Levels and Alternative Splicing of Amyloid β Protein Precursor (APP) Transcripts in Brains of APP Transgenic Mice and Humans with Alzheimer’s Disease*

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Abnormal expression of human amyloid precursor protein (hAPP) gene products may play a critical role in Alzheimer’s disease (AD). Recently, a transgenic model was established in which platelet-derived growth factor (PDGF) promoter-driven neuronal expression of an alternatively spliced hAPP minigene resulted in prominent AD-type neuropathology (Games, D., Adams, D., Alessandri, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., Guido, T., Haggopian, S., Jhun, F., Leibowitz, P., Lieberburg, I., Little, S., Masliah, E., McConlogue, L., Montoya-Zavala, M., Mucke, L., Paganini, L., and Penniman, E. (1995) Nature 373, 523-527). Here we compared the levels and alternative splicing of APP transcripts in brain tissue of hAPP transgenic and nontransgenic mice and of humans with and without AD. PDGF-hAPP mice showed severalfold higher levels of total APP mRNA than did nontransgenic mice or humans, whereas their endogenous mouse APP mRNA levels were decreased. This resulted in a high ratio of mRNAs encoding mutated hAPP versus wild-type mouse APP. Modifications of hAPP introns 6, 7, and 8 in the PDGF-hAPP construct resulted in a prominent change in alternative splice site selection with transcripts encoding hAPP770 or hAPP751 being expressed at substantially higher levels than hAPP695 mRNA. Frontal cortex of humans with AD showed a subtle increase in the relative abundance of hAPP751 mRNA compared with normal controls. These data identify specific intron sequences that may contribute to the normal neuron-specific alternative splicing of APP pre-mRNA in vivo and support a causal role of hAPP gene products in the development of AD-type brain alterations.

A number of observations indicate that the disregulated expression or processing of human amyloid β protein precursor (hAPP)1 gene products may play a critical role in the development of Alzheimer’s disease (AD). Increased cerebral deposition of the hAPP-derived peptide Aβ in the form of amyloid plaques constitutes a hallmark of AD (2). Persons with Down’s syndrome who carry an additional copy of the hAPP gene on their third chromosome 21 show an overexpression of hAPP (3, 4) as well as a prominent tendency to develop AD-type pathology early in life (5). A number of hAPP point mutations that are tightly associated with the development of familial AD encode amino acid changes close to either side of the Aβ peptide (for review, see Refs. 6 and 7), and in vitro studies indicate that aggregated Aβ can induce neurodegeneration (for review, see Ref. 8).

To assess the neuropathogenic potential of hAPP gene products in vivo, a variety of transgenic models have been developed that were engineered to overexpress either full-length hAPP or hAPP derivatives in the CNS using different promoters (1, 9–12). So far, only one transgenic model has reproducibly developed prominent AD-type neuropathology: neuronal expression of an alternatively spliced minigene, driven by the platelet-derived growth factor (PDGF B chain) promoter and encoding (Val17 → Phe)-mutated hAPP770, hAPP751, and hAPP695, resulted in an age- and brain region-dependent development of typical amyloid plaques, dystrophic neurites, loss of presynaptic terminals, astrocytosis, and microgliosis (1). In contrast, previous transgenic models with neuron-specific enolase (NSE) promoter-driven expression of individual hAPP isoforms, such as wild-type or (Val17 → Ile)-mutated hAPP695 or hAPP751, showed no (12) or only minimal (13) pathology.

The APP gene contains 19 exons (Fig. 1A), three of which (exons 7, 8, and 15) are subject to alternative splicing. The different APP isoforms that are derived from this gene are designated according to their number of amino acids. The longest isoform, APP770, contains a 56-amino acid domain (encoded by exon 7), which shares sequence homology with, and can function like Kunitz-type serine protease inhibitors (KPI) as well as an adjacent 19-amino acid domain (encoded by exon 8) with homology to the MRC OX-2 antigen, which is found on the surface of neurons and certain immune cells (for review, see Refs. 12, 14, and 15). APP695 lacks both of these domains (Fig. 1A) and is produced primarily by neurons, which constitute the primary source of APP within the CNS (16, 17). Brain tissue expresses little APP770 and, depending on the animal species and brain region analyzed, low, intermediate, or high levels of APP751 (16, 18–21). The latter isoform contains the KPI domain of Alzheimer’s disease. The APP gene contains 19 exons (Fig. 1A), three of which (exons 7, 8, and 15) are subject to alternative splicing. The different APP isoforms that are derived from this gene are designated according to their number of amino acids. The longest isoform, APP770, contains a 56-amino acid domain (encoded by exon 7), which shares sequence homology with, and can function like Kunitz-type serine protease inhibitors (KPI) as well as an adjacent 19-amino acid domain (encoded by exon 8) with homology to the MRC OX-2 antigen, which is found on the surface of neurons and certain immune cells (for review, see Refs. 12, 14, and 15). APP695 lacks both of these domains (Fig. 1A) and is produced primarily by neurons, which constitute the primary source of APP within the CNS (16, 17). Brain tissue expresses little APP770 and, depending on the animal species and brain region analyzed, low, intermediate, or high levels of APP751 (16, 18–21). The latter isoform contains the KPI domain of Alzheimer’s disease.
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**Materials and Methods**

**Mouse Tissues**—Frontal cortex (midfrontal gyrus) from humans with mild (n = 2; age range in years, 73–76), moderate (n = 6; age range, 71–86), or severe (n = 5; age range, 66–88) AD, and normal human controls (n = 5; age range, 65–78) was obtained at autopsy and snap-frozen within less than 8 h after death. The midfrontal gyrus was chosen because this region typically shows prominent formation of β amyloid plaques in AD. AD severity was graded clinically using the Blessed score and clinical dementia rating scale (CDR) and histologically according to the density of thioflavine S-stained plaques and tangles in the neocortex and hippocampus, as described previously (28–31). The following categories were used here: mild AD (CDR, 1; Blessed score, 2–14; Braak stage, I–II), moderate AD (CDR, 2; Blessed score, 15–25; Braak stage, IV), and severe AD (CDR, 3; Blessed score, 26–30).

**A** diagrammatic comparison of the hAPP gene and hAPP encoding transgenes. A, exon-intron organization of the hAPP gene based on Ref. 34. Boxes represent exons, closed and hatched boxes represent isoform-specific exons as shown. Horizontal lines indicate introns. The whole gene is approximately 400 kb in length. Elements are not drawn to scale. B, fusion gene constructs used to express hAPPs in neurons of transgenic mice. The regulatory sequences incorporated into the transgenes were derived from either the rat NSE gene or the human PDGF B chain gene as described in Refs. 12 and 1, respectively. Numerical boxes represent hAPP exons. Elements are not drawn to scale. The NSE-driven hAPP DNAs were either wild-type or point mutated to encode the familial AD-associated valine to isoleucine substitution (85). The PDGF-driven hAPP minigene was point mutated to encode a valine to phenylalanine change at the same position; this change is also tightly linked with familial AD (86). Besides the introduction of a point mutation into exon 17, hAPP coding sequences were not modified. Note, however, that the three introns incorporated into the PDGF-hAPP construct were modified as described under “Materials and Methods” and panel C below. As indicated, alternative splicing of pre-mRNA derived from the PDGF-hAPP transgene results in transcripts encoding hAPP770, hAPP751, or hAPP695. C, intron modifications of the PDGF-hAPP transgene. Compared with the authentic hAPP gene (top), all three introns of the PDGF-hAPP transgene (bottom) were modified. B, authentic BamHI sites. a and b, engineered BamHI sites introduced via PCR primer modifications. X, Xho site. R, EcoRI site. Crossed out line, deleted intron sequence. Elements are not drawn to scale. Numbers indicate distances in base pairs; *, based on Ref. 34; †, based on Ref. 24; ‡, based on Ref. 34; ‡‡, size estimates from Ref. 34; ††, based on Ref. 34.

**In the current study,** we compared the levels and alternative splicing of transcripts encoding APP695, APP751, and APP770 in brain tissue of PDGF-hAPP and NSE-hAPP mice and of humans with and without AD to address the following questions in vivo. (i) Could the development of prominent AD-type pathology in PDGF-hAPP mice be related to the level of total APP expression achieved in this model or to the relative proportion of mutated hAPP versus wild-type mouse APP (mAPP) expressed in their brains? (ii) Does overexpression of hAPP in the murine CNS alter the expression of the endogenous mAPP gene? (iii) How is the neuron-specific splicing of hAPP exons 7 and 8 affected by specific modifications of hAPP intron sequences made in the construction of the PDGF-hAPP transgene? (iv) Does expression of hAPP transcripts in brains of hAPP transgenic models resemble the expression of hAPP transcripts found in the CNS of humans with AD?
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TABLE I  Human cases with good RNA preservation

| Patient | Age | Sex | CNS assessment | PMI | Cause of death |
|---------|-----|-----|----------------|-----|----------------|
| 94-06   | 66  | F   | Normal         | 4.0 | Non-Hodgkin's lymphoma |
| 94-25   | 77  | F   | Normal         | 3.5 | Cardiac arrest   |
| 94-35   | 78  | F   | Normal         | 1.25| Lung cancer      |
| 95-03   | 77  | F   | Normal         | 2.5 | Emphysema        |
| 4176    | 03  | M   | Normal         | 3   | Pneumonia        |
| 4817    | 71  | F   | Moderate AD    | 3   | Cardiac arrest   |
| 4839    | 83  | F   | Moderate AD    | 7   | Congestive heart failure |
| 4811    | 75  | M   | Severe AD      | 2   | Cardiac arrest   |
| 4814    | 80  | M   | Severe AD      | 6   | Myocardial infarction |

“Normal”, no clinical or neuropathological evidence for AD or other neurodegenerative diseases. AD was graded as described under “Materials and Methods.”

PMI, death to autopsy interval.

26–33: Braak stage, V-VI). All dementia cases and one of the normal controls (case 4176) were from the Alzheimer’s Disease Research Center of the University of California at San Diego; the other four normal control cases were obtained from Dr. J. Rogers at the Sun Health Research Institute (Sun City, AZ) where they were analyzed neuropathologically by Dr. W. H. Civen.

**RNA Structure**—The construction of NSE-hAPP695 and NSE-hAPP751 transgenes has been described elsewhere (12). Although the basic design of the PDGF-hAPP fusion gene has also been described (1), no details were provided in this initial report with respect to the modified hAPP intron sequences contained in these constructs. The PDGF-driven hAPP minigene represents a fusion product of (from 5’ to 3’): (i) hAPP RNA spanning exon 1 through the Xmol site in intron 6 including 180 bp of exon 6; (ii) a 166-bp PCR-generated fragment of genomic hAPP sequence extending from the Xmol site in exon 6 to an engineered BamHI site in intron 6 (introduced just downstream of position 143 of intron 6 using PCR primer modification); (iii) a 6.8-kb BamHI fragment of hAPP genomic DNA (obtained from a human placental genomic library via a phage cloning) containing exons 7 and 8 and extending from the BamHI site located 1,658 bp upstream of exon 7 to the first BamHI site of intron 8 (according to our analysis, this latter BamHI site is located 2,329 bp downstream of exon 8, see Results); (iv) a 313-bp PCR-generated fragment of genomic hAPP sequence extending from an engineered BamHI site (introduced just upstream of the last 236 bp of intron 8 by PCR primer modification) to the Xmol site in exon 9 (i.e., the hAPP cDNA sequence extending from the Xmol site in exon 9 to 135 bp downstream of the first hAPP polya (signal in the hAPP 3’-untranslated region (UTR)). The unique Xhol site in hAPP intron 7 was destroyed as described in the legend to Fig. 1C.

Generation of Riboprobes—DNA templates for riboprobes were generated from mRNA extracted from nontransgenic mouse brains (mAPP770, mAPP, total APPα, NFL, SYN, GAP-43, MAP-2 probes) or from cDNA expression construct (hAPP770, hAPPSV40, total APPα, actin probes) using reverse transcriptase PCR or conventional PCR, respectively. Reverse transcriptase reaction (200 μl) was as follows: 10–20 μg RNA, 2.5 μM random hexamers, 5 μM MgCl2, dNTP mix (10 mM each), 5 μM RNasin, 2.5 μM of Maloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in 1× PCR buffer (Perkin-Elmer) incubated sequentially at room temperature (10 min), 37 °C (15 min), and 95 °C (5 min). PCR (50 μl) was as follows: 100–200 ng of template DNA or 2 μl of reverse transcriptase reaction, 10 ng of each primer, 2 μM MgCl2, dNTP mix (200 μM each), 1% Tween 20, 2.5 units of Taq (Life Technologies, Inc.). PCR cycles were as follows: 95 °C × 5 min; 5 × (95 °C × 30 s, 55 °C × 30 s, 72 °C × 30 s); 25 × (90 °C × 1 min, 50 °C × 1 min, 72 °C × 2 min); 72 °C × 10 min. Nine pairs of oligonucleotide primers (15–28-mers) were used to amplify DNA templates complementary to the following sequences (GenBank accession numbers in parenthesis): nucleotides 2468–2657 (X06989) of hAPPα, nucleotides 2532–2566 (M24914) of SV40 (mAPP probe); nucleotides 1041–1174 (X59379) of mAPPα probe; nucleotides 1215–1348 (M24915) of hAPP (total APPα probe); nucleotides 2818–3020 (X59379) of mAPP (mAPP probe); nucleotides 1312–1560 (M13016) of mouse neurofilament (68 kDa chain) (NFL probe); nucleotides 1805–2077 (M21041) of mouse microtubule-associated protein-2 (MAP-2 probe); mouse synaptophysin corresponding to nucleotides 487–696 (M17367) (GAP-43 probe); and nucleotides 480–559 (X03672) of mouse β-actin (actin probe). See Fig. 2, B and C, for information on the hAPP770 and mAPP770 probes. Amplification products were subcloned in pCRII (Invitrogen, La Jolla, CA) and T7 or SP6 RNA polymerase was used to generate P32-labeled antisense riboprobes from 100 ng of linearized plasmids.

DNA Analysis and PCR Amplification of Genomic DNA—The complete sequence of the PDGF-driven hAPP minigene and of DNA templates for all riboprobes used in this study was determined using Sequenase kits (Amersham Corp.). For the identification of transgenic mice, genomic DNA extracted from tail biopsies was slot blotted and hybridized with P32-labeled DNA probes that recognize specific sequences in NSE-hAPP and PDGF-hAPP transgenes. Genomic DNA was amplified using the Expand Long Template PCR System (Boehringer Mannheim), and genomic amplions were subcloned using the Prime PCR Cloner cloning system (5 Prime → 3 Prime, Inc., Westchester, PA), by manufacturers’ instructions. PCR cycles were as follows: 92 °C × 2 min; 10 × (92 °C × 10 s, 55 °C × 30 s, 72 °C × 15 min); 5 × (92 °C × 10 s, 55 °C × 30 s, 72 °C × 10 min); 20 × (92 °C × 20 s, 55 °C × 30 s, 72 °C × 10 min) × 25 min; 72 °C × 15 min. DNA was purified using the QIAquick spin column (QIAGEN) and used as template for PCR reactions. PCR products were visualized on agarose gels and subsequent restriction fragments were analyzed by Southern blotting using the QuantiBlot DNA detection system (Ambion). PCR products were labeled by 5′-end labeling using [32P]dCTP (Amersham Corp.) and T4 polynuclease (Boehringer Mannheim) according to the manufactures’ instructions. Probes were purified using microspin columns (Pharmacia) and were labeled with [32P]dCTP to a specific activity of 108 cpm/μg.

OH) and stored in Formazol buffer (Molecular Research Center) at −20 °C. Poly(A)−enriched RNA was isolated as described previously (32) using diigo(dT)-cellulose from Invitrogen. The quality of total RNA extracted from human postmortem tissues was assessed by electrophoretic fractionation on 1% agarose/formaldehyde gels, Northern blotting and densitometric comparison of 28 S and 18 S ribosomal RNA bands on methylene blue-stained nylon membranes using the Quantity one software package (Bio-Rad, Hercules, CA). Final values were expressed as ratios of (specific signal − background)/(actin signal − background) to correct for differences in RNA content/loading across samples. For comparisons of signals representing distinct components of the same probe (hAPP versus SV40 fragments of the hAPPSV40 probe or isofom-specific APP fragments of the hAPP770 and mAPP770 probes), readings were corrected for differences in the amount of radioactive label incorporated into specific probe fragments based on the number of urinary bases contained in each fragment. For groups of cases analyzed, results were expressed as mean ± S.E. The statistical significance of differences between experimental and control groups was assessed by unpaired, two-tailed Student’s t test or analysis of variance. All reported results were confirmed in two or more independent RPAs.

**RESULTS**

Characterization of PDGF-hAPP Intron Modifications—As illustrated in Fig. 1, B and C, the PDGF-hAPP fusion gene contains three modified hAPP introns that differ from the corresponding authentic hAPP gene introns by large deletions (introns 6 and 8) or insertion of four nucleotides (intron 7). Sequencing of the 6.8-kb genomic BamHI fragment of the PDGF-hAPP transgene containing exons 7 and 8, intron 7 and portions of introns 6 and 8 of the hAPP gene (see “Materials and Methods” and Fig. 1C), revealed that intron 7 is 2,601 bp long and that the first BamHI site in intron 8 (5′-end of deletion in PDGF-hAPP intron 8) is located 2,329 bp downstream of exon 8 (Fig. 1C). This does not coincide with the hAPP restriction map published by Yoshikai et al. (34), which placed a 1.48-kb EcoRI fragment in intron 7 upstream of a 1.6-kb EcoRI

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FIG. 2. Regions of endogenous and transgene-derived APP mRNAs protected by antisense riboprobes. A: hAPPSV40, mAPP, and total APP probes. The 3'-ends (coding region, 3'-UTR, poly(A) signal, and tail) of NSE-hAPP (top), PDGF-hAPP (middle) and endogenous murine APP (bottom) mRNAs are depicted diagrammatically. The hAPP 3'-UTR of NSE-hAPP constructs was truncated at the HindIII site (nucleotide 3023 in Genbank accession number X06989), whereas the hAPP 3'-UTR of the PDGF-hAPP construct ends 135 bp downstream of the first hAPP poly(A) signal (87). In RPA, only the regions that hybridize with complete or (depending on the conditions used) near-complete complementarity with specific riboprobes will be protected (black, stippled, or hatched), whereas noncomplementary regions will be degraded (open). Numbers indicate the sizes of protected fragments in nucleotides. The hAPPSV40 probe was produced by ligating hAPP with SV40 sequences via a NotI linker. This probe protects an hAPP fragment that is present in all endogenous hAPP mRNAs as well as in the hAPP transgenes; because of significant sequence variation in the 3'-UTR of these transcripts, this probe does not protect mAPP or APLP2 mRNAs under the conditions used here. The hAPPSV40 probe also protects a smaller fragment of SV40 sequence, which is present in hAPP transgenes but not in endogenous human or murine transcripts. Because the NotI linker is absent from hAPP transgenes and the hAPPSV40 probe is cleaved into two fragments (Fig. 3A), the hAPP770 probe specifically protects a region of mAPP 3'-UTR present in all mAPP mRNAs; it does not protect hAPP or APLP2 mRNAs. The total APP probes protect a fragment of APP coding region in which hAPP and mAPP transcripts show a high degree of homology; under appropriate conditions, this probe protects both hAPP and mAPP mRNAs (see Fig. 4 for further details).

B, alignment of sequences protected by hAPP770 and mAPP770 riboprobes. The DNA template for the hAPP770 probe was amplified by PCR from a cloned hAPP770 cDNA (a generous gift from Dr. D. Goldgaber, SUNY, Stony Brook, NY). The mAPP770 probe template was generated by reverse transcriptase PCR from mouse brain RNA (see "Materials and Methods"). Sequence analysis of two different mAPP770 clones, amplified from a Balb/c or a SJ L/J mouse, respectively, gave the same results (shown above). This sequence is consistent with previous publications reporting on different components of this region (37, 38, 88). Because there are numerous mismatches (*) between hAPP and mAPP sequences in this region, the hAPP770 probe generally protects only hAPP and the mAPP770 only mAPP transcripts. While neither of these probes would be expected to protect APLP2 mRNAs (36–40), these probes cannot differentiate between APP transcripts that contain or lack exon 15; however, the latter (L-APP mRNAs) are expressed in brain at very low levels (27).

C, APP mRNA regions protected by hAPP770 (h) and mAPP770 (m) riboprobes. To quantitate the three main APP splice products (APP770, APP751, and APP695), two probes were generated from human or murine APP770 cDNAs,
fragment containing exon 8 and the first BamHI site of intron 8 within 500 bp downstream of exon 8 (approximate sizes). Our sequence analysis indicates that the 1.6-kb EcoRI fragment containing exon 8 is actually located upstream of the 1.48-kb EcoRI fragment. To further assess this point, genomic DNA was extracted from a human cell line (HeLa cells) as well as from tail tissues of PDGF-hAPP mice and subjected to PCR amplification using a pair of primers specific for hAPP exons 7 and 8, respectively (see “Materials and Methods” and Fig. 1D). Electrophoretic fractionation of the resulting amplification products revealed that, consistent with our sequencing results, the PDGF-hAPP intron 7 and the endogenous hAPP intron 7 were both approximately 2.6 kb in size (Fig. 1D).

Comparison of hAPP mRNA Levels—hAPP mRNA levels were quantitated using the hAPPSV40 probe, which protects a fragment of hAPP 3′-UTR that is present in all hAPP mRNA isoforms (Fig. 2A). Total hAPP mRNA levels in PDGF-hAPP mice were approximately 10-fold higher than those achieved in the highest NSE-hAPP expresor line and 20–27-fold higher than those found in the majority of NSE-hAPP lines (Fig. 3, A and B). hAPP mRNA levels in brains from the highest NSE-hAPP expressor line were only about 50% of those found in the frontal cortex of humans with or without AD, whereas PDGF-hAPP brains showed 7.5–8-fold higher levels of hAPP mRNA expression than the human cases (Fig. 3, A and B). Overall hAPP RNA levels in the frontal cortices of humans with and without AD were similar (Fig. 3, B and C), with no statistically significant differences in signals being revealed between these two groups by PhosphorImager quantitation (not shown).

Low Efficacy of the hAPP Poly(A) Signal in the PDGF-hAPP Construct—The hAPPSV40 probe protects two fragments of NSE-hAPP and PDGF-hAPP messages, namely an upstream fragment of hAPP 3′-UTR and a downstream fragment of SV40 sequence (Figs. 2A and 3A). Because the first hAPP poly(A) signal, which is present in the PDGF-hAPP transgene but not in NSE-hAPP constructs, is located between these two protected fragments (Fig. 2A), the hAPPSV40 probe can be used to assess the effectiveness of this signal. After correcting for the difference in radioactivity incorporated into the corresponding probe fragments, the average relative signal intensities of the hAPP:SV40 bands were found to be 1.1 for NSE-hAPP695 and NSE-hAPP751 but 1.075 for PDGF-hAPP mice (3–4 mice analyzed per group). As there was no evidence for partially protected, truncated SV40 fragments (Fig. 3A), this suggests that approximately 25% of PDGF-hAPP-derived mRNAs terminate within the 135-bp fragment that separates the hAPP poly(A) signal from the downstream SV40 sequence, whereas roughly 75% of PDGF-hAPP-derived messages terminate downstream of the SV40 poly(A) signal. These data are consistent with results from a comparison of three authentic hAPP poly(A) signals, which also revealed the first hAPP poly(A) signal to be relatively ineffective (35).

Comparison of Total APP mRNA Levels—Because of the strong but incomplete homology between hAPP and mAPP sequences (36–38) and the weaker but still considerable homology between mRNAs encoding APP and APP-like protein (APLP2) (39, 40), accurate quantitations of total APP transcript levels in hAPP transgenic mice are difficult. The following strategies were used to address this issue: (i) for the generation of total APP probes, a region of APP coding sequence was selected that shows strong homology between hAPP and mAPP but significant divergence from APLP2 sequences (Fig. 2A); (ii) total APP probes, a region of APP coding sequence was selected that shows strong homology between hAPP and mAPP but significant divergence from APLP2 sequences (Fig. 2A). The expected sizes of the protected mRNA fragments for the different isoform-specific transcripts are indicated (nt, nucleotides). Solid lines, protected probe regions (bands on RPA autoradiograph). Dotted lines, unprotected probe regions (degraded by RNase treatment).
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Fig. 4. Comparison of total APP mRNA levels in brains of transgenic mice and of humans with AD. Total RNA was extracted from hemibrains of nontransgenic controls (Non-Tg) and of transgenic mice from lines NSE-hAPP751m-57, NSE-hAPP695m-19, and PDGF-hAPP-109 (three mice/group). Levels of endogenous mAPP transcripts were measured by RPA using a mAPP-specific probe (Fig. 2A) and RNase condition 2. Results indicate means ± S.E. of radioactive signals as determined by PhosphorImager quantitations. Note the significant decrease in mAPP mRNA levels in the PDGF-hAPP mouse (p value determined by Student's t test comparing PDGF-hAPP mice with nontransgenic controls). Similar results (not shown) were obtained in two additional experiments using independent sets of comparable samples and quantitations of signals generated with either the mAPP probe or the mAPP770 probe (86-nucleotide fragment of mAPP exon 9; see Figs. 2C and 6A).

In contrast, the highest NSE-hAPP expressor line showed on average only a 1.3–1.6-fold increase in total APP mRNA levels over levels found in nontransgenic controls. This increase is lower than the approximately 2.9-fold increase in mAPP mRNA expression determined for the same line of NSE-hAPP mice by Northern blot analysis in a previous study using a large hAPP751 cDNA probe (12). It is likely that this cDNA probe showed substantially greater bias toward transgene-derived hAPP transcripts than the total APP riboprobes chosen for the above RPA analysis and, hence, overestimated the level of total APP mRNA overexpression in NSE-hAPP mice. The 1.3–1.6-fold increase in total APP mRNA levels determined here is clearly more consistent with the 1.33–1.52-fold increase in total APP protein expression determined for this line by PhosphorImager analysis of radiolabeled Western blots (12).

Effect of hAPP Overexpression on Endogenous Neuronal mRNAs—To assess what effects different levels of hAPP overexpression have on the expression of endogenous mAPP transcripts, a mAPP probe (Fig. 2A) was used that specifically protects a fragment of mAPP 3'-UTR that is present in all mAPP mRNA isoforms. Compared with nontransgenic controls, PDGF-hAPP mice showed a significant 30% decrease in overall endogenous mAPP levels, whereas NSE-hAPP mice did not (Fig. 5). To determine whether other neuronal mRNAs were also decreased in PDGF-hAPP mice, brain RNA from PDGF-hAPP mice (n = 6) and nontransgenic controls (n = 6) was analyzed by RPA and PhosphorImager quantitation of radioactive signals using probes for mRNAs encoding MAP-2, GAP-43, synaptophysin, or neurofilament (see “Materials and Methods”). Compared with nontransgenic controls, PDGF-hAPP mice showed no significant alterations in the levels of any of these neuronal messages (data not shown).

Alternative APP Splice Site Selection in Transgenic and Nontransgenic Mice—As outlined above, mAPP transcripts were more abundant than hAPP transcripts in brains of NSE-hAPP mice, whereas the opposite was true for brains of PDGF-hAPP mice. Two different probes, hAPP770 and mAPP770 (Fig. 2, B and C), were used to assess the relative abundances of specific human or murine APP splice products (APP770, APP751, and APP695). The results from this analysis are exemplified in Fig. 6 and summarized in Table II. In brains of nontransgenic
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FIG. 6. Quantitation of mAPP and hAPP splice products. RNA was extracted from hemibrains of nontransgenic controls (Non-Tg) and of transgenic mice from lines NSE-hAPP751m-S7, NSE-hAPP695m-19, and PDGF-hAPP-109 (three to four mice/group) (A and B) and from the frontal cortex of humans without (Controls; n = 5) or with AD (n = 6) (C). Ten μg of total RNA was analyzed per sample using RNase conditions 2 (C) or 3 (A and B); mAPP (A) or hAPP (B and C) splice products (indicated on the right) were detected using the mAPP770 probe or the hAPP770 probe, respectively (see Fig. 2, B and C). Representative autoradiographs are shown. For a statistical analysis of the relative abundances of isoform-specific APP mRNAs, see Table II. The mAPP (exon 9) band in panel A represents an 86-nucleotide fragment of mAPP exon 9 (Fig. 2C). To distinctly reveal the prominent hAPP770 and hAPP751 bands in PDGF-hAPP brain samples, the autoradiograph in panel B was developed after a 20-min exposure; because of this short exposure, the actin bands appear faint.

and NSE-hAPP mice, mAPP695 transcripts were much more abundant than either mAPP770 or mAPP751 mRNAs, consistent with previous results obtained in nontransgenic rodents (for example, see Ref. 18). No significant differences were seen with respect to any of these isoforms across nontransgenic and NSE-hAPP751 or NSE-hAPP695 transgenic mice (Fig. 6A and Table II). In contrast, PDGF-hAPP-derived mRNAs showed an inverted splicing pattern with hAPP770 and hAPP751 mRNAs being substantially more abundant than hAPP695 transcripts (Fig. 6B and Table II). Notably, the levels of hAPP695 mRNA expression in PDGF-hAPP mice were similar to those achieved in the high expressor NSE-hAPP695 line (Fig. 6B); PhosphorImager quantitations (not shown) revealed no statistically significant differences in hAPP695 signals between these two groups (seven mice analyzed per group).

A comparison of endogenous mAPP splice products in brains of nontransgenic and PDGF-hAPP mice (n = 3/group) using the mAPP770 probe and RNase conditions 3 or 4 indicated that the significant drop in mAPP mRNA levels in PDGF-hAPP mice (Fig. 5) was due primarily to a prominent decrease in mAPP695 levels, whereas alterations in mAPP770 and mAPP751 mRNA levels were less pronounced (data not shown). Although the overall trends were clear, precise quantitations of these changes were difficult because degradation products of the abundant hAPP770 and hAPP751 mRNAs interfered with the reliable measurement of isoform-specific mAPP signals in the PDGF-hAPP mice.

Alternative Splicing of hAPP PremRNA in Humans With and Without AD—The hAPP770 probe was also used to compare the relative abundances of hAPP770, hAPP751, and hAPP695 mRNA isoforms in the frontal cortex of humans with and without AD. Consistent with previous reports (18–20), humans expressed substantially higher levels of KPI-encoding APP transcripts than rodents, particularly hAPP751 (Fig. 6C and Table II). Compared with normal human controls, patients with moderate to severe AD showed a 5% increase in the relative abundance of hAPP751 mRNA that was significant at a level of p < 0.05 (Table II). Otherwise, cortical hAPP mRNA isoform levels were similar in humans with and without AD (Table II).

DISCUSSION

The current study demonstrates that 2–4-month-old PDGF-hAPP mice from a line that consistently develops prominent AD-type pathology between 6 and 8 months postnatally express substantially higher hAPP and total APP steady-state mRNA levels than NSE-hAPP mice from lines that fail to develop such alterations, suggesting that high levels of hAPP expression may be requisite for the induction of robust pathology. PDGF-hAPP mice were analyzed before the development of amyloid deposits to help differentiate potentially causal factors from secondary responses that could follow amyloid-induced neuronal injury. The high levels of hAPP expression in PDGF-hAPP mice were associated with a statistically significant 30% reduction in mAPP mRNA levels (Fig. 5). Because levels of other neuronal messages encoding MAP-2, GAP-43, synaptophysin, or NFL were not significantly altered in PDGF-hAPP mice at this developmental stage, it is unlikely that the decrease in mAPP mRNA levels resulted from a generalized decrease in the expression of endogenous neuronal gene products. It is tempting to speculate that the decrease in mAPP message levels reflects an active counterregulatory decrease in the expression of the endogenous mAPP gene in cells exposed to high levels of hAPP. The findings that total APP levels in the highest NSE-hAPP expressor line were increased 1.3–1.6-fold over levels found in nontransgenic controls (Fig. 4), while endogenous mAPP levels were not significantly altered (Fig. 5), indicates that the brains of these mice, ap-
APP mRNAs in Brains of Transgenic Mice and Humans with AD

RNA was extracted from mouse hemibrains or from human frontal cortices (midfrontal gyrus) and analyzed by RPA using either the mAPP770 or hAPP770 probe (see Fig. 2, B and C, and Fig. 6). The NSE-hAPP group included transgenic mice from lines NSE-hAPP751m-57 (n = 6) and NSE-hAPP695m-19 (n = 6). Humans without AD were nondemented controls without neuropathological evidence for AD or other neurodegenerative diseases, the AD group consisted of cases with neuropsychologically documented dementia and neuropathological evidence for moderate or severe AD (see Table I, and "Materials and Methods"). Isoform-specific signals were quantitated by PhosphorImager analysis, adjusted for the different amounts of radioactive label incorporated into the corresponding probe fragments, and expressed as percent of the sum of the signals for APP770 + APP751 + APP695 (% of total). Data shown represent means ± S.E.

| Group (probe) | APP770 (% of total) | APP751 (% of total) | APP695 (% of total) | Cases (n) |
|---------------|----------------------|----------------------|----------------------|-----------|
| Non-tg mice (mAPP770) | 2.6 (±0.2) | 3.1 (±0.3) | 94.3 (±0.5) | 7 |
| NSE-hAPP mice (mAPP770) | 2.3 (±0.2) | 3.3 (±0.4) | 94.4 (±0.5) | 12 |
| PDGF-hAPP mice (hAPP770) | 45.8 (±12.2) | 46.7 (±0.5) | 7.5 (±1.3) | 7 |
| Humans without AD (hAPP770) | 4.7 (±0.2) | 36.7 (±1.5) | 58.6 (±0.3) | 5 |
| Humans with AD (hAPP770) | 3.9 (±0.3) | 41.7 (±1.4) | 54.4 (±1.3) | 4 |

* p = 0.0467 by unpaired, two-tailed Student's t test (compared with humans without AD).

approximately 83–88% of APP transcripts encode mutated hAPP. In concert with the substantial increase in total APP mRNA levels, this prominent shift toward mutated hAPP transcripts can be expected to strongly promote the synthesis of amyloidogenic hAPP molecules in the CNS of PDGF-hAPP mice. Consistent with this prediction, significant increases in Aβ production have been detected in brains of PDGF-hAPP mice (1). Experiments are in progress to further complement the RNA analysis presented here with characterizations of these models at the protein level.

It is interesting that PDGF-hAPP-derived pre-mRNA was spliced differently than endogenous hAPP pre-mRNA in humans or mAPP pre-mRNA in mice. In nontransgenic mice and normal human controls, APP695 transcripts were substantially more abundant than APP770 transcripts with APP751 mRNA levels being similar to APP770 mRNA levels in mice and closer to APP695 mRNA levels in humans (Fig. 6 and Table II). In contrast, alternative splicing of PDGF-hAPP pre-mRNA resulted in roughly similar levels of hAPP770 and hAPP751 transcripts, both of which were expressed at high abundance, and in comparatively low levels of hAPP695 mRNA (Fig. 6B and Table II). In fact, hAPP695 mRNA levels in PDGF-hAPP mice were similar to those achieved in our highest NSE-hAPP695 expressor line (Fig. 6B). This implies that the difference between these lines with respect to hAPP and total APP mRNA levels (Figs. 3 and 4) is due primarily to the substantial overexpression of KPI-containing isoforms achieved in the PDGF-hAPP model.

In contrast to the hAPP splicing observed in the PDGF-hAPP model, the hAPP splicing pattern in the cerebral cortex of mice expressing the entire hAPP gene via yeast artificial chromosomes resembled that of the endogenous hAPP gene in humans, with transgenic mice showing substantially higher ratios of APP751/APP695 mRNA levels than nontransgenic controls and low levels of hAPP770 expression (41). This indicates that the higher levels of APP751 mRNA expression in humans compared with mice is probably related to regulatory sequences within the hAPP gene itself rather than to differences in the neuronal splicing machinery of humans versus mice.

Although the PDGF-hAPP transgene is also expressed in peripheral organs, PDGF-B chain promoter-driven gene expression within the CNS is restricted predominantly to neurons (Ref. 42 and data not shown). Hence, the unusual alternative splicing of PDGF-hAPP pre-mRNA (Fig. 6B and Table II) suggests that the normal neuron-specific skipping of APP exons 7 and 8 in vivo depends, at least in part, on the hAPP intrinsic sequences that were modified in the construction of this fusion gene (Fig. 1C). It is conceivable that these sequences normally participate in the regulation of splice site selection, for example, by interacting with cell-specific RNA-binding proteins that may block the use of certain exons (43). Notably, studies on the β-tropomyosin pre-mRNA have demonstrated that alternative splicing patterns can be altered not only by deletions of intron sequences but also by clustered point mutations within introns that do not alter the distance between 3'-splice sites and upstream branchpoints (43). Hence, while it is tempting to speculate that the large deletions within hAPP introns 6 and 8 had a greater effect than the Xhol site elimination in hAPP intron 7, it is possible that both types of modifications contributed to the difference in alternative splice site selection and hAPP exon usage observed between the PDGF-hAPP and endogenous APP pre-mRNAs. Because there is evidence suggesting that APP splicing within neurons can be regulated by factors that influence neuronal differentiation and activity (44–49), it should be noted that no obvious abnormalities in CNS development have been identified in PDGF-hAPP mice and that their neurons show no morphological evidence of dedifferentiation (data not shown). We therefore believe that the hAPP intron modifications discussed above provide the most plausible explanation for the hAPP770/751-predominant splicing pattern identified in this model.

As an aside, our comparison of PDGF-hAPP and endogenous human genomic DNA (Fig. 1, C and D) revealed that the authentic hAPP intron 7 is actually shorter than indicated by a previous study (34). As outlined under "Results," the previously published map can be reconciled with our data if one assumes that two neighboring EcoRI fragments of the hAPP gene, one of which contains exon 8, are arranged in an order opposite to the one reported by Yoshikai et al. (34).

Does a shift toward the expression of KPI-containing hAPP play a causal role in amyloidogenesis, and can similar alterations be identified in humans with AD? As summarized in Tables III and IV, the pertinent literature is complex. Many of the controversies in this field probably relate to specific differences among studies with respect to stages of AD selected, specific CNS regions examined, integrity of RNA samples analyzed, and RNA detection methods used. When analyzing RNA extracted from autopsy material, it is frequently difficult to ensure that alterations in the levels of specific transcripts reflect effects of the disease state under study and not of the death process or postmortem alterations (50, 51). Our RPA analysis of relatively well-preserved RNA extracted postmortem from the midfrontal gyri of humans with or without AD revealed no significant change in total hAPP mRNA levels in patients with moderate to severe AD compared with normal controls (Fig. 3C) and a subtle increase in the relative abundance of hAPP751 mRNA (Table II). While this study was not designed to differentiate whether this shift reflects an increase in neuronal versus glial hAPP751 mRNA expression, others have reported that reactive glia in AD brains show no clear overexpression of APP mRNAs (17, 26). It should also be noted...
in this context that our hAPP770 probe does not differentiate between transcripts encoding APP751 or amyloid precursor-related protein-563 (52); the latter closely resembles APP751 in its N-terminal portion but does not encode Aβ as it differs substantially from APP isoforms in its C-terminal region (52). Amyloid precursor-related protein-563 has been found to be increased in AD brains by reverse transcriptase-PCR and in situ hybridization (53).

It has been postulated that the relative increase in KPI-containing APP isoforms in the aging human brain may increase the risk of amyloid formation (21, 54–57) and that the rarity of β amylloidosis in the aged rodent brain and the cerebellum of higher mammals is related to the low levels of KPI-containing APP isoforms in these tissues (20). It has further been speculated that an overexpression of KPI-containing APP could disturb the balance between biosynthesis and degradation of APPs in the brain and lead to β amyloid formation (21, 57, 58). The pronounced overexpression of KPI-encoding hAPP mRNAs and development of prominent AD-type pathology in the PDGF-hAPP model might be consistent with these postulates. However, to determine the relative neuropathogenic potential of individual hAPP isoforms more conclusively, transgenic models should be compared in which the PDGF promoter is used to overexpress selectively hAPP695, hAPP751, or hAPP770 at levels similar to those achieved in the original PDGF-hAPP mice; the generation of such models is underway.

In contrast to the PDGF-hAPP model, transgenic mice expressing the entire hAPP gene via yeast artificial chromosomes (41, 59, 60) have apparently so far failed to develop prominent AD-type pathology. Although the difference in hAPP splicing pattern in these distinct models could contribute to the difference in neuropathological readout (see above), the presence/absence of amyloidogenic mutations in the hAPP molecules expressed and differences in the overall levels of hAPP expression may be of equal or even greater pathogenetic importance. Notably, the overall hAPP mRNA levels in hAPP yeast artificial chromosome mice do not appear to have been significantly higher than those found in human brains (41, 59).

In conclusion, the differences in APP isoform-specific mRNA levels identified among humans and nontransgenic and PDGF-hAPP mice suggest that the neuron-specific skipping of APP exons 7 and 8 depends, in part, on the hAPP intron sequences

| RNA studied | AD vs. control | Method | CNS region(s) analyzed and references |
|-------------|----------------|--------|--------------------------------------|

### TABLE III

Studies comparing levels of hAPP (isoform) mRNAs in humans with and without AD

| Control | Method | CNS region(s) analyzed and references |
|---------|--------|--------------------------------------|

- **Total APP**: Unchanged
  - **Quantitative RT-PCR**: Frontal gray matter (15)
  - **Northern blot analysis**: Frontal, temporal, parietal, occipital cortex (14)
  - **In situ hybridization**: Neurons of the hippocampal subiculum, basis pontis and occipital cortex (75)

- **hAPP770**: Unchanged
  - **Quantitative RT-PCR**: Frontal gray matter (15)
  - **RNA slot blot analysis**: Frontal, temporal, parietal, occipital cortex (14)

- **hAPP751**: Unchanged
  - **Quantitative RT-PCR**: Frontal gray matter (15)
  - **Northern blot analysis**: Frontal, temporal, occipital cortex (14)

- **hAPP(KPI)**: Unchanged
  - **Northern blot analysis**: Cerebral cortex (79–81); hippocampus (81)

- **hAPP695**: Unchanged
  - **S1 nuclease protection assay**: Parietal cortex (14)
  - **Northern blot analysis**: Frontal gray matter (74); cerebellum (81)

- **hAPP770**: Decreased
  - **Northern blot analysis**: Frontal cortex (74, 77); parietal cortex (78)

- **hAPP751**: Increased
  - **In situ hybridization**: Neurons of nucleus basalis of Meynert, locus ceruleus, basis pontis, hippocampal subiculum and occipital cortex (75)

- **hAPP(KPI)**: Increased
  - **Quantitative RT-PCR**: Frontal white matter (15)
  - **In situ hybridization**: Neurons of nucleus basalis of Meynert, hippocampal formation, occipitotemporal gyrus (53)

- **hAPP695**: Decreased
  - **Northern blot analysis**: Frontal cortex (14)

- **hAPP770**: Decreased
  - **Northern blot analysis**: Frontal cortex (74)
  - **RT-PCR for APRP-563**: Nucleus basalis of Meynert (53)

- **hAPP751**: Increased
  - **Quantitative RT-PCR**: Frontal white matter (15)
  - **RT-PCR for APRP-563**: Nucleus basalis of Meynert, hippocampal formation, occipitotemporal gyrus (53)

- **hAPP(KPI)**: Increased
  - **Quantitative RT-PCR**: Frontal white matter (15)
  - **In situ hybridization**: Neurons of nucleus basalis of Meynert, hippocampal formation, occipitotemporal gyrus (53)
  - **In situ hybridization**: Neurons of nucleus basalis of Meynert, parahippocampal gyrus, occipitotemporal gyrus (53)
  - **In situ hybridization**: Nucleus basalis of Meynert (53)
  - **In situ hybridization**: Cerebellum (15)

- **hAPP695**: Increased
  - **S1 nuclease protection assay**: Frontal, temporal, occipital cortex (14)
  - **In situ hybridization**: Neurons of nucleus basalis of Meynert and locus ceruleus (75)

- **hAPP770**: Decreased
  - **Northern blot analysis**: Frontal cortex (3, 16)

- **hAPP751**: Increased
  - **In situ hybridization**: Neurons of nucleus basalis of Meynert, hippocampal subiculum, basis pontis and occipital cortex (75)

- **hAPP(KPI)**: Increased
  - **Quantitative RT-PCR**: Frontal white matter (15)
  - **In situ hybridization**: Hippocampal neurons (CA1, CA3, subiculum) (82)

- **hAPP695**: Decreased
  - **Northern blot analysis**: Frontal, temporal, occipital cortex (14)
  - **In situ hybridization**: Neurons of nucleus basalis of Meynert, hippocampal subiculum and occipital cortex (75)
  - **In situ hybridization**: Neurons of hippocampal subiculum, occipital cortex and basis pontis (75), nucleus basalis of Meynert (53) and hippocampus (17)

- **hAPP770**: Increased
  - **RT-PCR for APRP-563**: Nucleus basalis of Meynert (53)
  - **In situ hybridization for APRP-563**: Nucleus basalis of Meynert (53)

- **hAPP751**: Decreased
  - **Northern blot analysis**: Frontal cortex (74, 77); parietal cortex (78)
  - **Northern blot analysis**: Hippocampus (80, 81, 83)
that were modified in the construction of the PDGF-hAPP transgene. In addition, the results of the current study indicate that the development of prominent AD-type pathology in PDGF-hAPP mice could be related to (i) the substantial level of hAPP overexpression achieved in this model, (ii) the relatively high proportion of mutated hAPPs versus wild-type mAPPs expressed in their brains, and (iii) the prominent shift toward decreases in the relative abundance of hAPP751 and no significant increase in overall hAPP levels compared with normal controls. However, to condense a disease process that probably takes decades to develop in humans into a substantially shorter time period, potential etiologic factors, such as APP(derivatives), may have to be expressed at significantly higher levels than those encountered in the human disease. This postulate is supported directly by the development of AD-type pathology in the high expressor PDGF-hAPP model (1) and indirectly by the earlier development of AD-type pathology in persons with Down’s syndrome (S) who show roughly 1.5-fold higher levels of cerebral hAPP mRNA expression than controls (3, 4). In addition, evidence is accumulating that the development of AD may involve complex interactions between different pathogenetic pathways, including alterations in the function and/or expression of glia-derived factors such as apoE (61–67), cytokines (68–70), and extracellular matrix components (71–73). The further in vivo dissection of the pathogenetic potential of specific hAPP isoforms, derivatives and mutations as well as of their interactions with such co-factors constitutes a promising field for future investigations.

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