (11) since its content in specific AVP mRNA was considerably lower. In contrast, the poly(A)$^+$ fraction, which contains at least 200 times more AVP mRNA per μg than the poly(A)$^-$ fraction, was nevertheless less effective at any concentration tested. When digested with RNase H, the poly(A)$^+$ fraction increased urine osmolarity as high as the poly(A)$^-$ fraction, suggesting that poly(A) tail removal restored functional activity. In this assay, injections with as little as 1 ng of hypothalamic poly(A)$^-$ RNA or 1 fg of synthetic AVP RNA lacking polyadenylylation (both containing $\approx 4 \times 10^{-21}$ mol of AVP RNA) were effective in increasing urine osmolarity, indicating a very high translation efficiency. The increase in urine osmolarity was much briefer with injections of synthetic RNA as compared to hypothalamic RNA. One explanation for this shorter response could be that the mass of the hypothalamic poly(A)$^-$ fraction buffers cytoplasmic degradation and provides a longer-lasting effect.

RIAs also showed massive synthesis of immunoreactive vasopressin plasma levels, >1000-fold above Brattleboro baselines, after injection of 1 μg of hypothalamic poly(A)$^-$, reaching nearly half the average level found in a normal rat ($\approx$3 pg/ml). Poly(A)$^+$ was 10-fold less active at the same RNA concentration but still caused a small increase in vasopressin plasma levels. We do not understand the nature of this effect, since it cannot be increased further by higher doses, suggesting a saturation mechanism. The maximum tail length that results in vasopressin expression remains to be determined.

Our results offer a physiological explanation to the recent study of Mohr *et al.* (9) showing that the poly(A) tail length of AVP mRNA is progressively shortened during axonal transport in the hypothalamo–neurohypophysial tract. Further data are necessary to determine whether the poly(A)$^-$ AVP can be directly translated or whether it requires repolyadenylylation prior to translation.

In a previous study, we demonstrated that the immunoprecipitates of nonradioactive *in situ* hybridization oxytocin mRNA was associated with secretory vesicles of the posterior lobe during axonal transport (1). The same tail shortening happens for oxytocin mRNA in the posterior lobe (9). We propose that for the RNA to enter neurons by some sort of endocytotic process through the axons of magnocellular neurons, its poly(A) tail must be short or absent. Possibly this structural form facilitates interaction with organelles, proteins, or other factors involved in RNA stabilization and in centripetal transport. Recent studies showing transport of some RNAs to the dendrites (26–28), or transport of olfactory marker protein mRNA to the axon terminals of the olfactory bulb (29), suggest that the mechanism of RNA transport might be generalized in the nervous system beyond the magnocellular neuropeptidic neurons. Moreover, active polysomes have been found in the squid axoplasm (30), suggesting the possibility of *de novo* protein synthesis in the axonal compartment, at least in this species. Although it has yet to be established what molecular mechanisms mediate the specificity of RNA accumulation and transport, the present data suggest that the length of the poly(A) tail must be among the signals involved.

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