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Genetics

Expression of the human β-amyloid precursor protein gene from a yeast artificial chromosome in transgenic mice

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ABSTRACT **One hallmark of Alzheimer disease is the formation in the brain of amyloid plaques containing a small peptide derived from the β-amyloid precursor protein (APP). The APP gene exhibits a complex pattern of expression in peripheral tissues and in the brain. The entire human APP gene was introduced into embryonic stem (ES) cells by co-lipofection of a 650-kb yeast artificial chromosome (YAC). Three ES lines containing an essentially intact YAC were isolated, and expression of human APP mRNAs at levels comparable to those of endogenous mouse APP transcripts was obtained. A transgenic mouse line was established by germ-line transmission of the APP YAC. RNase protection analysis of human APP mRNAs demonstrated appropriate splicing of the primary APP transcript in ES cells and in the brain of a transgenic animal. These mice may be useful for elucidating the function of the various APP isoforms *in vivo*.**

The senile plaques characteristic of Alzheimer disease (AD) consist of abnormal neurites around an amyloid core primarily composed of a 4-kDa polypeptide, β-amyloid. The β-amyloid peptide is derived from the cellular processing of β-amyloid precursor protein (APP). Mutations in the APP gene have been identified in certain cases of familial AD, suggesting a causal role for β-amyloid deposition in at least some forms of AD (1, 2). The human APP gene is located on chromosome 21, and Down syndrome (DS) individuals overexpress the wild-type APP protein (3). Those who survive past their thirties invariably develop an AD neuropathology (4), and immunoreactive β-amyloid depositions can be found prior to any detectable neuronal degeneration in the DS brain (5). Although APP is expressed at normal levels in non-DS cases of AD, the phenotype seen in DS individuals suggests that AD may be initiated or accelerated by overexpression of APP.

The APP gene is composed of 18 exons distributed over several hundred kilobases (6). Alternative splicing of the primary transcript results in the production of at least five distinct transcripts. Of the three predominant APP isoforms, APP770 and APP751 contain a Kunitz-type protease inhibitor (KPI) domain, and are found at varying levels in many tissues (7, 8). APP695, lacking the KPI domain, is the predominant splice form in the brain and is less abundant in the periphery (7). Other APP variants have been detected at lower levels (9–11). A possible role for the various isoforms in the progression of AD is suggested by changes in AD brains of the ratio of the KPI-containing APP variants to APP695 (12–15). However, it is clear that the APP gene exhibits complex tissue- and cell type-specific expression in human (10) and mouse (16) brain.

The lack of a small-animal model for AD has hampered investigation of the early events in the progression of AD. Expression of human APP751 from a neuron-specific promoter in transgenic mice resulted in extracellular β-amyloid deposits in the cortex and hippocampus but did not result in plaque formation (17). Transgenic mice expressing APP695 (18), the β-amyloid peptide (19), or the C-terminal 100 amino acids (including the β-amyloid region) of APP (20) did not duplicate the pathology of AD. Since these transgene constructs were all cDNA-based, none was capable of producing the full complement of APP isoforms present in various areas of the brain.

Recently, methods for the introduction of yeast artificial chromosomes (YACs) into mice have been developed (21–24). Because genomic-DNA-based transgenes permit expression of alternatively spliced transcripts and are more likely to be appropriately regulated than cDNA transgenes (25), we have introduced a 650-kb YAC containing the entire human APP gene into embryonic stem (ES) cells by the colipofection procedure (24). These ES cells were used to generate transgenic mice expressing the human APP gene.

MATERIALS AND METHODS

Colipofection of ES Cells. The YAC clone B142F9 (Washington University YAC library) contains the entire human APP gene on a 650-kb YAC (26). Yeast chromosomes from this clone were prepared in agarose blocks (3×10^9 cells per ml), and the APP YAC DNA was isolated as described (24). ES cells (AB-1) were maintained on the mitomycin C-treated STO feeder line SNL76/7 (27). Colipofection of ES cells was performed essentially as described (24). Each of the 10 lipofection mixtures contained 12 ng of a linearized selectable marker plasmid [pPGKneoA+R (24)] and 800 ng of the APP YAC. No carrier DNA was used. Genomic DNA was prepared from ES-cell clones as described (28).

Analysis of DNAs from ES-Cell Clones. ES-clone DNAs were pooled by rows and columns (see Fig. 2). DNA pools were screened for human APP promoter and exon 17 sequences by PCR by methods adapted from Fidani *et al.* (29). Oligodeoxynucleotide primers, with sequences given 5′ to 3′, were as follows: promoter forward, pGCTTTTGACGTTGGGGGTTA; promoter reverse, pTTCGTGAACAGTGGGAGGGA; exon 17 forward, pATAACCTCATCCAAATGTCCCC; exon 17 reverse, pGTAACCCAAGCATCATGGAAGC.

Chromosomal DNA was prepared in agarose blocks (24) from the YAC clone B142F9, the parent ES cell line (AB1), and the six ES clones containing both promoter and exon 17 sequences. Partial *Sfi* I digestion analysis of YAC DNA was performed as follows. The yeast chromosome blocks were equilibrated in *Sfi* I buffer lacking Mg^{2+}. *Sfi* I restriction endonuclease was added at various dilutions (0, 0.1, 0.1, and 1 unit), Mg^{2+} was added to 4 mM, and the blocks were incubated for 60 min at 50°C. The digestions were stopped

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Abbreviations: AD, Alzheimer disease; APP, β-amyloid precursor protein; DS, Down syndrome; ES, embryonic stem; KPI, Kunitz-type protease inhibitor; PFGE, pulsed-field gel electrophoresis. YAC, yeast artificial chromosome.

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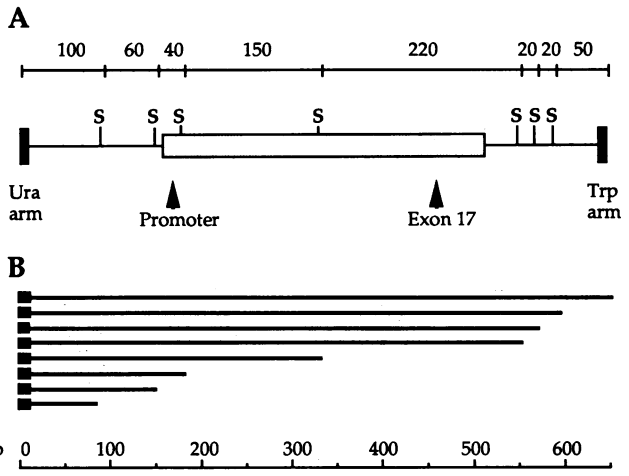

FIG. 1. *Sfi* I restriction map of the 650-kb YAC B142F9. (*A*) Fragments from complete digestion with *Sfi* I were probed with total human DNA. The APP structural gene is ≈400 kb long and is depicted as a box. Orientation of the APP gene was determined by using probes for the promoter and exon 17. (*B*) The *Sfi* I fragments were ordered by partial-digestion mapping. The cartoon depicts the ladder of partial-digest fragments visualized with a probe specific for the acentromeric (Ura) vector arm. The fragment order was confirmed by partial-digest mapping using a centromeric arm-specific probe.

with 50 mM EDTA and analyzed by pulsed-field gel electrophoresis (PFGE) followed by Southern blot analysis with probes specific for the centromeric or acentromeric YAC vector arms (the 2.5-kb and 1.6-kb *Bam*HI–*Pvu* II fragments of pBR322, respectively). PFGE Southern analyses using total human DNA as probe were performed as described (24), except that sheared denatured mouse DNA was added at 0.25 mg/ml to the hybridization solution. ES cell lanes contained 10 μg of genomic DNA, whereas the B142F9 lane contained ≈100 ng of yeast genomic DNA.

Analysis of APP mRNA Expression. Total RNAs were prepared from ES cells by the guanidinium isothiocyanate procedure (30) and from mouse tissues as described (31). Human brain total RNA was purchased from Clontech. Northern blots were prepared with 10 μg of total RNA per lane (30). Reverse transcription–PCR analysis for mouse and human APP alternative splice products was performed with 10 μg of total RNA per reaction mixture and the following primers: mouse APP forward, pCAGGAATTCCACCACTGAGTCCGTGGAG; mouse APP reverse, pCAGGATCCGTGTCTCCAGGTACTTGTCG; human APP forward, pCAGGAATTCCACCACAGAGTCTGTGGAA; human APP reverse, pCAGGATCCGTGTCTCGAGATACTTGTCA. Three PCR products of 377 bp, 322 bp, and 152 bp, corresponding to APP770, APP751, and APP695 transcripts, respectively, were observed. The mouse and human 322-bp PCR products were isolated, digested with *Eco*RI and *Bam*HI, and cloned into plasmid pSP72 (Promega). Northern analysis was performed with an equal mixture of the mouse and human 322-bp fragments described above. RNase protection assays were performed (30) with 25 μg (ES cell) or 10 μg (cerebral cortex) of total RNA with mouse and human APP-specific probes. The 459-bp cDNA fragments of mouse and human APP770 encompassing exons 6–9 were amplified by reverse transcription–PCR from total RNA of ES clone 24 by using the following primers: mouse APP forward, pGCAGTCGAATTCACGAGGTGGAGGAGGAGGCC; mouse APP reverse, pGCTGACGGATCCTCTCTCGGTGCTTGGCTTCC; human APP forward, pGCAGTCGAATTCATGAGGTAGAGGAAGAGGCT; human APP reverse, pGCTGACGGATCCTCTCTCGGTGCTTGGCCTCA. The products were cloned into the *Eco*RI and *Bam*HI sites of pSP72 and linearized at the *Hpa* I site, and 574-nt RNase protection probes were transcribed from the SP6 promoter.

RESULTS

***Sfi* I Restriction Fingerprint of B142F9.** An *Sfi* I restriction fingerprint of the YAC clone B142F9 was produced by PFGE Southern analysis using total human DNA as probe (Fig. 1). The APP gene was ordered within the YAC by probing the complete *Sfi* I digest with probes specific for the promoter and exon 17 (data not shown). The *Sfi* I fragments ranged in size from 20 to 220 kb, and were ordered by partial *Sfi* I digest mapping (Fig. 1) using probes specific for the centromeric or acentromeric YAC vector arms.

Colipofection of ES Cells. Of the 330 primary G418-resistant colonies obtained from the APP YAC colipofections, 240 were picked and expanded in microwell plates for frozen storage and DNA analysis. The clone DNAs were initially pooled by rows and columns and then screened by PCR for APP promoter sequences and exon 17 sequences. Seven of 16 row pools and 6 of 18 column pools were positive for both regions, while 5 row pools and 2 column pools were positive for either promoter or exon 17 sequences (Fig. 2). The clones located at the intersection of double-positive row and column pools were screened individually by PCR. Of the 42 clones analyzed, 6 contained both regions, while 2 contained only the promoter region.

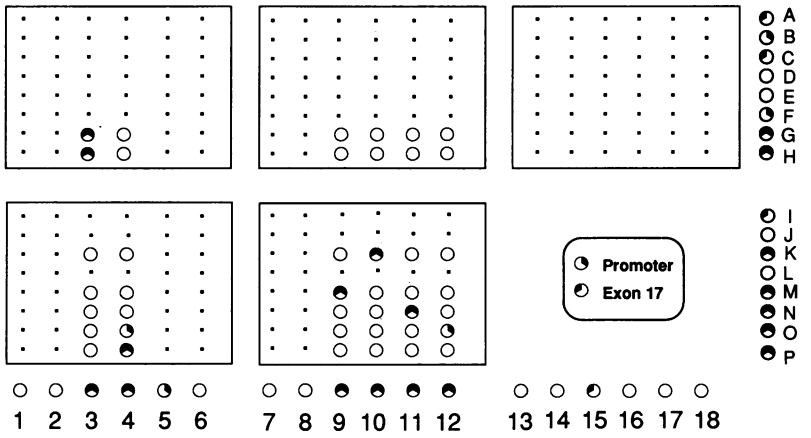

FIG. 2. PCR screen of 240 ES clone DNAs for promoter and exon 17 sequences. ES clone DNAs were pooled by rows and columns. PCR-positive pools are denoted by a filled-in segment of a circle marked by a row pool letter (A–P) or a column pool number (1–18). Clones not analyzed further are represented by a dot. The 42 clones located at the intersection of double-positive row and column pools were analyzed individually and are depicted as per the pool analysis.

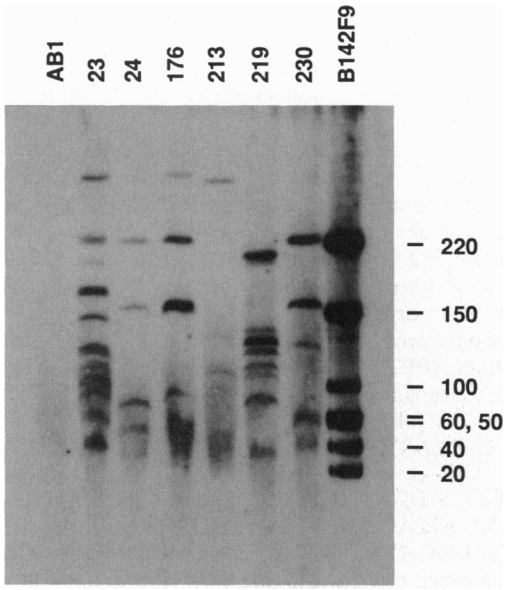

FIG. 3. *Sfi* I restriction fingerprint of ES cell clones. AB1 denotes the parent ES cell line and B142F9 denotes the parent YAC clone. The APP YAC-containing ES lines are denoted by clone number. *Sfi* I fragment sizes are denoted in kb. Total human DNA was used as probe.

Structural Analysis of B142F9 in ES Cells. The *Sfi* I restriction fingerprint of an ES cell line containing an intact YAC would contain the internal *Sfi* I bands, whereas the terminal bands would be shifted in size in accordance with neighboring *Sfi* I sites in the flanking genomic DNA. The six doubly positive clones (nos. 23, 24, 176, 213, 219, and 230) were analyzed by *Sfi* I restriction fingerprint analysis (Fig. 3). Lines 23 and 219 display a clearly aberrant *Sfi* I banding pattern containing many novel bands, presumably arising from integration of YAC fragments. Line 213 lacks most of the *Sfi* I bands found in the parent B142F9 YAC. Lines 24, 176, and 230 all show an *Sfi* I restriction fingerprint consistent with integration of an intact YAC. The 220-kb and 150-kb bands are clearly present, whereas the 100-kb terminal fragment is absent. Although loss of resolution and mobility shifting of bands below 100 kb due to overloading was evident, the 60-kb and 40-kb bands are present, while the 20-kb bands are not resolvable or are missing. Unique bands of 300 and 130 kb are found in lines 176 and 230, respectively, and may be the novel bands expected from integration of the terminal YAC fragment. Other terminal bands are not detected and may be lost in the unresolved region below 50 kb.

The *Sfi* I restriction fingerprints of lines 24, 276, and 230 are consistent with integration of an essentially intact B142F9 YAC, although some sequences may have been lost from the ends of the YAC, as has been described (32). Although it is possible that these ES cell lines contain more than one copy of the intact YAC, previous lipofections of YACs into ES cells indicated that, in most cases, one or less than one copy of the YAC had integrated (21, 24). Since the mouse APP gene is expressed in the preimplantation embryo, the ES cell lines were screened for expression of mouse and human APP mRNAs to confirm integrity of the APP transgene.

Expression Analysis of Human APP in ES Cells. Total RNAs were prepared from the six clones and the parent ES cell line (AB1) and analyzed by reverse transcription–PCR for human APP transcripts. The APP primary transcript is processed into three predominant splice products encoding species of 770, 751, and 695 amino acids. APP770 contains exons 7 and 8, APP751 lacks exon 8, and APP695 lacks exons 7 and 8. By use of primers specific for exons 6 and 9, all three mRNA forms can be detected by reverse transcription–PCR (10). Because human and mouse APP cDNAs are >90% similar at the nucleotide level, species-specific primers were chosen which differed at the 3′-terminal nucleotide. Human APP770, APP751, and APP695 splice products were detected by reverse transcription–PCR in the three ES lines (nos. 24, 176, and 230) with an intact *Sfi* I restriction fingerprint (data not shown). No human APP specific transcripts could be detected in the parental ES line or in the three ES lines with an aberrant *Sfi* I pattern, in agreement with the restriction fingerprint analysis.

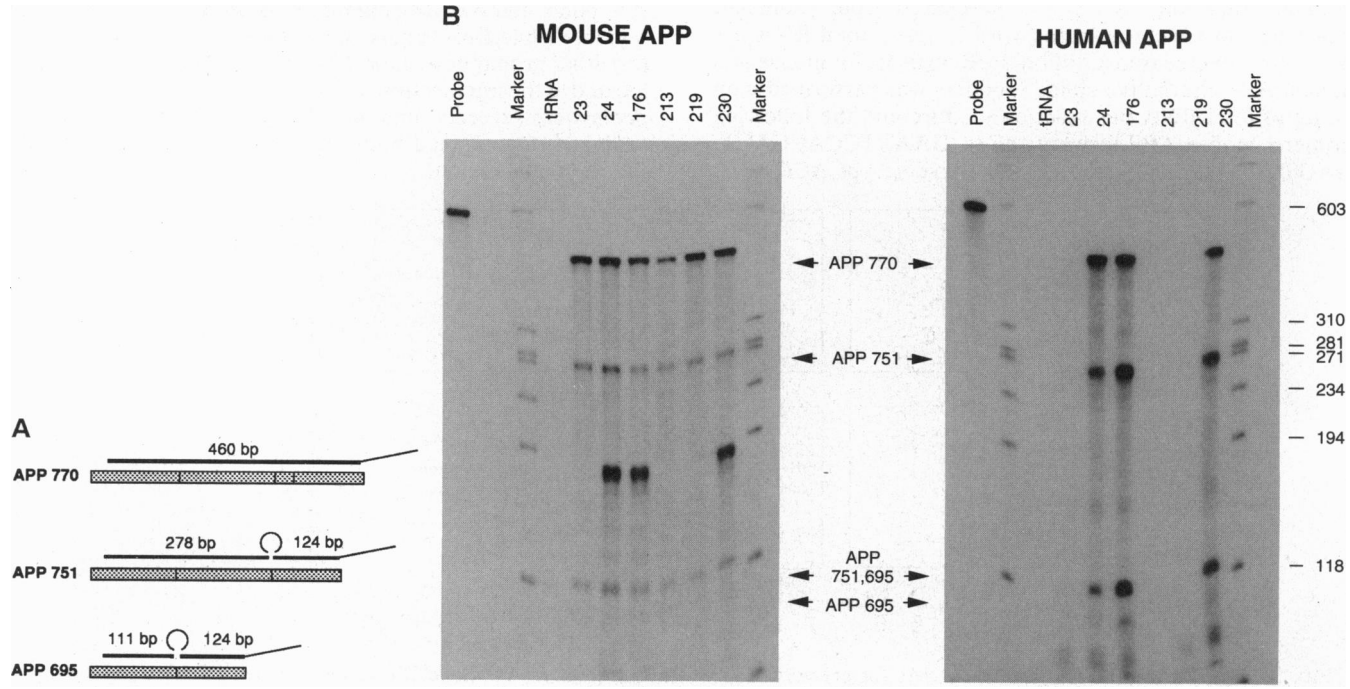

FIG. 4. APP expression in ES cells. (*A*) Expected RNA probe fragments from the three predominant APP transcripts. (*B*) RNase protection analysis of mouse and human APP transcripts in ES lines. Marker lanes show end-labeled *Hin*fI-digested ϕX174 DNA fragments.

Northern analysis of the six ES cell lines was performed with a mixed probe against both mouse and human APP mRNAs (*Materials and Methods*). A single band of 3.4 kb was detected in all lines, consistent with full-length human APP transcript production. The 3.4-kb band was approximately twice as intense for lines 24, 176, and 230 as for the other three lines (data not shown).

To better quantitate the APP transcripts produced from the human and mouse APP genes, RNase protection assays were performed. The APP770 transcript is expected to produce a 460-nt protected fragment, whereas the APP751 transcript would produce 278- and 124-nt protected fragments, and the APP695 transcript would produce 111- and 124-nt protected fragments (Fig. 4*A*). RNase protection analysis of the six ES lines with the mouse APP probe demonstrated that all six lines produced a high level of APP770 transcript, a low level of APP751 transcript, and traces of APP695 transcript (Fig. 4*B*). The ≈190-nt band seen in samples from lines 24, 176, and 230 are presumably due to partial protection of human APP transcripts. As expected, human APP transcripts were seen only for ES lines 24, 176, and 230 (Fig. 4*B*). The level of human APP mRNA was equivalent in all three ES lines, suggesting position-independent expression of human APP in ES cells. Further, the overall level of human APP transcription was comparable to that of mouse APP expression. Although highly suggestive of completely authentic APP expression, assessment of position independence and copy-number dependence in different tissues and stages of development awaits analysis of multiple transgenic lines containing one or more copies of the human APP transgene. As was seen for mouse APP, a high relative level of APP770 transcript and a trace relative level of APP695 transcript were evident. A slightly higher relative level of human APP751 transcript was detected than was seen for mouse APP751.

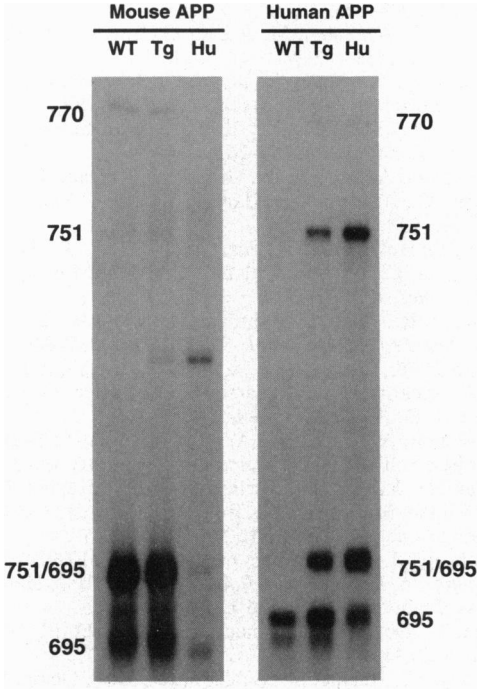

FIG. 5. APP expression in transgenic mouse cerebral cortex. The RNA probe used is indicated by human APP and mouse APP, respectively. WT, Tg, and Hu denote an 8-week-old nontransgenic mouse, a line 230 transgenic littermate, and total human brain RNA, respectively. The mouse- and human-specific RNA probes are >90% similar in APP nucleotide sequence, and weak cross-hybridization can be detected with the mouse APP RNA probe against the human brain control RNA and with the human APP RNA probe against the wild-type mouse cerebral cortex RNA. The lower band for human APP695 is slightly larger than the corresponding mouse APP695 band and is presumably an artifact of the assay, since the size variation is also seen in the human brain RNA.

Expression Analysis of Transgenic Mice. ES cells from lines 24, 176, and 230 were injected into blastocysts to generate six, three, and four chimeric founder animals, respectively. Coat color chimerism ranged from 5% to >90%, and seven of these animals have been paired with wild-type mates. Germline transmission has been obtained from a line 230 chimeric founder. Of the eight F_2 offspring, four contained the YAC (data not shown). Southern analysis for plasmid sequences demonstrated a single large (>20-kb) *Eco*RI band which cosegregated with the YAC (data not shown), indicating that the colipofected plasmids had inserted at or near the YAC integration site, as has been observed previously for colipofection (24). RNase protection assays revealed human APP expression in the cerebral cortex of a line 230 transgenic mouse (Fig. 5). In the frontal brain, APP695 is the predominant splice product for human and mouse APP (10, 33). Abundant expression of human APP695 mRNA was evident in the transgenic brain, consistent with that seen for the endogenous mouse APP gene. Although not strictly comparable, the relatively high level of human APP751 mRNA in the transgenic mouse brain RNA is more akin to that seen in the control human brain RNA than to that seen in the wild-type mouse brain RNA (Fig. 5) and may reflect a more human pattern of brain APP expression from the YAC (10).

DISCUSSION

Understanding the functional role of the APP has been hindered by the complex expression pattern of APP isoforms in the periphery and in the various substructures of the brain. Experiments which perturbed the balance of APP isoforms in transgenic mice by expression of particular APP splice products (17, 18) or APP fragments (20) have met with limited success in producing an AD-like pathology. However, the finding that the APP gene is located on chromosome 21 and that DS individuals develop a neuropathology indistinguishable from AD in their thirties or forties suggests that overexpression of a normal complement of APP isoforms may lead to an acceleration of AD.

Mouse chromosome 16 is syntenic with human chromosome 21 over a broad region, including the APP gene and the region implicated in DS. Transplants of embryonic brain tissue from trisomy 16 mice (which do not survive to term) into normal adult mouse brain resulted in some of the characteristics seen in the degenerating neurons of AD brains (34). Development of an AD-like phenotype by overexpression of APP may require rigorously authentic expression of the various APP isoforms in the various cell types of the brain. Because genomic-DNA-based transgenes permit expression of differential splice products and are more likely to be appropriately expressed (25), we have introduced the entire human APP gene into mouse ES cells and transgenic mice.

Essentially equivalent expression of human APP mRNA was observed in the three ES cell lines with an intact APP YAC. Position-independent expression has rarely been achieved with conventional transgenes (35). However, among the four recent reports of transgenic mice carrying YAC (21–24), two (21, 22) have shown position-independent transgene expression, and three (21–23) have shown transgene expression comparable to that of the endogenous gene.

The predominant APP transcripts in the ES cell are the KPI domain-containing APP770 and APP751 splice products, with only a trace level of APP695 mRNA. ES cells are derived from the inner cell mass of 3.5-day blastocyst-stage mouse embryo, and in a survey of APP expression during early mouse development, Fisher *et al.* (36) found that the APP695

isoform constituted ≈60% of APP mRNA in the 3.5-day mouse embryo. Presumably, other non-ES cell types in the blastocyst (such as the trophectoderm) contribute significant amounts of APP695 transcript. Thus, differential expression of APP isoforms may occur as early as the 64- to 128-cell blastocyst stage of development.

Although the levels of mouse and human APP770 transcripts were comparable in the three ES cell lines the level of human APP751 transcript was slightly higher than the level of mouse APP751 transcript. This difference may reflect either a bias for processing of the primary human APP transcript toward the APP751 splice product or a difference in stability between mouse and human APP751 mRNAs. A tissue-specific pattern of differential splice products implies that there are both cell type-specific, trans-acting factors which modulate the processing of the primary transcript or the stability of the processed transcript, as well as sequences on the primary or processed transcript recognized by these factors, and it is possible that the higher relative level of human APP751 mRNA in the mouse cellular environment reflects a more human pattern of APP expression. This bias in APP751 expression is also seen in the transgenic cerebral cortex. The consistency in the pattern of expression of the human and mouse APP genes in cell types as diverse as ES cells and those of the brain suggests authentic tissue-specific expression of human APP isoform mRNAs from the YAC transgene.

We have demonstrated human APP transgene expression at the level of transcription. If expression of human APP isoforms is obtained at the protein level as well, these transgenic mice may be useful for studying the role of the different APP isoforms in the context of the whole animal. No significant loss of viability or behavioral phenotype has been observed in the chimeric founder mice or in the fully transgenic offspring. As these animals age, it will be of great interest to track human and mouse APP expression in the transgenic brain by immunohistochemistry. Homozygosis of the YAC transgene and interbreeding with mice carrying the other two integration sites of the APP YAC would permit the derivation of mice carrying up to six copies of the human APP gene. If copy-number-dependent expression of the transgene is obtained, multiple copies of the human APP transgene may result in an accelerated development of an AD-like phenotype in mice.

Note Added in Proof. Lamb *et al.* (26) have reported on transgenic mice expressing the APP gene from a YAC.

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