ORIGINAL CONTRIBUTION

Polymorphisms in the Promoter of the Human *APP* Gene

Functional Evaluation and Allele Frequencies in Alzheimer Disease

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Background: Missense mutations in the amyloid precursor protein (*APP*) gene cause early-onset Alzheimer disease (AD). However, little is known regarding the effects of polymorphisms in regulatory sequences of *APP* on AD susceptibility.

Objectives: To identify polymorphisms in the *APP* promoter, to test these for associations with AD, and to assess their influence on *APP* promoter activity in transfected cells.

Setting: Community study of 1013 people of white, African American, or Caribbean Hispanic ethnicity, 65 years and older, residing in northern Manhattan.

Main Outcome Measures: The diagnosis of AD was established by stringent criteria, with multiple follow-up examinations over 7 years.

Results: We identified 2 polymorphisms in the *APP* promoter: a rare G→C variant at –9 and a frequent G→C variant at +37 relative to the transcription start site. The +37C allele was most frequent in African American patients (18% frequency), followed by Caribbean Hispanic patients (10%) and white patients of European descent (3%). This allele was overrepresented among patients with AD compared with elderly controls (odds ratio [OR], 1.57; 95% confidence interval [CI], 1.08-2.27 in the combined ethnic groups), but this was not significant after adjusting for age, sex, and education (OR, 1.41; 95% CI, 0.93-2.12). A stronger association was found in participants lacking any apolipoprotein-E ε4 allele (OR, 2.12; 95% CI, 1.36-3.32 [univariate analysis]; OR, 2.08; 95% CI, 1.26-3.45 after adjusting for age, sex, and education). The –9C allele was not frequent enough to be evaluated for a disease association. Both variants were tested in promoter-reporter assays in U-87 glioma cells, and no differences in promoter activity were detected.

Conclusions: The –9G/C and +37G/C *APP* promoter polymorphisms are unlikely to contribute strongly to AD susceptibility or to cause major differences in *APP* expression, but the +37C allele warrants further study for association with AD in larger population samples.

Arch Neurol. 2002;59:1793-1799

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THE NEUROTOXIC and amyloidogenic peptide Aβ is generated by proteolytic cleavage of the amyloid precursor protein (APP).[1] Genetic and functional studies have assigned a pivotal role for increased Aβ production in the neuropathologic characteristics of Alzheimer disease (AD). Several factors account for the increased secretion of Aβ and the accelerated aggregation of this peptide in AD. These include missense mutations in the *APP* gene and in the genes encoding presenilin-1 and presenilin-2, which increase the proteolytic conversion of APP into the fibrillogenic Aβ42 peptide and lead to early-onset AD.[2-4] A coding change in a third locus, the apolipoprotein-E (*APOE*) ε4 variant, acts to increase Aβ aggregation and is a significant risk factor for late-onset AD.[5,6]

Since the production of Aβ is predicted to depend both on the amount of APP protein and on factors involved in its processing, a link between increased *APP* gene expression and AD has been examined. Increased expression of the *APP* gene correlates with Aβ accumulation in severe head injury in humans, and overexpression, but not low-level expression, of *APP* missense alleles in transgenic mice mimics some aspects of AD.[7-9] Perhaps most convincingly, *APP* gene duplication in trisomy 21 leads to elevated levels of circulating Aβ peptide[10] and to premature accumulation of Aβ in amyloid plaques in the brain,[11,12] a process that likely contributes to the observed approximately 40-year decrease in age of onset of AD in people with Down syndrome.[13,14]

Although the molecular mechanisms governing *APP* gene expression are

Table 1. Oligonucleotidic Sequences and Primer Annealing Conditions*

Primer	Sequence	Annealing, °C	Position in GenBank Accession D87675	PCR Fragment Size, bp
1F	GCTCGTGCCTGCTTTTGACGTTGG	69	8428-8451	356
IR	CCTGGGCTTCGTGAACAGTGGGA		8784-8762	
2F	AGCCTCAGCGTCCTAGGACTCAC	65-60; Touchdown PCR	8693-8715	432
2R	AGTGCGCTGCTGTGCGAGTGGGAT		9125-9102	
3F	GATCAGCTGACTCGCCTGGCT	65	8949-8969	96
3R	GCACGCTCCTCCGCGTGCTCT		9024-9044	
4F	GCTGAGCTCTGCTTTTGACGTTGGGGG	65	8436-8454	733
4R	CAGAGATCTCAGTGCCAAACCGGGCAG		9168-9151	
ASO-C	ACGCGGAGCAGCGTGCG	42; Hybridization (+37G/C)	9029-9045	NA
ASO-G	ACGCGGAGGAGCGTGCG		9029-9045	

*PCR indicates polymerase chain reaction; NA, not applicable; and bp, base pair.

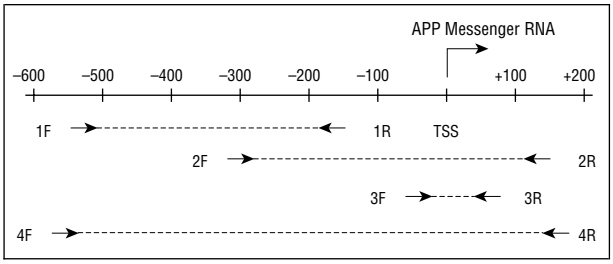

Figure 1. Segments of the amyloid precursor protein (*APP*) promoter examined in this study. Numbering is relative to the transcription start site (TSS). Arrows indicate polymerase chain reaction primers (see Table 1).

not fully understood, the *APP* promoter is an essential regulatory element that is highly conserved between species. It resembles promoters of housekeeping genes in that the proximal region has a high GC content and lacks typical CAAT and TATA boxes. The *APP* promoter contains consensus binding sites for several transcription factors that respond to signals from extracellular ligands and cell stress and an initiator sequence that is essential for start site selection.[15-26] Little information is available concerning genetic variation in *APP* regulatory sequences. To date, the screening for variants in the *APP* promoter identified a C→G substitution at position –209 relative to the transcription start site, which was stated as not associating with AD, although data on allele frequencies were not shown.[27,28] Another polymorphic marker, a microsatellite sequence in the first intron of APP, showed weak association with AD in a recent sibling study,[29] but a tetranucleotide repeat in intron 7 did not associate with AD.[30] To address this issue more fully, we have screened for *APP* promoter variants in a large tri-ethnic population sample of elderly Caribbean Hispanic, African American, and white participants. We report functional and genetic association data for 2 *APP* promoter polymorphisms found in this population.

METHODS

PARTICIPANTS AND DIAGNOSIS

Participants were individuals older than 65 years residing in the Washington Heights–Inwood neighborhood of Manhattan. For those who agreed to participate, an in-person interview and a standardized assessment, including a medical history, physical and neurological examination, and neuropsychological battery,[31] were completed. Individuals who qualified for initial inclusion in the community study (n=1401) all had at least one subsequent follow-up evaluation. Participants included 282 (20%) non-Hispanic whites, 462 (33%) African Americans, 646 (46%) Caribbean Hispanics, and 11 (1%) from other ethnic groups. For this study we excluded individuals with other forms of dementia or Parkinson disease. We also excluded individuals with questionable dementia (possible AD). This left 1077 eligible individuals, of whom 169 (16%) had a history of stroke. Of these, DNA from 1013 people was used for genotyping. For patients with AD, the diagnosis was established at a consensus conference of physicians and neuropsychologists and required evidence of cognitive deficit on the neuropsychological battery and evidence of impairment in social or occupational function. When available, all medical records and imaging studies were used in the evaluation, as were data from the initial and follow-up examinations. Patients with AD included individuals with probable AD and those with a Clinical Dementia Rating Scale score of 1.0 or higher.[32]

POLYMERASE CHAIN REACTION, DNA SEQUENCING, AND GENOTYPING

Oligonucleotide primers for polymerase chain reaction (PCR) amplification of the *APP* promoter were based on GenBank (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md) accession D87675 (**Table 1** and **Figure 1**). Fragments were amplified from genomic DNAs using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, Calif), with cycling parameters of denaturation at 94°C for 30 seconds, annealing at a specific temperature for 45 seconds (primer sequences and temperatures in Table 1), and extension at 72°C for 1 minute. Direct sequencing of the PCR products was performed with dye terminators (ABI PRISM 377 DNA Sequencer; Applied Biosystems, Foster City, Calif). To improve accuracy, the polymorphisms were scored by multiple partially redundant methods. For denaturing high-performance liquid chromatography (DHPLC), sequences were amplified as described herein using primers 2F and 2R, except the final extension in the PCR was followed by denaturation and reannealing to allow heteroduplex formation. Of the PCR product, 15 µL was injected into the WAVE (Transgenomics, Omaha, Neb) DNA fragment analysis system. The DHPLC parameters were calculated using a predictive algorithm supplied by the manufacturer. The +37G/C polymorphism in the heterozygous configuration produced a common DHPLC variant, while heterozygosity for the –9G/C polymorphism produced a rare DHPLC variant. For confirming heterozygotes and detecting homozygotes at the +37G/C

polymorphism, the PCR products were resolved on duplicate 1% agarose gels, blotted, and hybridized with ^{32}P end-labeled, allele-specific oligonucleotides ACGCGGAGCAGCGTGCG and ACGCGGAGGAGCGTGCG. End labeling of probes was conducted with γ[^{32}P]adenosine triphosphate using T4 polynucleotide kinase (Promega, Madison, Wis), and hybridization conditions have been described.[33] For definitive genotyping of the –9G/C polymorphism, the region flanking the polymorphism was amplified by PCR with primers 3F and 3R to generate a 95 base pair (bp) product. This was digested with 2 U of the restriction enzyme *Ava*I (Roche, Indianapolis, Ind) at 37°C overnight and resolved on 3% Metaphor (BioWhittaker Molecular Applications, Rockland, Md) agarose gels stained with ethidium bromide. The G allele is cleaved to fragments of 57 and 39 bp, distinguishable from the 96 bp fragment representing the C allele.

CELL CULTURE AND PROMOTER-REPORTER ASSAYS

A 750-bp promoter fragment, spanning from –573 to +177 relative to the transcription start site, was amplified from templates corresponding to homozygotes for each allele using primers 4F and 4R into which *Sac*I and *Bgl*II restriction sites were introduced. The PCR products from individuals with –9G;+37G, –9G;+37C, and –9C;+37G haplotypes were directionally cloned between *Sac*I and *Bgl*II sites of the pGL3-Basic vector (Promega) upstream of the luciferase reporter gene. Negative and positive control constructs were pGL3-Basic, lacking any promoter sequences, and pGL3-Control, containing the SV40 promoter and enhancer sequences. A β-galactosidase expression plasmid (pSV-beta-galactosidase; Promega) was cotransfected to allow normalization for transfection efficiency. U-87 MG glioma cells (American Type Culture Collection, Rockville, Md) were grown in EMEM medium with Earle's balanced salt solution and 2mM L-glutamine containing 10% heat-inactivated fetal calf serum. The cells were transfected at 70% confluence using FuGene 6 reagent (Roche) according to the manufacturer's specifications. When decreasing amounts of the experimental reporter constructs were used, the total amount of transfected DNA per well was kept constant by adding pGL3-Basic plasmid to achieve a final DNA amount of 1 µg per 35 mm^2 plate. The transfected U87 cells were washed with phosphate-buffered isotonic sodium chloride solution and lysed in the plate using 250 µL of Reporter Lysis Buffer (β-Galactosidase Enzyme Assay System; Promega). The cell extract was centrifuged for 5 minutes at 10000*g*, and the supernatant was collected. An aliquot (20 µL) was used for determining luciferase activity with 100 µL of Luciferase Assay Buffer (Promega) in a Berthold luminometer. β-Galactosidase assays (β-Galactosidase Assay System; Promega) were performed according to the manufacturer's protocol using 10 to 20 µL of the cell lysate. Luciferase values were then normalized to β-galactosidase activity.

STATISTICAL ANALYSIS

Allele frequencies were determined by counting each allele and by calculating sample proportions. For comparison of cases and controls within and across ethnic groups, allele frequencies were calculated for all participants and compared using χ^2 analysis. Logistic regression was used to compute the odds ratio for the association between AD and the *APP* promoter polymorphisms. Data were stratified by the presence or absence of an *APOE* ε4 allele and by adjusting for differences in age and education. Logistic regression analyses were conducted separately for each ethnic group. We tested for Hardy-Weinberg equilibrium using a χ^2 analysis. Multivariate logistic regression was used to compute the odds ratio for the association between AD and *APP* promoter polymorphisms, adjusting for age, sex, and education.

RESULTS

SEQUENCE POLYMORPHISMS IN THE APP PROMOTER

To screen for *APP* promoter variants in a tri-ethnic population, the proximal promoter region, from –573 to +125 relative to the transcriptional initiation site, was amplified from genomic DNA of 20 individuals, approximately equally divided among African American, Caribbean Hispanic, and white ethnic groups. We focused on this region since functional analysis and deletion mapping of the human and murine *APP* promoters have shown it to be sufficient for high level expression in various cell types.[18,20,22,23] The initial PCR strategy generated overlapping amplicons with primers 1F and 1R and 2F and 2R (Table 1 and Figure 1). Sequencing revealed a single polymorphism: a G→C substitution in the first (nontranslated) exon, at position +37. To extend this search to detect rare variants, the amplicon from –308 to +124 (primers 2F and 2R; Table 1 and Figure 1) was generated from genomic DNAs of 1019 individuals, including patients with AD and elderly controls, from the tri-ethnic population sample. These PCR products were analyzed by DHPLC, a highly sensitive method that we have previously employed for detecting allelic variants without a prior knowledge of sequence variation.[34] Sequencing of PCR products that produced rare DHPLC variants revealed a second polymorphism, a G→C substitution at position –9. No other variants were found. The DHPLC analysis also provided preliminary scoring of heterozygosity at the +37G/C site. For definitive genotyping of the +37G/C polymorphism, the PCR products generated with primers 2F and 2R (Table 1 and Figure 1) were analyzed by Southern blottings followed by hybridization with allele-specific oligonucleotides. Since the –9G/C polymorphism fell within an *Ava*I restriction site, definitive genotyping of this marker was performed by *Ava*I digestion of the PCR products made with primers 3F and 3R (Table 1 and Figure 1).

ALLELE FREQUENCIES IN PATIENTS WITH AD AND CONTROLS

Overall allele frequencies for the +37G/C and –9G/C polymorphisms in the combined ethnic groups did not deviate significantly from Hardy-Weinberg equilibrium. The genotype distributions for the +37G/C and –9G/C polymorphisms in patients with AD and controls are given in **Table 2** and **Table 3**. While the –9C allele was not frequent enough to allow statistical conclusions (Table 3), the +37C allele was overrepresented among AD cases overall. This trend was significant only in the univariate analysis of the combined ethnic groups and was not significant after correcting for age, sex, and education (Table 2). Since the frequency of the +37C allele was highest in African Americans, we also analyzed this group separately. This showed a similar trend, but again, the re-

Table 2. Allele Frequencies of the –9G/C and +37G/C Polymorphisms and Rates of AD in 3 Ethnic Groups*

Group	–9G	–9C	+37G	+37C	Control Cases, %	Patients With AD, %
White	310 (100.0)	0	366 (96.8)	12 (3.2)	91.6	8.4
Caribbean Hispanic	800 (99.3)	6 (0.7)	871 (89.6)	101 (10.4)	80.2	19.8
African American	544 (99.3)	4 (0.7)	556 (82.3)	120 (17.8)	80.1	19.9
Total	**1654** (99.4)	**10** (0.6)	**1793** (88.5)	**233** (11.5)	. . .	. . .

*Individuals with uncertain clinical diagnosis (possible Alzheimer disease) are excluded (see the "Methods" section). Values are given as No. (%) except where indicated.

Table 3. Distributions of +37G/C and –9G/C Genotypes in Patients With Alzheimer Disease and Controls*

Genotype	AD Cases	Controls	Univariate OR (95% CI)	Multivariate OR (95% CI)
+37G/C Genotypes				
GG	124 (71.7)	671 (79.9)	1.00	1.00
GC	45 (26.0)	158 (18.8)	1.54 (1.05-2.26)	1.34 (0.88-2.05)
CC	4 (2.3)	11 (1.3)	1.97 (0.62-6.28)	2.55 (0.70-9.21)
Risk of AD given ≥1 +37C alleles	. . .	. . .	1.57 (1.08-2.27)	1.41 (0.93-2.12)
+37G/C Genotypes (African American Patients)				
GG	39 (59.1)	191 (70.2)	. . .	. . .
GC	23 (34.8)	73 (26.8)	. . .	. . .
CC	4 (6.1)	8 (2.9)	. . .	. . .
Risk of AD given ≥1 +37C alleles	. . .	. . .	1.63 (0.94-2.84)	1.10 (0.58-2.07)
+37G/C Genotypes: Participants Without *APOE* ϵ4				
GG	74 (67.3)	493 (81.4)	1.00	1.00
GC	34 (30.9)	105 (17.3)	2.16 (1.37-3.41)	2.01 (1.20-3.37)
CC	2 (1.8)	8 (1.3)	1.67 (0.35-8.00)	3.85 (0.74-19.98)
Risk of AD given ≥1 +37C alleles	. . .	. . .	2.12 (1.36-3.32)	2.08 (1.26-3.45)
+37G/C Genotypes: Participants With ≥1 *APOE* ϵ4 Allele				
GG	46 (82.1)	163 (78.0)	1.00	1.00
GC	8 (14.3)	43 (20.6)	0.66 (0.29-1.50)	0.54 (0.22-1.31)
CC	2 (3.6)	3 (1.4)	2.36 (0.38-14.56)	1.48 (0.19-11.54)
Risk of AD given ≥1 +37C alleles	. . .	. . .	0.77 (0.36-1.64)	0.61 (0.27-1.40)
–9G/C Genotypes†				
GG	141 (100)	685 (98.7)	. . .	. . .
GC	0	8 (1.2)	. . .	. . .
CC	0	1 (0.1)	. . .	. . .

*The univariate analysis shows the crude odds ratio (OR) for Alzheimer disease (AD), with GG as the reference genotype. The multivariate analysis corrects for age, sex, and education. CI indicates confidence interval; *APOE* ϵ4, apolipoprotein-E ϵ4. Values are for all ethnic groups, except where indicated.
†Slightly fewer genotypes were determined for this genotype than for the +37G/C genotype.

sults were not significant in the multivariate analysis (Table 3). Of interest, in both the combined ethnic groups and in the African American group, homozygosity for the +37C allele was more common among patients with AD, and, while the number of participants with this genotype was small, there was an apparent allele dosage effect (Table 3). Also of interest is that the +37C allele was significantly associated with AD in participants lacking an *APOE* ϵ4 allele (combined ethnic groups), and this remained significant in the multivariate analysis (Table 3). Although the numbers were small, a significant association was not seen in participants with one or more *APOE* ϵ4 alleles (Table 3).

Since the allele frequencies differed by ethnicity, we considered the possibility that the observed association of the +37C allele with AD might be trivially explained by genetic admixture in the 3 ethnic groups. Such confounding effects would be expected if the frequencies of AD differed by ethnicity. As given in Table 2, the white group had the lowest rate of AD, but the rates of AD did not differ between the African American and Caribbean Hispanic groups. Since most of the +37C genotypes occurred in the latter 2 groups, genetic admixture is not a likely explanation for the AD associations seen with this marker.

ASSAYS FOR FUNCTIONAL EFFECTS OF THE –9G/C AND +37G/C POLYMORPHISMS

The location of the 2 polymorphisms within regulatory elements in the proximal 5′-flanking region of *APP*, which accounts for most of the basal transcriptional activity of the promoter,[35] suggests that they might influence transcription. Moreover, alignment of human and mouse sequences shows that these polymorphisms were embedded in strongly conserved sequences (**Figure 2**). A search

of the TRANSFAC[36] (http://www.gene-regulation.com) and TESS[37] (http://www.cbil.upenn.edu/tess/) databases using a 100-nucleotide sequence centered on these changes was performed to determine if the polymorphisms affected predicted transcription factor binding sites. As expected, several potential transcription factor binding sites were altered: 276 sites were detected by TESS with the –9G;+37G sequence, and 266 sites were detected with the –9C;+37C sequence; the TRANSFAC search returned fewer sites and showed a loss of 2 SP1 sites with the –9C;+37C sequence compared with the –9G;+37G sequence. In addition, the –9G/C polymorphism is located 1 bp downstream of the sequences comprising the initiator box, which can determine transcription initiation sites and transcription efficiency.[38]

To test whether these sequence variants could cause differences in *APP* transcription, we cloned a series of matched 750 bp promoter fragments, containing the common –9G;+37G allele, or each of the 2 variant alleles (–9C;+37G and –9G;+37C), upstream of the luciferase reporter gene. These were transfected into human U-87 astrocytoma cells, a cell type that, like neurons, expresses the *APP* gene.[39,40] The minimal promoter region strongly stimulated expression of the luciferase reporter gene, but luciferase activity was not significantly altered by either of the sequence variants (**Figure 3**).

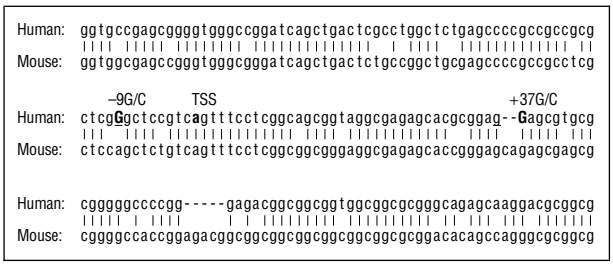

Figure 2. Alignment of the human amyloid precursor protein (*APP*) promoter sequence spanning the –9G/C and +37G/C polymorphisms with the corresponding murine sequence. Both polymorphisms (bold uppercase letters) are embedded in well-conserved sequences. TSS indicates transcription start site (bold lowercase letters). The murine sequence is from GenBank (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md) accession D10603.

The fact that linear changes in luciferase were observed paralleling the amount of transfected plasmid DNA confirmed that this assay was not in the saturating range and was therefore giving a valid readout of promoter activity (Figure 3).

COMMENT

In principle, variations in promoter sequences can alter gene expression directly by altering a transcription factor binding site or indirectly by changing the organization of chromatin. Promoter variants with effects on the transcriptional activity of certain human genes have been identified, and genetic association studies have suggested that some of these variants may be disease risk factors. Examples include promoter polymorphisms in the tumor necrosis factor α gene, with effects on transcription that are associated with increased morbidity in infections, including malaria and leishmaniasis[41]; in the interleukin 6 gene, which is associated with risk of coronary heart disease and systolic blood pressure[42,43]; in the interferon regulatory factor 1 gene, which can affect allergy and responses to interferons[44,45]; in the beta-fibrinogen gene, which contributes to regulation of plasma fibrinogen concentration[46]; and in the insulin gene, which is associated with type I diabetes mellitus.[47] In *PS1* and *APOE*, genes that have strong effects on the risk of AD when they contain coding changes, several promoter variants have also been identified. Although consistent findings have yet to emerge from multiple studies, at least one *APOE* polymorphism, –219G/T, may be associated with altered promoter activity and an altered risk for AD.[48-50] Screening of the *PS1* upstream region has identified several polymorphisms. Notably, promoter-reporter analysis demonstrated a decrease in promoter activity for 2 of the variant alleles, and 1 of these variants, –48C/T, was associated with early-onset AD.[51,52] In another study, polymorphisms in the PS1 promoter and intron 8 were not associated with late-onset AD.[53]

In the current study, we have identified and characterized 2 genetic variants, a common +37G/C polymorphism and a rare –9G/C variant, in the core sequences of the proximal APP promoter. The +37C allele

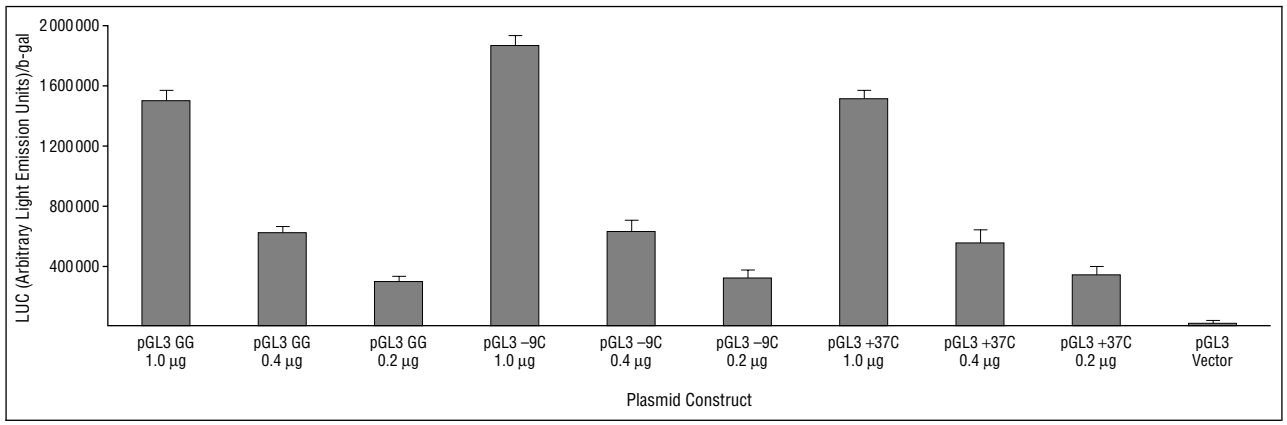

Figure 3. Activity of the amyloid precursor protein (*APP*) promoter containing the variant alleles in promoter-reporter assays. Plasmid constructs and the amounts of specific plasmid DNA transfected are indicated on the x-axis. The –9C and +37C constructs differ from the control (–9G;+37G) constructs only at these single positions. Although the activity of the –9C allele is slightly higher than the other constructs at the highest plasmid concentration tested, this difference is not observed at lower concentrations. LUC indicates luciferase-catalyzed luminescence; b-gal, the optical density obtained from the β-galactosidase reactions.

was weakly associated with AD in a univariate analysis, and there was a suggestion of an allele-dosage effect. However, that association became nonsignificant in the multivariate analysis. A significant association, in the univariate and multivariate analyses, was observed in participants lacking any *APOE* ε4 allele. Although both promoter polymorphisms were embedded in highly conserved sequences, neither the –9G/C nor the +37G/C variants affected basal promoter activity.

Future studies might include expanding the genetic studies to larger cohorts and assessing the functional effect of these polymorphisms on inducible, as opposed to basal, expression of *APP* messenger RNA. Functional characterization has shown that the region that we examined accounts for the bulk of the basal promoter activity.[18,19,35,38] This region also accounts for inducible expression of *APP* messenger RNA in response to stimuli and cell stress.[15,54] However, physiological regulation of the *APP* gene is also influenced by sequences situated more distally. The "APPB" sites, in the more distal promoter region at –1837/–1822 and –2250/–2241, were shown to interact with a complex containing the p50 subunit of NF-κB, which is constitutively expressed in neurons and acts as a positive regulator of gene expression.[55,56] The distal APP promoter also harbors at least one negative regulatory element, the upstream regulatory element between –2257 and –2234. This binds to an unknown transcription factor present in neural lineage cell lines and in brain extracts.[25] These regions may warrant genetic analysis in future studies.

Accepted for publication June 28, 2002.

Author contributions: Study concept and design *(Drs Athan, Lee, Mayeux, and Tycko);* acquisition of data *(Drs Athan, Mayeux, and Tycko);* analysis and interpretation of data *(Drs Lee, Mayeux, and Tycko and Mr Arriaga);* drafting of the manuscript *(Drs Athan, Lee, Mayeux, and Tycko);* critical revision of the manuscript for important intellectual content *(Drs Athan, Lee, Mayeux, and Tycko);* statistical expertise *(Drs Lee and Mayeux and Mr Arriaga);* obtained funding *(Dr Mayeux).*

We thank Alejandra Ciappa, MD, and Martha Posada for DNA preparations.

This study was supported by federal grants AG07232 and AG08702 from the National Institutes of Health (Bethesda), the Charles S. Robertson Memorial Gift for Alzheimer's Disease Research from the Banbury Fund, and the Blanchette Hooker Rockefeller Foundation.

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