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Discovery of a brain promoter from the human transferrin gene and its utilization for development of transgenic mice that express human apolipoprotein E alleles

BARBARA H. BOWMAN*†, LAILA JANSEN*, FUNMEI YANG*, GWENDOLYN S. ADRIAN*, MING ZHAO*, SALLY S. ATHERTON*, JAMES M. BUCHANAN*, RHONDA GREENE*, CHRISTI WALTER*, DAMON C. HERBERT*, FRANK J. WEAKER*, LAURA K. CHIODO‡, KATHLEEN KAGAN-HALLET§, AND JAMES E. HIXSON*¶

Departments of *Cellular and Structural Biology, ‡Medicine, and §Pathology, University of Texas Health Science Center, San Antonio, TX 78284-7762; and ¶Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX 78284

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ABSTRACT **Transgenic mice carrying heterologous genes directed by a 670-bp segment of the regulatory sequence from the human transferrin (TF) gene demonstrated high expression in brain. Mice carrying the chimeric 0.67kbTF-CAT gene expressed TF-CAT in neurons and glial cells of the nucleus basalis, the cerebrum, corpus callosum, cerebellum, and hippocampus. In brains from two independent TF-CAT transgenic founder lines, copy number of TF-CAT mRNA exceeded the number of mRNA transcripts encoding either mouse endogenous transferrin or mouse endogenous amyloid precursor protein. In two transgenic founder lines, the chloramphenicol acetyltransferase (CAT) protein synthesized from the TF-CAT mRNA was estimated to be 0.10–0.15% of the total soluble proteins of the brain. High expression observed in brain indicates that the 0.67kbTF promoter is a promising director of brain expression of heterologous genes. Therefore, the promoter has been used to express the three common human apolipoprotein E (apoE) alleles in transgenic mouse brains. The apoE alleles have been implicated in the expression of Alzheimer disease, and the human apoE isoforms are reported to interact with different affinities to the brain β-amyloid and tau protein *in vitro*. Results of this study demonstrate high expression and production of human apoE proteins in transgenic mouse brains. The model may be used to characterize the interaction of human apoE isoforms with other brain proteins and provide information helpful in designing therapeutic strategies for Alzheimer disease.**

The complete human transferrin (TF) gene demonstrates strong expression in liver and brain and to a lesser extent in other tissues (1–4). In transgenic mice, we studied expression of heterologous genes directed by different size constructs of the 5′ regulatory region of the TF gene (5). The 0.67-kb and 1.2-kb fragments of the 5′ regulatory region of the TF gene directed high expression of heterologous genes in brains of different independent transgenic founder lines (5, 6). High expression of the TF-CAT gene persisted and increased slightly in transgenic mice brains throughout the aging process (5).

Because of the reproducibility of the 0.67kb TF promoter to direct high expression of heterologous genes in the brain, it was utilized to direct expression of the human apolipoprotein E (apoE) alleles apoE-2, -3, and -4 in brains of transgenic mice. The development of transgenic mice carrying human apoE genes was performed because of recent studies that demonstrated a correlation between the presence of the apoE-4 allele with development and decreased age of onset of Alzheimer disease (AD). In AD families, the presence of a single E-4 allele was reported to increase the member's risk of developing AD from 20% to 47% (7, 8). In 115 AD cases, homozygous apoE-4/4 was carried by 16%, while apoE-4/4 occurred in only 2% of 243 controls. ApoE-3/4 was carried by 49% of AD subjects, while 22% of controls had this genotype (8). ApoE-4 protein binds β-amyloid protein *in vitro* to a greater extent than apoE-2 or apoE-3 (9). β-amyloid, a proteolytic fragment of the amyloid precursor protein (APP), is implicated in the pathology of AD.

In contrast, evidence that the apoE-2 allele is associated with human longevity and may be protective against AD has been reported (10). In 115 AD patients the heterozygous apoE-2/3 genotype occurs in 1% of the cases, while 16% of controls carry this genotype. ApoE-3 is also reported to have some protective properties. Evans *et al.* (11) demonstrated that at physiological concentrations apoE-3 inhibits amyloid nucleation *in vitro*. Nucleation is the rate-limiting phase in fiber formation. Thus, apoE-3 may protect against the formation of neurotoxic amyloid fibrils in the brain.

Another idea was put forward by Roses (12) that implicates tau, an intracellular brain protein found in tangles within specific neurons of most AD patients. Roses' suggestion was that tau is the interacting target for apoE-2 and -3 and that apoE-2 and apoE-3 but not apoE-4 protect tau so that hyperphosphorylation of tau occurs slowly. Otherwise, hyperphosphorylated tau forms tangles within neurons. There is supporting data of tau binding to apoE-3 but not to apoE-4 *in vitro* (13). High brain expression of the chloramphenicol acetyltransferase (CAT) reporter gene directed by the 0.67kbTF construct is described here. Application of the 0.67kb TF promoter to express the three human apoE alleles and to synthesize human apoE isoforms in transgenic brains is described.

MATERIAL AND METHODS

TF-ApoE Constructs. The preparations of the 0.67kbTF-CAT constructs were described previously (5). Human apoE-3 cDNA was placed under the transcriptional control of the TF promoter by insertion into a plasmid (p0.67kbTF) containing a segment of the TF promoter (position −621 to +46 in the 5′ untranslated region), and the simian virus 40 (SV40) polyadenylylation signal and small intron from the *Sca* I to *Bam*HI sites in pSV0CAT (14). The plasmid p0.67kbTF was con-

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Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein; apoE, apolipoprotein E; TF, human transferrin; mTf, mouse transferrin; SV40, simian virus 40; RT-PCR, reverse transcription–PCR.

†To whom reprint requests should be addressed at: Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7762.

structed from p0.67kbTF-CAT (5) that originally contained a CAT gene. To remove the CAT gene, 0.67kbTF-CAT was digested with *Sal* I and *Sca* I. Synthetic *Xho* I linkers were added at the *Sca* I site, and synthetic *Sal* I/*Xho* I adapters were added at the *Sal* I site, resulting in a single cloning site for *Xho* I and *Sal* I. The apoE-3 insert of pE368 plasmid containing full-length cDNA (15) was amplified by using a forward primer from the apoE 5′ untranslated region with a mismatch to introduce a *Sal* I site (primer A: 5′-CCAGGAGTCGACTG-GCCAATCAC-3′) and a reverse primer from the apoE 3′ untranslated region with mismatches to create an *Xho* I site (primer D: 5′-GGTCGCATGGCTCGAGGCTTCG-3′). PCR reactions used Vent polymerase (New England Biolabs) for 25 cycles of denaturation (95°C for 1 min), and combined annealing and elongation (70°C for three min). The amplified apoE-3 cDNA was then ligated into the *Xho* I/*Sal* I site of p0.67kbTF between the TF promoter and the SV40 sequences.

To generate the apoE-4 construct, apoE-3 cDNA (pE368) was subjected to site-specific mutagenesis using the PCR-based overlap extension method (16, 17). The 5′ overlapping DNA fragment was generated by PCR amplification with forward primer A and a mismatched reverse primer containing the T → C substitution encoding arginine at position 112 (primer B: 5′-GCACCAGGCGGCCGCGCACGTCCTC-CATGTC-3′). PCR conditions included 25 cycles of denaturation (95°C for 40 sec), annealing (55°C for 40 sec), and elongation (72°C for one min). The 3′ overlapping DNA fragment was generated by PCR amplification with a mismatched forward primer from apoE (primer C: 5′-GACAT-GGAGGACGTGCGCGGCCGCCTGGTGC-3′) and reverse primer D by using the same PCR conditions. The two overlapping PCR fragments were gel purified, mixed together (0.5 μg of each), and used for PCR amplification with primers A and D. The resulting PCR product was digested with *Xho* I and *Sal* I (sites in primers A and D), and ligated into p0.67kbTF. The apoE-2 construct was made in a similar fashion. All of the TF-apoE constructs were sequenced to verify the desired apoE substitutions and to exclude any clones with PCR-generated errors.

Identification of Transgenic Mice by Southern Blot Analysis. Identification of the TF-CAT gene in transgenic mouse DNA has been described (5, 18). Transgenic mice carrying the human apoE genes were identified by PCR of the *Hha* I-digested apoE products (19). Estimation of copy numbers of the human apoE genes in transgenic mice was determined by Southern analysis (20) of DNA from mouse tails digested with *Sac* I and *Hpa* I. This generated a specific 2-kb DNA fragment in transgenic mice. The TF-apoE constructs were used for probes in Southern blot hybridization.

Transgenic Mice. Transgenic mice were developed by using the techniques of Gordon and Ruddle (21). The procedures used to develop TF-CAT lines in a background of C57BL/6 have been described (5). The percentage of TF-CAT protein produced in TF-CAT transgenic mouse brains was calculated on the basis of the known specific activity of pure CAT protein, which is 125 units per mg of CAT protein (22). One unit converts 1 μmol of [^{14}C]chloramphenicol per minute.

In this study, we describe the development of human apoE transgenic mice directed by 0.67kbTF. Four independent founder lines carry the human apoE-3 transgene, six founder lines carry human apoE-4 transgene, and three founder lines carry human apoE-2 transgene. The human apoE-4 transgene is in the congenic mouse C57BL/6J background, and the apoE-3 and -2 transgenes are in the hybrid CB6/F2 background. In hybrid lines, the proportion of embryos that are suitable for microinjection is higher than in congenic lines and more transferred embryos are born in hybrids. The apoE-3 transgene has recently been transferred into the C57BL/6J mouse line. The apoE-2 will also be transferred into the congenic C57BL/6J mouse line for future studies because of this line's genetic uniformity. The apoE-3 lines contain from 12 to 30 copies of the human apoE-3 gene. The apoE-4 lines contain from 10 to 40 copies of the human apoE-4 gene. ApoE-2 transgenics contain 2–4 copies of the human apoE-2 gene. Only hemizygous apoE mice were used in this study.

Sequencing of Brain RNA from ApoE-3 Transgenic Mice. To characterize human apoE transcripts in brains from four apoE-3 transgenic mice, total RNA was isolated from brain tissues and used for reverse transcription (RT) and PCR (RT-PCR). The RT-PCR products were cloned into the pGEM-T vector (Promega), and two clones from each of the transgenic mice were sequenced.

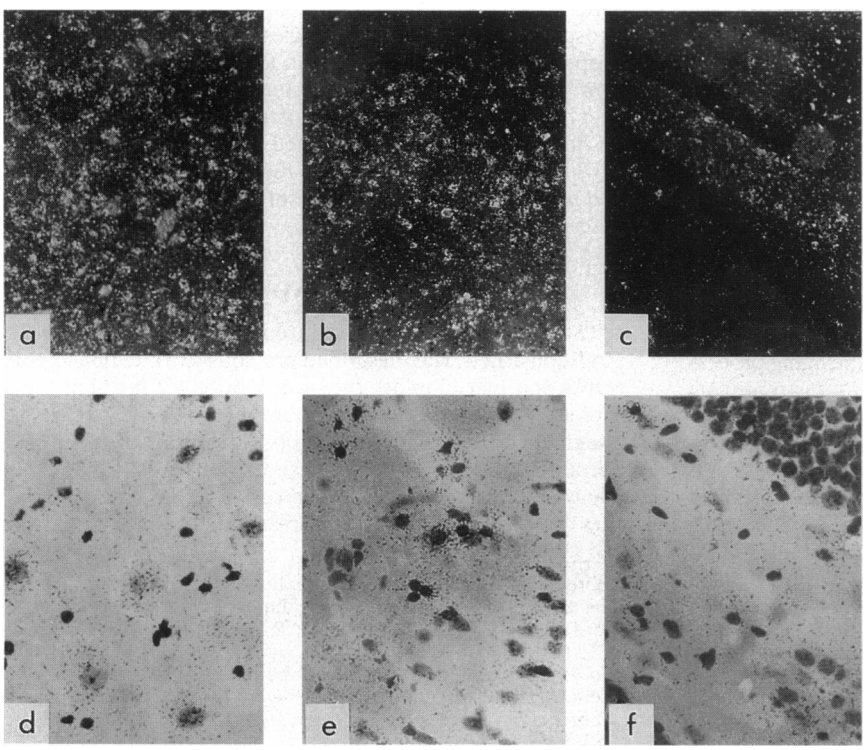

FIG. 1. TF-CAT expression in distinct areas of the brain in transgenic mice. *In situ* hybridization was carried out in sagittal sections of the brain of a 0.67kbTF-CAT transgenic mouse. In dark-field microscopy, labeling of mRNA appears as white grains in the nucleus basalis (*a*), in the cerebral cortex (*b*), and around the hippocampus (*c*). (*a–c*, ×900.) In bright-field microscopy, labeling of mRNA appears as dark grains in the nucleus basalis (*d*), the cerebral cortex (*e*), and around the hippocampus (*f*). Controls included hybridization with sense TF-CAT probes, hybridization of nontransgenic brains with anti-sense TF-CAT probes, and hybridization of anti-sense probes with sections of transgenic mouse liver (not shown). (*d–f*, ×3600.)

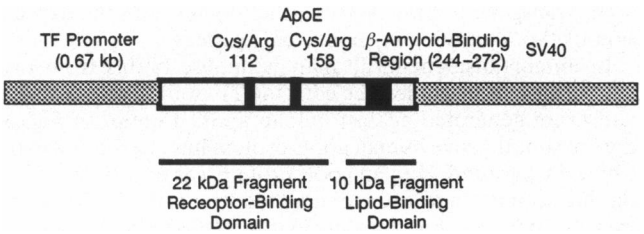

FIG. 2. Constructs microinjected into mouse embryos contained the DNAs encoding the three common human apoE alleles directed by the 0.67kbTF promoter. Genetic changes of arginine and cysteine at residues 112 and 158 account for the three allelic products. Construction of transgenes is described in *Material and Methods.*

Analysis of the Expression of the TF-CAT and the TF-ApoE Genes in Transgenic Mouse Brains by *in Situ* Hybridization. The expression of TF-CAT and TF-apoE genes in brains of the transgenic mice was analyzed by *in situ* hybridization histochemistry by using the technique of Zeller and Rogers (23).

Northern Blot Analysis. Expression of the human apoE transgenes was also followed by analysis of apoE RNA levels in brain following the method of Chomczynski and Sacchi (24). RNA was prepared from tissue (25), and the probe used for Northern analysis was human apoE cDNA. The TF-CAT probe used for Northern blot analysis has been described (5). Probes for mouse APP and mouse endogenous transferrin (mTf) were generated by PCR. Quantitation of RNA bands was performed using the Betascope 603 (Betagen, Waltham, MA) and the PhosphorImager (Molecular Dynamics).

Immunoblot. Extracts from mouse brains were prepared by methods previously described (26, 27). The primary antibody was polyclonal human anti-apoE from Calbiochem, and the secondary antibody was biotinylated rabbit anti-goat IgG (Pierce). The human apoE antibody preparation did not react with mouse serum or tissue. The recombinant human apoE used as a standard was from Calbiochem.

Immunohistochemical Analysis. A modification of the immunohistochemical method of Hsu *et al.* (28) in which staining was with alkaline phosphatase substrate was used. Brains from two apoE-3 transgenic mice and their nontransgenic littermates were examined. Appearance of stain, indicative of human apoE protein, was monitored microscopically, and the reaction was stopped when the red color development was maximal.

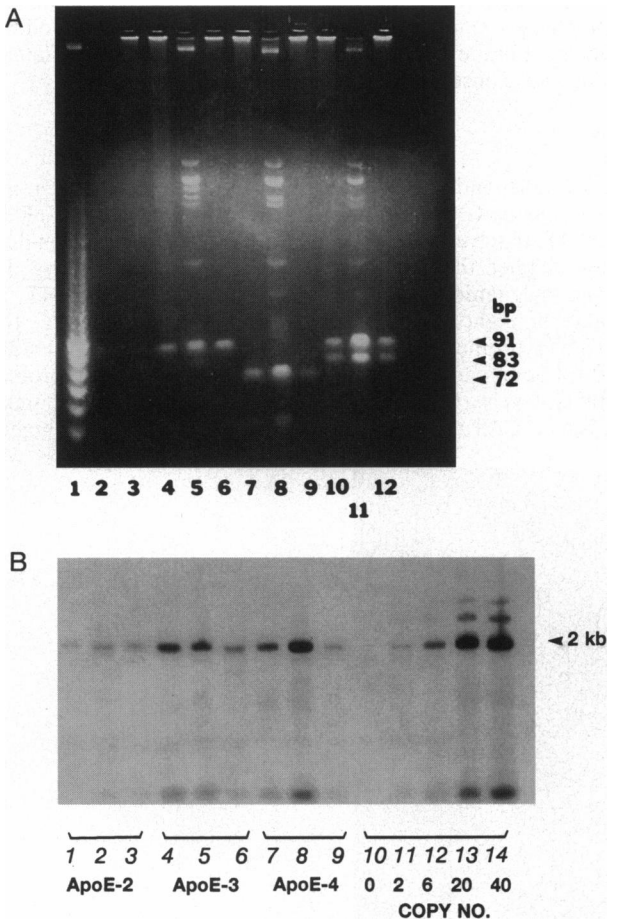

FIG. 3. (*A*) Identification of human apoE alleles in transgenic mice by PCR product patterns. DNA from transgenic mouse tails amplified by PCR gave specific patterns previously described (19) with apoE-3 in lanes 4 and 6 (one 91-bp band), apoE-4 in lanes 7 and 9 (one 72-bp band), and apoE-2 in lanes 10 and 12 (one 83-bp band). (*B*) Estimation of transgene copy numbers by Southern analysis. DNA from three transgenic founders, each carrying one of the three human apoE alleles, was used to estimate the copy number of the genes in each founder by comparison with the band densities of apoE plasmid dilutions shown at the right side of the figure. The approximate copy number of human apoE-2 was 1–4 (lanes 1–3); apoE-3 copy number was 5–15 (lanes 4–6); and apoE-4 copy number was 5–40 (lanes 7–9).

RESULTS

The construction of 0.67kbTF-CAT has been described (5). Data from Northern blot analysis demonstrated the expression of the 0.67kbTF-CAT chimeric gene in brains and livers of two independent lines of transgenic mice. The 0.67kbTF-CAT expression was compared with two endogenous mouse genes: mouse APP and mTf. The TF-CAT gene expression was measured by quantitating copy numbers of 0.67kbTF-CAT mRNA, mouse APP mRNA, and mTf mRNA. In transgenic line 243, brain expression of TF-CAT was 3.7-fold higher than endogenous mouse APP and 3-fold higher than mTf. In transgenic line 262, TF-CAT expression was 2.5-fold higher than mTf and 1.9-fold higher than the mouse APP. The results verified that the 0.67kbTF promoter drives the expression of heterologous genes at a high level in brain. Of the total soluble protein extracted from brains of two different TF-CAT trans-

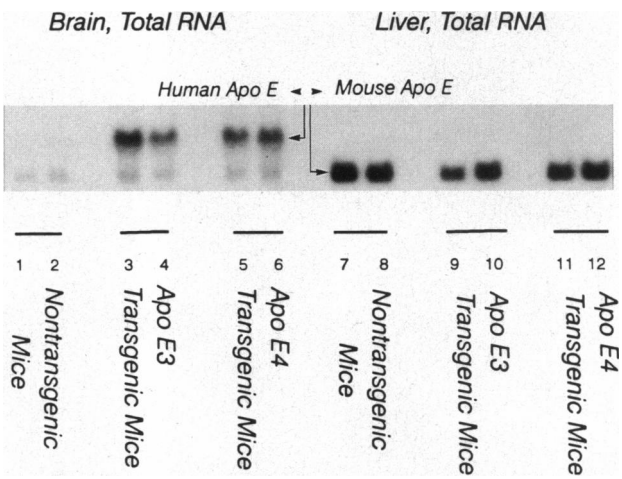

FIG. 4. Expression of the human apoE-3 and apoE-4 transgenes directed by the 0.67kbTF promoter in transgenic brains. Northern blot analysis demonstrates the expression of apoE in brain and liver of transgenic mice compared with expression in nontransgenic littermates. Size difference between the human apoE mRNA and the endogenous mouse apoE mRNA bands is due to the presence of the untranslated SV40 sequences and untranslated TF RNA sequences in the human apoE transgene mRNA. RNA patterns from two transgenic mice of each apoE-3 and -4 genotypes and two nontransgenic littermates are shown.

genic founder lines (A15III and A19III), CAT protein was calculated to be 0.15% and 0.10%, respectively. Expression of TF-CAT in lines A15III and A19III has been described (5).

The mTf gene is expressed mainly in oligodendrocytes (29). *In situ* histohybridization analysis, in contrast, showed that the TF directed CAT gene was expressed in glial and neuronal cells in the nucleus basalis, in the cerebral cortex, around the hippocampus (Fig. 1), and in other brain regions, including cerebellum (data not shown).

The map of the 0.67kbTF-apoE DNA constructs introduced into the mouse genome is shown in Fig. 2. Constructs of the three human apoE alleles directed by the 0.67kbTF promoter contained the signal peptide of human apoE and cDNAs containing the codons specific for the apoE-2, -3, and -4 alleles. Each construct contained the SV40 polyadenylylation signal and small intron. DNA in transgenic mice specifying each human apoE allele was confirmed by the technique described by Hixson and Vernier (19). The nucleotide sequences of the brain RT-PCR products from four transgenic apoE-3 mice were identical with the sequence of human apoE-3 cDNA.

Transgenic mice carrying the TF-apoE alleles were produced and were analyzed by PCR and Southern blots of DNAs taken from their tails. PCR analyses identifying the PCR product pattern for the three human apoE alleles (apoE-2, -3, and -4) demonstrated specific PCR product patterns because of restriction sites introduced by the nucleotide base substitutions in the polymorphic genes (19). Fig. 3 shows PCR patterns and Southern blot analyses of representative transgenic mouse lines carrying human apoE-2, -3, or -4 alleles. Human apoE gene copy numbers in transgenic mice are given in the legend of Fig. 3.

In situ tissue hybridization demonstrated that the 0.67kbTF promoter directed apoE-3 expression in the brain of a 3-month-old mouse in glial cells and, to a lesser extent, neurons from the cerebral cortex, fimbria hippocampi, and the cerebellum (data not shown).

Expression of human apoE-3 and apoE-4 in brains of transgenic mice was validated by Northern blot analysis. Fig. 4 shows overexpression of the human apoE-3 and -4 transgenes in brains of transgenic mice. The expression of the human apoE transgene in brain was ≈2-fold higher than the expression of the mouse endogenous apoE gene.

In immunohistochemical analysis, mouse brains from two 3-month-old transgenic mice carrying TF-apoE-3 reacted with antiserum generated against human apoE protein. In Fig. 5, the immunoreactive human apoE protein has a red color upon a blue background. Human apoE protein was observed in both the intracellular and extracellular regions in the brain of the transgenic mouse. Fig. 5*A* demonstrates cells in the hippocampus that contain human apoE protein. Red color stains cells around the hippocampus where the human apoE protein is synthesized. Human apoE protein was also observed in the cerebellum and other areas of the brain. No indication of human apoE was observed in the nontransgenic littermate (Fig. 5*B*).

Immunoblot analysis of brain homogenates with the polyclonal antibody preparation directed against human apoE revealed human apoE protein in the brain of two 3-month-old transgenic mice carrying the human apoE-3 allele. An immunoblot from brain extracts from a transgenic mouse and a nontransgenic littermate is shown in Fig. 6. The nontransgenic littermate did not demonstrate human apoE immunoreactivity. The concentration of the human apoE protein in the transgenic brain extract is estimated to be 0.13% of the extracted protein. The estimate is based on comparisons on the same membrane of immunoblots of known amounts of human apoE and mouse brain extracts.

DISCUSSION

In 10 independent lines of transgenic mice in which the heterologous CAT gene was directed by either 0.67kbTF or 1.2kbTF, tissue expression of CAT in brains was approximately 6-fold higher than in liver (5). When used to express the O^6-methylguanine-DNA methyltransferase gene (MGMT) in brains of eight independent lines of transgenic mice, the 0.67kbTF promoter increased MGMT protein activity in brain 150-fold compared with endogenous mouse MGMT protein activity levels (6). In this study, expression of the human 0.67kbTF-CAT transgene was shown to be significantly higher

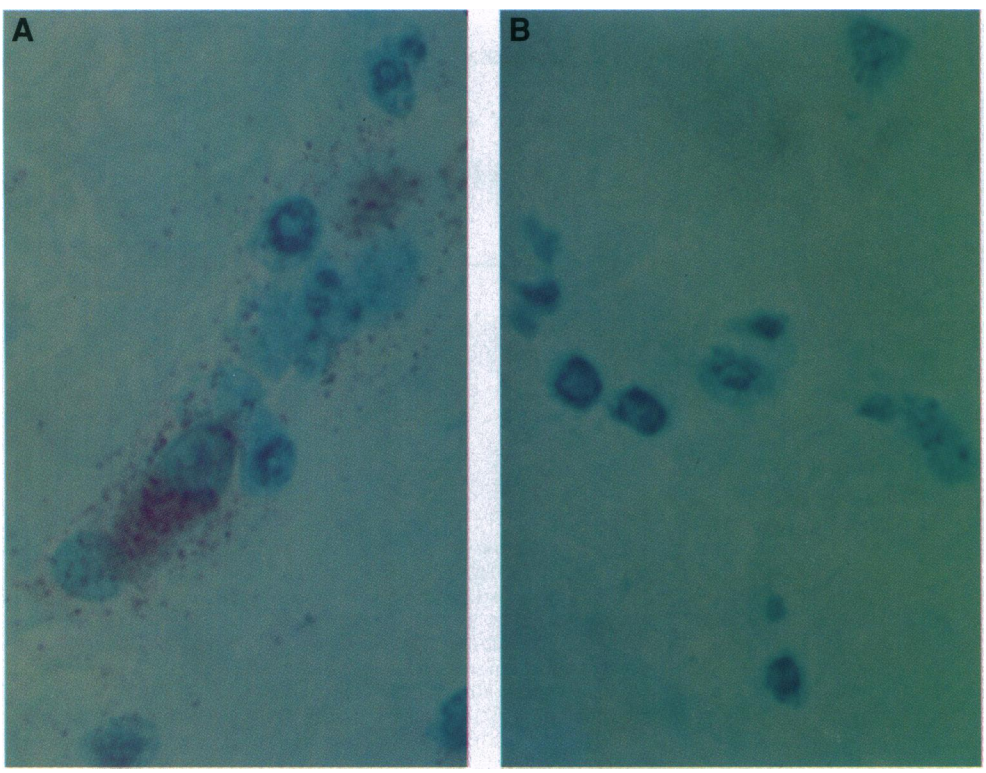

FIG. 5. Human apoE protein is detected in transgenic brains by immunohistochemistry. Human apoE protein–antibody reactions have a red color on a blue background. (*A*) Immunoreaction with human apoE-3 protein and the antibody preparation to human apoE is shown in the hippocampus of the brain of a 3-month-old transgenic mouse. (*B*) No human apoE is observed in the brain of a nontransgenic littermate. (*A* and *B*, ×1600.)

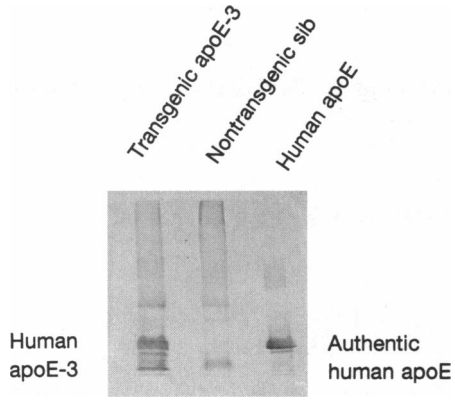

FIG. 6. Human apoE protein is present in extracts of brains of transgenic mice. Immunoblot of a brain extract from a human apoE transgenic mouse illustrates the immunoreaction between apoE antibodies specific for human apoE and human apoE-3 protein in the transgenic mouse brain. Human apoE was absent from the brain of a nontransgenic littermate.

when compared with the expression of the mTf and mouse APP genes. An mTf transgene constructed with the mouse 3kbTf directing the human growth hormone DNA was expressed in brain also, mainly in the oligodendrocytes (29). Unlike the TF-CAT and TF-apoE transgenes, the mTf gene is expressed mainly in oligodendrocytes (29). This is not surprising since the human 0.67kbTF and the corresponding mTf gene 5′ DNA sequences differ significantly (5, 30). The elevated brain expression and lower liver expression directed by the 0.67kbTF promoter may be due to the absence of a liver enhancer, which is located at approximately −3.5 kb 5′ to the 0.67kbTF sequence (31, 32).

Endogenous apoE is produced mainly by the astrocytes of the brain (27) and is the major transport protein of lipids in the cerebral spinal fluid (33). The most prevalent apoE alleles in human populations are apoE-2, -3, and -4 (14, 34). ApoE-4 differs from apoE-3 in that the arginine at residue 112 in apoE-4 has been replaced by cysteine in apoE-3. ApoE-2 differs from apoE-3 at residue 158, where cysteine in apoE-2 is replaced by arginine in apoE-3. The basis of the positive effect of the apoE-4 allele upon late onset or sporadic AD in susceptible individuals is unknown but may relate to a difference in protein folding as a result of the amino acid substitution at residue 112. The carboxyl-terminal domain of apoE-4 binds β-amyloid, which may be transported into neurons where it is toxic. The amino-terminal apoE domain binds to the α_2-macroglobulin receptor, the apoE receptor on neuronal membranes (35). Cholesterol, which is bound and transported by apoE, is required for the normal function of brain cells. If the β-amyloid bound to the apoE carboxyl-terminal domain prevents apoE-4 from binding cholesterol, failure of obtaining adequate levels of cholesterol or fat could prevent normal fat metabolism in neurons or could promote apoptosis, which is reported to occur in AD brain cells (36). Alternately, in the brain, apoE-4 may fail to protect β-amyloid from forming neurotoxic fibrils (11) and/or fail to protect tau from hyperphosphorylation leading to neuronal fibrillary tangles (12).

In summary, the results of this work (*i*) identified the human 0.67kbTF promoter as an efficient director of brain expression in transgenic mice; (*ii*) located where the 0.67kbTF directed genes are expressed in brain; and (*iii*) identified newly synthesized human apoE in brains of transgenic mice.

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