double-stranded templates using the dideoxy chain termination method (Sequenase, U.S. Biochemical Corporation). For single-strand sequencing a sequential series of overlapping clones were prepared by selective digestion with T4 DNA polymerase followed by religation into M13 (Cyclone 1 Biosystem, IBI).

23. Primers used in PCR studies were as follows: LTR (bp 561–582) 5′TGACCCTGCTTGCTCAACTCTA3′, (bp 602–634) 5′TCTCTCCTAGGAGTGCTATAG3′; *gag* (bp 1402–1423) 5′CCATCACCAGCAGCTAGATAGC3′, (bp 1513–1535) 5′GCTGGTATTCTCGCCTTAATCC3′; *pol* (bp 4757–4778) 5′CCCTACAATCCAACCAGCTCAG3′, (bp 4919–4942) 5′GTGGTGAAGCTGCCATCGGGTTTT3′; *env* (bp 5405–5424) 5′TACCATGCCACCTATTCCCT3′, (bp 5659–5678) 5′GCTCGACTAGAAGGGAGAAG3′; p*X* (bp 7574–7595) 5′CCAATCACTCATACAACCCCCA3′, (bp 7719–7700) 5′CTGGAAAAGACAGGGTTGGGAG3′. Corresponding internal oligonucleotide probes for the LTR, *gag*, *pol*, *env*, and p*X* amplified products were 5′GGGTGGAACTTTCGATCTGTAACGGCGCAGAA3′, 5′TAATACCTCGGGTTTCGCCTCTGATATAAG3′, 5′GTACTTTACTGACAAACCCGACCTAC3′, 5′TCCCTCATTGGACTAAGAAGCCAAACCG3′, and 5′GTGCTGCCCAAGGGTGGGTTCCATGTATCC3′, respectively. Oligonucleotides were obtained from Research Genetics, Huntsville, AL. Amplification of DNA was performed in a total volume of 50 μl in a reaction mixture containing 225 μM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTp), and deoxythymidine triphosphate (dTTP), 125 pmol each of primer, 50 mM KCl, 2.5 mM $MgCl_2$, and 10 mM tris-HCl (pH 8.3), and 2 units of *Taq* polymerase (Cetus). Solutions were covered with mineral oil to prevent condensation. Thirty cycles of denaturation for 60 s at 94°C, primer annealing for 90 s at 54°C, and chain elongation for 120 s at 72°C were carried out in a DNA thermal cycler (Perkin-Elmer Cetus). Aliquots (10 μl) of amplified products were separated by electrophoresis on 1.5% agarose gels and transferred to nitrocellulose membranes (Schleicher & Shuell) that had been presoaked in 6× SSPE. [1× SSPE contains 0.15 M NaCl, 0.01 M NaH_2PO_4, and 0.001 M EDTA (pH 7.4)]. Membranes were dried in a vacuum oven for 1 hour at 80°C and prehybridized in 4× SSPE, 5× Denhardt's solution, 25% formamide, 0.5% SDS for 1 hour at 42°C. Hybridization was continued overnight at 42°C in the same solution containing the corresponding oligonucleotide probe end-labeled with [γ-^{32}P]ATP. After hybridization, membranes were washed twice at room temperature with 2× SSPE containing 0.1% SDS for 5 min, twice with 0.2× SSPE containing 0.1% SDS for 15 min, and finally once with 0.2× SSPE containing 0.1% SDS at 56°C for 15 min. Membranes were exposed to XAR-2 Kodak film with intensifying screens at −70°C overnight.

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Inhibition of Entry of HIV-1 in Neural Cell Lines by Antibodies Against Galactosyl Ceramide

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Although the CD4 molecule is the principal cellular receptor for the human immunodeficiency virus (HIV), several CD4-negative cell lines are susceptible to infection with one or more HIV strains. These findings indicate that there are alternate modes of viral entry, perhaps involving one or more receptor molecules. Antibodies against galactosyl ceramide (galactocerebroside, or GalC) inhibited viral internalization and infection in two CD4-negative cell lines derived from the nervous system: U373-MG and SK-N-MC. Furthermore, recombinant HIV surface glycoprotein gp120 bound to GalC but not to other glycolipids. These results suggest a role for GalC or a highly related molecule in HIV entry into neural cells.

THE CD4 MOLECULE IS THE PRINCIpal cellular receptor for the type 1 HIV (HIV-1) (*1*). However, many cell lines of nervous system, liver, and fibroblast origin do not express CD4 but can be infected, albeit less efficiently, with one or more strains of HIV-1 (*2–4*). These findings suggest that, in some cells, HIV entry is mediated by one or more alternate receptor molecules (*3*). To identify the HIV-1 receptor in cells of nervous system origin, we began screening antibodies against cell surface components for their ability to inhibit viral internalization and infection in two infectible cell lines, U373-MG and SK-N-MC (*3, 4*).

The SK-N-MC and U373-MG lines were used for these experiments because they represent two ends of the spectrum of CD4-negative cells susceptible to HIV infection. The U373-MG line was derived from a glioma (*3*); it can be infected with several HIV-1 strains, but infection can only be detected by cocultivation with CD4-positive cells or by polymerase chain reaction (PCR) analysis for viral DNA (*5*). In contrast, the SK-N-MC line, derived from a peripheral neuroblastoma, is much more permissive for viral replication (*4*). Both $p24^{gag}$ antigen and infectious virus can be detected in the supernatant of infected SK-N-MC cells, although the amount is much lower than in CD4-positive lymphoblastoid cells or in a HeLa line (HeLaT4) that constitutively expresses CD4 (*4, 6*).

We tested the inhibition of viral uptake in the U373-MG cell line with rabbit antisera raised against neural cell adhesion molecule, whole glioma membranes, gangliosides, GalC, and irrelevant antigens. Three antisera, 4BGC2, 8586, and 674, raised against GalC, a glycolipid common to oligodendrocytes and Schwann cells (*7–10*), inhibited viral entry, whereas other rabbit antisera did not (Fig. 1). None of these antisera affected the uptake of virus into HeLaT4 cells (Fig. 1), and we did not detect significant viral uptake in two noninfectible lines, HTB-138 and HeLa (Fig. 1). Using both enzyme-linked immunosorbent assay (ELISA) and high-performance thin-layer chromatography (HPTLC), we determined that the three antibodies against GalC (anti-GalC) reacted equally well with GalC and with galactosyl sulfatide, a nervous system glycolipid that differs from GalC by a single sulfatide group (*11*). To confirm the specificity of inhibition, we incubated one of the anti-GalC with GalC liposomes, decreasing its titer in ELISA and concomitantly diminishing its inhibition of viral uptake (*6*).

We then examined the effect of the anti-GalC on infection of U373-MG and SK-N-MC cells. Because infection of U373-MG results in low or undetectable production of virus unless the cells are stimulated (*3*), we used a PCR assay to determine the presence of viral DNA 3 days after inoculation with HIV-1 (strain III_B) (Fig. 2A). Treatment of the cells with immunoglobulin G (IgG) prepared from antiserum 4BGC2 (*12*) resulted in absence of the PCR *gag* signal in U373-MG cells, but treatment with IgG from a rabbit immunized against an irrelevant antigen did not. However, 4BGC2 IgG had no effect on the signal obtained from infected HeLaT4 cells. Similar results were obtained with antibody 8586.

Because HIV-1 infection of the SK-N-MC line is less restrictive than in the U373-MG cells, a different assay was used to measure inhibition by the antibodies against GalC (Fig. 2B). SK-N-MC cells were infected and maintained in the presence of the test IgG, and their effect on viral $p24^{gag}$ production was determined (*13*). In the infected cells, there was a burst of p24 production 2 days after infection that rapidly decreased to a low, chronic level. The anti-GalC resulted in a marked decrease in the early peak of $p24^{gag}$, then a rapid return to undetectable levels. Even at the highest concentration (6 mg/ml before infection, then 0.1 mg/ml

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throughout the experiment), the anti-GalC did not inhibit viral replication in infected HeLaT4 (*6*).

These findings indicated that the antibodies against GalC were capable of inhibiting viral uptake and infection in neural cells but not in HeLaT4 cells. To establish that GalC was present at the cell surface of U373-MG and SK-N-MC, we used two monoclonal antibodies (MAbs) against GalC. R-MAb has been analyzed extensively, detects both GalC and galactosyl sulfatide, and is widely used as a marker of oligodendrocyte differentiation. I6G1 was prepared against purified GalC and has also been used for identifying central nervous system (CNS) cells (*14, 9*).

Immunofluorescence microscopy (*15*) with either MAb showed qualitatively similar results. The U373-MG cell line demonstrated occasional (<1%) strongly positive cells that were frequently clustered in small groups. In the more permissive SK-N-MC, 20% of the cells were GalC-positive, and the fluorescence was much more intense. Neither HeLaT4 nor HTB-138 (a glioma line that is not susceptible to infection with HIV-1) expressed GalC on the cell surface.

R-MAb and I6G1 also bind to closely related glycolipids, including galactosyl sulfatide, which may likewise be present on the surface of some CNS cells (*16*). To establish which molecule was identified by the immunofluorescence assay, we performed thin-layer chromatography (TLC) on lipid extracts from the two cell lines and identified the GalC and galactosyl sulfatide bands by their differing mobilities and ability to bind anti-GalC. The results indicated that U373-MG and SK-N-MC contain GalC, whereas galactosyl sulfatide was not detectable (*17*).

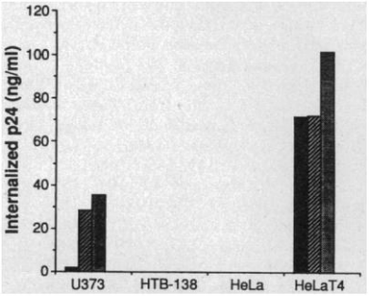

Fig. 1. Internalization of viral proteins is inhibited by antibodies against GalC. U373-MG, HeLaT4, HTB-138, and HeLa cells were incubated with purified IgG (*12*) (0.25 mg/ml) for 30 min at 0°C. Stock HIV-1–III_B (clarified supernatant from infection of SUP-T1 cells) was added at a multiplicity of approximately 1.12 μg of $p24^{gag}$ per 1.25×10^5 cells. The virus-cell mixture was maintained at 0°C for 30 min to allow binding without internalization, and aliquots were then maintained an additional 30 min at either 0°C or 36°C (to allow internalization). The cells were washed, treated with trypsin (25 μg/ml for 20 min at 23°C) to remove uninternalized virus, then lysed with 1% Triton. The lysate, representing cytoplasmic viral antigen, was assayed for $p24^{gag}$ by an antigen-capture ELISA (*13*). Internalization at 0°C was insignificant. These results are representative of six experiments with either purified immunoglobulin or whole sera (*30*). Solid bars, 4BGC-2 IgG (36°C); hatched bars, NRS IgG (36°C); stippled bars, PBS (36°C).

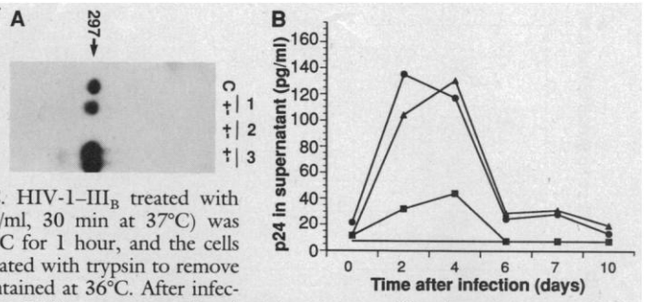

Fig. 2. (**A**) Inhibition of HIV infection of U373 cells by anti-GalC IgG. U373 (lane 1), HeLa (lane 2), or HeLaT4 (lane 3) cells were incubated with either nonimmunized rabbit IgG (−) or anti-GalC IgG (+) (0.18 mg/ml) for 30 min at 0°C. HIV-1–III_B treated with deoxyribonuclease I (20 U/ml, 30 min at 37°C) was added and incubated at 36°C for 1 hour, and the cells were washed extensively, treated with trypsin to remove residual inoculum, and maintained at 36°C. After infection (72 hours), total cellular DNA was extracted and subjected to amplification. A two-step PCR protocol was used (*31*). Initially, 2.5 μg of cellular DNA was amplified with primer pair 4331 and 4332, then one-tenth of the product was amplified with primer pair K3-K7. The amplified nested product was subjected to electrophoresis in a 1.5% agarose gel, transferred to a nylon membrane, and probed with a 600–base pair HIV-1 fragment encompassing the amplified sequences (*27*). C, amplification of control positive DNA. (**B**) Inhibition of $p24^{gag}$ production in SK-N-MC cells. The cells were incubated with purified IgG (6 mg/ml) from a rabbit immunized with GalC (8586) (■) or from a rabbit immunized against an irrelevant antigen (●), infected with HIV-1 (strain III_B) (*13*) in the presence of the test IgG, and then fed with media containing antibody. Aliquots of the supernatant were removed at regular intervals and replaced with fresh media containing the antibody, and then assayed for supernatant $p24^{gag}$ with an antigen capture assay. Solid triangles indicate cells treated with PBS, and the line without symbols indicates the sensitivity of the assay (8 pg/ml).

Studies with recombinant proteins and with different antibodies have clearly established that, of the two HIV-1 glycoproteins, gp120 is responsible for binding to CD4 (*18, 19*). To determine whether HIV-1 gp120 could bind to GalC, an HPTLC binding assay was used (*20*). GalC and control glycolipids were separated by HPTLC, and their ability to bind ^{125}I-labeled, recombinant gp120 from HIV-1 (strain III_B) was determined (*21*). ^{125}I-labeled gp120 bound to GalC and to galactosyl sulfatide, but not to glucosyl ceramide, G_{M1}, G_{D1a}, or neutral glycolipids extracted from erythrocytes (*22*) (Fig. 3B). To control for nonspecific binding of recombinant glycoproteins, we radioiodinated herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) (*21*) at a similar specific activity and showed that there was minimal binding to GalC and galactosyl sulfatide (Fig. 3C). The ability of radioiodinated gp120 to bind to GalC in the HPTLC plate was reduced by exposure to either antibody 4BGC2 or to unlabeled gp120. To obtain a saturation curve for the gp120-GalC interaction, 5 μg of GalC were spotted on an HPTLC plate, a binding assay was performed as described (Fig. 3), and the amount of binding was quantitated with a gamma spectrometer (Fig. 4A). The maximum binding capacity (B_{max}) for gp120 binding was 1.4 pM, and the dissociation constant (K_d) was 11.6 nM, which is between 2.3- and 6-fold higher than the binding of gp120 to CD4 (K_d = 2 to 5 nM) (*19, 23*).

Binding of HIV to carbohydrates on cell membranes may have a role in viral interaction with CD4-negative cells (*24*). Our results implicate a prominent brain and peripheral nervous system glycolipid in HIV infection of neural cells in culture. Because synthesis of GalC involves complex biochemical pathways, it is difficult to express this molecule in cells not susceptible to HIV infection, thus converting them to infectible lines. Therefore, although two lines of evi-

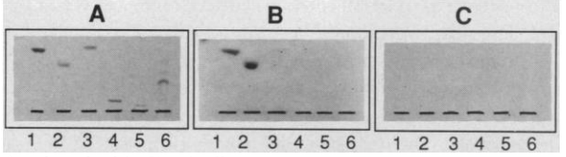

Fig. 3. Binding of HIV-1 gp120 to GalC immobilized on a TLC plate. Glycolipids were separated by chromatography on alumina-backed silica gel G-60 (EM Science, Gibbstown, New Jersey) in chloroform-methanol-water (60:35:8) on three identical plates. The plates were then incubated with GTS buffer [1% gelatin in 50 mM tris-HCl (pH 7.4) and 0.15 M NaCl] for 45 min. HIV-1 (strain III_B) gp-120 or HSV-1 gD were radioiodinated with ^{125}I-labeled Bolton-Hunter reagent (ICN Biochemicals) to a specific activity between 8×10^3 and 13×10^3 cpm per nanogram and incubated with the HPTLC plates (10^6 cpm per milliliter) in GTS buffer for 1 hour. The plates were washed three times for 10 min with PBS containing 0.3 M NaCl, and the chromatograph was exposed to x-ray film overnight. Lane 1, GalC; lane 2, galactosyl sulfatide; lane 3, glucosyl ceramide; lane 4, GM_1; lane 5, G_{D1a}; lane 6, neutral glycolipids from human erythrocytes (glucosyl ceramide, lactosyl ceramide, ceramide trihexoside, and globoside). (**A**) Chromatographed lipids stained with orcinol; (**B**) chromatographed lipids exposed to ^{125}I-labeled gp120, demonstrating binding of gp120 to GalC and to galactosyl sulfatide; and (**C**) chromatographed lipids exposed to ^{125}I-labeled gD (*17*), showing minimal binding of gD to lipids.

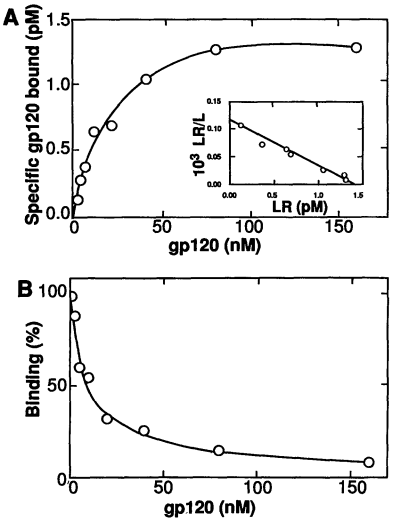

Fig. 4. (**A**) Saturation curve of gp120 binding to GalC. An experiment similar to that described in Fig. 3 was performed, but the quantity of gp120 added to the chromatographic plate was varied, and the lipid spot was quantified by exposing it to iodine vapors and counting with a gamma spectrometer. (**Inset**) Scatchard plot obtained from the data (LR, bound gp120; L, free gp120). The K_d was 11.6 nM and the B_{max} was 1.4 pM. (**B**) Inhibition of binding to GalC by unlabeled gp120. The chromatogram was incubated with 1.0 pM ^{125}I-labeled gp120 and unlabeled gp120 in a 1.0 ml volume as indicated (*23*).

dence point to involvement of GalC in viral entry in these cells, proof of the role of GalC or of a related glycolipid as a receptor molecule will have to await these experiments.

The pathogenesis of the primary neurologic complications of HIV infection, particularly the HIV-associated dementia complex (HADC), is likely to be complicated, and, as in the immune system, may involve infection of several cell types (*25*). Pathologic studies of the brain in some patients with HIV infection have shown demyelination (*26*). More commonly, there is considerable infiltration by macrophages, which are clearly the most productively infected cell type in the CNS (*27*), with much less prominent infection of other CNS elements (*28*). Limited detection of HIV antigens in glial cells may be due to difficulties inherent in the detection of latent retroviral infections (*29*). The results presented here suggest that GalC could serve as a receptor for infection of oligodendrocytes, which were noted to be infected in one study of brain biopsies (*28*). Alternatively, the gp120-GalC interaction could affect oligodendrocyte function in the absence of a full infection, as has been noted with some GalC antibodies (*8*).

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12. IgG was purified with Affi-Gel Blue (Bio-Rad, Richmond, CA) for the internalization assays and with Staph-A Sepharose (Sigma) for the infection experiments.
13. SK-N-MC or U373-MG cells were incubated with purified IgG for 30 min at 0°C, then infected with HIV-1 (stain III_B) for 60 min at 36°C. The multiplicity of infection, calculated by titering the stock virus in a CD4-positive cell line (SUP-T1) was 0.1 median tissue culture infectious dose ($TCID_{50}$) per cell. At the end of the incubation period, the cells were washed extensively and treated with trypsin (25 μg/ml, 5 min at room temperature), and media containing the appropriate IgG (0.1 mg/ml) were added. Samples were collected at the indicated time points, and media containing antibody were added back. The samples were assayed for viral $p24^{gag}$ protein by means of an antigen capture assay. The antigen capture assay (Coulter Immunology, Hialeah, FL) was used as per the manufacturer's instructions. The sensitivity of the assay was at least 8 pg/ml.
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30. A similar assay was performed with antibody 8586; in addition, internalization in the SK-N-MC cell line was measured. The results ($p24^{gag}$ concentration in nanograms per milliliter of lysate of cells incubated with HIV-1 (strain III_B) at 37°C) were as follows: for cell-type SK-N-MC, serum 8586 yielded 1.6 ng/ml, and an irrelevant antibody (NRS) yielded 21 ng/ml; for U373, serum 8586 yielded 1.6 ng/ml, and NRS yielded 24.6 ng/ml; and for HeLaT4, serum 8586 yielded 142 ng/ml, and NRS yielded 110 ng/ml. (NRS was preincubated with GalC liposomes.)
31. The PCR amplification was done in a 100-μl reaction mix with reagents provided by Cetus and *Taq* polymerase. Reagents were cycled 30 times at 94°C for 1 min, at 55°C for 1 min, and at 70°C for 2 min in a Perkin-Elmer cycler. Primer pair 4331 5′-CGAGAGCGTCAGTCTTAAGC (*gag* sense beginning at position 795 of the pHXB2D sequence) and 4332 5′-AAGTCCTAGGTGATATGGCC (*gag* an-

tisense 1220) were used for the initial amplification, and K3 5′-TAGAACGATTCGCAGTTAAT (*gag* sense 913) and K7 5′-CCTGGATGTTCTGCACTATA (*gag* antisense 1207) were used for the nested amplification. The transferred product was probed with a fragment of pHXB2D (nucleotides 631 to 1258) labeled with deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanidine triphosphate by the random priming method to a specific activity of 1.14×10^8 cpm per milligram of DNA.

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Deposits of Amyloid β Protein in the Central Nervous System of Transgenic Mice

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Alzheimer's disease is characterized by widespread deposition of amyloid in the central nervous system. The 4-kilodalton amyloid β protein is derived from a larger amyloid precursor protein and forms amyloid deposits in the brain by an unknown pathological mechanism. Except for aged nonhuman primates, there is no animal model for Alzheimer's disease. Transgenic mice expressing amyloid β protein in the brain could provide such a model. To investigate this possibility, the 4-kilodalton human amyloid β protein was expressed under the control of the promoter of the human amyloid precursor protein in two lines of transgenic mice. Amyloid β protein accumulated in the dendrites of some but not all hippocampal neurons in 1-year-old transgenic mice. Aggregates of the amyloid β protein formed amyloid-like fibrils that are similar in appearance to those in the brains of patients with Alzheimer's disease.

ACCUMULATION OF AMYLOID β PROTEIN is a characteristic and diagnostic feature of brains from individuals with Alzheimer's disease (AD) and Down syndrome (DS) (*1*). The 4-kD amyloid β protein is a truncated form of a larger amyloid precusor protein (APP), which has features typical of a cell surface integral membrane glycoprotein (*2*). At least five different APP isoforms containing 563, 695, 714, 751, and 770 amino acids (*3*) can be generated by alternative splicing of primary transcripts of a single gene on chromosome 21 (*3*). The 40– to 42–amino acid β protein segment comprises half of the transmembrane domain and the first 28 amino acids of the extracellular domain of APP (*2*), and is encoded within two exons (*4*).

The mechanism by which the amyloid β protein is derived from its precursor is not known. APP is processed in vitro by a proteolytic cleavage within the amyloid β protein region (*5*). Generation of the amyloid β protein, therefore, involves an alternative processing pathway, possibly as a result of post-translational modifications such as phosphorylation (*6*).

Although the deposition of amyloid appears to be an early event in the progression of AD (*7*), its role in neurodegenerative processes remains unknown. Amyloid β protein can be neurotrophic for undifferentiated hippocampal neurons in culture and, at high concentrations, neurotoxic to differentiated neurons (*8*). Mutant forms of APP have been implicated in hereditary cerebral hemorrhage with amyloidosis of Dutch origin (*9*) and in at least two families with familial forms of AD (*10*). In addition, overexpression of one or more forms of APP may be responsible for the AD-like pathologies of individuals with DS (*11*). These findings suggest that accumulation of amyloid β protein may be a critical step in the neurodegenerative processes of AD.

The lack of experimental animal models for AD has limited the elucidation of the mechanism of amyloid formation and its role in the pathogenesis of AD. Nonhuman primates provide the only in vivo model for investigating amyloid formation in the central nervous system (CNS) (*12*). The high cost and limited availability of aged primates, however, restricts their use as practical model systems. Transgenic rodent models may provide a useful alternative. The expression of native or mutant forms of APP in transgenic mice may help to identify aberrant APP processing pathways that lead to the accumulation of amyloid β protein and clarify the role of amyloid β protein in neuronal degeneration. We therefore initiated a series of experiments to express various forms of APP in the brain of transgenic mice.

We have introduced into mice a construct that encodes the 42–amino acid amyloid β protein, regulated by a 4.5-kb fragment from the 5′ region of the human APP gene (Fig. 1). This APP regulatory region directs neuron-specific expression of the reporter gene *lac Z* from *Escherichia coli* in the CNS of transgenic mice in a pattern that is similar to the pattern of endogenous mouse and human APP mRNA expression (*13*).

Two lines of transgenic mice, AE101 and AE301, expressed human amyloid β protein mRNA in the brain (Fig. 2) and transmitted the transgene in a Mendelian fashion. Steady-state amounts of the transgene mRNA were lower than steady-state amounts of the endogenous mouse APP mRNA. In both transgenic lines, however, human amyloid β protein was synthesized and accumulated in the CNS of 1-year-old mice (Fig. 3).

We examined immunocytochemical and ultrastructural features of brains from several F1 generation transgenic mice from lines AE101 and AE301 at approximately 1 year of age. When sections of brain from 1-year-old control mice were stained with antibodies to the amyloid β protein (*14*), no immunoreactivity was detected (*15*). In contrast, sections of brain from transgenic mice showed amyloid β protein immunoreactivity (Fig. 3). Amyloid β protein staining was located predominantly in the hippocampus, where it appeared as clusters of dots that were symmetrically distributed on both sides of the brain. Within the hippocampus, amyloid β protein immunoreactivity was most prominent in the molecular layer of CA1 and CA2; only occasional amyloid β protein–positive clusters were detected in CA3 regions of the hippocampus and dentate gyrus. Amyloid β protein was not detected in cerebral cortex. We found similar patterns of amyloid β protein immunoreactivity in four F1 generation mice from both transgenic lines by four different amyloid β protein–specific rabbit polyclonal antibodies (*15*). Occasional clusters of amyloid β protein immunoreactivity were found in other regions of the CNS but not in a consistent pattern. Amyloid β protein immunoreactiv-

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