mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM Hepes, 4 mM adenosine triphosphate, and 0.3 mM guanosine triphosphate (pH 7.4). Cells were bathed in a solution of 138 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM Hepes, and 10 mM glucose (pH 7.4). Odor stimuli were delivered with the SF77 Fast Perfusion system (Warner Instruments), allowing precise concentrations to be applied for steps of various durations.

19. Five animals were infected with virus containing only the *lacZ* gene. In each animal the EOG electrode was positioned in several areas over the epithelium. After recording, the epithelia were reacted with X-gal and 18 electrode positions were determined to have been within areas of high infection. Responses to octanal at those positions were not different from those of uninfected animals.
20. T. Sato, J. Hirono, M. Tonioke, M. Takebayashi, *J. Neurophysiol.* **72**, 2980 (1994). K. Mori and Y. Yoshihara, *Prog. Neurobiol.* **45**, 585 (1995).
21. K. Raming *et al.*, *Nature* **361**, 353 (1993).
22. P. Sengupta, J. H. Chou, C. I. Bargmann, *Cell* **84**, 899 (1996); Y. Zhang, J. H. Chou, J. Bradley, C. I. Bargmann, K. Zinn, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12162 (1997).
23. C. Wellerdieck *et al.*, *Chem. Senses* **22**, 467 (1997).
24. J. Ostrowski, M. Kjelsberg, M. Caron, R. Lefkowitz, *Annu. Rev. Pharmacol. Toxicol.* **32**, 167 (1992).
25. J. Ngai, M. M. Dowling, L. Buck, R. Axel, A. Chess, *Cell* **72**, 657 (1993).
26. M. Hashimoto *et al.*, *Hum. Gene Ther.* **7**, 149 (1996).
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Frameshift Mutants of β Amyloid Precursor Protein and Ubiquitin-B in Alzheimer's and Down Patients

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The cerebral cortex of Alzheimer's and Down syndrome patients is characterized by the presence of protein deposits in neurofibrillary tangles, neuritic plaques, and neuropil threads. These structures were shown to contain forms of β amyloid precursor protein and ubiquitin-B that are aberrant (+1 proteins) in the carboxyl terminus. The +1 proteins were not found in young control patients, whereas the presence of ubiquitin-B^{+1} in elderly control patients may indicate early stages of neurodegeneration. The two species of +1 proteins displayed cellular colocalization, suggesting a common origin, operating at the transcriptional level or by posttranscriptional editing of RNA. This type of transcript mutation is likely an important factor in the widely occurring nonfamilial early- and late-onset forms of Alzheimer's disease.

In Alzheimer's disease (AD) and Down syndrome (DS) patients, intracellular and extracellular deposits of proteins in tangles, neuropil threads, and neuritic plaques are correlated with neuronal dysfunction leading to dementia (*1*). In particular, the familial types of AD have been investigated thoroughly and are due to mutations in genes located on chromosomes 1, 14, and 21, and the apolipoprotein E genotype (chromosome 19) is a risk factor (*2*). However, at least 60% of AD patients do not have a family history of the disease (*3*). For these frequently occurring, sporadic cases, a more general mechanism must exist, ultimately leading to neuronal degeneration.

Messenger RNA editing is a means of producing phenotypic variability (*4*). Moreover, we have identified another type of mutation in vasopressin transcripts (*5*). Homozygous Brattleboro rats have a single base deletion in the vasopressin gene, and newborn rats do not have a functional vasopressin mRNA and protein. Surprisingly, functional RNA and protein are found in a small but increasing proportion of hypothalamic cells as the animals age (*6*). This apparent reversion is due to a dinucleotide deletion (ΔGA) within GAGAG motifs of the mutant RNA (*5*). Thus, genetic information in neurons is not stable but subject to modification through an as yet unknown mechanism. We surmised that the opposite process may take place in other neuronal genes, resulting in mutant transcripts from wild-type genes, and so we looked for dinucleotide deletions in two genes associated with the pathogenesis of AD. The genes encoding β amyloid precursor protein (βAPP) and ubiquitin-B (Ubi-B) protein (*1*, *7*) each contain several GAGAG motifs.

In βAPP mRNA, seven GAGAG motifs are present in regions corresponding to exons 4, 6, 9, 10, and 14. Because three motifs are clustered in exons 9 and 10, this part of the transcript, encoding a putative growth-promoting domain (*8*), was selected for the detection of a +1 frameshift mutation resulting in truncated βAPP (βAPP^{+1}) with a novel COOH-terminus (Fig. 1A). In two of the three repeats of Ubi-B mRNA, a single GAGAG motif is present (Fig. 1A). The predicted +1 frameshift results in an aberrant COOH-terminus of Ubi-B of the first or second repeat (Ubi-B^{+1}). As a result, the glycine moiety essential for multiubiquitylation (*9*) would be lacking. To examine the occurrence of the predicted +1 proteins, we generated antibodies to the novel COOH-termini of βAPP^{+1} and Ubi-B^{+1} and used them to evaluate the presence of the abnormal proteins in tissue sections of cerebral cortex from AD, DS, and control patients by immunocytochemistry (*10*) and immunoblot analysis (*11*) and to assess reading frame mutations by selecting cDNA clones expressing +1 immunoreactivity.

Immunoreactivity for βAPP^{+1} and Ubi-B^{+1} was prominent in early- and late-onset AD cases and even more prominent in DS patients compared with controls matched for age, sex, postmortem delay, and duration of fixation (*12*) (Fig. 2 and Table 1). When the three brain areas studied were taken together, βAPP^{+1} immunoreactive structures were present in 71% and Ubi-B^{+1} immunoreactive structures in 100% of the AD patients (*12*). In young controls and one nondemented DS patient devoid of neuropathology in the frontal and temporal cortices and hippocampus, no Ubi-B^{+1} immunoreactivity was found (*12*). When Ubi-B^{+1} immunoreactivity was found in elderly, nondemented controls (>72 years), their neuropathological diagnosis revealed the presence of some plaques and tangles (*12*). Furthermore, no βAPP^{+1} and Ubi-B^{+1} immunoreactivities were found in the substan-

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tia nigra and striatum of 11 patients suffering from Parkinson's disease, except for the striatum and temporal cortex of one patient with AD neuropathology.

In the frontal and temporal cortices and the hippocampus of AD and DS patients, both βAPP^{+1} and Ubi-B^{+1} immunoreactivities were present in neurofibrillary tangles, neuropil threads, and dystrophic neurites (Fig. 2). In many cases, βAPP^{+1} and Ubi-B^{+1} immunoreactivities coexisted (Fig. 3, A and B), especially in neurons located in layers 2, 3, and 5 (Fig. 2, A and D). The βAPP^{+1} or Ubi-B^{+1} immunoreactive tangles and neuropil threads formed a considerable subpopulation of classical Bodian silver and Alz-50– or MC-1–stained neuropathological structures (Fig. 3, C to F). The presence of βAPP^{+1} and Ubi-B^{+1} in a subpopulation of Alz-50 or MC-1 immunoreactive neurons excludes the possibility that they cross-react with hyperphosphorylated tau protein. In a subpopulation of the wild-type βAPP and Ubi-B immunoreactive neurons, accumulation of βAPP^{+1} and Ubi-B^{+1} immunoreactivities was found in neurofibrillary tangles. βAPP^{+1} immunoreactivity was often found in restricted areas of the sections, whereas Ubi-B^{+1} immunoreactivity was distributed much more widely throughout the section and was present in a higher percentage of AD patients. In the hippocampus, intense βAPP^{+1} and Ubi-B^{+1} immunoreactivities were prominent in neurofibrillary tangles present in pyramidal cells of CA1 (Fig. 2H) and the subiculum, whereas, in the more intensely stained cases (*12*), immunoreactivity was frequently seen as cytoplasmic staining in CA4, CA3, CA2, and the hilus. In the entorhinal cortex tangle, staining was often observed in the pre-α layers (*13*) and concentrated in cellular islands.

To characterize the immunoreactive products detected in immunocytochemistry by molecular size, we used immunoblots of homogenates of the temporal cortex of AD and DS patients to reveal the presence of immunoreactive proteins with sizes [38 kD for βAPP^{+1} (Fig. 1C) and 11 kD for Ubi-B^{+1} (*14*, *15*)] predicted by the open reading frames of transcripts with a dinucleotide deletion. In young, nondemented controls, these +1 protein bands were absent. The eukaryotically (βAPP^{+1}) or bacterially expressed (Ubi-B^{+1}) recombinant proteins corresponding to the +1 mutant proteins served as positive controls (*14*, *15*). Antibodies to wild-type sequences of βAPP (Fig. 1C) and Ubi-B, located in the unaffected region near the COOH-terminus with the +1 reading frameshift, revealed bands of the same size.

Preimmune and solid-phase adsorbed antisera showed no reaction in paraffin sections or immunoblots. In contrast to the

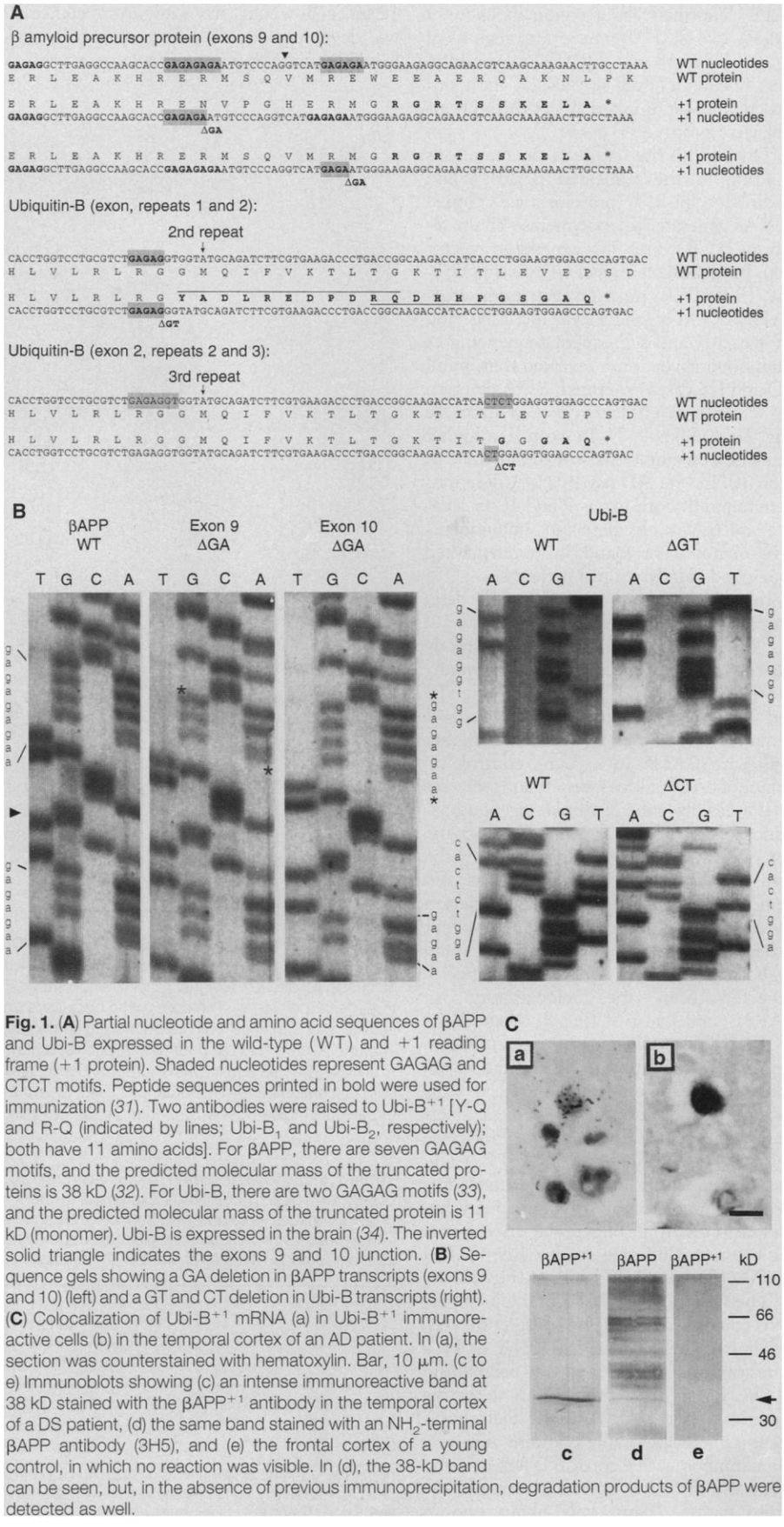

Fig. 1. (**A**) Partial nucleotide and amino acid sequences of βAPP and Ubi-B expressed in the wild-type (WT) and +1 reading frame (+1 protein). Shaded nucleotides represent GAGAG and CTCT motifs. Peptide sequences printed in bold were used for immunization (*31*). Two antibodies were raised to Ubi-B^{+1} [Y-Q and R-Q (indicated by lines; Ubi-B_1 and Ubi-B_2, respectively); both have 11 amino acids]. For βAPP, there are seven GAGAG motifs, and the predicted molecular mass of the truncated proteins is 38 kD (*32*). For Ubi-B, there are two GAGAG motifs (*33*), and the predicted molecular mass of the truncated protein is 11 kD (monomer). Ubi-B is expressed in the brain (*34*). The inverted solid triangle indicates the exons 9 and 10 junction. (**B**) Sequence gels showing a GA deletion in βAPP transcripts (exons 9 and 10) (left) and a GT and CT deletion in Ubi-B transcripts (right). (**C**) Colocalization of Ubi-B^{+1} mRNA (a) in Ubi-B^{+1} immunoreactive cells (b) in the temporal cortex of an AD patient. In (a), the section was counterstained with hematoxylin. Bar, 10 μm. (c to e) Immunoblots showing (c) an intense immunoreactive band at 38 kD stained with the βAPP^{+1} antibody in the temporal cortex of a DS patient, (d) the same band stained with an NH_2-terminal βAPP antibody (3H5), and (e) the frontal cortex of a young control, in which no reaction was visible. In (d), the 38-kD band can be seen, but, in the absence of previous immunoprecipitation, degradation products of βAPP were detected as well.

βAPP^{+1} antibody to a region in exons 9 and 10, two βAPP^{+1} antisera to regions of exons 14 and 18 and a βAPP^{+2} antiserum (part of exon 17) did not display immunocytochemical staining in any of the sections that were positive for βAPP^{+1}.

To establish the nature of the mutations resulting in the truncated βAPP^{+1} and modified Ubi-B^{+1} proteins, we cloned cDNAs generated by polymerase chain reaction (PCR) into an expression vector (*16*). Immunoscreening of reverse transcriptase (RT)–PCR products generated from βAPP cDNAs of young and elderly AD and DS patients and subsequent sequencing of immunopositive clones revealed that, in all AD and DS cases examined, a GA deletion was present in either exon 9 or exon 10 (Fig. 1B and Table 2). In exon 9, a higher frequency of mutations was found than in exon 10. In one AD patient, GA deletions were found in both exons 9 and 10 in separate mRNA molecules. No immunopositive clones were found in nondemented young and elderly control patients.

In addition, another dinucleotide deletion (ΔGT) was found in the first repeat of Ubi-B. This mutation is located directly adjacent to the GAGAG motif in young and elderly AD and DS patients (Fig. 1B and Table 2). The frequency of Ubi-B^{+1} immunopositive clones was much higher than with βAPP. In a young control, no immunopositive clones were found, whereas in the elderly control displaying neuropathology and Ubi-B^{+1} immunoreactivity, not only was a GT deletion found in the first repeat, but a CT deletion was also found in the third repeat. A CT deletion was also found in a DS patient. In four patients, dinucleotide deletions were found in both βAPP and Ubi-B transcripts as expected from the colocalization of βAPP^{+1} and Ubi-B^{+1} (Fig. 3, A and B).

To exclude PCR and cloning artifacts as a possible explanation for the mutation, we confirmed the presence of mutated RNA by in situ hybridization (*17*). PCR artifacts were also excluded by genomic PCR (see below). The GA deletion in βAPP^{+1} and the GT deletion in Ubi-B^{+1} transcripts were both detected by stringent in situ hybridization with an oligoprobe discriminating between wild-type and mutant (ΔGA) βAPP (*17*) and (ΔGT) Ubi-B (Fig. 1C).

The mutations in βAPP and Ubi-B proteins could be caused at two different levels, either by a deletion in the DNA or by a transcriptional defect or editing mechanism in the RNA. The problem in detecting mutations in the DNA is the fact that a tissue sample inevitably contains not only the immunoreactive cortical layers 2, 3, and 5 but also the other layers and a part of the white matter. Thus, only about one in 10,000 cells would carry a mutation. Hence, we developed two PCR protocols that would be sufficiently sensitive to detect this frequency of mutation (*18*).

With the assumption that the human haploid genome contains 3×10^9 base pairs

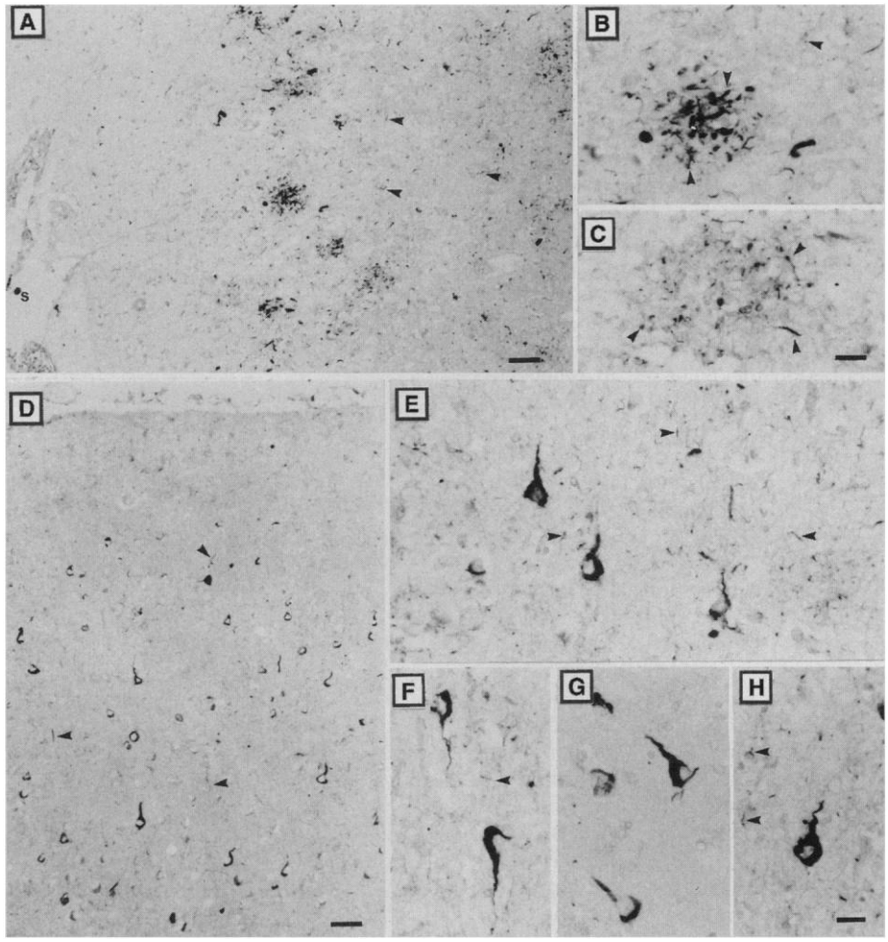

Fig. 2. Neuritic plaques. (**A** and **B**) βAPP^{+1} and (**C**) Ubi-B^{+1} immunoreactivities in the frontal cortex of an AD patient are present in neuritic plaques. In (A), neuropil threads can be seen (arrowheads); s, sulcus. In (B) and (C), a higher magnification shows immunoreactivity in dystrophic neurites and neuropil threads (arrowheads). The core of the plaques is unstained as seen in (B) and (C). Ubi-B^{+1} immunoreactivity was obtained with two different antibodies (that is, Ubi-B$_1$ and Ubi-B$_2$). Ubi-B$_2$ was most immunoreactive and used for all immunohistochemistry. (**D** to **H**) Neurofibrillary tangles. Ubi-B^{+1} (D to F) and βAPP^{+1} (G and H) immunoreactivities in tangle-shaped structures in the temporal (D) and frontal (E to G) cortex and the hippocampus (CA1 area) (H) of two different AD patients (D and E to H). Neuropil threads are indicated with arrowheads. Bar in (A) and (D), 50 μm; bar in (B) and (C) and (E) to (H), 20 μm.

Table 1. Immunoreactivities in the human frontal and temporal cortices and hippocampus for βAPP and Ubi-B, for which the mRNA is expressed in the +1 reading frame (resulting in βAPP^{+1} and Ubi-B^{+1} protein). Tissues were obtained from controls and neuropathologically confirmed AD and DS cases (*12*). Immunoreactivity present in tangles, dystrophic neurites, and neuritic plaques of patients is expressed as a percentage of the total number of patients studied.

Disease	Frontal cortex (area 11)		Temporal cortex (area 38)		Hippocampus	
	βAPP^{+1}	Ubi-B^{+1}	βAPP^{+1}	Ubi-B^{+1}	βAPP^{+1}	Ubi-B^{+1}
Nondemented controls* (n = 12)	0	0	0	8†	0	50†
AD‡ (n = 21)	19	80	43	95	50	95
DS (n = 7)	86	86	86	86	71	86

*Young (n = 6) and elderly (n = 6) nondemented controls. Controls were matched for sex, age, and postmortem delay. †In elderly, nondemented patients with age-related neuropathology (tangles and plaques). ‡Early- (<65 years, n = 10) and late- (>65 years, n = 11) onset AD.

(bp) for the RT-PCR (*19*) and the genomic PCR for βAPP, an equivalent of 50,000 cells (that is, 0.5 μg of total RNA and 300 ng of genomic DNA, respectively) was assayed. Given the estimate of one immunopositive cell out of 10,000, we should have found about five out of 50,000 positive clones. In the immunoscreening assay after the RT-PCR of the RNAs, we found a minimum of 2 and a maximum of 12 positive clones, resulting in a mean of 6 (Table 2). However, we were not successful in finding immunopositive clones after the genomic DNA PCR in two AD and two DS patients. A total of 400,000 clones were negative with immunoscreening, which also excluded PCR artifacts in the cDNA screening. A similar result was obtained with the RT-PCR and genomic PCR for Ubi-B of one of the DS patients: 100,000 clones were negative with immunoscreening. A comparable amount of RNA and genomic DNA resulted in 2 to 138 positive clones (Table 2) after RT-PCR compared with none after genomic PCR. In a best case estimate of the variables of the genomic PCR-immunoscreening approach, if screening 100,000 colonies we should have found five positives [for both βAPP and Ubi-B, on the basis of the assumption of a heterozygous genotype (6)]. Because we found no positive clones and in a worst case estimate this method would not be sufficiently sensitive, we developed a more sensitive method.

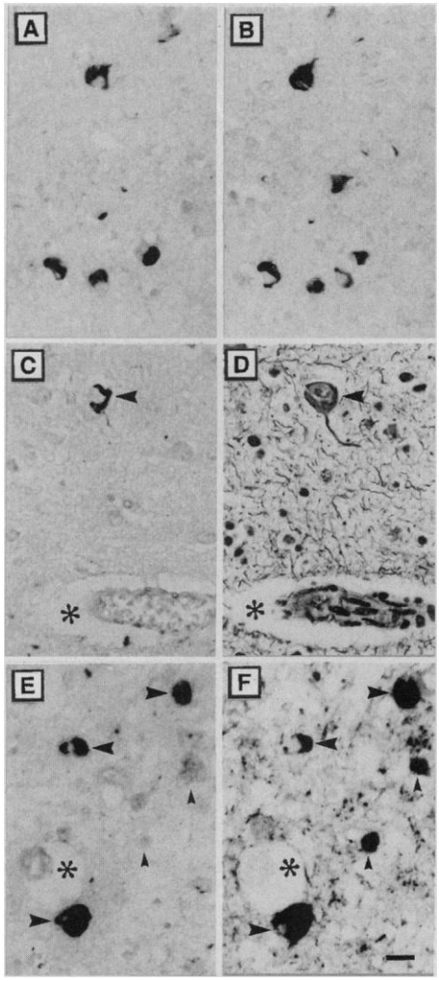

Fig. 3. Colocalization of (**A**, **C**, and **E**) βAPP^{+1} and (**B**) Ubi-B^{+1} immunoreactivities, with (**D**) Bodian- and (**F**) Alz-50–stained cell bodies (large arrowheads) in consecutive sections of the frontal cortex (A to D) and subiculum (E and F) of an AD patient. Alz-50 (F) stains neurons and neuropil threads more abundantly than βAPP^{+1} (E). Small arrowheads, Alz-50–positive neurons and βAPP^{+1}–negative neurons; *, capillary. Bar, 20 μm.

A direct discrimination between mutant and wild-type DNA was based on the use of primers specific for the mutation. Using 5′ oligonucleotides ending in the expected deletions and 3′ oligonucleotides, which hybridize 100 and 674 bp downstream of the βAPP and Ubi-B mutations, respectively, we reached a detection level of 10 copies of mutant cDNA mixed into 500 ng of genomic DNA (80,000 cells expressing βAPP or Ubi-B). Under the same sensitive conditions, that is, one copy of the mutant DNA in 8000 cells, genomic PCR was performed on human brain DNA derived from an AD, a DS, and a control patient.

These experiments repeatedly failed to detect any amplification products for either βAPP^{+1} or Ubi-B^{+1}. We repeated the PCR 10 times, checking at least 5 μg of genomic DNA from each patient (that is, 1.6×10^6 copies of βAPP and Ubi-B), but none of the PCRs showed a specific amplification product for the mutation. A PCR with oligonucleotides hybridizing to the wild-type sequences of βAPP and Ubi-B gave the expected products. Although it is difficult to base conclusions on a negative result, the mixing control experiments show that there is only a very small chance that a positive DNA amplification product was not detected. Thus, it is likely that frameshift mutations introduced in the transcripts and not in the DNA are responsible for the +1 proteins observed by immunocytochemistry.

Here, in the cerebral cortex of AD, DS, and control patients, two novel βAPP- and Ubi-B–derived proteins generated by mutations of βAPP and Ubi-B transcripts were detected. The mutations were in all instances a dinucleotide deletion (ΔGA or ΔGT) occurring preferentially in or adjacent to GAGAG motifs. Because we also found a CT deletion in a CTCT motif of Ubi-B transcripts, it seems that other dinucleotide deletions in simple dinucleotide repeats occur as well. The much higher frequency of βAPP^{+1} and Ubi-B^{+1} proteins in AD patients compared with their age- and sex-matched controls indicates that transcript mutation is a critical factor for initiating neuropathology in nonfamilial forms with early- and late-onset AD.

The absence of these +1 proteins in patients with Parkinson's disease, except for one patient who also showed AD neuropathology, suggests that these +1 proteins correlate strongly with AD. Furthermore, the +1 proteins occurred in areas known to be severely affected in AD [for example, CA1 and the subiculum in the hippocampus (*20*)]. The DS patients revealed intense βAPP^{+1} and Ubi-B^{+1} immunoreactivities,

Table 2. Immunoscreening and sequencing of cDNA of βAPP and Ubi-B for dinucleotide deletions in the cortex and hippocampus of AD and DS patients and nondemented control patients.

Disease	Total number of clones	Number of positive clones	Dinucleotide deletion
		βAPP^{+1}	
Control 1*	20,000	0	–
Control 2†	20,000	0	–
AD‡	20,000	10	ΔGA (exon 9)
AD 1‡§	20,000	2	ΔGA (exon 10)
AD 2§,‖	20,000	5	ΔGA (exons 9 or 10)
AD 3§,‖	20,000	5	ΔGA (exon 9)
DS 1§	20,000	2	ΔGA (exon 9)
DS 2§	20,000	12	ΔGA (exon 9)
		Ubi-B^{+1}	
Control 1*	20,000	0	–
Control 2†	20,000	13	ΔGT or ΔCT
AD‡	5,000	15	ΔGT
AD 1‡§	1,500	138	ΔGT
AD 2§‖	2,000	32	ΔGT
DS 1§	800	44	ΔGT
DS	1,000	2	ΔGT or ΔCT
DS 2§	20,000	93	ΔGT

*Young, nondemented control patient. †Elderly, nondemented control patient with age-related neuropathology. ‡Early-onset AD (<65 years). §Patients with a dinucleotide deletion in both βAPP and Ubi-B transcripts. ‖Late-onset AD (>65 years).

except for one DS patient, who did not display any neurodegeneration in the three areas studied and did not suffer from dementia (*12*). Consistent with the idea that the transentorhinal cortex is an early target for neuropathological changes in AD (*13*), in the nondemented DS patient, βAPP^{+1}, Ubi-B $^{+1}$, and Alz-50 immunoreactivities coexisted in Bodian-stained tangles in cellular islands of the pre-α layers. The age-related presence of Ubi-B^{+1} immunoreactivity in the hippocampus of nondemented controls indicates that the Ubi-B^{+1} peptide may contribute to initial stages of neurodegeneration in AD. The Ubi-B^{+1} protein may therefore be a valuable diagnostic tool for the early detection of AD.

Because enhanced transcriptional activity may be correlated with the presence of +1 proteins (*5*), it is possible that a transcript mutation resulting in βAPP^{+1} and Ubi-B^{+1} proteins occurred. This idea is best illustrated in DS, in which βAPP gene expression is much higher than expected on trisomy 21 alone (*21–23*). On the other hand, the lower frequency of βAPP^{+1} protein in AD patients (Table 1) is consistent with the fact that their βAPP transcript levels are not essentially increased (*24*, *25*). The high frequency of Ubi-B^{+1} protein in AD and DS is in accordance with overexpression of the Ubi-B gene (*7*, *26*).

The coexisting βAPP^{+1} and Ubi-B^{+1} proteins, as well as other +1 proteins, may impair neuronal functioning and amplify or induce neuropathology in an as yet unknown manner. For instance, the Ubi-B^{+1} molecules may be responsible for the lack of multiubiquitylation of the hyperphosphorylated tau-rich neurofibrillary tangles that accumulate during the long period of neurodegeneration (*9*). One explanation for these findings is that the Ubi-B^{+1} molecules are unable to bind to lysine residues in target molecules, because they lack the COOH-terminal glycine residue in the first repeat, which is essential for subsequent multiubiquitylation and activation of the proteasomal machinery (*27*). This process does not seem to occur efficiently in cells with tangles in AD. No Ubi-B–associated COOH-terminal hydrolase and 26*S* proteasomal immunoreactivity have been found in compact tangles (*28*, *29*).

This study shows that βAPP and Ubi-B transcripts can be modified by dinucleotide deletions (ΔGA, ΔGT, or ΔCT). The GA deletion is similar to the one reported in vasopressin transcripts of the homozygous Brattleboro rat (*5*). The frequently mutated motif in exon 9 of the βAPP gene transcript is in fact an extended version of GAGAG (that is, GAGAGAGA) (Fig. 1). We also addressed the issue of whether the dinucleotide deletions occur at the transcript or the genomic level. Although we used two different sensitive approaches to reveal a genomic mutation, we failed to find any indication of a mutation at the genomic level. Support for the possibility of a general process of transcript mutation was provided by the present results showing that βAPP^{+1} and Ubi-B^{+1} proteins are coexpressed within the same neurons. In addition, in individual AD and DS patients, two or three different dinucleotide deletions were found in two different transcripts, which makes a genomic event unlikely. We thus tentatively conclude that these modifications may take place during or after transcription. In view of the finding that frameshift mutations occur in multiple proteins within the same neuron, we postulate that a common denominator in the transcription-propagating events is involved. The mechanism of transcript mutation (ΔGA, ΔGT, or ΔCT) is, however, unclear. It is most probably not restricted to postmitotic cells, because we were able to show that an ectopically expressed rat vasopressin transgene undergoes a similar process in dividing cells (*30*).

Transcript mutation may thus be a widely occurring phenomenon. In principle, each transcript containing a susceptible motif, such as GAGAG, could undergo such a process. However, postmitotic neurons are less capable of compensating for transcript-modifying activity and are thus particularly sensitive to the accumulation of frameshifted proteins. Accumulation of +1 proteins together with the consequent lack of functional proteins is probably critical for cellular functioning. Thus, during aging, single neurons may generate and accumulate abnormal proteins, consequently leading to cellular disturbances and causing degeneration. The mechanism of dinucleotide deletion at the transcript level may well underlie a number of neurodegenerative pathologies.

REFERENCES AND NOTES

1. R. D. Terry, E. Masliah, L. A. Hansen, in *Alzheimer Disease*, R. D. Terry, R. Katzman, K. L. Bick, Eds. (Raven, New York, 1994), pp. 179–196.
2. J. Hardy, *Trends Neurosci.* **20**, 154 (1997).
3. C. L. van Broeckhoven, *Eur. Neurol.* **35**, 8 (1995); R. Tanzi *et al.*, *Neurobiol. Dis.* **3**, 159 (1996).
4. B. Sommer, M. Köhler, R. Sprengel, P. H. Seeburg, *Cell* **67**, 11 (1991); L. Simpson and R. B. Emeson, *Annu. Rev. Neurosci.* **19**, 27 (1996).
5. D. A. P. Evans, A. A. M. van der Kleij, M. A. F. Sonnemans, J. P. H. Burbach, F. W. van Leeuwen, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6059 (1994).
6. F. van Leeuwen, E. van der Beek, M. Seger, P. Burbach, R. Ivell, *ibid.* **86**, 6417 (1989).
7. G. Perry *et al.*, *J. Neurochem.* **52**, 1523 (1989).
8. L. W. Jin *et al.*, *J. Neurosci.* **14**, 5461 (1994).
9. M. Morishima-Kawashima *et al.*, *Neuron* **10**, 1151 (1993).
10. Paraffin-embedded and frozen brain material of the cerebral cortex, that is, the orbitofrontal cortex (area 11), temporal pole (area 38), and hippocampus, from nondemented control (n = 12), AD (n = 21), and DS (n = 7) patients was obtained from the Netherlands Brain Bank (coordinator R. Ravid). Informed consent was obtained for the use of autopsy material for research. All brains were neuropathologically investigated (*12*). The cases were matched for age, sex, postmortem delay, and fixation duration. The Brain Bank also supplied the substantia nigra and striatum of 11 Parkinson patients, of whom only one displayed AD neuropathology as well (male, 72 years). The +1 peptides (Fig. 1A) were coupled with glutaraldehyde to thyroglobulin, mixed in a 1:1 ratio with complete Freunds adjuvant, and injected once both intramuscularly and subcutaneously. No homology was found for these peptide sequences in the European Molecular Biology Laboratory database. The affinities were evaluated on nitrocellulose paper with antigen spots and on tissue sections. Paraffin sections (6 μm) were deparaffinized, treated in 100% formic acid for 30 min, and incubated overnight at 4°C with rabbit antibodies raised to βAPP^{+1} and Ubi-B^{+1} (Fig. 1). Antibodies recognizing the more NH_2-terminal parts of wild-type sequences were used as well. These antibodies include Ubi-B 3-39 (*7*), recognizing residues 50 to 65, and βAPP 22C11, recognizing residues 66 to 81 [C. Hilbich, U. Mönning, C. Grund, C. L. Masters, K. Beyreuther, *J. Biol. Chem.* **268**, 26571 (1993)]. Furthermore, +1 reading frame antisera to parts of exon 14 (RSPAVAFFWG) (*31*) and exon 18 (HSSWCGGG) of βAPP were raised, and a +2 reading frame antiserum to exon 17 (LDSWWAVLG) was raised. The βAPP^{+1} and Ubi-B^{+1} antibodies were diluted 1:250 and 1:400, respectively. The peroxidase-antiperoxidase method was followed (*8*). In some patients, the βAPP^{+1} and Ubi-B^{+1} immunolocalizations were matched with Bodian silver, Alz-50, and MC-1 staining [G. A. Jicha, R. Bowser, I. G. Kazam, P. Davies, *J. Neurosci. Res.* **48**, 128 (1997)]. When sections were incubated with preimmune serum and with antiserum absorbed with the specific antigen, no staining was seen.
11. A small piece (~0.1 g) of frozen temporal lobe, frontal lobe, or hippocampus was homogenized in 0.5 ml of 100% formic acid. The proteins were extracted from the dried samples by boiling for 10 min in 200 μl of 10% SDS. The extract was loaded on a 12.5% SDS-polyacrylamide gel, transferred onto nitrocellulose, and, after an overnight fixation in 5% formaldehyde, probed with rabbit antisera to βAPP^{+1} and Ubi-B^{+1} (Fig. 1). Additionally, antisera were used that recognized residues 229 to 252 of the NH_2-terminus of βAPP {3H5 [B. Philippe, J.-P. Brion, A.-F. Macq, J.-N. Octave, *Neuroreport* **5**, 289 (1993)]} and residues 50 to 65 of Ubi-B [3 to 39 (*7*)].
12. For clinical and pathological information on control and AD patients, see www.nih.knaw.nl
13. H. Braak *et al.*, *J. Neural Transm.* **103**, 455 (1996).
14. F. W. van Leeuwen *et al.*, data not shown.
15. As a positive control, wild-type βAPP^{+1} and Ubi-B^{+1} proteins were expressed in stably transfected AtT20 cells and in bacteria [M15 (pREP4)], respectively. AtT20 cells were stably transfected with βAPP^{+1} pcDNA3 by electroporation at 260 V and 170 μF. Resistant clones were expanded and assayed for expression of βAPP^{+1} after the formic acid treatment.
16. Pieces of frozen human frontal and temporal cortex or hippocampus (CA1 and the subiculum) (~0.1 g) were homogenized in 1 ml of Trizol (Gibco-BRL), and total RNA was isolated according to the manufacturer's instructions. Total RNA (2 μg) was primed with random hexamers and reverse transcribed with Expand RT. With the Expand High Fidelity PCR system (Boehringer Mannheim), βAPP cDNA (fragments 897 to 1405 of Y00264: 5′ primer GCGGGATCCTGGTGATGAGGTAGAGGAAGAGGCT and 3′ primer CTTACATACAAGCTTAGCATATTGAACACGTGACGAGGCC) and Ubi-B cDNA were amplified (fragments 1094 to 1765 of X04803: 5′ primer GGGGATCCGGTCAAAATGCAGATCTTCGTGAAA and 3′ primer AGAAGAAGCTTTTAACAGCCACCCCTCAGG). The cycle temperature and timing were as follows: for βAPP^{+1}, 92°C, 30 s; 62°C, 30 s; and 72°C, 40 s (30 cycles); and for Ubi-B^{+1}, 92°C, 30 s; 64°C, 30 s; and 72°C, 40 s (40 cycles). For both PCRs in the last cycle, the extension was carried out for 7 min. The amplification products were digested with Bam HI and Hind III and cloned in frame in the expression vector pQE-31. Bacterial clones were

screened for mutant proteins with βAPP^{+1} and Ubi-B^{+1} antibodies (5). Immunopositive clones were isolated, and their inserts were sequenced with Sequenase version 2.0 (USB, Cleveland, OH), with ^{35}S-labeled deoxyadenosine triphosphate (dATP).

17. Paraffin sections (6 μm) were deparaffinized, rehydrated, and deproteinated for 20 min with 0.2 M HCl followed by a 15-min treatment with proteinase K. The sections were again dehydrated, delipidated by a 5-min treatment with chloroform, rehydrated, and incubated for 20 min in 0.1 M phosphate-buffered saline and 0.1% v/v Triton X-100. Sections were prehybridized with hybridization buffer [30% formamide, 0.1 M NaCl, 5× Denhardt's solution, 10 mM tris-HCl (pH 7.5), 1 mM EDTA, 10% dextran sulphate, and 50 mM dithiothreitol (DTT)] containing herring sperm DNA (100 μg/ml) and tRNA (500 μg/ml) for 1 hour at room temperature. To detect the βAPP mutant mRNA, we labeled a 20-nucleotide oligomer complementary to the βAPP mRNA containing the GA deletion in exon 9 at position 1130 (5′-GGGACATTCTCTCGGTGCTT-3′, corresponding to nucleotides 1123 to 1144) at the 3′ end using terminal deoxynucleotidyl transferase (Boehringer Mannheim) and ^{35}S-labeled dATP (NEN/Dupont, Boston, MA) to provide an average tail length of five nucleotides (*14*). To detect the Ubi-B mutant mRNA, we similarly labeled a 20-nucleotide oligomer complementary to the Ubi-B mRNA containing the GT deletion at position 1317 (5′-CGCAGACTCTC-CCATACGTC-3′, corresponding to nucleotides 1307 to 1327). The labeled probes were applied to the sections at a concentration of 10^7 counts per milliliter hybridization buffer [βAPP^{+1}: 30% formamide, 0.0125 M NaCl, 5× Denhardt's solution, 10 mM tris-HCl (pH 7.5), 1 mM EDTA, 10% dextran sulfate, 50 mM DTT; and Ubi-B^{+1}: 30% formamide, 0.025 M NaCl, 5× Denhardt's solution, 10 mM tris-HCl (pH 7.5), 1 mM EDTA, 10% dextran sulphate, and 50 mM DTT]. The sections were incubated under stringent conditions overnight in a humid chamber: βAPP^{+1} at 38.5°C (hybridization at T_m + 15°C) and Ubi-B^{+1} at 40.5°C (hybridization at T_m + 10°C) (T_m is the temperature at which 50% of double-stranded DNA or DNA-RNA hybrids are denatured). Slides were washed once at 55°C in 1× standard saline citrate (SSC) followed by two washings at 55°C in 0.3× SSC. Sections were dehydrated in graded ethanols containing 300 mM ammonium acetate and after drying dipped in NTB-2 photoemulsion (Kodak). After 2 weeks, the sections were developed, counterstained with hematoxylin, dehydrated, and mounted.

18. To check if the dinucleotide deletions in βAPP and Ubi-B occur in the genome or exclusively during transcription, we pursued two strategies: (i) genomic PCR with intron primers followed by immunoscreening and (ii) genomic PCR with a 5′ primer that hybridizes only to the complementary DNA template with the mutation and thus can discriminate between the wild-type gene and the mutated gene. In the first strategy, genomic DNA was isolated from frozen human brain tissue and amplified with the Expand High Fidelity System (Boehringer Mannheim) with PCR primers recognizing intron sequences adjacent to exons 9 and 10 of βAPP (5′ primer, exon 9, 5′-GCGGATCCCCTCCTTCTCTTCTACTTTATAG-3′, and 3′ primer, exon 9, 5′-AGGGGAGCTCGATG-GAAGAGCCAGACTTAC-3′; 5′ primer, exon 10, 5′-AAATTCAAGAGCTCCCTTTATTTATTGGTC-3′, and 3′ primer, exon 10, 5′-TGTTAAGCTTGAAAT-GGGTTCAGGTTTTAC-3′). The PCR products were cloned in frame in the Bam HI and Sac I sites (exon 9) or in the Sac I and Hind III sites (exon 10) of the expression vector pQE-31. Ubi-B was amplified with a 5′ primer recognizing the intron sequence adjacent to the start codon (5′-GGGGATCCCGCT-TATGTTTTACTTTTAAACAAG-3′) and a 3′ primer recognizing the exon sequence (5′-AGAA-GAAGCTTTTAACAGCCACCCCTCAGG-3′, corresponding to nucleotides 1765 to 1783). The cycle temperature and timing were as follows: for βAPP^{+1} (exon 9), 92°C, 30 s; 50°C, 30 s; and 72°C, 40 s (40 cycles); and for Ubi-B^{+1}, 92°C, 30 s; 55°C, 30 s; and 72°C, 40 s (40 cycles). For both PCRs in the last cycle, the extension was carried out for 7 min. The Ubi-B fragment was digested with Bam HI and Hind III and cloned in frame in pQE-31 expression vector. Bacterial clones were screened for the production of recombinant proteins as described above. With this method, we were able to detect 10 copies of plasmid DNA containing the dinucleotide deletion of βAPP in a background of 100,000 copies of wild-type βAPP DNA. In the second strategy, genomic DNA isolated from the frontal cortex of frozen brain material from AD, DS, and control patients was amplified with a 20-nucleotide oligomer complementary to the βAPP gene containing a GA deletion in exon 9 at position 1135 (5′ primer, 5′-AGGCCAAGCACCGAGAGAAT-3′, corresponding to nucleotides 1110 to 1138) and an intron primer (3′ primer, 5′-CTGTGGGGAGACT-GAGGCAG-3′) hybridizing to the exon 9 adjacent intron sequence. For the mutant Ubi-B gene with a GT deletion in the first repeat, we chose an appropriate primer (5′ primer, 5′-GGTCCTGCGTCTG-AGAGGGT-3′, corresponding to nucleotides 1300 to 1321) and a 3′ primer hybridizing downstream to the polyadenylate signal (3′ primer, 5′-GCGTACCC-ACACATCACTAG-3′, corresponding to nucleotides 1973 to 1954). Genomic DNA (0.5 μg) was amplified by PCR with Goldstar DNA Polymerase (Eurogentec, Seraing, Belgium) in 0.3 M tris-HCl (pH 9.5), 10 mM deoxynucleotide triphosphate, 35 pmol of each primer, 7.5 mM $MgCl_2$, and 75 mM ammonium sulfate. The cycle temperature and timing were as follows: 93°C, 1 min and 56°C, 1.5 min (35 cycles). In the last cycle, the extension was carried out for 10 min. PCR products were checked on a 2% agarose gel {for details, see S. Glisic and D. Alavantic [*Trends Genet.* **12**, 391 (1996)]}.

19. T. Strachan and A. P. Read, *Human Molecular Genetics* (Bios Scientific, Oxford, UK, 1997).
20. M. J. West, P. D. Coleman, D. G. Flood, J. C. Troncoso, *Lancet* **344**, 769 (1994).
21. R. L. Neve, E. A. Finch, L. R. Dawes, *Neuron* **1**, 669 (1988).
22. B. Rumble *et al.*, *N. Engl. J. Med.* **320**, 1446 (1989).
23. F. Oyama *et al.*, *J. Neurochem.* **62**, 1062 (1994).
24. P. J. Harrison, W. H. Wighton-Benn, J. M. Heffernan, M. W. Sanders, R. C. A. Pearson, *Neurodegeneration* **5**, 409 (1996).
25. J. A. Johnston *et al.*, *Mol. Brain Res.* **43**, 85 (1996).
26. Z. Ali-Khan, J. Normand, K. Alizadeh-Khiavi, Y. Robitaille, S. Chronopoulos, *Neurosci. Lett.* **139**, 24 (1992).
27. W. Hilt and D. H. Wolf, *Trends Biochem. Sci.* **21**, 96 (1996).
28. J. Lowe, H. McDermott, M. Landon, R. J. Mayer, K. D. Wilkinson, *J. Pathol.* **161**, 153 (1990).
29. S. Kwak, T. Masaki, S. Ishiura, H. Sugita, *Neurosci. Lett.* **128**, 21 (1991).
30. F. W. van Leeuwen., J. P. H. Burbach, D. Murphy, unpublished results.
31. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
32. S. Yoshikai, H. Sasaki, K. Dohura, M. Furuya, Y. Sakaki, *Gene* **87**, 257 (1990).
33. R. T. Baker and P. G. Board, *Nucleic Acids Res.* **15**, 443 (1987).
34. M. D. Adams *et al.*, *Nature* **355**, 632 (1992).
35. We thank J. Verhaagen, D. F. Swaab, C. W. Pool, J. J. van Heerikhuize, T. P. van der Woude, J. Wortel, A. Holtrop, J. Wouda, M. Kooreman, O. Pach, and G. van der Meulen for their help and R. T. Baker, I. Grundke-Iqbal, P. Davies, T. Hartmann, and J. M. Octave for generously providing us with plasmid antisera and advice. Supported by Het Stimuleringsfonds Nederlandse Organisatie voor Wetenschappelijk Onderzoek-prioriteitsorogramma Geheugen processenen Dementie, an Koninklijke Nederlandse Akademie van Wetenschappen grant, the Matty Brand Foundation, and the Jan Dekker and Ludgardine Bouwman Foundation.

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