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The mutant vasopressin gene from diabetes insipidus (Brattleboro) rats is transcribed but the message is not efficiently translated

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The vasopressin gene from normal and diabetes insipidus (Brattleboro) rats has been isolated and sequenced. Except for a single deletion of a G residue in the region coding for the neurophysin carrier protein the ~2300 nucleotides of both genes are identical. Blot analysis of hypothalamic RNA as well as transfection and microinjection experiments indicate that the mutant gene is correctly transcribed and spliced, however the resulting mRNA is not efficiently translated.

Key words: vasopressin gene/Brattleboro rats/diabetes insipidus/single base deletion/gene transfection/microinjection

Introduction

Vasopressin is one of a number of neuropeptides considered to be multifunctional, serving either as hormones in the circulation or as modulators in the central nervous system, where they may coordinate complex behavioral responses (Douglass *et al.*, 1984). To understand how these various functional activities are controlled it is essential to know more about the biosynthetic pathways of these peptides. This understanding is invaluable in situations where organs are malfunctioning, as is the case with hereditary hypothalamic diabetes insipidus (Sokol and Valtin, 1982). This disease is due to a lack of vasopressin, which as a hormone normally controls water resorption in the distal kidney tubules.

Patients deficient in vasopressin drink considerable quantities of water and excrete proportionally large amounts of very dilute urine. This disorder is known as vasopressin-sensitive diabetes insipidus in contrast to the nephrogenic form which is due to vasopressin-resistance of the kidney.

In man, X-linked and autosomal dominant forms of diabetes insipidus exist (Green *et al.*, 1967). In some patients neurones of the supraoptic and paraventricular nucleus of the hypothalamus were strikingly decreased – both areas are known to synthesize vasopressin (Braverman *et al.*, 1965). An appropriate model for studying the genetic defect is the so-called Brattleboro rat with an autosomal recessive form of diabetes insipidus (Sokol and Valtin, 1982).

The first detailed clarification of this defect came from studies of the vasopressin gene in wild-type and mutant rats (Schmale *et al.*, 1983; Schmale and Richter, 1984). Based on recombinant DNA methods it is now clear that vasopressin is initially synthesized as a much larger precursor which includes – besides the hormone – its carrier protein, neurophysin and a glycoprotein of as yet unknown function.

Analysis of the gene encoding vasopressin showed that the functional domains of the protein precursor are located on three exons separated by two introns. Basically the first exon encodes the hormone, the second exon most of the carrier protein and the last exon the glycoprotein. In extending these studies to a diabetes insipidus rat it became evident that both the wild-type and mutant genes differed in the second exon by a single base deletion. The data presented here show that the defective gene is still transcribed, but that the translation of the resulting mRNA is greatly impaired.

Results

Gene sequence

Figure 1 shows a sequence comparison of the vasopressin gene from wild-type and homozygous diabetes insipidus rats. Except for a single deletion in the second exon the two sequences are completely identical including ~350 bp upstream of the transcription start site (Schmale *et al.*, 1983), the exon-intron junctions, the two introns and the 3′-untranslated region with its polyadenylation site. The deletion leads to a shift in the reading frame which no longer has a stop codon for terminating protein synthesis. Thus the predicted precursor not only has an entirely different C terminus but also could end theoretically in a polylysine tail yielding a protein with a higher mol. wt.

Transcription of the mutated gene

Northern blot analysis of hypothalamic mRNA from wild-type and diabetes insipidus rats showed that the mRNAs are of the same size implying that the mutated gene is correctly transcribed and spliced (Figure 2). Mouse 3T3 cells transfected with the two vasopressin genes also showed no differences in the size of the synthesized mRNA. Again similar results were obtained when oocyte nuclei (*Xenopus laevis*) were injected with the two genes. mRNA hybridizing to the vasopressin-specific DNA probe was, as anticipated, in the cytoplasm of the oocyte, though the size of both gene transcripts was smaller (570 bases) than that in the hypothalamus (700 bases). This difference might be explained by a different amount of polyadenylation.

Cell-free translation

In vivo, in spite of the defective gene being transcribed, no protein products have been detected in the Brattleboro hypothalamus and pituitary. This would imply that the single base deletion in the mutant gene and the consequent frame-shift lead to a block at the translational or post-translational level. Cell-free translation in a rabbit reticulocyte lysate system was performed, using [^{35}S]cysteine as radiolabel.

Inspection of the total translation products of hypothalamic mRNA (Figure 3, lanes 2 and 3) already indicates that a major cysteine-labeled product of mol. wt. 19 000, present in the normal rat, is absent in the mutant (arrowed). This product is specifically selected both by vasopressin and by neurophysin antisera (Figure 3, lanes 4 and 8) and is lacking entirely in the diabetes insipidus rats (Figure 3, lanes 6 and 10), even on prolonged exposure to film. That the hypothalamic mRNA from diabetes insipidus

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                                  -367 CCTGCTAGTCCTTGGTGAATGAGACCTGGGGACCCCTCTAGTCTGTTGAGAGCTGCTGAAATGCTCA
                                  -367 *******************************************************************

-300 ACTATGATTTCCAGGTGACCCTCAAGTCGGCTCACCTCCCTGATTGCACAGCACCAATCACTGTGGCGGTGGCTCCCGTCACACGGTGGCCAGTGACAGC
-300 ****************************************************************************************************

-200 CTGATGGCTGGCTCCCCTCCTCCACCACCCTCTGCATTGACAGGCCCACGTGTGTCCCCAGATGCCTGAATCACTGCTGACAGCTTGGGACCTGTCAGCT
-200 ****************************************************************************************************

-100 GTGGGCTCCTGGGGAGCCACTGGGGAGGGGGTTAGCAGCCACGCTGTCGCCTCCTAGCCAACACCTGCAGACATAAATAGACAGCCCAGCCCGCTCAGGC
-100 ****************************************************************************************************

                                     MetLeuAlaMetMetLeuAsnThrThrLeuSerAlaCysPheLeuSerLeuLeuAlaLeuThrSerAla
   1 AGCAGAGCAGAGCTGCACGCAGTGCCCACCTATGCTCGCCATGATGCTCAACACTACGCTCTCTGCTTGCTTCCTGAGCCTGCTGGCCCTCACCTCTGCC
   1 ****************************************************************************************************
                                     MetLeuAlaMetMetLeuAsnThrThrLeuSerAlaCysPheLeuSerLeuLeuAlaLeuThrSerAla

       V A S O P R E S S I N            N P         exon A     intron I
     CysTyrPheGlnAsnCysProArgGlyGlyLysArgAlaThrSerAspMetGluLeuArgGln
 101 TGCTACTTCCAGAACTGCCCAAGAGGAGGCAAGAGGGCCACATCCGACATGGAGCTGAGACAGGTACCACTGTGGTCCGTTCAGGGCTGCTGACAGTGCC
 101 ****************************************************************************************************
     CysTyrPheGlnAsnCysProArgGlyGlyLysArgAlaThrSerAspMetGluLeuArgGln

 201 GTAGGAAGGGTCATGGGCTAGGAGAGAGGGAAACCTTGTCTGAGCAGTCAGACTTTAGGGGAGGTTCCTGGAAGGAAGCAGTTATCTTATATGGAGTAGA
 201 ****************************************************************************************************

 301 TGGGTTTCCCAGAACGGTAAGAGGGGACCAGGTGCCAGAGAAGCCACATAAAGGACAGTGTCCCCAGGCAGGGGATATGCCAGAAAATGAGAGATACTTA
 301 ****************************************************************************************************

 401 TCACTGGGCTTGGGATGAGAACGGGTTAAACTGGGTACCCTGGCCTCCTCTGCACAGCTGGAGGTGGCCGGTGGTATGTTGGCTCACCAGGACTGGGTAG
 401 ****************************************************************************************************

 501 ATGGTACGAAACTGTTCTCGCCTGAGTACAAAGCCTTTCCCACCCAGCTCAAACTCTCTTAGCTCCTTTTTAGCCAGCTGCACCGGTTTCTTCCTGTCC
 501 ****************************************************************************************************

 601 ACGGAAGACGGCCATTGCCCTGTGTCTGAGCGGAGTATGTCCCACATCTAGCCTCAGCCTCGTGCCCAGATCTGCTGTACTGTATGTTCAGCTCTGAGTC
 601 ****************************************************************************************************

 701 TGCCCTTCCGGCAGGGCTGAAGGGAATCCAGTCACTAGGCTCAAATCTGGTCAGGTCACAGGTGGCTCAGTTTTGAACAAGCTCGATGGGCAGTAGGCAG
 701 ****************************************************************************************************

 801 TTCACCGAGTCTGCCTTCCGTTTGCTGAGTTCCTTTGGAGACTTCCGAGGCACTAGGTGTGTCTTGCACCCATCAGCCTAATTCGGTCCTTGCCACCTTC
 801 ****************************************************************************************************

 901 CTACTAGGGCATAATAGGTTGGCGGGAGGTAAAAGCCCACCAGCGTGGGGCAGGGGTAAGAGTGAGCGAGCCGTAGGTACAGGAAAGAGGATCTTGGAAT
 901 ****************************************************************************************************

1001 GTGTAGGGCCATCTGAATGTCGGAGAGGTAAGTCTCTGAGAGACTGCTGCACACCGGTGACACATCAGAGCTGAGGAGGTCCCCCAAGTGTTGTCTCCCC
1001 ****************************************************************************************************

1101 CGCCCCCCGCCCCATACGACTCTGTCAAAGCAGGAGAGGGTTTTGAGACCTCATGAGAACTGATCCTCCTGATAACCTAGCCGGTTAGATTTCCACTCTC
1101 ****************************************************************************************************

1201 GCCCTTTACGGCTGCTTCGTCCTAGATAGAGCCAGAGCATCTGGCCGGTGAAGCTGGGATAGCAGCAGGGTGACCTTAGGTTCCCAACGCCCCTCTTGGC
1201 ****************************************************************************************************

                                intron I     exon B
                                      CysLeuProCysGlyProGlyGlyLysGlyArgCysPheGlyProSerIleCysCysAlaAspGluL
1301 CTGGCTCCAGCTGACCCGCGTCCTTCCCCGCAGTGTCTCCCCTGCGGCCCTGGCGGCAAAGGGCGCTGCTTCGGGCCGAGCATCTGCTGCGCGGACGAGC
1301 ****************************************************************************************************
                                      CysLeuProCysGlyProGlyGlyLysGlyArgCysPheGlyProSerIleCysCysAlaAspGluL

     euGlyCysPheLeuGlyThrAlaGluAlaLeuArgCysGlnGluGluAsnTyrLeuProSerProCysGlnSerGlyGlnLysProCysGlySerGlyGl
1401 TGGGCTGCTTCCTGGGCACCGCCGAGGCGCTGCGCTGCCAGGAGGAGAACTACCTGCCCTCGCCCTGCCAGTCTGGCCAGAAGCCTTGCGGAAGCGGAGG
1401 ************************************************************************************************_***
     euGlyCysPheLeuGlyThrAlaGluAlaLeuArgCysGlnGluGluAsnTyrLeuProSerProCysGlnSerGlyGlnLysProCysGlySerG luA
                      exon B      intron II
     yArgCysAlaAlaAlaGlyIleCysCysSerAspG
1501 CCGCTGCGCTGCCGCGGGCATCTGCTGCAGCGATGGTGCGCACAAAGCCAGGCGGGCTGAGCATGGGGAATGGATGGGGTGGGTGGAGGTAAAGGGGGG
1500 ****************************************************************************************************
     laAlaAlaLeuProArgAlaSerAlaAlaAlaMet

1601 CTAAGTGGGGGACTGAGGAATCAGGACCGGAGATGGAGGGTGAGTAGTATGAAGGGGGTCGAGAGTTGGAACGTAGCAGGGTAGGATAAAGGGGATTGTG
1600 ****************************************************************************************************
                                                                         intron II     exon C
                                                                              luSerCysValAlaGluProGluCysArgGluGlyP
1701 GGGATGGCGCCCCTATAGGTGCGCCCACCCCAGGACGCCTGACCTCACACAGCCCTTCCTTCAGAGAGCTGCGTGGCCGAGCCCGAGTGTCGAGAGGGTT
1700 ****************************************************************************************************
                                                                  ArgAlaAlaTrpProSerProSerValGluArgVal
         N P          G L Y C O P R O T E I N
     hePheArgLeuThrArgAlaArgGluGlnSerAsnAlaThrGlnLeuAspGlyProAlaArgGluLeuLeuLeuArgLeuValGlnLeuAlaGlyThrGl
1801 TTTTCCGCCTCACCCGCGCTCGGGAGCAGAGCAACGCCACGCAGCTGGACGGGCCAGCCCGGGAGCTGCTGCTTAGGCTGGTACAGCTGGCTGGGACACA
1800 ****************************************************************************************************
     PheSerAlaSerProAlaLeuGlySerArgAlaThrProArgSerTrpThrGlyGlnProGlySerCysCysLeuGlyTrpTyrSerTrpLeuGlyHisL

     nGluSerValAspSerAlaLysProArgValTyrEnd
1901 AGAGTCCGTGGATTCTGCCAAGCCCCGGGTCTACTGAGCCATCGCCCCCCACGCCTCCCCCCTACAGCATGGAAAATAAACTTTTAAAAAA
1900 *******************************************************************************************
     ysSerProTrpIleLeuProSerProGlySerThrGluProSerProProThrProProProTyrSerMetGluAsnLysLeuLeuLysLy
```

Fig. 1. Nucleotide sequence of the vasopressin gene obtained for normal and homozygous diabetes insipidus (D.I.) rats. The sequence shown differs from that reported by Schmale *et al.* (1983) at five positions: −95 to −91 reads CTCCT (instead of CTCT); −85 to −81 reads GCCAC (instead of GCAC, also stated in Schmale and Richter, 1984); −57 (C instead of G); +1550 to +1553 reads AGGC (instead of AGCGC); +1733 (G instead of A). Amino acid +143 reads Arg (instead of Ala). The underlined nucleotide sequence indicates the modified TATA box, the underlined amino acid sequence shows a 14-mer peptide for which antibodies were raised. Asterisks indicate identical nucleotides. Nucleotides upstream of the transcription start site are given in negative numbers. Np, neurophysin.

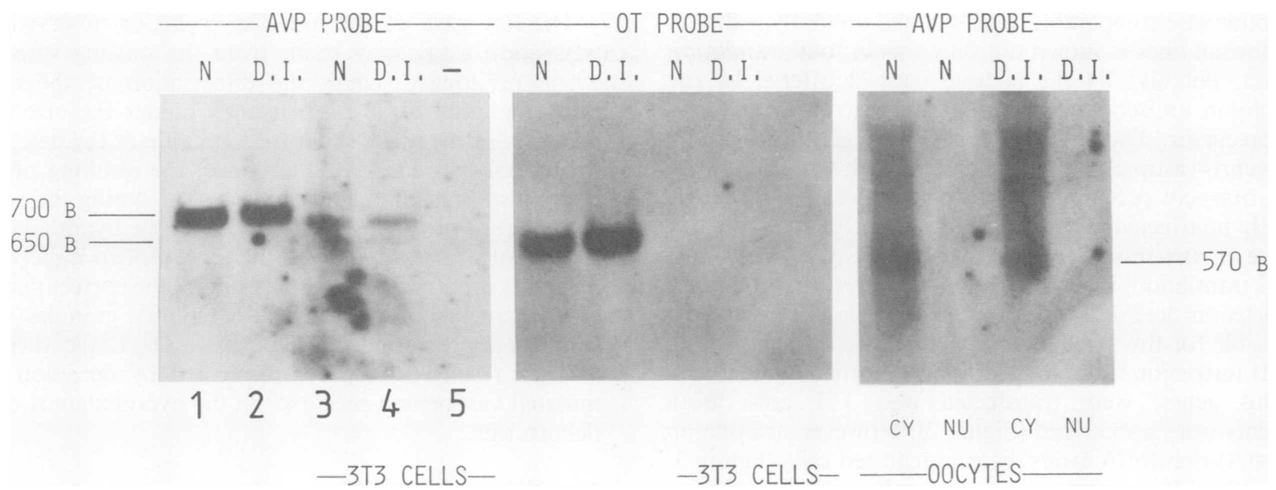

Fig. 2. Poly(A)$^+$ RNA prepared from 3T3 cells transfected with either the normal (N) or the diabetes insipidus (D.I.) gene and from untreated cells (–) was separated on a denaturing agarose/methylmercuric hydroxide gel (**lanes 3–5**). After transfer of the RNA onto a nitrocellulose filter, hybridization was performed firstly with an AVP-specific DNA probe derived from exon C of the rat vasopressin gene (pVλPstI; Schmale *et al.*, 1983) (**left blot**). Blot, hybridization and dehybridization conditions were the same as reported (Schmale and Richter, 1984). For comparison, hypothalamic poly(A)$^+$ RNA from normal (**lane 1**) and Brattleboro rats (**lane 2**) were run on the same gel. After dehybridization the same blot was rehybridized with an oxytocin (OT)-specific probe (*Ava*I-*Eco*RI; Ivell and Richter, 1984) (**central blot**). In the **right hand blot** total RNA (10 μg of cytoplasmic, cy, or 0.5 μg of nuclear, nu, RNA) from oocytes (injected with the respective gene as outlined in Materials and methods; the oocytes were incubated for 46 h at 20°C) were applied to the gel.

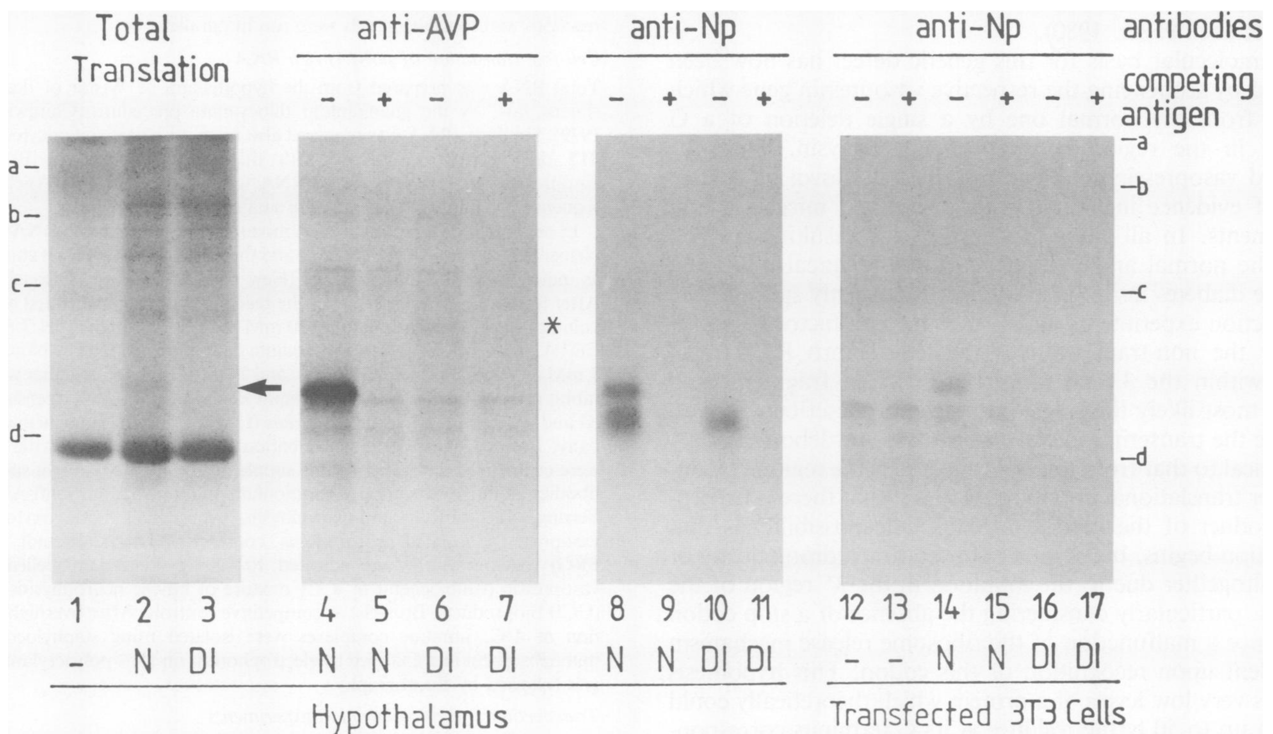

Fig. 3. Cell-free translation of poly(A)-rich RNA from normal and Brattleboro hypothalami, and from transfected 3T3 cells. **Lanes 1–3**: 2 μl of total translation products synthesized in the absence (**lane 1**) or presence of normal (**lane 2**) or Brattleboro (**lane 3**) hypothalamic mRNA. **Lanes 4–11**: immunoreactive normal (N) or Brattleboro (D.I.) translation products selected using anti-AVP (**lanes 4–7**) or anti-neurophysin (**lanes 8–11**) in the absence or presence of competing antigen as indicated. **Lanes 12–17**: translation products of mRNA from 3T3 cells transfected with the normal (**lanes 14** and **15**) or Brattleboro (**lanes 16** and **17**) genes or not (**lanes 12** and **13**), specifically selected by neurophysin antibodies. Similar results were obtained using anti-vasopressin (not shown). Exposure times: **lanes 1–3**, 1 day; **lanes 4–7**, 9 days; **lanes 8–17**, 4 days. Calibration proteins (left for **lanes 1–7**; right for **lanes 8–17**): a, bovine serum albumin, mol. wt. 69 000; b, ovalbumin, mol. wt. 46 000; c, carbonic anhydrase, mol. wt. 30 000; d, lysozyme, mol. wt. 14 300.

rats is otherwise comparable in quality and quantitiy with that from normal ones is shown not only by the total translation products, but also by the internal control offered by the neurophysin antisera, which also cross-react with the oxytocin precursor. The latter migrates in the gel more rapidly than prepro-vasopressin, to correspond with the smaller size of the oxytocin precursor (Ivell and Richter, 1984), and is evidently unaffected by the mutation (lane 10).

These *in vitro* results confirm the presence of a block at the level of translation; it can be argued, however, that the single base deletion detected in the mutant gene is not exclusively responsible for this phenomenon. To clarify this, the 3.8-kb *Hind*III restriction fragments from both normal and diabetes insipidus genes, were transfected into 3T3 cells. Both fragments were transcribed (Figure 2). However, translation of poly(A)-rich RNA from these transfected cells (Figure 3, lanes 12–17) reiterated the results of translating hypothalamic mRNA; only cells containing the normal gene produce a translatable message.

Discussion

Earlier studies of hereditary hypothalamic diabetes insipidus in rats have shown that *in vivo* vasopressin as well as its corresponding neurophysin carrier are absent (Sokol and Valtin, 1982); that neurones of the supraoptic and paraventricular nuclei are hypertrophied; that those cells supposed to produce vasopressin lack normal neurosecretory granules (Valtin *et al.*, 1978) and that a possible mutated vasopressin precursor, referred to as 'peptide X' has antigenic sites for vasopressin and neurophysin antibodies, while it lacks a carbohydrate chain (Russell *et al.*, 1980).

The molecular basis for this genetic defect has now been resolved by sequencing the respective vasopressin gene which differs from the normal one by a single deletion of a G residue in the region coding for neurophysin. That the mutated vasopressin gene is transcribed is shown by several lines of evidence including transfection and microinjection experiments. In all cases the sizes of the resulting mRNA from the normal and mutant genes are identical indicating that the diabetes insipidus transcript is correctly spliced. The transfection experiments also prove that the factor responsible for the non-translation of the Brattleboro RNA must reside within the 3.8-kb *Hind*III restriction fragment, and thus is most likely the single base deletion described above.

Since the transcription start site of the Brattleboro mRNA is identical to that from normal rats, and is the region responsible for translational initiation, why is it that there is no protein product of the modified gene? One possibility is that translation begins, but is either slowed down dramatically or stops altogether due to the mutation in the 3′ region of the mRNA, particularly considering the absence of a stop codon and hence a malfunction of the ribosome release mechanism dependent upon recognition of this codon. This hypothesis predicts very low levels of a protein which theoretically could contain up to 50 lysine residues at its C terminus corresponding to the 150 adenosines of the poly(A) tail and which should have a higher mol. wt. (~26 000). Close inspection of Figure 3 (lane 6, asterisk) does in fact suggest a faint band at this mol. wt., which is only visible after prolonged exposure. This band is effectively competed by an excess of cold antigen; it is absent among the products of normal hypothalamic mRNA (lane 4). A similar band has been detected using anti-neurophysin but again only after very long exposures.

The low yield of the mutated precursor observed in the translation assay may result from the missing stop codon which no longer signals the dissociation of the mRNA-ribosome complex and hence may hinder the effective reinitiation of the mRNA. Further, because of the basic nature of the polylysine at the C terminus, the resulting precursor might also precipitate and hence be lost during the isolation procedure. For the *in vivo* situation in the hypothalamus of Brattleboro rats, the lack of the glycoprotein moiety or the altered neurophysin carrier may hinder the correct packaging of the precursor into the neurosecretory granules and its subsequent processing. It will be interesting to see whether the *in vitro* results can be complemented by detection of the mutated vasopressin precursor in the hypothalamus of Brattleboro rats.

Materials and methods

Homozygous diabetes insipidus (Brattleboro) rats were obtained from Holland Centraal Proefdierenbedrij, Zeist, The Netherlands. Homozygosity of the rats was checked, and isolation of the DNA and RNA was carried out as reported (Schmale and Richter, 1984).

Sequence analysis

The 3.8-kb *Hind*III fragments subcloned in the plasmid pUC8 were used for sequence analysis. These fragments derived from both normal and diabetes insipidus rats, contained the entire vasopressin gene of 2.2 kb and ~1.5 kb of upstream genomic sequences (Schmale *et al.*, 1983; Schmale and Richter, 1984). Appropriate restriction fragments of the first intron either 5′-labelled with T4 kinase and [γ-^{32}P]ATP or 3′-labelled with terminal transferase and [^{32}P]cordycepin were chemically modified according to the sequencing method of Maxam and Gilbert (1980). All restriction sites used for fragment preparation and labelling were confirmed by sequence analysis. For optimum comparison of the wild-type and diabetes insipidus nucleotide sequences, all reactions and sequencing gels were run in parallel.

Cell-free translation of poly(A)-rich RNA

Total RNA was prepared from the hypothalami of Wistar or diabetes insipidus rats, by the guanidinium thiocyanate procedure (Chirgwin *et al.*, 1979). Similarly, RNA was prepared also from microinjected oocytes or from 3T3 cells transfected or not with the isolated normal or Brattleboro vasopressin genes. The extracted RNA was enriched for poly(A)-containing sequences by oligo(dT)-cellulose chromatography.

In each 50 μl translation reaction mixture, 1 μg poly(A)-rich RNA was supplemented with 40 μCi [^{35}S]cysteine and the rabbit reticulocyte kit components as indicated by the manufacturer (New England Nuclear, Dreieich, FRG). After incubation for 1 h at 37°C, the reactions were supplemented with three volumes of a solution containing 10 mM sodium phosphate (pH 7.6), 1 mM EDTA, 0.3% w/v SDS, 1% w/v sodium deoxycholate, 1% v/v Nonidet-P40, 5 mM cysteine, 2 mM dithiothreitol and 2% v/v Trasylol, together with 30 μg rabbit pre-immune IgG and 100 μl equivalent of a 10% w/v suspension of fixed and washed *Staphylococcus aureus* (IgSorb, The Immunochemical Company, Boston). After 30 min incubation with shaking at 4°C, the mixtures were centrifuged and supernatants supplemented with 30–50 μg specific antibodies as the 40% saturated ammonium sulfate fractions (anti-AVP from Ferring, Kiel, FRG; anti-neurophysin, recognizing both oxytocin and vasopressin associated neurophysins, courtesy of Dr. A. Weindl, Munich, FRG), together with, where indicated, 10–20 μg of either unlabelled arginine vasopressin (Calbiochem) or a 1:1 mixture of bovine neurophysins I and II (UCB Bioproducts, Brussels) as competitive controls. After overnight incubation at 4°C, immune complexes were isolated using staphylococcal immunoabsorbent and analysed by electrophoresis on 15% polyacrylamide/SDS gels followed by fluorography.

Transfection and microinjection experiments

For both types of experiment the 3.8-kb *Hind*III fragments from normal and Brattleboro rats were used. This fragment contained the entire vasopressin gene and additional 5′ sequences, subcloned in the plasmid pUC8.

Transfection. Mouse 3T3 cells were transfected (Stuhlmann, 1983) with the *Hind*III fragments, cleaved from the plasmid. Eight clones of cells transfected with the normal or the diabetes insipidus gene, were selected for further DNA or RNA analysis. High mol. wt. genomic DNA, total and poly(A)$^+$ RNA were prepared as described (Schmale and Richter, 1984). Southern blot analysis showed that all 16 clones contained ~20–50 copies of the vasopressin gene inserted at different sites of the 3T3 cell genome. The

transcription of these genes into RNA was determined by dot blot analysis of total RNA. Hybridization with a vasopressin-specific DNA probe (Schmale and Richter, 1984) showed different levels of expression for the individual clones. One clone out of both groups was used for preparation of poly(A)$^+$ RNA and subsequent Northern blot analysis.

Microinjection. Oocytes from *X. laevis* were isolated and the nuclei and cytoplasm prepared as described (Kalthoff and Richter, 1979; Richter *et al.*, 1982). 10–15 nl of DNA (0.5 ng/nl) were injected into the nucleus as reported (Breindl *et al.*, 1983). For the injection experiments circular DNA was used (Harland *et al.*, 1983) which included the 3.8-kb *Hind*III fragments from normal and diabetes insipidus rats subcloned in the plasmid pUC8.

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Note added in proof

We have recently isolated a vasopressin-encoding clone from a Brattleboro hypothalamic cDNA library. Sequence analysis shows the same nucleotide deletion as in the mutant gene, thus providing independent confirmation as to the cause of the Battleboro defect.