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Frameshift mutations at two hotspots in vasopressin transcripts in post-mitotic neurons

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ABSTRACT **Mutations in DNA underlie carcinogenesis, inherited pathology, and aging and are generally thought to be introduced during meiosis and mitosis. Here we report that in post-mitotic neurons specific frameshift mutations occur at high frequency. These mutations were identified in vasopressin transcripts in magnocellular neurons of the homozygous Brattleboro rat and predominantly consist of a GA deletion in GAGAG motifs. Immunocytochemistry provides evidence for similar events in wild-type rats. However, the diseased state of the Brattleboro rat, resulting in a permanent activation of vasopressin neurons, enhanced the mutational rate. These data reveal hitherto unrecognized somatic mutations in nondividing neurons.**

Somatic mutations can result in a changed gene function and have been implicated in carcinogenesis and aging. These mutations are generally thought to be introduced during mitosis (1). However, the detection of solitary magnocellular neurons with revertant phenotype in the homozygous diabetes insipidus (di/di) Brattleboro rat (2–4) suggested that post-mitotic cells may also be subjected to mutagenesis. Thus, the di/di rat may serve as a unique model to delineate genomic alterations in nondividing cells. The homozygous (di/di) Brattleboro rat suffers from severe diabetes insipidus due to the absence of the antidiuretic hormone vasopressin (VP) (5). The cause of the disease is the deletion of a single deoxyguanosine in the second exon of the VP gene resulting in a mutant VP precursor with an altered C-terminal amino acid sequence (6). The mutant VP precursor is synthesized in hypothalamic neurons but does not enter the secretory pathway (7). Surprisingly, in the di/di rat, a small number of magnocellular neurons exhibits a heterozygous (+/di) phenotype, expressing immunoreactivity for both the mutated precursor and the apparently normal VP gene products [VP, neurophysin (NP), and the glycoprotein (GP)] (2–4). The number of these neurons increases linearly with age from 0.1% of the total number of VP neurons at birth to 3% in 2-year-old di/di rats (8). Since these neurons do not display cell division after fetal day 15 (9), these findings indicate that after mitotic division has ceased, sequence alterations occur that restore the reading frame of the VP transcripts of the di/di rat.

In this study, sequence alterations have been identified that restore the reading frame of the mutant VP precursor (VP^{di}) mRNA. The results, revealing a preferred 2-nucleotide deletion in GAGAG motifs, show that nondividing neurons can be subject to somatic mutations at high frequency.

MATERIALS AND METHODS

Cloning and Immunoscreening. Total RNA (5 μg), isolated from hypothalami of individual female di/di rats of 90 weeks old using RNAzol (Biotecx Laboratories, Houston), was used for first-strand cDNA synthesis with Maloney murine leukemia virus H^- reverse transcriptase (BRL). VP cDNAs were amplified by PCR with *Pfu* DNA polymerase (Stratagene) (0.25 unit/μl) and 10% of the first strand cDNA reaction mixture/all four dNTPs (each at 200 μM)/0.5 μM of each primer/1.5 mM $MgCl_2$/10 mM KCl/6 mM $(NH_4)_2SO_4$/20 mM Tris·HCl, pH 8.2/0.1% Triton X-100. The cycle temperatures and timing were as follows: 95°C, 1 min; 50°C, 1 min; 75°C, 3 min (30 cycles). In the last cycle, the extension was carried out for 10 min [5′ oligonucleotide, 5′-TCTGCCTGCGAATTCCAGAACTGCCCAAGA-3′, corresponding to nucleotides 95–124 of the VP cDNA (10), including an *Eco*RI restriction site; 3′ oligonucleotide, 5′-AGTTTATTTTCCAAGCTTTAGGGGGGAGGCGT-3′, corresponding to nucleotides 583–552, including a *Hin*dIII restriction site]. PCR products were cloned in the *Eco*RI and *Hin*dIII sites of the expression vector pGEMEX-1 (Promega) and transformed to the bacterial strain BL21(DE3) (11) by electroporation. Bacterial clones were transferred to nitrocellulose filters (UV-Duralose; Stratagene) soaked in 200 mM isopropyl β-D-thiogalactoside. The bacterial clones were then incubated for 2 h at 37°C to induce expression of the cloned cDNAs. The bacterial proteins were precipitated on the nitrocellulose filters by heating at 95°C for 30 min, and the cell debris was washed from the filters in 10 mM Tris·HCl (pH 7.4). GP-positive clones were selected by immunoscreening with rabbit anti-rat GP antiserum [C3final (ref. 12), 1:1000 dilution] using the peroxidase-anti-peroxidase method. GP-immunoreactive clones were selected from the original agar plate and grown overnight in LB medium containing ampicillin. DNA was isolated using the alkaline lysis method (13), and sequence analysis was performed using Sequenase version 2.0 (United States Biochemical). Sequence reaction products were analyzed on denaturating polyacrylamide gels containing 50% (vol/vol) formamide.

***In Situ* Hybridization.** Female di/di rats were anesthetized with Nembutal (60 mg/kg, i.p.), perfused intracardially with 0.9% NaCl/0.1 M sodium phosphate-buffered 4% (wt/vol) paraformaldehyde, pH 7.4. After dehydration, hypothalami were embedded in paraffin. Consecutive 10-μm sections throughout the hypothalamus were used alternately for *in situ* hybridization and immunocytochemistry. For *in situ* hybridization, the slides were treated with 0.2 M HCl, dehydrated, and air-dried. A 20-mer oligonucleotide complementary to the VP mRNA containing a GA deletion at position 365 (5′-GCTACTCGACGCACCGGCTC-3′, corresponding to nucleotides 361–382) was labeled at the 3′ end using terminal deoxynucleotidyl-transferase (Boehringer Mannheim) and ^{35}S-labeled dATP (NEN/DuPont) to provide an average A-tail length of 5 nucleotides. The labeled probe, at 4×10^6 cpm/ml of hybridization buffer [50% deionized formamide/0.5 M NaCl/1× Denhardt's solution/10 mM Tris·HCl, pH 7.5/1 mM EDTA/tRNA (0.5 mg/ml)/10% (wt/vol) dextran

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Abbreviations: VP, vasopressin; NP, neurophysin; GP, glycoprotein; di, diabetes insipidus phenotype.

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sulfate/30 mM dithiothreitol/herring sperm DNA (30 μg/ml)] was incubated with the sections under coverslips at 42°C overnight in a humid chamber. Slides were washed at 55°C in SSC (1× SSC, 45 min; 0.3× SSC, three 45-min periods) and subsequently dehydrated (70% ethanol/300 mM ammonium acetate, 5 min; 95% ethanol/300 mM ammonium acetate, 5 min). The slides were dipped in Ilford K-5 emulsion, air-dried, and stored at 4°C. After 5 days of exposure, the slides were developed with Kodak D-19, counterstained with thionine, dehydrated, and mounted with coverslips.

Antisera. Antisera were raised in rabbits against synthetic peptides predicted from the +1 reading frame of wild-type VP mRNA (antisera VP474^{+1} and VP363^{+1}, see Fig. 1). The potency of the antisera VP474^{+1} and VP363^{+1} was tested on peptides immobilized on nitrocellulose filters. Both antisera (1:1000 dilution) could detect 10 ng of their antigen. The antibody specificity was checked by pre-absorbing the VP474^{+1} antiserum with its antigen. Subsequently, no immunoreactivity was detected.

Immunocytochemistry. Immunostaining with anti-GP antiserum (C3final) was done on 10-μm paraffin sections according to ref. 8. Male Wistar rats of 12, 44, and 80 weeks of age were perfused intracardially with 0.9% NaCl/Bouin's fluid for immunostaining with antisera VP363^{+1} (dilution 1:1000) and VP474^{+1} (dilution 1:1000) on 50-μm Vibratome sections (for methods, see ref. 8).

VP Substitution. Male di/di rats were randomly divided over two groups at the age of 24 weeks. After anesthesia with Aescoket-Plus [Aesculaap, Boxtel, The Netherlands; 0.1 ml/100 g (body weight) i.p.], one group received VP by subcutaneous placement of an osmotic minipump (Alzet; Alza; #2 ML4, releasing 120 ng of VP per h) and the control group received a similarly sized tube only. The rats were housed separately accompanied by an ovariectomized Wistar rat and checked for water consumption every day. Every 28 days the pumps were replaced and the controls were sham-operated. After 40 weeks of VP substitution, all animals were housed in metabolic cages for urine collection. The urine osmolality was measured. After anesthesia, perfusion fixation with 0.1 M sodium phosphate-buffered 4% paraformaldehyde, and embedding, 10-μm paraffin sections throughout the hypothalamus were incubated with anti-GP antiserum (C3final) as described (8) and the total number of cells was determined.

RESULTS

To determine the nature of the frameshift leading to GP immunoreactivity in solitary neurons of di/di rats, VP transcripts were cloned by PCR from the hypothalamus of di/di rats and screened for expression of GP immunoreactivity in a bacterial expression system. This cloning system was rigorously evaluated in view of the possible artifactual introduction of mutations by PCR. A cloned VP template containing the di deletion was amplified using the *Pfu* DNA polymerase, which has a proofreading activity, and cloned in a bacterial expression system. By immunoscreening with anti-GP antiserum, a low number of GP-positive clones was detected (0.5 ± 0.1 GP-positive clones per 1000 clones; n = 4). Immunoscreening of material amplified and cloned from di/di rats resulted in 8.8 ± 1.9 GP-positive clones per 1000 clones (n = 7). This number of GP-positive clones is in agreement with the number of reverted cells in di/di rats of the same age (7). A total of 42 cDNA clones, obtained from five individual di/di rats, was analyzed (Table 1). Thirty of the 42 VP cDNAs (70%) contained a GA deletion: 19 of which occurred within a GAGAG stretch at nucleotides 365–369 (deletion, GA^{365}) of the VP cDNA and 11 of the GA deletions within a GAGAG stretch at nucleotides 393–397 (deletion, GA^{393}). Both GA deletions were found together in all individual di/di rats. In addition, 12 mutations ranging from single nucleotide insertions to large deletions were found, but each occurred only in one di/di rat (Table 1). In all clones, the original di deletion (G missing at nucleotide 326) was present. The deletions GA^{365} and GA^{393} cause the coding for a VP precursor with a mutated region of 13 and 22 amino acids, respectively (Fig. 1). These results indicate that there are two hotspots for a GA deletion that both give rise to VP mRNAs with a +1 frameshift.

Table 1. The +1 frameshift mutations identified in VP transcripts of di/di rats

Mutation	Number of clones	Position(s)
− GA	19 (45%)	365–369
− GA	11 (26%)	393–397
+ A	1	501–502
− TT	1	400–405
+ ACA/+ C	1	507–508/383
+ T	2	400–405
+ A	1	300
− GG	2	397–399
Deletion		
26 nt	1	258–283
169 nt	1	145–313
65 nt	1	365–429
122 nt	1	331–452
Total	42 (100%)	

Position refers to the numbering of the nucleotide sequence of the VP cDNA according to ref. 10.

Through the use of high-stringency *in situ* hybridization with an oligonucleotide containing the GA^{365} deletion, solitary cells expressing the VP transcripts with this mutation were identified in the di/di rat. In 10-μm paraffin sections of the hypothalamus, high levels of VP transcripts with the mutation appeared in solitary neurons (Fig. 2*a*). Consecutive sections stained with anti-GP antiserum revealed that the same solitary neurons displayed the reverted phenotype (Fig. 2*b*). These results show that the mutation identified by PCR is endogenous to the di/di rat.

The VP mRNAs with a +1 frameshift encode VP precursors with a correct GP region. These predicted precursors explain the wild-type phenotype of the reverted magnocellular neurons of di/di rats as detected previously by immunocytochemistry (2–4). Whether the VP precursors are properly sorted and processed remains to be determined, but the presence of VP, NP, and GP immunoreactivity in axons projecting toward the neural lobe of di/di rats (2, 3, 8) suggests that this may be the case.

To determine whether +1 frameshift mutations also exist in VP transcripts of wild-type rats, antibodies were raised against a synthetic peptide predicted by the amino acid sequence that would result from the deletion GA^{365} or GA^{393}, which creates a stop codon at position 474 (antiserum VP474^{+1}, Fig. 1). A second antiserum was raised against a truncated VP precursor that would be created if a +1 frameshift occurred before nucleotide 363 (antiserum VP363^{+1}, Fig. 1). When antiserum VP474^{+1} was used, heavily stained solitary magnocellular neurons were found in the supraoptic and paraventricular nuclei of normal rats (Fig. 3). The number of these immunoreactive cells was 6.6 ± 3.9 (mean ± SD) in 12-week-old wild-type rats (n = 5) and 30.0 ± 4.8 in 44-week-old wild-type rats (n = 5). But when antiserum VP363^{+1} against the hypothetical mutated precursor terminating at position 363 was used, no immunoreactive cells were found in 12-week-old wild-type rats (n = 5) and only a few (1.2 ± 0.8) were found in 80-week-old wild-type rats (n = 5). The difference in the number of immunoreactive neurons displaying a +1 frameshift as detected by the two

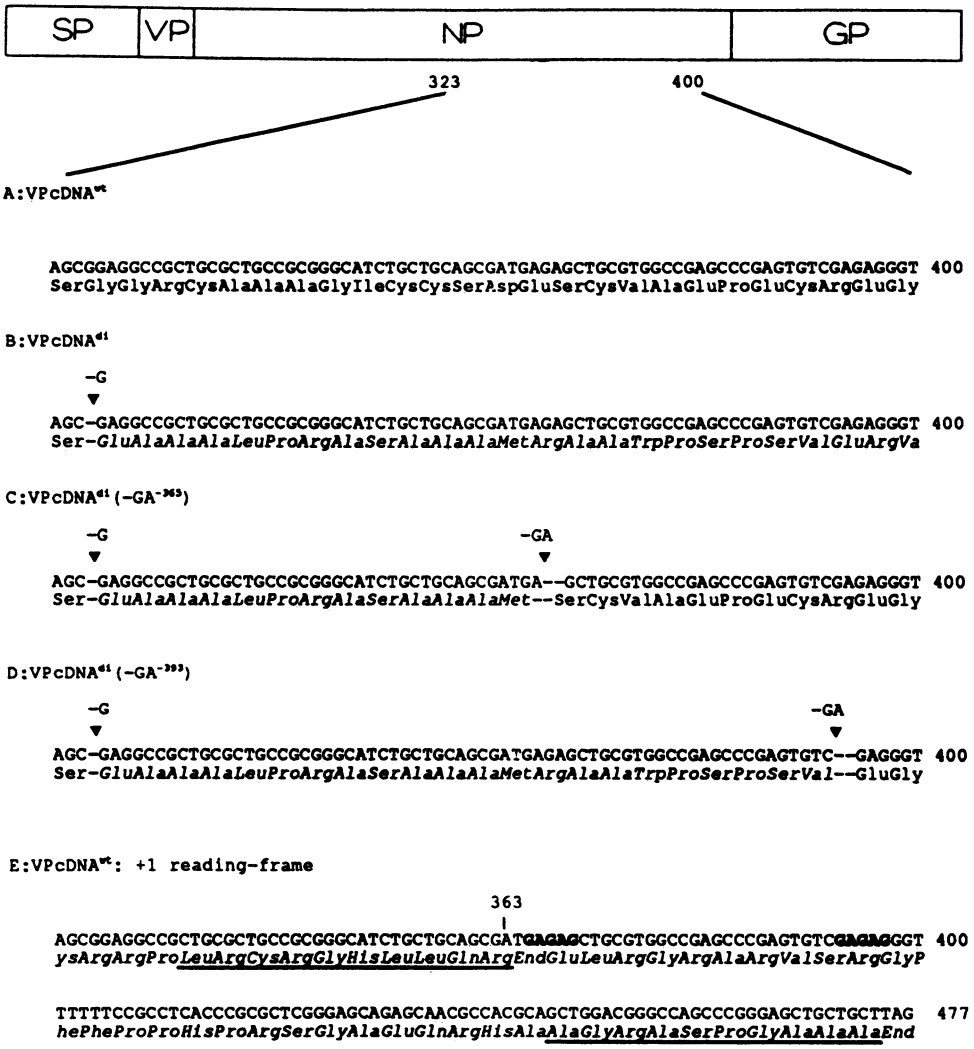

FIG. 1. Structure of the wild-type VP precursor and mutant precursor forms. The VP gene encodes a protein consisting of a signal peptide (SP), VP, NP, and GP. The diabetes insipidus (di) VP gene contains a G deletion in exon B that alters the C-terminal part of NP and the GP region into a different amino acid sequence. Part of the VP cDNA sequence (nucleotides 323–382; numbered according to ref. 10) is shown. Sequences: A, wild-type VP cDNA and amino acid sequence; B, VP cDNA of the di allele and altered amino acid sequence from nucleotide 326, the position of the di deletion; C, VP cDNA of the di allele with a GA deletion at positions 365–369, showing an incorrect reading frame from nucleotides 326 to 369 and the predicted precursor structure; D, VP cDNA of the di allele with a GA deletion at positions 393–397 causing an incorrect reading frame from nucleotides 326 to 397 and the predicted precursor structure; E, wild-type VP cDNA translated into the +1 reading frame. The two GAGAG stretches are shown in boldface type. The amino acid sequences of the synthetic peptides to which the rabbit anti-rat antisera $VP363^{+1}$ and $VP474^{+1}$ are raised are underlined.

antisera is in agreement with the frequencies of mutations at the various positions in VP transcripts of di/di rats. As predicted from the cloned cDNAs (Table 1), there are more than 10 times as many neurons immunoreactive to antiserum $VP474^{+1}$ than to antiserum $VP363^{+1}$.

These data, showing that +1 frameshift mutations also occur in VP transcripts of wild-type rats, suggest that the events leading to the mutations are not caused by the diseased state of the di/di rat *per se*. Since in the di/di rat magnocellular VP neurons are chronically activated due to the lack of the antidiuretic hormone VP, we addressed the question whether the rate at which these mutations are generated is affected by the diseased state. The high metabolic activity of the magnocellular neurons of the di/di rat is reflected by an enlarged volume of the nucleoli, which can be normalized by VP substitution (14). Therefore, the number of GP-immunoreactive neurons was determined in di/di rats treated continuously with exogeneous VP via osmotic minipumps for 40 weeks. This treatment established a normal water intake and urine osmolality (Table 2). These chronically VP-substituted animals displayed a significant 25% decrease in the number of reverted neurons compared to nontreated di/di rats (Table 2), indicating that the mutation rate is affected by cellular activity.

DISCUSSION

The presently identified frameshift mutations in VP transcripts in magnocellular neurons explain the phenomenon of solitary neurons in the di/di rat and reveal mutational events with hitherto unknown mechanism that also exist in wild-type rats. Our experiments do not tell whether these mutations are introduced in the gene or in transcripts. However, a number of observations argue against a form of RNA "editing." (*i*) Frameshifted transcripts and reverted precursors are only found at high levels in solitary cells. (*ii*) The modifications lead to an irreversible phenotype as shown by the constant increase in number of solitary neurons with age (8). (*iii*) Solitary neurons display a heterozygous phenotype (8)—i.e., have both the di and frameshifted transcripts coexisting in

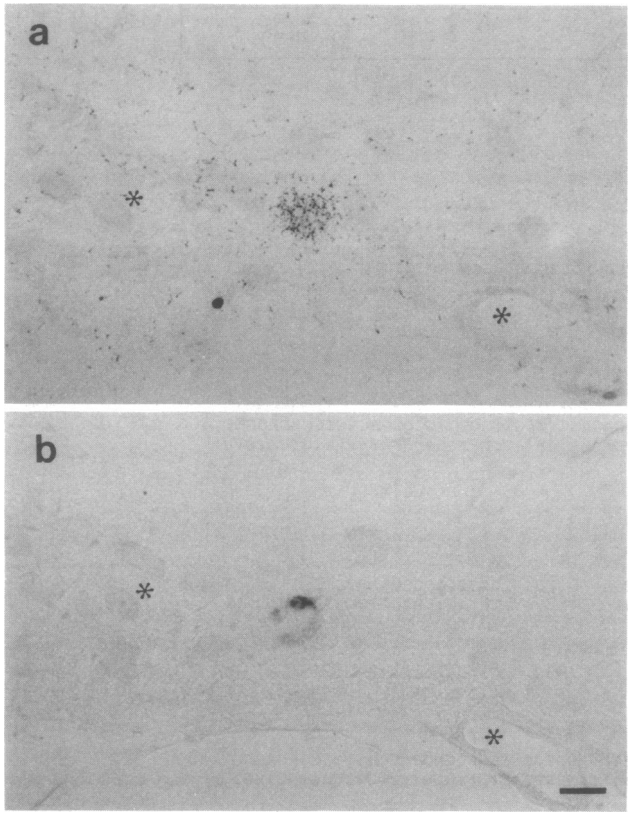

FIG. 2. VP transcripts containing the GA^{365} deletion detected by *in situ* hybridization (*a*) and reverted VP precursors stained with anti-GP antiserum (*b*) on serial hypothalamic sections. High levels of frameshifted VP transcripts and precursors are expressed in the same solitary neuron of the supraoptic nucleus of the di/di rat. Asterisks indicate the same capillaries within the supraoptic nucleus. (Bar = 25 μm.)

one cell. These data suggest the introduction of the deletion in one allele, thus involving DNA modification.

A possible implication of DNA modification is the existence of somatic instability of the genome in neuronal cells, resulting in mutagenesis operating at a relatively high frequency. As estimated from the 30-fold increase in number of GP-positive neurons in di/di rats during 2 years (8), the mutation frequency of the VP gene is on the order of 10^{-6} to 10^{-5} per base pair. As compared to the overall mutation rate in somatic cells (which is on the order of 10^{-10} to 10^{-8} per base pair), as determined by a number of *in vivo* and *in vitro* assays (15), the frequency of frameshift mutations at the VP locus is considerably higher.

The mechanism by which frameshift mutations occur in general is unknown. However, it has been suggested that a number of frameshifts are caused by misalignment of complementary or palindromic sequences or by slippage of DNA

Table 2. Number of GP-immunoreactive cells, water intake, and urine osmolality in di/di rats after 40 weeks of VP substitution

Rats	GP-positive neurons, no.	Water intake, ml per 100 g (body weight) per 24 hr	Urine osmolality, milliosmoles/kg
VP-substituted di/di (n = 6)	47.7 ± 2.5*	5.6 ± 0.8*	1456 ± 294
Control di/di (n = 4)	63.5 ± 3.5	59.8 ± 5.7	140 ± 18
+/+	—	6.9 ± 0.4	1931 ± 112

Data for +/+ rats are from ref. 14. All data are the mean ± SD.
*Statistical significance ($P < 0.05$; test of Wilcoxon) as compared to di/di rats.

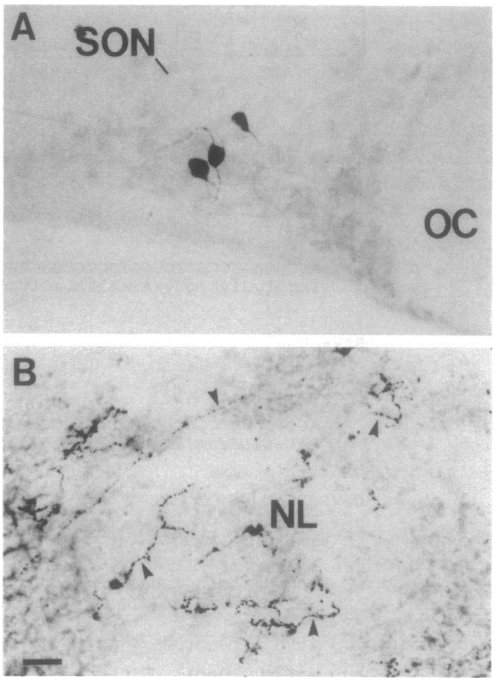

FIG. 3. (*A*) Immunocytochemical staining of solitary neurons within the supraoptic nucleus (SON) of a Wistar rat using rabbit anti-rat antiserum $VP474^{+1}$ (Fig. 1, sequence E). OC, optic chiasm. (*B*) These cells project toward the neural lobe (NL) where immunoreactive fibers are detected (arrowheads). (Bar = 50 μm.)

polymerase at monotonous runs of a base during DNA replication in meiosis and mitosis (16). Since neurons are postmitotic cells, processes related to regular DNA replication are not likely to be involved in the mechanism generating the frameshift mutations identified here. Alternatively, a role for gene conversion and recombination processes cannot be excluded. Indeed, somatic DNA recombination has been reported in transgenic mouse brain (17). In addition, the observed genetic instability of the VP locus may be a consequence of error-prone DNA repair processes. Although a DNA repair machinery in the brain has yet to be identified, the brain possesses a number of enzymes involved in DNA repair, including β-DNA polymerase (18, 19), which is the most error prone of the known mammalian DNA polymerases (20, 21). The consequence of defective repair of DNA damage in neurons is well illustrated by premature death of neurons in xeroderma pigmentosa patients (22). This might indicate that the nervous system is particularly sensitive to disrupted DNA repair mechanisms.

In this study we provide evidence for the generation of sequence-specific frameshift mutations in post-mitotic cells. In the VP neurons of the di/di rat, these mutations partly restore the wild-type phenotype. However, in wild-type rats it leads to disruption of normal functioning and should, therefore, be considered to be a deleterious mechanism. The finding that the mutational rate is enhanced in neurons in a permanent state of (hyper)activity suggests that the process of mutagenesis can be affected. To test the implications and underlying mechanism of these findings, it would be of importance to determine whether this somatic instability is intrinsic to the VP gene or the neurons that express it or whether it is a property of neurons in general. The finding that two GAGAG-containing regions are particularly prone to GA deletions may allow development of *in vivo* and *in vitro* models to gain a better understanding of the role and mechanism of similar or related events in processes underlying carcinogenesis, inherited pathology, and aging.

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