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10. Radioactive signals from blots causing different autoradiogram exposures were estimated with the LKB Ultroscan XL soft laser scanning densitometer. Areas under optical density peaks over a 0.8-mm-wide path in the center of an autoradiographic band were measured. First, the dose-response curve of the film as detected by the densitometer was determined by measuring the areas due to 1.5-fold increments of ^{32}P-labeled DNA spotted onto small rectangular Whatman 3-mm filter strips. The fractional increase in area per fractional increase in radioactivity was determined at each average optical density between two bands. The inverse of this, varying from 0.80 to 1.15 over a range of 0.5 to 2.5 optical density units, was used to convert area ratios into radioactivity ratios.
11. We thank J. Amos and G. Stewart for providing DS DNAs and S. Orkin for the HL60 cDNA library. Supported by NIH grants HD18658 (R.L.N. and S.A.L.) and MH/NS 31862 (E.D.B.) and by the Howard Hughes Medical Institute (S.A.L.). This work was done during R.L.N.'s tenure as a National Down Syndrome Society Scholar.

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Gene Dosage of the Amyloid β Precursor Protein in Alzheimer's Disease

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The progressive deposition in the human brain of amyloid filaments composed of the amyloid β protein is a principal feature of Alzheimer's disease (AD). Densitometric analysis of Southern blots probed with a complementary DNA for the amyloid protein has been carried out to determine the relative dosage of this gene in genomic DNA of 14 patients with AD, 12 aged normal subjects, and 10 patients with trisomy 21 (Down syndrome). Whereas patients in the last group showed the expected 1.5-fold increase in dosage of this gene, none of the patients with AD had a gene dosage higher than that of the normal controls. These results do not support the hypothesis that the genetic defect in AD involves duplication of a segment of chromosome 21 containing the amyloid gene. Alternative mechanisms for the brain-specific increase in amyloid protein deposition in AD should be considered.

THE DEPOSITION OF EXTRACELLULAR amyloid filaments within clusters of degenerating neurites (neuritic plaques) and in cerebral and meningeal blood vessels is a constant accompaniment of Alzheimer's disease (AD) (*1*). The major proteinaceous component of this brain amyloid is a 4- to 5-kD hydrophobic protein designated the amyloid β protein (β-AP) (*2–5*). The recent isolation of complementary DNA (cDNA) clones for the β-AP precursor (*6–9*) has shown that it is a fragment of a larger precursor and that its gene is on the long arm of human chromosome 21. Patients with trisomy 21 [Down syndrome (DS)] almost invariably develop amyloid deposits in the brain at a premature age—perhaps due in part to increased dosage of the β-AP gene.

Because vascular and plaque amyloid deposits in AD are morphologically and biochemically similar if not identical to those in DS (*1, 10, 11*), an increased gene dosage for the β-AP precursor could also be operative in AD (*12*). In view of the potential importance of this issue for understanding amyloidogenesis in AD and for diagnosing subjects at risk, we isolated cDNAs for the β-AP precursor and determined its relative gene dosage in genomic (leukocyte) DNA from 14 patients with AD, 10 patients with DS, and 12 normal aged humans. Our results do not support recent reports (*13, 14*) that a subsegment of chromosome 21 containing the amyloid gene is duplicated in AD.

A λgt11 cDNA library derived from normal adult human brain (*15*) was screened with a 29-base oligonucleotide corresponding to bases 1849 to 1877 of the full-length cDNA clone for the β-AP precursor reported by Kang *et al.* (*7*). Of seven clones isolated, the largest clone (β-6) contained a 1.6-kb insert, which, by partial sequencing, corresponded to nucleotides 849 to 2451 of the sequence of Kang *et al.* Digestion of β-6 with Eco RI produced two fragments of ~0.9 and ~0.7 kb (*6, 7*). The 0.7-kb fragment encodes the carboxyl terminal of the precursor and contains an additional 366 bases of 3′ untranslated region; this fragment coincides to a large extent with the probe used in the AD gene dosimetry study of Delabar *et al.* (*13*). The 0.9-kb fragment encodes a major portion of the precursor protein that is on the amino-terminal side of the amyloid peptide. Each Eco RI fragment was purified and used independently to probe Southern blots. As a control probe not localized to chromosome 21, we used a cDNA for the human microtubule-associated protein 2 (MAP 2) (*16*). This gene is on chromosome 2 and is not linked to the familial AD defect in four pedigrees with autosomal dominant AD (*17*).

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Table 1. Results of the relative gene dosage of the β-AP in normal controls (C), Alzheimer's disease (AD), and Down syndrome (DS) shown for each Southern blot used in this study. The relative gene dosage was determined as described in Fig. 2. Each value represents the mean relative gene dosage (± SD) derived from *n* samples in a particular diagnostic category (C, AD, or DS) examined on that blot. *n* is defined in Fig. 2. *N* is the total number of DNA samples for diagnostic category run on blot.

Patient groups		Blot number																
		1	3	5	7	8	9	11	12	13	16	17	18	23	28	30	31	32
C	Mean	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	SD	0.1	0.2	0.2	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.2	0.2	0.0	0.2	0.1	0.1	0.2
	n/N	6/6	8/8	6/6	6/6	5/5	4/6	5/6	4/6	5/5	5/5	4/6	5/5	3/5	3/5	4/5	4/6	5/6
AD	Mean	1.0	0.9	0.9	1.1	1.0	1.1	1.0	1.0	1.0	0.9	0.9	1.0	1.0	0.9	1.0	1.0	0.9
	SD	0.1	0.1	0.1	0.2	0.2	0.3	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.3
	n/N	8/8	8/8	5/5	6/8	8/8	8/9	7/9	8/8	2/2	6/7	5/6	6/7	5/7	5/6	4/6	4/4	3/5
DS	Mean	1.5	1.5	1.6	1.5	1.6	1.6	1.6	1.5	1.3	1.6	1.5	1.6	1.4	1.4	1.5	1.6	1.5
	SD	0.1	0.4	0.5	0.4	0.6	0.2	0.2	0.2		0.2	0.0	0.1	0.2	0.2	0.3	0.1	0.3
	n/N	3/3	3/4	3/5	3/3	2/3	2/3	3/3	3/3	1/1	6/7	2/6	6/6	5/5	3/5	4/5	5/7	5/6

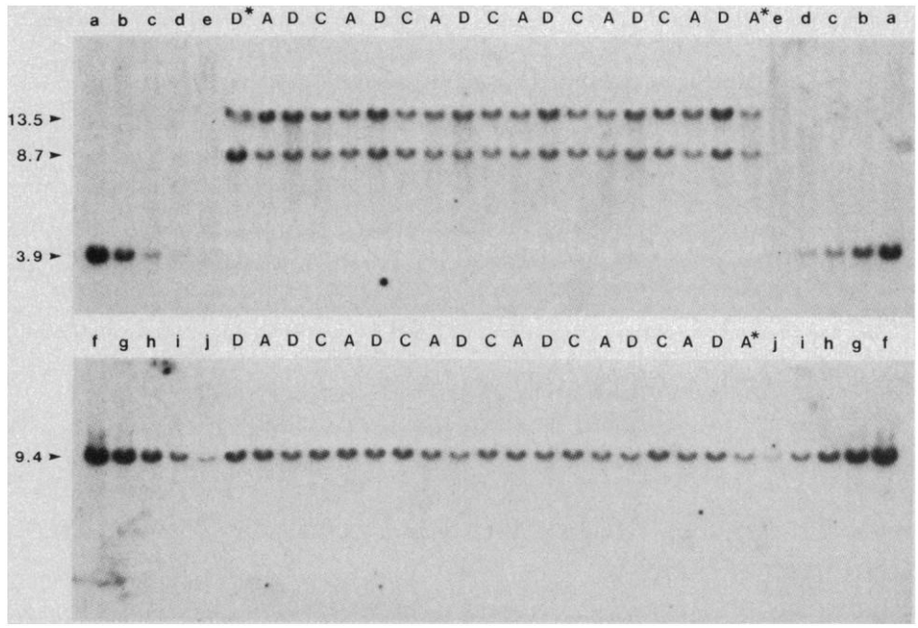

Fig. 1. Representative autoradiographs used to establish the relative gene dosage of the β-AP. The same Southern blot was hybridized consecutively with one of two probes. (**Top**) β-6 (0.9 kb) probe for the β-AP; (**bottom**) MAP 2 as a control probe. Numbers indicate fragment size (in kilobases); note that the MAP 2 plasmid standard comigrates with the human genomic fragment recognized by the MAP 2 probe. Lanes marked A, D, or C are purified genomic DNA samples from different patients: Alzheimer's disease (A), Down's syndrome (D), or normal aged controls (C). The five outside lanes on each side were loaded with standards consisting of the plasmids that contain the DNA inserts used as probes. Lanes a, b, c, d, and e contain 19.0, 9.5, 4.8, 2.4, and 1.2 pg, respectively, of the linearized β-6 (0.9 kb) plasmid; only standard e was outside the linear dose-response range of the film. Lanes f, g, h, i, and j contain 46.0, 23.0, 11.5, 5.8, and 2.9 pg, respectively, of the linearized MAP 2 plasmid; standards f and j were outside the linear dose-response range. Asterisks indicate patient samples that were deleted from analysis because they were outside the linear dose-response range or showed artifacts in or near the hybridization bands. Leukocyte DNA was quantitated (A_{260}) after Eco RI digestion. Four-microgram samples (in 40 μl) were electrophoresed on an 0.65% agarose gel in Howley's buffer (*25*) and transferred to nitrocellulose (*26*). Filters were incubated in 10× Denhardt's solution, 5× standard sodium citrate (SSC), 0.1% SDS, and single-stranded calf thymus DNA (150 μg/ml) (*25*) at 42°C for 2 to 4 hours. Hybridization was then carried out in 50% formamide, 10× Denhardt's solution, 5× SSC, 0.1% SDS, and single-stranded DNA (150 μg/ml) at 42°C for 38 to 42 hours. Gel-isolated insert DNA was labeled with α^{32}P-labeled deoxycytidine 5′-triphosphate by using random primers (*27*) to a specific activity of ~4×10^8 cpm/μg; hybridization was carried out at 1×10^7 to 2×10^7 cpm/ml. Filters were washed in 50% formamide and 5× SSC (42°C, 30 minutes) followed by 0.1% SDS and 0.1× SSC (42°C, 2 hours), and exposed for 3 to 30 hours with one or two intensifying screens. Autoradiographs were quantified on a Bio-Rad model 620 laser densitometer. Bound probe was removed completely (50% formamide and 5× SSC at 68°C for 2 hours) before hybridizing with the next probe.

All 36 subjects whose DNA was analyzed were assessed neurologically by one examiner (D.J.S.). The 14 AD patients were diagnosed by this examiner and followed for 1 to 6 years. NINCDS-ADRDA criteria for the clinical diagnosis of AD (*18*) were fulfilled in all 14 cases, and alternative diagnoses were excluded by laboratory tests and follow-up examinations. All 12 nondemented control subjects were also assessed and followed here (*19*). We included nondemented subjects of advanced age to reduce the likelihood that some controls carried the AD trait but had not yet developed dementia. Mean ages (range) were 79 years (64 to 89) for AD, 78 years (62 to 90) for controls, and 33 years (25 to 54) for DS. Eight of 14 AD patients had a known family history of senile dementia compared to 1 of 12 controls. Fresh, heparinized blood was used to prepare leukocyte nuclei from which genomic DNA was purified (*20*). To determine amyloid gene dosage, Eco RI digests were Southern blotted and hybridized successively with the MAP 2, β-6 (0.9 kb), and β-6 (0.7 kb) probes. Our β-6 (0.9 kb) probe detects Eco RI fragments of human genomic DNA at 13.5, 8.7, and 1.4 kb; the two major bands at 13.5 and 8.7 kb (Fig. 1) were quantitated densitometrically and the values summed. The β-6 (0.7 kb) probe recognizes 8.7- and 2.9-kb fragments; the former was used for quantitation. The MAP 2 probe detects fragments at 9.4 and 1.5 kb; the major band at 9.4 kb (Fig. 1) was quantitated. We selected MAP 2 as a reference probe in part because its genomic Eco RI fragment is similar in size to those detected by the amyloid probes and should be transferred with similar efficiency during Southern blotting.

To assure the accuracy of the gene dosage quantitation, several steps were taken. On each Southern blot, duplicate sets of five internal standards containing increasing amounts of β-6 and MAP 2 plasmids were used to construct dose-response curves that established the linear range of the x-ray film for that blot (Fig. 1). DNA samples with hybridization signals outside the linear range were omitted from further analysis. The duplicate standards were electrophoresed in the outside lanes of the gel (Fig. 1), so that differences in sample loading or transfer between left and right sides could be detected. On most blots, AD, control, and DS DNA samples were alternated across the blot. The DNA of each of the 36 subjects was examined 5 to 11 times on at least five different blots, except for three subjects with DS examined three to four times. Lanes that showed autoradiographic artifacts near the hybridization signals or were otherwise distorted were not analyzed. Hybridization signals were quantitated by transmission densitometry; duplicate scans were highly reproducible (≤5% variance). To control for differences among patient samples in the amount of DNA loaded, we calculated a ratio of the area of the hybridization signal for the β-6 amyloid probe to that of the MAP 2 control probe for each sample. The relative gene dosage for amyloid in each AD or DS patient was established by comparing his or her β-6:MAP 2 signal ratio to the mean signal ratio of all control subjects on the same blot, with the latter value normalized to 1.0.

All ten patients with trisomy 21 showed an approximately 1.5-fold increase in dosage for the amyloid gene compared to the mean normalized control value of 1.0 (Fig. 2). In contrast, none of the 14 AD patients had a relative gene dosage above that of the normal subjects. We also analyzed the results per blot, that is, as a function of patient groups on each blot (Table 1). Again, the AD patients showed normal gene dosage. Table 2 summarizes our study. The results in Fig. 2 and Tables 1 and 2 were obtained primarily with the 0.9-kb β-6 probe. Similar results were obtained with the 0.7-kb β-6 probe (for example, blots 1, 11, 12, 31, and 32 in Table 1). The mean β-AP gene dosage for the 10 subjects with DS differed significantly from the means of the 12 controls and the 14 AD patients. In contrast, the means of the control and AD groups were indistinguishable.

Our study demonstrates a normal dosage of the amyloid gene on chromosome 21 in patients with AD, in contrast to an earlier

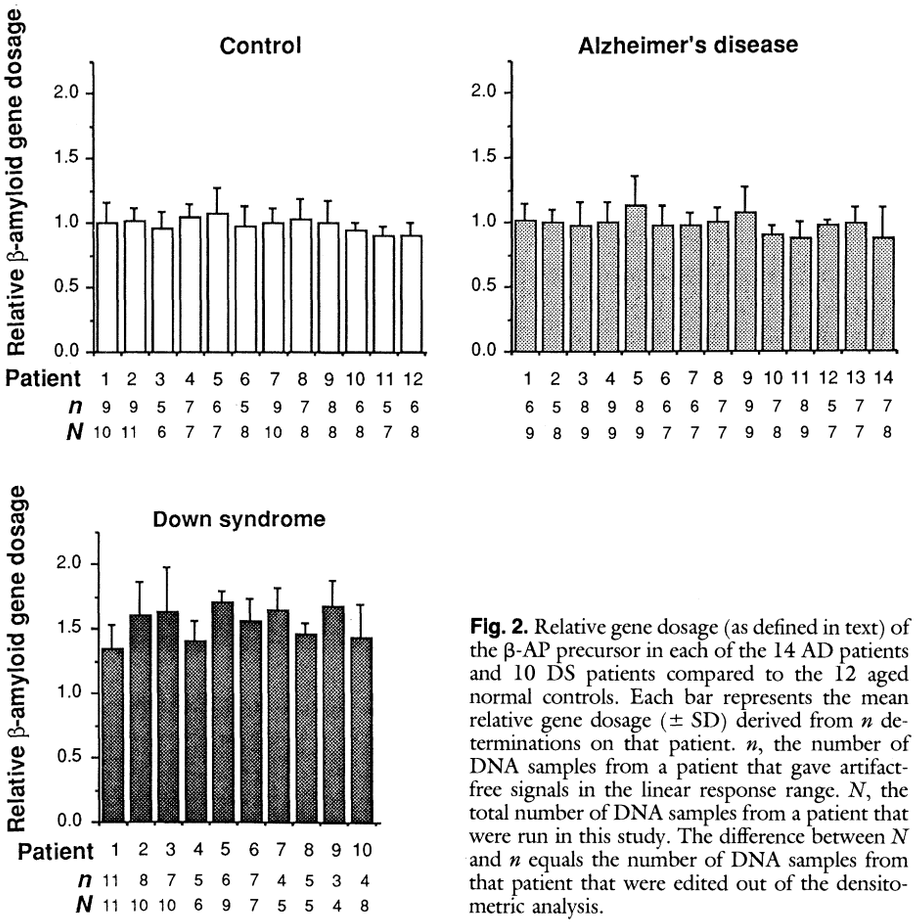

Fig. 2. Relative gene dosage (as defined in text) of the β-AP precursor in each of the 14 AD patients and 10 DS patients compared to the 12 aged normal controls. Each bar represents the mean relative gene dosage (± SD) derived from *n* determinations on that patient. *n*, the number of DNA samples from a patient that gave artifact-free signals in the linear response range. *N*, the total number of DNA samples from a patient that were run in this study. The difference between *N* and *n* equals the number of DNA samples from that patient that were edited out of the densitometric analysis.

report (*13*). The 0.7-kb β-AP probe we used corresponds exactly to the first two-thirds of the λAm4 probe used in the study of Delabar *et al.* (*13*). Fresh leukocyte DNA from clinically diagnosed AD patients was used in both studies. In contrast to the earlier report, we used internal standards and interspersed DS and normal DNA samples as positive and negative controls on each blot. The accuracy of gene dosage quantitation depended highly on these steps and on the editing of artifactual signals based on analysis of both the film and the densitometric tracings. Autopsy and biopsy examinations of putative AD patients who were diagnosed with criteria similar to ours have demonstrated histological confirmation of the diagnosis in 80 to 100% of subjects (*21, 22*). We recently reviewed the pathological diagnoses in 150 putative AD brains received consecutively at our center from many community sources (*22*); 131 (89%) met NIA-AARP histopathological criteria (*23*) for AD. Of the 14 AD patients in the present study, one has died and was found to have severe AD. It is unlikely that more than a few of our 14 patients will turn out not to have AD; however, none of them showed evidence of microduplication of the β-AP gene.

Table 2. Summary of all determinations of the relative gene dosage of the β-AP carried out in the study. The values represent grand means (± SD) derived from *n* patients. The mean for DS was significantly different from those of controls ($P < 0.001$) and AD ($P < 0.001$) (two-tailed *t* test). The means for controls and AD were indistinguishable ($P > 0.85$).

Value	Controls	AD	DS
Mean	0.99	0.98	1.55
SD	0.05	0.07	0.13
n	12	14	10

If duplication of a subsegment of chromosome 21 containing the amyloid gene and certain flanking genes were the defect underlying AD, as has been postulated (*12–14*), one might expect a higher rate of concordance for AD between monozygotic twins than has been reported. The limited studies currently available suggest that this concordance rate is less than 0.5 and is not higher in monozygotic than in dizygotic twins (*24*). In view of the results of our study, alternative mechanisms, whether transcriptional or post-translational, that could explain the regionally selective increase in brain β-AP deposition and neuritic plaque formation in AD patients should be sought.

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