Title	THE DEVELOPMENT AND THE PRACTICAL APPRECIATION OF NEURAL CELL-TYPE-SPECIFIC EXPRESSION SYSTEM WITH REPLICATION-DEFECTIVE ADENOVIRUS VECTORS
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Citation	大阪大学, 1996, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/3109898
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THE DEVELOPMENT AND THE PRACTICAL APPLICATION OF NEURAL CELL-TYPE-SPECIFIC EXPRESSION SYSTEM WITH REPLICATION-DEFECTIVE ADENOVIRUS VECTORS

（アデノウイルスベクターを用いた

神経細胞型特異的遺伝子導入法の

開発と応用）

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<CONTENTS>

CHAPTER I

GENERAL INTRODUCTION

SECTION 1 INTRODUCTION OF VIRAL VECTORS
SECTION 2 ADENOVIRUS BIOLOGY

CHAPTER II

MATERIALS AND METHODS

CHAPTER III

A NEURAL CELL-TYPE SPECIFIC EXPRESSION SYSTEM USING RECOMBINANT ADENOVIRUS VECTORS

INTRODUCTION
RESULTS
DISCUSSION
ACKNOWLEDGEMENTS

CHAPTER IV

CONSTRUCTION OF GLIAL CELL-TYPE-SPECIFIC ADENOVIRUS VECTORS AND GENETICAL MODIFICATION OF PRIMARY ASTROCYTES TO EXPRESS TYROSINE HYDROXYLASE

INTRODUCTION
RESULTS
DISCUSSION
ACKNOWLEDGEMENT

ABBREVIATIONS

AADC, aromatic L-amino acid decarboxylase; **AAV**, adeno-associated virus; **Ad2**, human adenovirus serotype 2; **Ad5**, human adeovirus serotype 5; **β-Gal**, β-galactosidase; **BH₄**, tetrahydrobiopterin; **bp**,base pair; **CNS**, central nervous system; **DMEM**, Dulbecco's modified Eagle's medium; **FBS**, fetal bovine serum; **FCS**, fetal calf serum; **FI**, cerebellar fissure; **FITC**, fluorescein isothiocyanate; **GFAP**, glial fibrillary acidic protein; **GL**, granule cell layers; **HCMV**, human cytomegalovirus; **HSV-1**, Herpes simplex virus type 1; **hTH-1**, human tyrosin hydroxylase type-1; **IP₃R-1**, inositol 1,4,5-trisphosphate receptor type 1; **kbp**, kilobase pair; **L-DOPA**, dihydroxyphenylalanine; **MBP**, myelin basic protein; **MHC**, major histocompatibility complex; **m.o.i.**, a multiplicity of infection; **m.u.**, map units; **NL1**, nuclear locarization signal from the glucocorticoid receptor; **PBS**, phosphate-buffered saline; **p.f.u.**, plaque-forming unit; **PL**, Purkinje cell layers; **PLP**, pyridoxal phosphate; **WM**, white matter;

CHAPTER I

GENERAL INTRODUCTION

SECTION 1 INTRODUCTION OF VIRAL VECTORS

Several viruses to have been used as vectors for investigating gene functions and therapies. The viruses used as vectors are classified into two types, RNA virus [retrovirus] and DNA virus [herpesvirus, vaccinia virus, adeno-associated virus (AAV) and adenovirus]. The retrovirus vectors are most commonly used both in basic studies (Cepko et al., 1993) and gene therapies (Morgan and Anderson, 1993). Retroviruses reverse-transcribe their RNA genome into proviral DNA that integrates into the genomic DNA of host cells. Viral genetic informations are transferred to progenies as the proviral DNA, but it also enhances the risk for cellular transformation caused by insertional mutagenesis or activation of oncogenes. Furthermore, retrovirus can not transfer a foreign gene into non-dividing cells. Herpes simplex virus type 1 (HSV-1) vectors have the nature of transneuronal transfer and accommodate up to 15 kbp of foreign genes. However, recombinant HSV-1 retain several functional viral genes, which may be cytotoxic to the recipient cells and can potentially reactivate latent viruses which exist within most

adults (Johnson et al., 1992; Isacson, 1995). Vaccinia viruses are capable of accommodating large inserts (25 kbp or more), but genetic manipulation and obtaining recombinant virus is difficult and exogenous promoters can not be used in vaccinia virus vectors (Weisz and Machamer, 1994). AAV appears to integrate in a site-specific manner in human chromosome 19 (19q13.3qter) (Kotin et al., 1991). In case of AAV vectors, the size of inserts was limited to 4 kbp and its safety is unclear. Adenovirus vectors are better studied than other DNA virus vectors. Human adenovirus serotypes 5 (Ad5) is attractive candidate for use as a vector in basic studies and gene therapy (reviewed in Miller, 1992; Morgan and Anderson, 1993; Crystal, 1995). The reason is as follows. 1) The double-stranded DNA genome of adenovirus is relatively easy to manipulate by recombinant DNA techniques. 2) The viral particle is relatively stable. 3) Adenoviruses can be grown to high titer, purified, and concentrated to 10^{11} plaque-forming units (p.f.u.)/ml (Kanegae et al., 1994). 4) Adenoviruses have a broad host range and a variety of different cell-type specificity. 5) The infection with adenoviruses is independent of cell division. Thus, adenovirus vectors are high effective in transfering a foreign gene into non-dividing cells, including neural cells (Le Gal La Salle et al., 1993; Davidson et al., 1993; Akli et al., 1993; Bajocchi et al., 1993; Caillaud et al., 1993; Hashimoto et al., 1996), hepatocytes (Kay et al., 1995) and muscle cells (Stratford-

Perricaudet et al., 1992). 6) Adenovirus genome exists as an episome in the nucleus of host cells and integrates very rarely in host genome. 7) Cytotoxicity of adenovirus is low (Neve, 1993). 8) There is a safety of adenovirus to humans. Human adenovirus serotypes 5 (group C adenoviruses) very rarely cause malignant disease in human. Most children are immunized against human adenovirus serotype 5 up to three years old without accompanying serious disease and adenovirus vaccines have been used extensively in the military without accidents (reviewed in Horwitz 1995). The most common recombinant adenovirus is Ad5 deleted of the E1 and E3 regions. It can not replicate in wild-type cells because of lacking E1A which is essential for the expression of all other viral genes (see SECTION 2 of CHAPTER I). Thus, a recombinant adenovirus propagates only in 293 cells which are derived from human embryonic kidney cells transformed with adenovirus E1 genes, because 293 cells supply E1 proteins to recombinant adenoviruses in *trans* (Fig. I-4). Recombinant adenoviruses were deleted 2.9 kbp of E1 region (1.3-9.3 m.u.) and 1.9 kbp of E3 region (79.6-84.8 m.u.) from Ad5 genome, and moreover, adenoviruses can package about 105 % of the wild-type genome length (Bett et al., 1993). Thus, up to 7 kbp of inserts can be accommodated in the E1 deleted region. Recently, the replication-defective adenovirus vectors which can contain up to 8 kbp of insert were constructed by the deletion of 2.7 kbp of E3 region (78.3-85.8 m.u.) (Bett et al., 1994). However, it

is still unclear whether we can apply adenovirus vectors for a long term gene expression, because episomal adenovirus genomes are diluted upon cells division. However, in rats injected with a adenovirus vector which included *lacZ* gene as a reporter gene, *lacZ* gene expression persisted in rat brain for a month. (Le Gal La Salle et al., 1993). For the purpose of studying neuroscience or gene therapies of neural disorders, it is necessary to express a functional molecule in mature neural cells which do not divide and to deliver a foreign gene into brain *in vivo*. The nature of adenovirus vectors is available for studying the function of cloned genes in mature neural cells *in vitro* and *in vivo* as well as for use in gene therapy in human.

SECTION 2 ADENOVIRUS BIOLOGY

Wild-type adenovirus is categorized into DNA virus. The nature of adenovirus is shown in Figure I-1. Adenovirus is an icosahedral particle 70-80 nm in diameter with a dense core containing the DNA genome of about 36 kbp (Horowitz, 1985). The virus was originally isolated from human adenoid tissue (Rowe et al., 1953). Over 100 members of the adenovirus group have been identified and they infect a wide range of mammalian and avian hosts. It has been associated with a number of human respiratory illnesses. Human adenovirus serotypes 2 (Ad2) and 5 (Ad5) (Fig. I-3), which are included in group C adenoviruses, are most extensively studied for the genetic characters (Fig. I-3), the gene function and the viral life-cycle (Fig. I-4 and I-5) (Horowitz 1985).

A life cycle of wild-type adenovirus can be divided into early and late phases (Fig. I-3 and I-4) which are defined as occurring before and after the onset of viral DNA replication, respectively. Genes expressed during the early phase are E1A, E1B, E2A, E2B, E3 and E4 (Fig. I-3). E1A is the first viral gene to be expressed after viral infection. E1A is particularly important because it appears to be required for activation of all other early genes (E1B, E2, E3, and E4). E2A and E2B encode DNA binding and DNA polymerase activities, respectively, and are essential for viral DNA replication. E3 appears to have no essential function for adenovirus growth or DNA replication, and

may be involved in modulating host responsiveness to adenovirus infection. A 19-kDa protein encoded by the E3 region has been shown to bind to major histocompatibility complex (MHC) polypeptides in the endoplasmic reticulum, thereby inhibiting appearance of MHC peptides at the cell surface (Pääbo et al., 1986). The E4 region is currently not well understood. After the replication of viral DNA caused by the expression of early genes, the late mRNA are expressed and virions are assembled. The viral DNA replication is necessary to synthesize the late mRNAs.

Thus the transcription of early mRNAs (E1B-E4), the replication of viral DNA, and the expression of late genes did not occur in viral mutants with defective E1A genes (Berk et al., 1979) (shown as replication-defective adenovirus vector in Fig. I-4).

Fig. I-1 The properties of animal DNA viruses.

(Fig. I-1)

Animal DNA Viruses (Deoxyribovirus)

	Parvo(Picodna) virus	Papovavirus	Adenovirus	Herpesvirus
Nucleic acid core	DNA	DNA	DNA	DNA
Capsid symmetry	Cubic	Cubic	Cubic	Cubic
Virion: naked or enveloped	Naked	Naked	Naked	Enveloped
Site of capsid assembly	Nucleus	Nucleus	Nucleus	Nucleus
Site of nucleocapsid envelopment				Nuclear membrane
Reaction of ether treatment	Resistant	Resistant	Resistant	Sensitive
Number of capsomers	12 or 32	42 or 72	252	162
Diameter of virion, nm	18-24	40-55	70-80	110
Molecular weight of nucleic acid in virion (x10^6)	3.0-3.6	2-4	23	40-84
Virus group	Parvo(Picodna) virus	Papovavirus	Adenovirus	Herpesvirus

Fig. I-2 The properties of human adenovirus serotype 5 (Ad5).

(Fig. I-2)

Adenovirus
(Human; Serotype 5)

- Nucleic acid core : DNA (36 kb)
- Capsid symmetry : Icosahedral
- Number of capsomers : 252
- Diameter of virion : 70-80 nm
- Site of capsid assembly : Nucleus
- Site of viral genom : episomal
- Tumors in animals : Low or none

Fiber

Pentonbase

Hexon
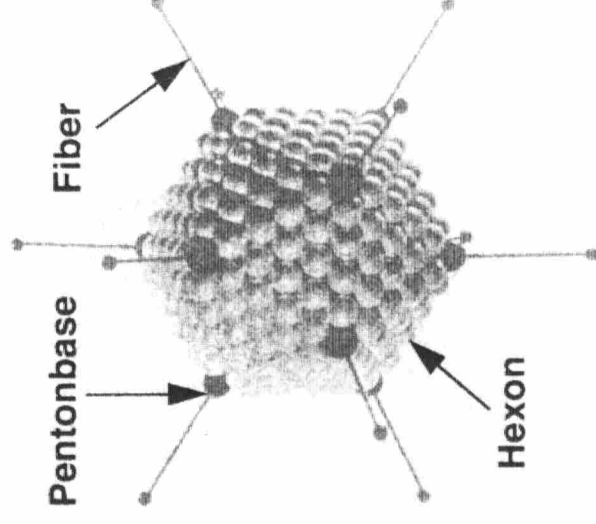
Fig. I-3 Transcription and translation map of adenovirus. The early mRNAs are designated E and are shown in thin lines. The late mRNAs are designated L and are shown in heavy lines. All mRNAs originate at the map units of 16.3 (major late promoter) and contain the tripartite leader sequence. The translations from early mRNAs induce to be synthesized late mRNAs. The tripartite leader sequence is designated 1, 2 and 3 (at map units 16.5,19.5 and 26.5, respectively). The polypeptides are designated by roman numerals for the virion structural components and in kilodaltons for nonstructural virus-coded translation products. The arrow heads shown are restriction sites of *Eco* T22I. Cited and revised from Horowitz (1985).

(Fig. I-3)

Proteins

14K
IX   core   PTNase   non-virion   14.5K
13K 21K   11K
26K 16K   52- penton   hexon   33K 10K   fiber
32K 55K   13.6K   55K IIIa III pVII V pVI II 23K   100K pVIII 16K IV

RNAs   1 2 i   3   x y   z
                                                                                                          L5
                                                                                               L4

                                                                                            L3

                                                                              L2

                                                                   L1
                                                                I II
EIA   EIB   VA
                                                                                        E3

r-strand 3'   5'
ℓ-strand 5'   3'
          0   10   20   30   40   50   60   70   80   90   100
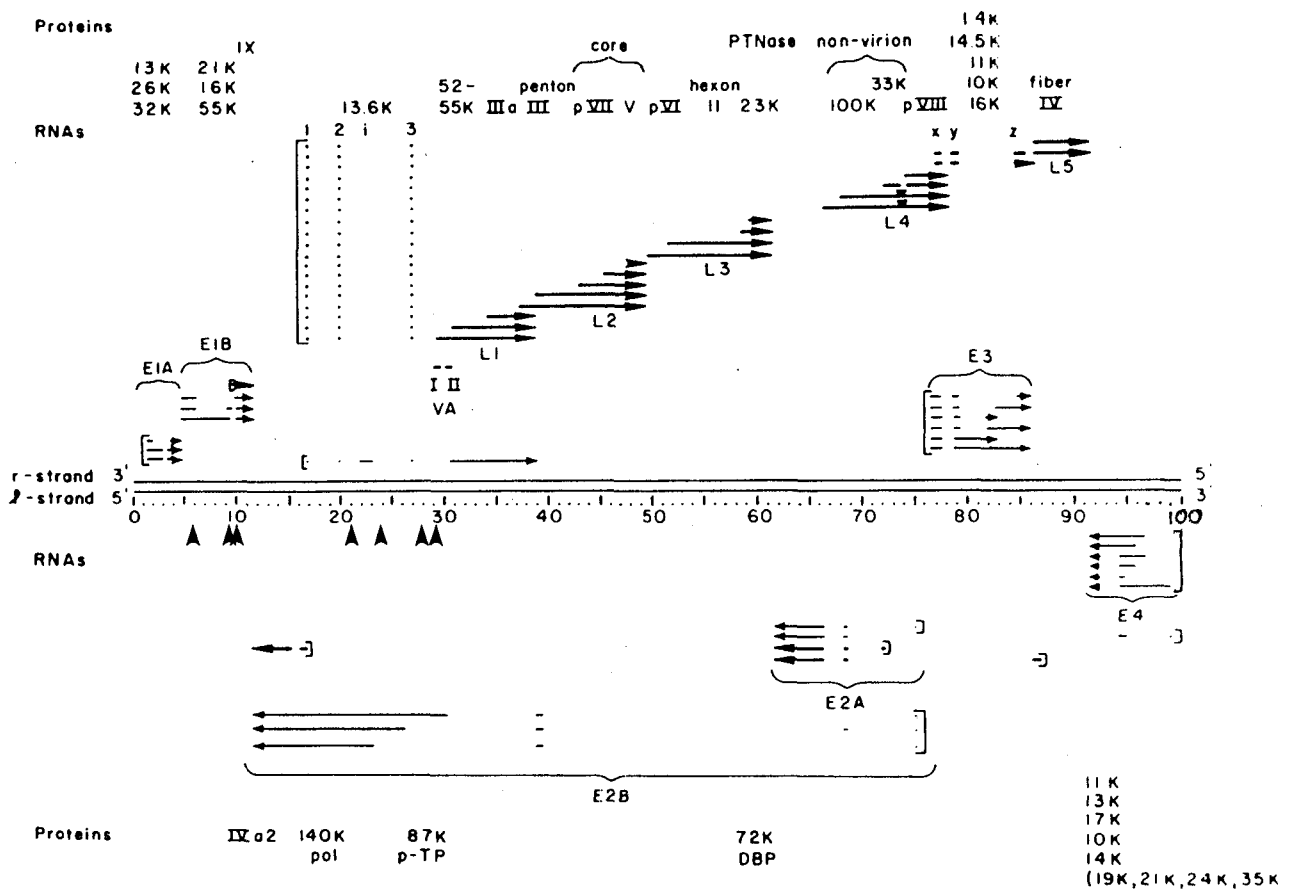
RNAs
                                                                                                        E4

                                                                              E2A

                                                                   E2B

                                                                                                        11K
                                                                                                        13K
                                                                                                        17K
Proteins   IVa2 140K   87K   72K   10K
                  pol   p-TP   DBP   14K
                                                                                                        (19K,21K,24K,35K

Fig. I-4 Life cycle of wild type adenoviruses, replication-defective adenovirus vectors and replication-defective adenovirus vectors in 293 cells. 293 cells are human embryonic kidney cells transformed with Ad5 E1 genes. Thus, replication-defective adenovirus vectors can propagate in 293 cells, because E1 proteins are provided from recombinant adenoviruses in *trans*.
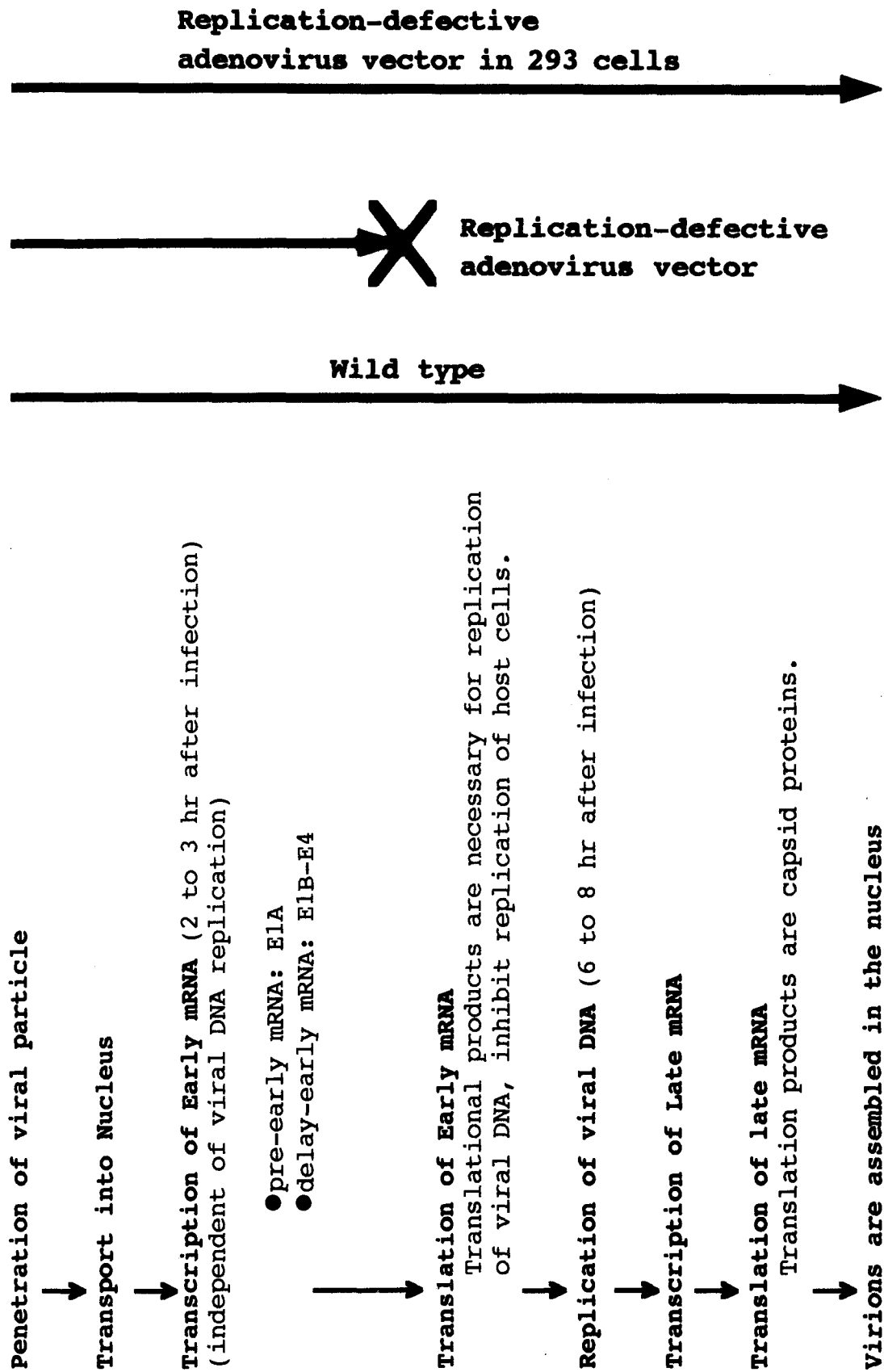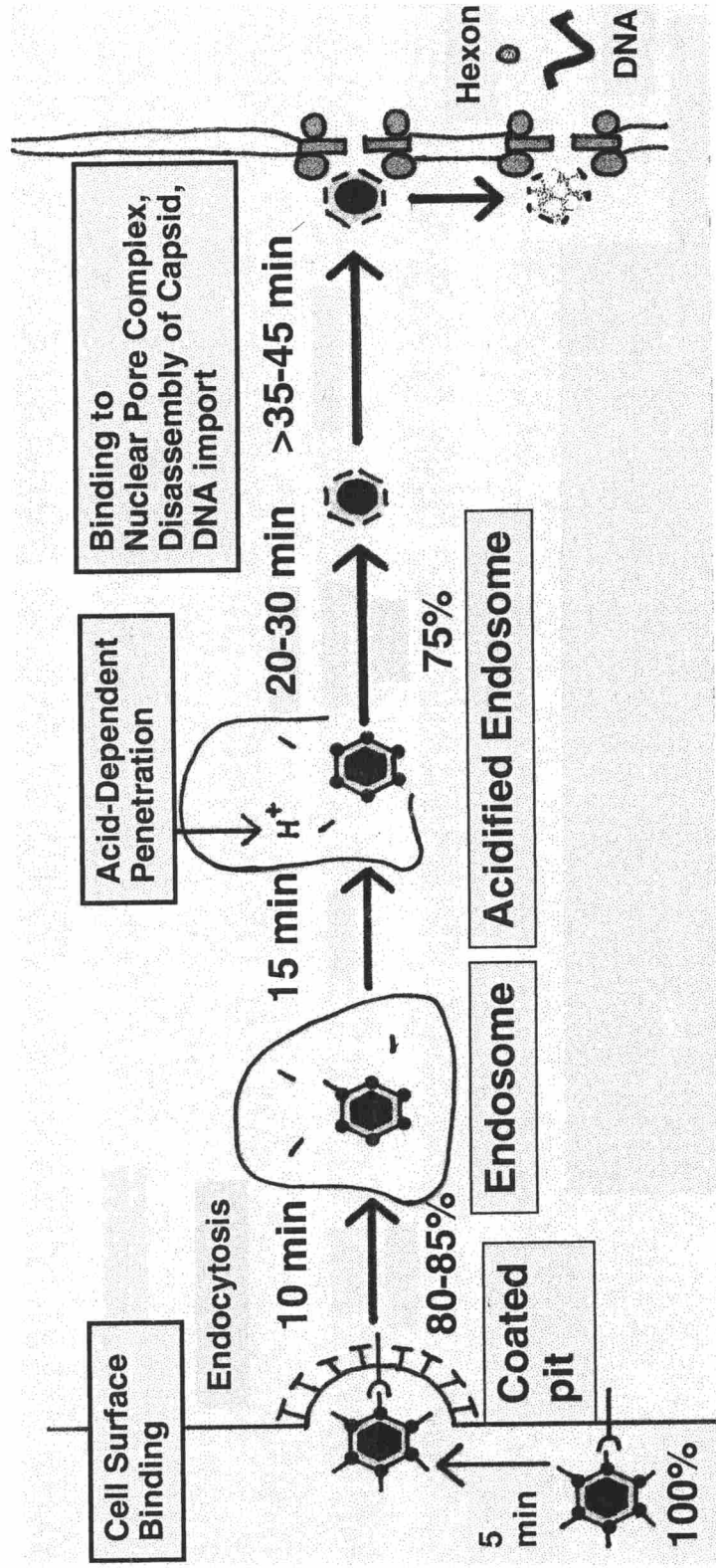
(Fig. I-4)

Replication-defective adenovirus vector in 293 cells →

→ ✕ **Replication-defective adenovirus vector**

Wild type →

Penetration of viral particle →

Transport into Nucleus →

Transcription of Early mRNA (2 to 3 hr after infection) (independent of viral DNA replication) →
- pre-early mRNA: E1A
- delay-early mRNA: E1B-E4

Translation of Early mRNA
Translational products are necessary for replication of viral DNA, inhibit replication of host cells. →

Replication of viral DNA (6 to 8 hr after infection) →

Transcription of Late mRNA →

Translation of late mRNA
Translation products are capsid proteins. →

Virions are assembled in the nucleus

Fig. I-5 Stepwise transport of adenoviruses from cell surface to the nucleus. Adsorption of adenovirus to a cell is a two-step process. First, the carboxy-termimal knob of the fiber protein binds to the cellular receptor that has not been identified. Then, the penton base protein binds to members of integrins (Wickham et al., 1993). The second interaction occurs through an arg-gly-asp sequence present in penton base. The same motif present in a variety of extracellular adhesion molecules that bind to integrins. After adsorption, viral particles are internalized by highly efficient receptor-mediated endocytosis. 80-85 % of adsorbed virus is internalized (Greber et al., 1993) and 75 % of adsorbed virus is penetrated in acidified endosome. About 40 min after adsorption, virus particles can be visualized at nuclear pores. Then, viral genome is imported into nuclei of host cells.

(Fig. I-5)

Cell Surface Binding

Endocytosis

Acid-Dependent Penetration

Binding to Nuclear Pore Complex, Disassembly of Capsid, DNA import

5 min

100%

10 min

80-85%

15 min

H⁺

20-30 min

75%

>35-45 min

Coated pit

Endosome

Acidified Endosome

Hexon

DNA

CHAPTER II

MATERIALS AND METHODS

A. *Viral genome construction*

The recombinant adenovirus constructs are shown in Figure II-1 and II-2.

Adex1SRLacZ, Adex1EFLacZ and Adex1CALacZ have been described by Kanegae et al. (1994), Chang, H. et al. (1995) and Nakamura et al. (1994), respectively. Adex1SRLacZ has *lacZ* inserted between the SRα promoter (Takebe et al., 1988) and the SV40 polyadenylation signal (0.4 kbp). Adex1EFLacZ has *LacZ* inserted between the elongation factor 1α promoter and the polyadenylation sequence (0.15 kbp) (a part of pEF321-T in Kim et al., 1990). In Adex1CALacZ, the *lacZ* gene was inserted into the pAdex1pCAw vector (Kanegae et al., 1995), which has an expression unit including the CAG promoter (Niwa et al. 1991), composed of the cytomegalovirus enhancer plus the chicken β-actin promoter and the rabbit β-globin polyadenylation signal (0.57 kbp) (Niwa et al., 1991).

AdexCAG-NL-LacZ was constructed by inserting a blunt-ended fragment containing an NLlacZ unit into the *Swa*I site of the

pAdex1pCAw vector. In the NLlacZ unit, the *lacZ* gene was fused with a 5' untranslated region and the initiation codon from Herpes simplex virus thymidine kinase gene (*HSV tk leader & AUG*) followed by a nuclear localization signal (*NL1*) from the glucocorticoid receptor (a part of 497-524.Z in Picard and Yamamoto, 1987).

AdexL7-NL-LacZ. The pPCP2S vector (a gift from Dr. T. Nakagawa) included the L7/PCP2 promoter (0.8 kbp) and the L7/PCP2 gene polyadenylation signal *L7 pA* (0.5 kbp), which was generated by the polymerase chain reaction. A *SalI-Bam*HI linker was inserted between the two fragments. The NLlacZ unit, which was modified to have *Sal*I and *Bam*HI on its ends, was inserted into the pPCP2S vector. The resultant expression unit was blunt-ended and inserted into the *Cla*I site of pAdex1cw (Kanegae et al., 1995), which is a 42 kbp cosmid vector containing the human adenovirus type 5 genome lacking the E1A, E1B (1.3-9.3 m.u.) and E3 regions (79.6-84.8 m.u.). The direction of the inserts was antiparallel to E1A and E1B to obtain higher levels of expression (Miyake et al., 1996).

AdexMBP-NL-LacZ. The *Bgl*II/*Mro*I 1.3 kbp fragment, which contains the 5' sequence (1297 bp) from the transcription initiation site of the mouse myelin basic protein (MBP) gene (Miura et al., 1989), was subcloned from cosBJAB5 (Okano et al., 1991). The fragment was modified to have *Spe*I sites on

both ends by linker insertion. The resultant *Spe*I fragment was inserted into pBluescript II KS(-) which also has a *Hind*III fragment (5.5 kbp) from 497-524.Z (Picard and Yamamoto, 1987) (I call it the NLlacZIVSpA unit), which contained NLlacZ unit followed by the second intron and the polyadenylation site of the rabbit β-globin gene (*rabbit β-globin IVS2 & pA*) (Pääbo et al., 1983). The unit that contained the MBP promoter followed by the NLlacZIVSpA expression unit was excised, blunt-ended and inserted into the *Swa*I site of the pAdexlcw vector.

AdexGFAP-NL-LacZ. The *Hind*III 256 bp fragment from pGF8L (Miura et al, 1990), which was the promoter region of glial fibrillary acidic protein (GFAP), was subcloned into pBluescript II KS (-) (I called that pL8). The NLlacZIVSpA unit changed its both ends into *Sal*I site and the *Sal*I fragment (5.5 kbp) was inserted into *Sal*I site of pL8. The fragnent which include the GFAP promoter linked nuclear targeted β-galactosidase gene and rabbit β-globin IVS2 & pA was inserted into the *Swa*I site of pAdexlcw (descrived in Hasimoto et al., 1996), this was termed pAdexGFAP-NL-LacZ.

AdexGFAPhTH1. The *Eco*RI 1.9 kbp fragnent from pHTH-1 (a gift from Prof. T. Nagatsu), which included cDNA of human tyrosine hydroxylase type 1 (hTH-1) was modifided to have blunted on both ends by T4 DNA polymerase. The fragment was inserted into *Sma*I site of pPA, which was including rabbit

β-globin IVS2 & pA (*Bam*HI-*Hind*III 2 kbp fragment from 497–524Z) in pBluescript II KS (-). The hTH-1 conjugated rabbit β-globin IVS2 & pA unit (hTH1pA) was insterted into *Sal*I site of pL8. Then, the GFAP promoter linked hTH1pA unit (GFAPhTH1pA) was inserted into *Swa*I site of pAdex1cw, this was termed pAdexGFAPhTH1.

AdexΔP-NL-LacZ contained an NLlacZIVSpA unit inserted into the *Swa*I site of pAdex1cw.

Adex1w did not have an expression unit (Miyake et al., 1996).

B. Preparation of recombinant adenovirus

I generated recombinant adenovirus by means of a modification of the method of Saito et al. (1985). Details of the procedure will be published elsewhere (Miyake et al., 1996). Briefly, expression cosmid vectors and *Eco*T22I digested DNA-terminal protein complex of Ad5-dℓX (Saito et al. 1985; Chang, H. et al. 1995), which is the human type 5 adenovirus lacking the E3 region, were co-transfected into 293 cells (ATCC; CRL1573) by calcium phosphate co-precipitation. *Eco*T22I digests Ad5-dℓX genome at seven sites between 0 m.u. and 29 m.u. (including E1A and E1b region) and this precedure prevents the generation of parent adenovirus (Ad5-dℓX). The recombinant adenovirus was generated through a homologous recombination in 293 cells

between the expression cosmid vector and the *Eco*T22I degested Ad5-d*l*X. The incorporation of the expression units into the isolated recombinant adenoviruses and their construction were verified by digesting the viral genomes with the appropriate restriction enzymes (Saito et al., 1985). Contamination of the parent adenovirus was carefully examined by restriction analyses and Southern blot analyses using the *Hind*III/*Sma*I fragment (1.8 kbp) including E1A region as a probe. The recombinant adenoviruses were further propagated in 293 cells and the viral solution was stored at -80 °C. For each vector, a single batch of high titer adenoviral stock (1 x 10^{11} p.f.u./ml) was prepared by double caecum step gradient purification (Kanegae et al., 1994). The titers of viral stocks were determined by plaque assay on 293 cells.

C. Cell culture

Monolayer cultures of B104, C6, Neuro2a and NIH3T3 were cultured in Dulbecco's modified Eagle medium (DMEM), 10 % heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomysin. 0.02 % EDTA / PBS(-) was used to detach the cells for subculture, trypsine did not have to be used. After cells were grown to 80 % confluence, they were infected with adenovirus vectors at a multiplicity of infection (m.o.i.) 20 and 100. Twe days After infection, the

tissue cultuer was fixed with 2 % paraformaldehyde 0.1 % glutaraldehyde for 10 min.

D. Serum-free primary cultures of mouse cerebellum

ICR mice cerebella at postnatal day 0 (P0) were primary-cultured in serum-free medium (Fischer et al., 1982) essentially as described by Weber and Schachner (1984). Seven cerebella were dissected out of the littermates at P0, freed of meninges and dissociated into single cells following incubation in Ca^{2+}-, Mg^{2+}- free Hanks' balanced salt solution containing 1 % trypsin and 0.05 % DNase I for 13 min. The cells were seeded on poly-L-lysin-coated glass slides and cultured in serum-free medium containing Eagle's minimum essential medium with 1 mg/ml bovine serum albumin, 10 μg/ml bovine insulin, 0.1 nM L-Thyroxine, 0.1 mg/ml human transferrin, 1 μg/ml aprotinin, 30 nM sodium selenite, 0.25 % glucose, 2 mM L-Glutamine and 2 mg/ml sodium bicarbonate. Cultures were then infected with the recombinant virus for 1 hr at 37 °C. Two days later, the cells were fixed with 2% paraformaldehyde and 0.1 % glutaraldehyde in PBS(-) for 10 minutes.

E. Serum-free primary culture of oligodendrocyte

Primary mixed mouse brain cell cultures were prepared using previously published procedures (Bottenstein, 1986; Knapp et al., 1987). The pallium cerebri removed from newborn ICR mouse were enzymatically dissociated in a solution of 0.25 % trypsin (Difco) and 12.5 μg/ml DNase in Hank's balanced salt solution (HBSS) at 37 °C for 30 min. Then, 5 x 10^5 cells in DMEM with 10 % FCS were seeded on poly-$_L$-lysine coated 15 mm glass coverslips. After 48 hr, the medium was exchanged for the serum free medium which contained 50 % DMEM, 50 % Ham's F-12, 5 μg/ml insulin (Sigma), 1.5 nM sodium selenite (Wako), 50 μg/ml transferrin (Sigma), 10 ng/ml biotin (Sigma), 6 g/l glucose (Sigma), 50 U/ml penicillin (PCG) and 50 μg/ml streptomysin (SM) (Knapp et al., 1987). Cultures were fed every 3 days by replacing half of the existing medium with fresh medium. After 18 days *in vitro*, cultures were infected with adenoviruses, following that cultured for another 2 days. These cultures were then fixed with 2 % paraformaldehyde and 0.1 % glutaraldehyde in PBS(-) for 10 minutes.

F. Primary culture of astrocytes from rat striatum

Astrocytes were prepared from striatum of 2- to 3-day-old neonatal Wister rat by modification of methods described previously (McCarthy and deVellis, 1989). Striatum was

carefully removed from brain and it was treated with 0.25 %
trypsin solution. The resulting mixed glial cells were
cultured in 24 cm^2 flasks in DMEM with 10 % fetal bovine
serum (FBS) at 37 °C. After 8-9 days, nearly confluent,
primary astrocyte cultures were obtained after shaking to
remove cells adhering to the astrocyte layer. After mild
trypsinization of primary astrocyte cultures, second passage
astrocytes, cultured in six-well plates for 3-4 days to
become confluent, were washed vigorously to remove adhering
cells.

G. Slice culture of cerebellum

Slice culture of rodent cerebellum was modified from
Tanaka et al. (1994). Cerebella were dissected from 17-day-
old Wister rats. The vermes of the cerebella were cut
parasagittally into about 600 μm-thick slice with knives. In
some cases, the slices were subsequently trimmed to a
slightly smaller size. That is, the slices were mounted on a
collagen-coated, porous (2.0 μm) polycarbonate membrane
(Nuclepore) which was located at the gas-liquid interface in
a petri dish. The culture medium was modified from that used
by Jaeger et al. (1988) and that by Battenstein et al.(1980).
It consisted of 15 % heat-inactivated horse serum (Gibco),
25 % Earle's balanced salt solution, 60 % Eagle's basal
medium (Earle's salt) (Gibco), 5.6 g/l glucose, 3 mM L-

glutamine, 5 μg/ml human transferrin (Sigma), 5 μg/ml bovine insulin (Sigma) 1 mM sodium pyruvate (Sigma), 50 U/ml penicillin G potassinm, and 100 μg/ml streptomysin sulfate. The cultures were incubated at 33 °C in 5 % CO_2/ 95 % air. Half the volume of the medium was replaced with fresh medium every 2 days. After 3 days, slices were infected with 20 μl AdexCAG-NL-LacZ or AdexΔP-NL-LacZ (1 x 10^{11} pfu) for 1 hour at 33 °C and another 2 days incubated. Then, these slices were washed by PBS(-) and fixed with 2 % paraformaldehyde, 0.1 % glutaraldehyde in PBS(-) for 20 min, following that stained with Bluo-gal solution.

H. In vivo infection with adenovirus

Sprague-Dawley female rats (150-180g ; 7-8 weeks old; Nihon SLC, Shizuoka , Japan), were anesthetized with pentobarbital (50 mg per kg) and secured on a murine stereotaxic platform. Using sterile technique, the skull was exposed and a 2 mm burr hole was made at a point 4 mm caudal from a lambda suture, using a high speed drill. Adenovirus [1μl, 1 x 10^{11} p.f.u./ml in 10 % sucrose / PBS(-)] was injected into the cerebellum using a 1 μl Hamilton syringe and a 26S gauge needle (Fig. II-3). The needle was inserted 4 mm from the dura and adenovirus was injected at a rate of 0.125 μl / 0.5 mm along the needle track as the needle was withdrawn. The needle was then kept in place for 10 min. The

rats were monitored daily for abnormal behavior or seizures. Three or 7 days later, the animals were anesthetized and sacrificed by intracardiac perfusion with 2 % paraformaldehyde, 0.1 % glutaraldehyde in PBS(-). The brains were removed, post-fixed overnight at 4 °C. and stained with Bluo-gal (described below) overnight at 37 °C. The brains were cryoprotected for 3 days by serial equilibration in sucrose [10 %, 15 %, and 20 % in PBS(-)] for 24 hr at 4 °C, then frozen in OCT compound (Sigma) and sectioned sagittally at 10 μm using a cryostat. The sections were placed onto gelatin coated slides, air-dried and stained as described below.

I. β-galactosidase cytochemistry

The fixed cells and brain tissues were briefly rinsed with PBS and stained with 5mM $K_4[Fe(CN)_6]$, 5mM $K_3[Fe(CN)_6]$, 1mM $MgCl_2$, 0.01 % sodium deoxycholate, 0.02 % Nonidet P-40, and 0.1 % halogenated indoyl-β-D-galactoside (Bluo-gal ; Gibco) in PBS for 12 hr at 37 °C.

J. Immunohistochemistry

Tissue cultures and cryo-sections were immunostained with cell-type specific antibody. Purkinje cells were identified using 4C11, a monoclonal antibody against

inositol 1,4,5-trisphosphate receptor type 1 (IP_3R-1) which is highly restricted in Purkinje cells (Maeda et al., 1988 and 1989). Oligodendrocytes were identified by staining with antibody against MBP (Ikenaka et al., 1992). Glial cells were identified with monoclonal antibody agaist GFAP (Amarsham, RPN 1106). Tissue cultures which were infected with AdexGFAPhTH1 were immunostained with TH-17, a monoclonal antibody against tyrosine hydroxyrase (TH-17 was gifted from Prof. Nishino). The second antibodies were fluorescein isothiocyanate (FITC) conjugated anti-Rat IgG (Vector Lab.) and FITC conjugated anti-Rabbit IgG (Vector Lab.).

Fig. II-1 Constructs of recombinant adenoviruses.

The replication-defective adenovirus expression vector (Adex),

consists of the human adenovirus type 5 (Ad5) genome lacking the

E1A, E1B (1.3-9.3 m.u.) and E3 regions (79.6-84.8 m.u.).

Adex1SRLacZ has the *E.coli* β-galactosidase gene (*lacZ*) inserted

between the SRα promoter (*SRα*) and the SV40 polyadenylation

signal (*pA*). Adex1EFLacZ has an expression unit including

elongation factor 1α promoter (*EF1α*), LacZ and *pA*. Adex1CALacZ

has an expression unit including the CAG promoter (*CAG*), composed

of the cytomegalovirus enhancer plus the chicken β-actin promoter,

LacZ and the rabbit β-globin polyadenylation signal (*G pA*). The

expression cassette for AdexCAG-NL-LacZ contains an NLlacZ unit

(See CHAPTER II) between *CAG* and *G pA*. The expression cassette

for AdexL7-NL-LacZ contains NLlacZ unit between the promoter (0.8

kbp) (*L7*) and the polyadenylate signal (0.5 kbp) (*L7 pA*) from

L7/PCP2 gene. AdexMBP-NL-lacZ contains MBP promoter (1.3 kbp)

(*MBP*) upstream of the NLlacZIVSpA unit (See CHAPTER II). The

expression cassette for AdexΔP-NL-LacZ contains NLlacZIVSpA unit

without promoter elements. Adex1w does not have an insert.

(Fig. II-1)

Fig. II-2 Constructs of AdexGFAP-NL-LacZ and AdexGFAPhTH1.

The expression cassette for AdexGFAP-NL-LacZ contains GFAP promoter (256 bp) (*GFAP*) upstream of the NLlacZIVSpA unit (See CHAPTER II). AdexGFAPhTH1 contains human tyrosine hydroxylase type 1 cDNA (hTH-1; 1.9 kbp) between GFAP promoter and rabbit β-globin IVS2 & pA (See CHAPTER II).

(Fig. II-2)

Ad5

Map units

100 90 80 70 60 50 40 30 20 10 0

ΔE3

ΔE1A,E1B

3.6kbp

Adex series

insert

<Name>

AdexGFAP-NL-LacZ

AdexGFAPhTH1

HSV tk leader & AUG

promoter

GFAP

GFAP

NL1

lacZ

hTH-1

poly A

rabbit β-globin IVS2 & pA

rabbit β-globin IVS2 & pA

500bp

Fig. II-3 *In vivo* injection with a stereotaxic technique.

A schematically shows the injection site of recombinant adenoviruses. B shows an actual experiment of *in vivo* infection with recombinant adenoviruses.
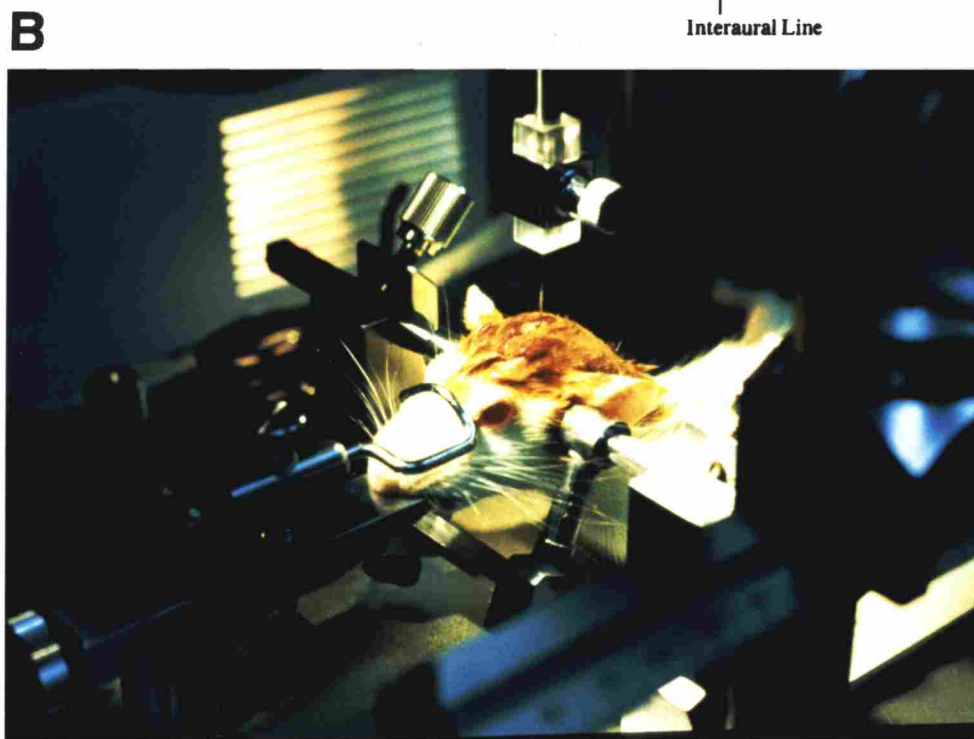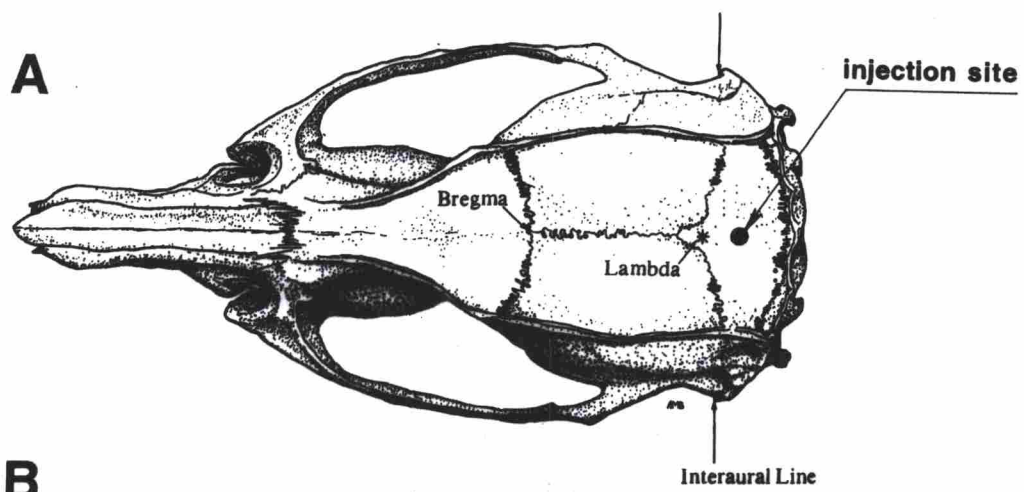
(Fig. II-3)

A

injection site

Bregma

Lambda

Interaural Line

B

CHAPTER III

A NEURAL CELL-TYPE SPECIFIC EXPRESSION SYSTEM USING RECOMBINANT ADENOVIRUS VECTORS

INTRODUCTION

Efficient gene transfer and expression into mature or immature neural cells is important for understanding brain function as well as for human gene therapy. Viral vectors can be used as efficient means of delivering foreign genes into neural cells. Among them, initial studies on herpes simplex virus (HSV-I) or retrovirus-mediated gene transfer have revealed their usefulness as tools in the study of brain function. However, there are still difficulties associated with gene transfer into quiescent neural tissue, as it often causes severe damage (Boviatsis et al. 1994).

Adenovirus mediated gene transfer has overcome some of these obstacles (Neve, 1993). Adenovirus vectors can deliver several kilobases of exogeneous genes and they have broad

host and cell-type specificity, including postmitotic neurons *in vitro* and *in vivo* (Le Gal La Salle et al., 1993; Davidson et al., 1993; Akli et al., 1993; Bajocchi et al., 1993; Caillaud et al., 1993). They can be grown to high titer, purified and concentrated to 10^{11} p.f.u./ml. These features are useful for studying the function of cloned genes in mature neural cells *in vitro* and *in vivo* as well as for use in gene therapy in humans. The previous studies using a recombinant adenovirus adopted ubiquitous, strong promoters to express high levels of genes (Bajocchi et al., 1993, Chen et al., 1994, Chang, M. W. et al., 1995).

When studying the mammalian central nervous system (CNS), it is desirable to express a protein molecule in a specific type of neural cell. This is because factors important for neural cell specific activities are often expressed in a neural cell-type specific manner. For gene therapy of neurological disorders, neural cell-type specific expression is also required.

In this study, we evaluated the cell-type dependence of gene transfer with replication defective adenovirus vectors carrying the *lacZ* reporter gene under the control of ubiquitous, strong promoters (*SRα*, *EF1α* and *CAG* promoter; in CHAPTER II). We tested neural cell-type specific expression in the rodent cerebellum as a model system. Using recombinant adenoviruses bearing *lacZ* fused with the

promoter elements of the L7/PCP2 gene (highly restricted to cerebellar Purkinje cells) and the myelin basic protein (MBP) gene (to oligodendrocytes), we recognized the neural cell-type specific gene expression of β-galactosidase *in vitro* and *in vivo*. These neural cell-type specific expression systems using a recombinant adenovirus provided a means with which to analyze the function of a cloned gene *in vitro* and *in vivo* and to determine a suitable therapy with which to treat cerebellar disease.

RESULTS

Viral constructs

Miyake et al. (1996) have established a procedure for constructing a replication-defective adenovirus using a *Eco*T22I digested DNA-terminal protein complex of Ad5-d*l*X (Saito et al., 1985) and a cosmid bearing human adenovirus type 5 (Ad5) genome. In the present study, we produced eight recombinant adenoviruses by this procedure (Fig. III-1). Basically, these constructs were derived from a cosmid vector (pAdex1cw ; Kanegae et al., 1995) that contains the Ad5 genome lacking E1A, E1B and E3. The expression cassettes were inserted into E1A and E1B deleted region. To evaluate the gene transfer and expression, *E. coli lacZ* gene was used as a histochemical reporter. The *lacZ* gene was placed under the control of various cis-acting regulatory elements.

The recombinant adenoviruses were divided into two categories. One contained the ubiquitous, strong promoters, Adex1SRLacZ, Adex1EFLacZ, Adex1CALacZ and AdexCAG-NL-LacZ (See CHAPTER II). Two adenoviruses (AdexCAG-NL-LacZ and Adex1CALacZ) containing the CAG promoter were constructed. AdexCAG-NL-LacZ contained the nuclear localization signal (*NL1*) whereas Adex1CALacZ did not. (See CHAPTER II).

The other recombinant adenoviruses consisted of AdexL7-NL-LacZ and AdexMBP-NL-LacZ which carries neural cell-type specific promoters from the mouse L7/PCP2 gene (Oberdick et

al., 1990; Vandaele et al., 1991) and the mouse MBP gene (Kimura et al., 1989) promoter, respectively. L7/PCP2 was identified as being located only in cerebellar Purkinje and retinal bipolar cells (Oberdick et al., 1988; Nordquist et al., 1988). Transgene expression is also highly restricted in the cerebellar Purkinje cells of transgenic mice (Oberdick et al. 1990; Vandaele et al., 1991). In AdexL7-NL-LacZ, the 0.8 kbp sequence of the L7/PCP2 gene, which includes the 5' flanking region (0.4 kbp) and the first exon and intron, was located upstream of the NLlacZ unit (See CHAPTER II) with a poly A sequence from L7/PCP2. The 1.3 kbp of the 5' flanking sequence of the mouse MBP gene directs the transgene-expression in oligodendrocytes in the CNS (Kimura et al., 1989) but not in the Schwann cells (Gow et al., 1992) of transgenic mice. Oligodendrocyte-specific expression was also detected by means of an assay using a recombinant retrovirus (Ikenaka et al., 1992). In AdexMBP-NL-LacZ, the MBP promoter (1.3 kbp) is located upstream of the NLlacZIVSpA unit (See CHAPTER II).

The controls were Adex1w not inserted with a foreign gene (Miyake et al., 1996) and AdexΔP-NL-LacZ carrying the NLlacZIVSpA unit without promoter elements.

Neural cell-type specific expression using recombinant adenovirus in vitro and in vivo

We tested the ability of Adenovirus-mediated gene transfer to infect several neural cell lines, such as B104, C6 and Neuro2a (data not shown). These cells were infected with Adex1CALacZ, Adex1EFLacZ and Adex1SRLacZ at a multiplicity of infection (m.o.i.) of both 20 and 100, respectively. About 70 % of the cells at an m.o.i. of 20 and about 100 % of those at an m.o.i. of 100 were positive for β-galactosidase activity without apparent toxicity.

As a model of mature neural tissue, we selected the rodent cerebellum because it is easy to discriminate the neural cell-types (Purkinje cells, granule cells, astrocytes and other neural cells). Purkinje cells grow *in vitro* according to a developmental schedule *in vivo* and neural function can be studied. In dissociated cultures, we identified Purkinje cells, granule cells, astrocytes and oligodendrocytes using specific antibodies. Adex1CALacZ was inoculated into cerebellar primary culture after 20 days *in vitro* when the neural cells are mature (Fig. III-2A). Two days after infection with Adex1CALacZ at an m.o.i. of 100, we detected β-galactosidase activities in almost all Purkinje cells, about 80 % of the granule cells and all astrocytes. Purkinje cells were positively stained for β-

galactosidase in their soma and dendrites. Adex1SRLacZ-infected, dissociated cultures were less intensely stained (data not shown). In particular, granule cells were almost β-galactosidase negative, which may reflect a difference in the CAG and SRα promoter activities. The activity of the CAG promoter may be more stable in neurons and glial cells than the SRα promoter in cerebellar primary culture. Infection with the adenovirus vector did not lead to morphological changes up to an m.o.i. of 100 *in vitro*. Labeling was undetectable when cultures infected with Adex1w at an m.o.i. of 100 were stained (Fig. III-2B) and morphological changes were not observed in each neural cell.

Based on the above findings, we tried to express an exogenous gene in a cell-type specific manner using AdexL7-NL-LacZ and AdexMBP-NL-LacZ. We tested the ability of AdexL7-NL-LacZ to express β-galactosidase specifically in Purkinje cells (Fig. III-2 C-E). At an m.o.i. of 100, we detected obvious nuclei-targeted β-galactosidase activity in large dissociated mouse cerebellar cells with relatively thick elaborated neurite processes, which are characteristic of Purkinje cells *in vitro* (Fisher 1982; Weber and Schachner, 1984). Neural cell-types were also identified by double staining with 4C11, anti-IP$_3$R-1 antibody and other cell-type specific antibodies. The cells were infected at 8 days *in*

(III-7)

vitro (Fig. III-2C) or at 20 days *in vitro* (Fig. III-2D and E), followed by 2 days incubation. All of the Purkinje cells were nuclear targeted β-galactosidase-positive. Other cells (including cerebellar neurons and astrocytes) were almost completely negative for β-galactosidase (Fig. III-2 C-E).

AdexL7-NL-LacZ could infect and express β-galactosidase in immature (Fig. III-2C) or mature (Fig. III-2D and E) Purkinje cells without morphological changes. In addition, this Purkinje cell-specific expression continued at least for 7 days after infection at 8 or 20 days *in vitro* (data not shown). When mouse brain cell cultures enriched with oligodendrocytes and astrocytes, were infected with AdexL7-NL-LacZ (an m.o.i of 100), β-galactosidase activity was undetectable (data not shown).

To test oligodendrocyte-specific expression with AdexMBP-NL-LacZ, a serum-free primary culture of oligodendrocyte was infected with AdexMBP-NL-LacZ (an m.o.i. of 100) at 18 days *in vitro* (Fig. III-3). After 2 days, this culture was stained for β-galactosidase and with anti-MBP antibody. Nuclear targeted β-galactosidase-positive cells showed the multiple processes to different directions, which mimic the morphology of oligodendrocyte. The β-

galactosidase-positive cells were also stained with anti-MBP antibody (Fig. III-3B).

To examine recombinant adenovirus-mediated gene transfer *in vivo*, AdexCAG-NL-LacZ was injected into the cerebellum of the adult rat using a stereotactic apparatus (Fig. III-4 A-D). At 3 or 7 days later, the tissue was fixed and stained for β-galactosidase. High levels of β-galactosidase activities were observed in the cells along the needle track and also in the fissures (arrowhead in Fig. III-4A) of the cerebellum. These activities in the fissures seemed to be derived from direct leakage of the recombinant adenovirus solution. Sections of the cerebellar tissue were cut on a cryostat in the sagittal plane at 10 μm. The sections near the injection site contained blue cells throughout all layers of the cerebellar cortex (Fig. III-4C and D) and these consisted of Purkinje cells, granule cells, astrocytes, oligodendrocytes and others. A group of Purkinje cells (arrow in Fig. III-4 B-D) had high levels of β-galactosidase activities in their cell bodies and dendrites, which were located near the surface (fissure) of the cerebellar lobules. After injection into the cerebellum of the rat, cytopathogenecity, characterized by local tissue necrosis and reactive gliosis, was restricted to the needle track (between the white dotted line in Fig. III-4C). This

phenomenon is primarily a result of the effect of the mechanical injury caused by the needle.

In the same manner, AdexΔP-NL-LacZ mixed with Fast-Blue, a blue fluorescent dye as marker (Bentivoglio et al., 1980), was injected into the rat cerebellum (Fig. III-4E and F). Under these conditions, the β-galactosidase positive cells were not detected (Fig. III-4E). In figure III-4F, the dye infiltration is shown in the same field view as that of figure 4E.

To test whether or not the neural cell-type specificity was retained *in vivo* as well as *in vitro*, we injected AdexL7-NL-LacZ (Fig. III-5) or AdexMBP-NL-LacZ (Fig. III-6) into the rat cerebellum . Seven days later, the rats were fixed and stained for β-galactosidase. Although β-galactosidase activities were restricted near the injection site (shown by an arrow head in Fig. III-5B and between the white dotted line in Fig. III-6A), the Purkinje cell layer (Fig. III-5C and D) and the white matter (Fig. III-6B) were stained blue. The stained neural cell-types were identified as Purkinje cells or oligodendrocytes by means of immunohistochemistry using 4C11, (Fig. III-5E) or anti-MBP antibodies (Fig. III-5C). Thus, the Purkinje cell or oligodendrocyte specificity was retained *in vivo*. None of the rats injected with these recombinant adenoviruses displayed any abnormal behavior or seizures. Furthermore, β-

galactosidase activities were undetectable in other tissues,

such as the kidney, liver and spleen (data not shown).

DISCUSSION

Adenovirus-mediated gene transfer using strong and ubiquitous promoters

Our results suggest that replication-deficient adenovirus mediated gene transfer in combination with ubiquitous, strong promoters is effective in neural cells irrespective of their cell-types in rodent cerebella *in vitro* or *in vivo*. Together with studies (Le Gal La Salle et al., 1993; Caillaud et al., 1993) in which it was shown that the adenovirus vector can transfer genes into sympathetic neurons and astrocytes *in vitro*, our findings confirmed the broad cell-type range of adenovirus mediated gene transfer.

Recombinant adenoviruses were grown to high titer, purified and concentrated to 10^{11} p.f.u./ml. We injected one microliter of viral stocks (total 10^8 p.f.u.), which were one fifth or one tenth the volume of that used in the previous studies (Akli et al., 1993; Bajocchi et al., 1993; Davidson et al., 1993; Le Gal La Salle et al., 1993). β-galactosidase active cells were found in each layer of the cerebellum, including white matter, the granule cell, Purkinje cell and molecular layers (Fig. III-4 A-D). The recombinant adenovirus seemed to spread easily into the cerebellum in comparison with other brain tissues, probably due to its finely folded structure and the loose tissue connectivity between each layers. The cerebellum was widely infected with

recombinant adenoviruses, independently of neural cell-type. Thus, we consider the cerebellum suitable for neural cell-type specific expression.

A group of Purkinje cells (arrow in Fig. III-4 B-D), located far from the needle track, had high levels of β-galactosidase activities in their cell bodies and dendrites, which were located near the surface (fissure) of the cerebellar lobules. They appeared to be stained as if the adenoviral vehicles were transported in a retrograde manner, from dendrites to cell bodies. This staining might have resulted from the retrograde transport of replication deficient adenovirus vectors. Ridoux et al. (1994) have also reported that adenovirus vectors can be used as a functional retrograde neuronal tracer on the substantia nigra.

A neural cell-type specific gene expression using recombinant adenovirus

We constructed a neural cell-type specific gene expression system using a replication deficient adenovirus vector and neural cell-type specific promoters *in vitro* and *in vivo*. AdexL7-NL-LacZ and AdexMBP-NL-LacZ carried neural cell-type specific promoters from the mouse L7/PCP2 gene (Oberdick et al., 1990; Vandaele et al., 1991) and the mouse MBP gene (Kimura et al., 1989) promoter, respectively. Under the control of the promoter of L7/PCP2 gene transgene

expression is highly restricted in the cerebellar Purkinje cells of transgenic mice (Oberdick et al. 1990; Vandaele et al., 1991). The 1.3 kbp of the 5' flanking sequence of the mouse MBP gene directs the transgene-expression in oligodendrocyte in the CNS (Kimura et al., 1989, Foran et al., 1992).

Our observations following the injection of AdexL7-NL-LacZ and AdexMBP-NL-LacZ were consistent with the results of the transgenic mice study. AdexL7-NL-LacZ directs the reporter gene expression specifically in the Purkinje cells and AdexMBP-NL-LacZ in oligodendrocytes. This cell-type specificity was maintained *in vitro* and *in vivo* and it was independent of the stage of maturity. To express these neural cell-type specific genes *in vivo* using AdexL7-NL-LacZ and AdexMBP-NL-LacZ, a total 10^8 p.f.u. of recombinant adenovirus were required. At this dose, cytopathogenecity was not manifested, except in the needle track.

This study indicated that adenovirus vectors are suitable for a neural cell-type specific gene expression because neural cell-type specificity was not concordant with the characteristics of adenovirus vehicles, but the activity of cell-type specific promoters in the recombinant adenovirus vectors. There have been studies of the viral vectors in combination with tissue specific promoters (retrovirus vector: Ikenaka et al., 1992; HSV-1 vector:

Andersen et al., 1992). We believe that there are some advantages in using the adenovirus mediated neural cell-type specific gene expression system.

The adenovirus vector can be used to analyze the cis-acting element which is important for neuronal subtype determination. One conventional means of studying this is to generate transgenic mice bearing the promoter-*lacZ* construct. However, it is difficult to analyze a promoter that is active in the early stage of development, due to the toxicity of the over-expressed transgene product. The procedure using a recombinant adenovirus developed by Miyake et al. (1996) can replace the transgenic mouse assay.

Another advantage is that functional molecules can be expressed in post mitotic neural cells without inflicting severe damage. Gene transfer into mature, quiescent neural cells, especially neurons, is very difficult due to poor efficiency. However, the adenovirus vector overcame this difficulty. Retroviral vectors have the problem of interference with foreign gene expression, whereas our study indicated that the level of inserted gene expression mainly depends on the strength and cell-type specificity of the promoter immediately upstream of the coding sequence of the foreign gene. Hayashi et al. (1994) have shown that a gene induced by thyroid hormone and retinoic acid can be expressed using adenovirus vectors. Together with our cell-

type specific system, foreign gene expression may be regulated *in vitro* and *in vivo* by adenovirus vectors. Our expression system would be of particular value for *in vitro* and *in vivo* studies of neural function with dominant-negative knockout or over-expressed functional molecules. We have generated several recombinant adenoviruses, in which neural cell-type specific promoters drive reporter genes and functional molecules for studying of neural function and gene therapy. As well as in physiological studies, our expression system will be useful in the study of pathogenesis and in the development of therapies to treat some diseases such as cerebellar degeneration, leukodystrophy and multiple sclerosis.

ACKNOWLEDGMENTS

Fig. III-1 Constructs of recombinant adenoviruses.

The replication-defective <u>ad</u>enovirus <u>ex</u>pression vector (Adex), consists of the human adenovirus type 5 genome lacking the E1A, E1B (1.3-9.3 m.u.) and E3 regions (79.6-84.8 m.u.). Adex1SRLacZ has the *E.coli* β-galactosidase gene (*lacZ*) inserted between the SRα promoter (*SRα*) and the SV40 polyadenylation signal (*pA*). Adex1EFLacZ has an expression unit including elongation factor 1α promoter (*EF1α*), *LacZ* and *pA*. Adex1CALacZ has an expression unit including the CAG promoter (*CAG*), composed of the cytomegalovirus enhancer plus the chicken β-actin promoter, *LacZ* and the rabbit β-globin polyadenylation signal (*G pA*). The expression cassette for AdexCAG-NL-LacZ contains an NLlacZ unit (See CHAPTER II) between *CAG* and *G pA*. The expression cassette for AdexL7-NL-LacZ contains NLlacZ unit between the promoter (0.8 kbp) (*L7*) and the polyadenylate signal (0.5 kbp) (*L7 pA*) from L7/PCP2 gene. AdexMBP-NL-lacZ contains MBP promoter (1.3 kbp) (*MBP*) upstream of the NLlacZIVSpA unit (See CHAPTER II). The expression cassette for AdexΔP-NL-LacZ contains NLlacZIVSpA unit without promoter elements. Adex1w does not have an insert.

(Fig. III-1)

Fig. III-2 The β-galactosidase histochemical staining of mouse cerebellar cells in primary culture. The cerebellar cells were infected with Adex1CALacZ (A), Adex1w (B) and AdexL7-NL-LacZ (C-E) at an m.o.i. of 100 at 20 days *in vitro* (A, B, D and E) or 8 days *in vitro* (C). Two days later, the cells were stained for β-galactosidase. E are higher magnifications of D. C to E were obserbed with Nomarski and fluorescent optics. A, Almost all cells were β-galactosidase-positive using Adex1CALacZ including ubiquitous, strong promoter (CAG). B, no cells were labeled using Adex1w. C to E, Nuclear targeted β-galactosidase-positive cells were found only immature (C) or mature (D and E) Purkinje cells which were immuno-stained with monoclonal antibody against IP$_3$R-1 (4C11) and FITC-coupled secondary antibody. The scale bar in B indicates 50 μm for A. The scale bar in D indicates 50 μm. The scale bar in C indicates 50 μm for E.
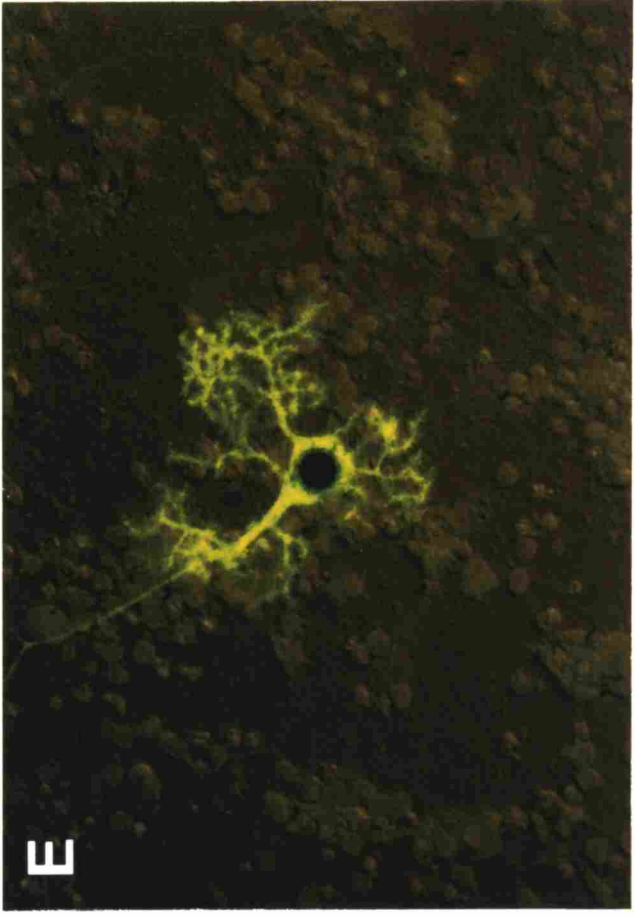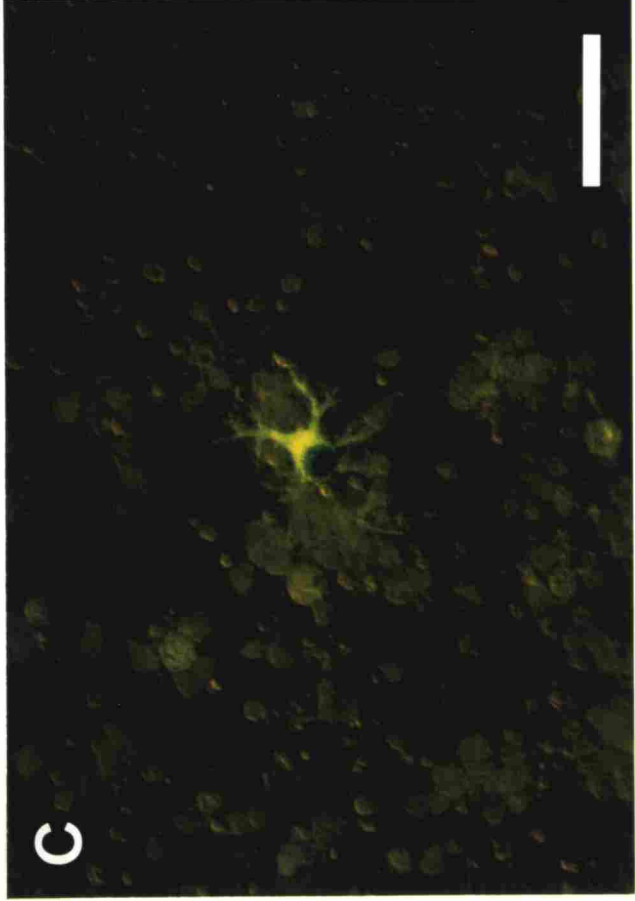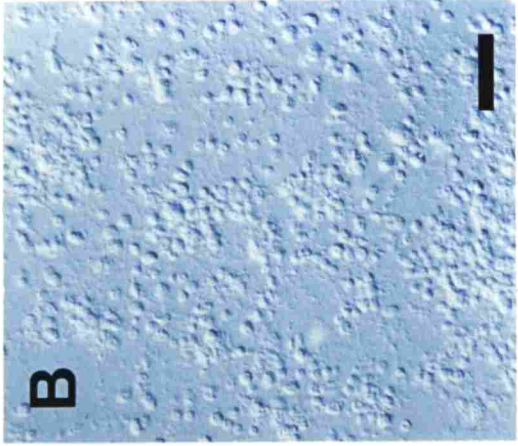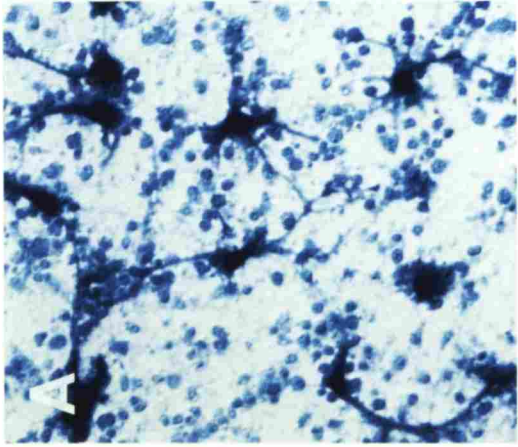
(Fig. III-2)

Fig. III-3. Oligodendrocyte-specific expression with AdexMBP-NL-LacZ in the serum-free primary culture of oligodendrocyte. After 18 days *in vitro*, cultures were infected with AdexMBP-NL-LacZ at an m.o.i. of 100, following that they were cultured for another 2 days and were stained for β-galactosidase. Figure III-3A indicated nuclear targeted β-galactosidase-positive oligodendrocyte which was also stained with anti-MBP antibody (Fig. III-3B). The scale bar in A indicates 50 μm for B.
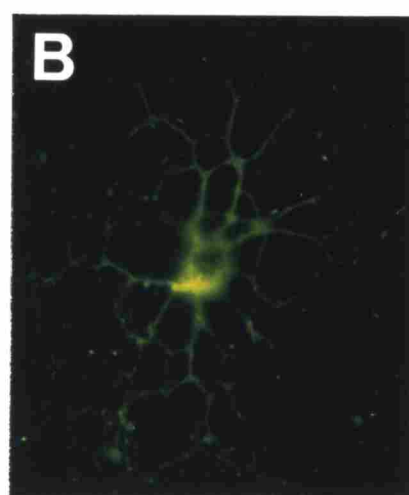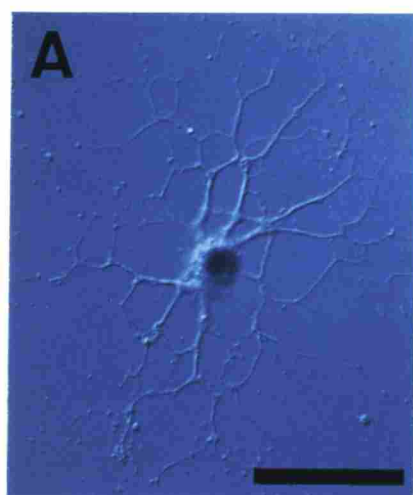
(Fig. III-3)

Fig. III-4. Expression of β-galactosidase activity *in vivo* following an injection of AdexCAG-NL-LacZ or AdexΔP-NL-LacZ, into rat cerebella. One microliter of AdexCAG-NL-LacZ (A-D) or AdexΔP-NL-LacZ (E and F) (1 x 10^{11} p.f.u./ml) was injected into the rat cerebella. Three (A-D) or 7 days (E and F) thereafter, the tissues were stained with Bluo-gal; B and D are higher magnifications of A and C, respectively; C and D were taken from a 10 μm sagittal cryosection near the injection site. **A-D,** β-galactosidase positive staining is apparent along the needle track indicated by white dotted lines in B and C and the fissure of the cerebella (arrowhead) throughout all layers of the cerebellar cortex. There was no staining in the brain except at the injected site. Some Purkinje cells on the other site from the needle track were blue-stained (arrow in B-D). The dendrites of Purkinje cells (arrow) were stained. **E** and **F,** In the control, there were no β-galactosidase-positive cells (E) and the injection site was illuminated with ultraviolet light (F). The white dotted line shows the outline of the cerebellum. The fluorescence-positive area was widely expanded, particularly in the Purkinje cell layer and fissure. The scale bar in A indicates 1 mm for A, E, F. The scale bars in B,C,D indicate 200 μm. FI, cerebellar fissure; ML, molecular layer; PL, Purkinje cell layer.
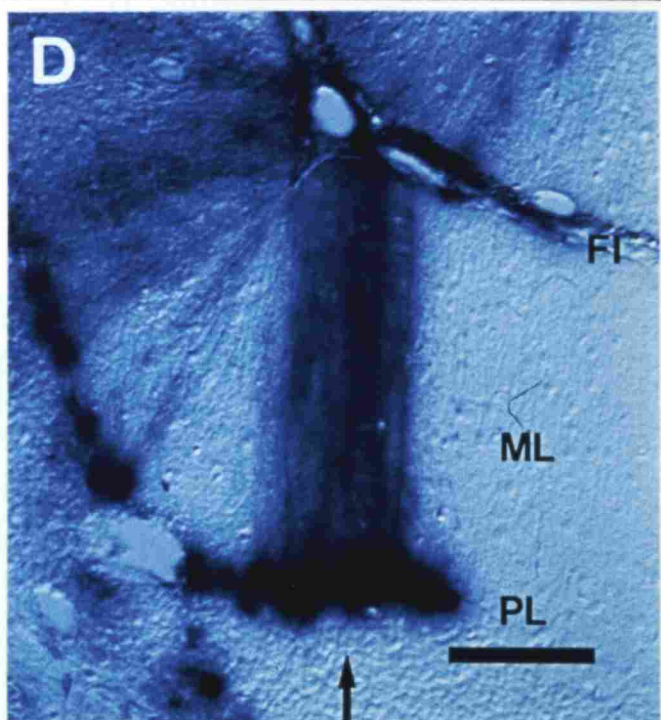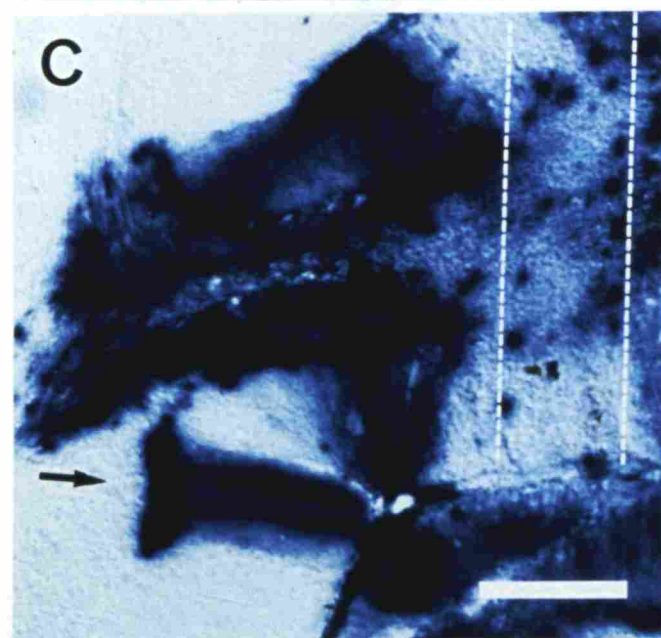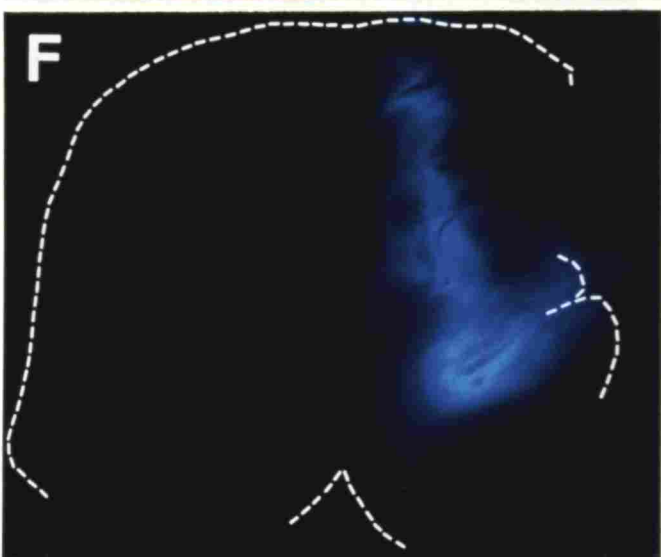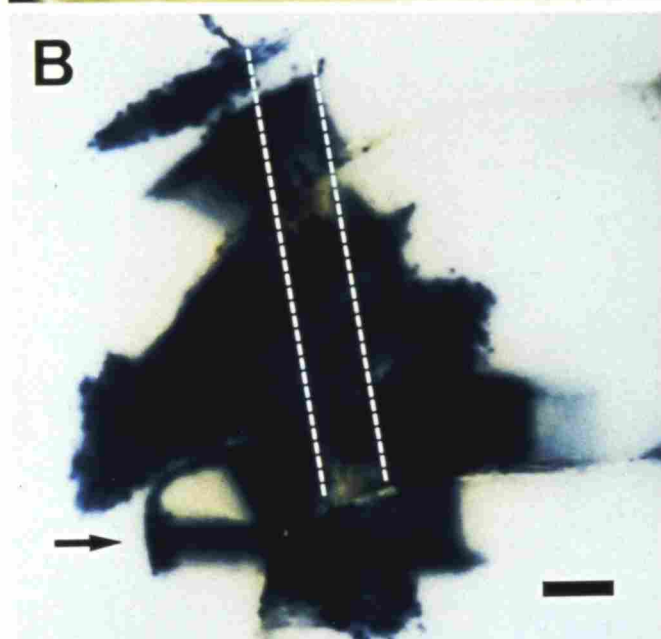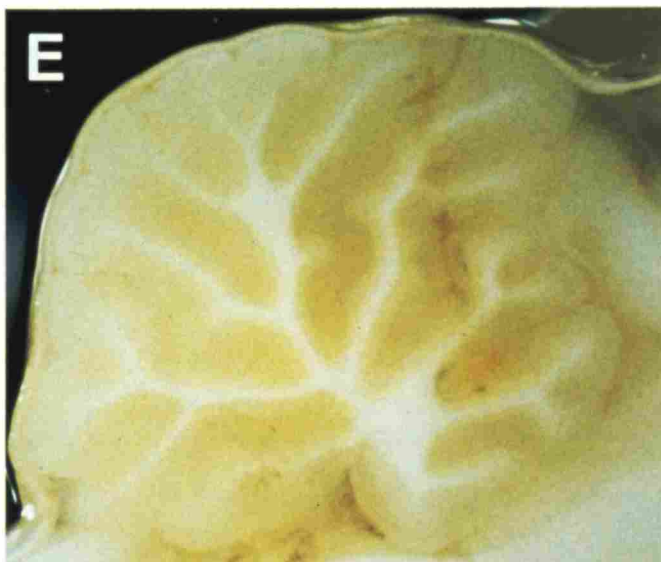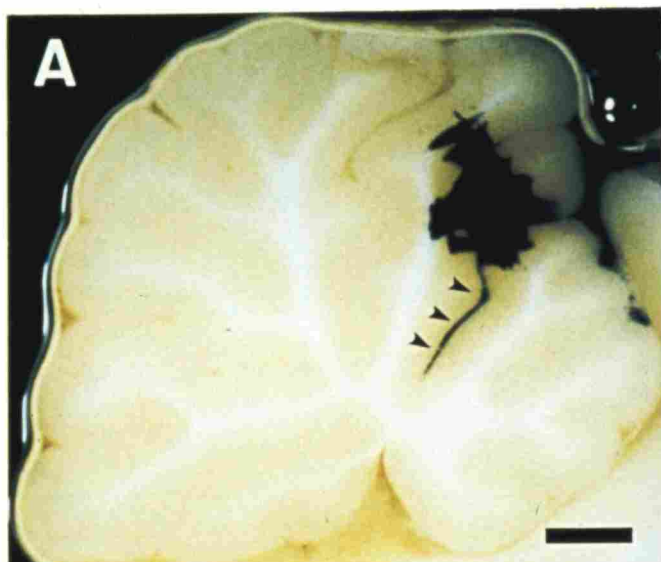
(Fig. III-4)

Fig. III-5. Purkinje cell specific gene expression by AdexL7-NL-LacZ. One microliter of AdexL7-NL-LacZ (1 x 10^{11} p.f.u./ml) was injected into the rat cerebellum. The brain tissue was stained 7 days thereafter. B was the dark field of A; C was higher magnification view of A; C and D were taken from a 10 μm sagittal cryosection near the needle track, which was indicated by arrowhead in B. **A,** Purkinje cell layers were β-galactosidase positive across the needle track. The Purkinje cell layers (PL) were -positive. There are no β-Gal-positive cells in the white matter (WM), granule cell (GL) and molecular layers (ML). The black dotted line indicates a cerebellar fissure. **D** and **E,** (D) indicates that nuclear targeted β-galactosidase positive cells in the PL, which were also stained with 4C11 antibody and FITC coupled secondary antibody (E). The scale bar in A indicates 1 mm for A and B. The scale bar in C indicates 200 μm. The scale bar in D indicates 50 μm for D and E.

(Fig. III-5)

A

B

C
WM
GL
—PL
ML
ML
—PL

D
GL
—PL
ML

E
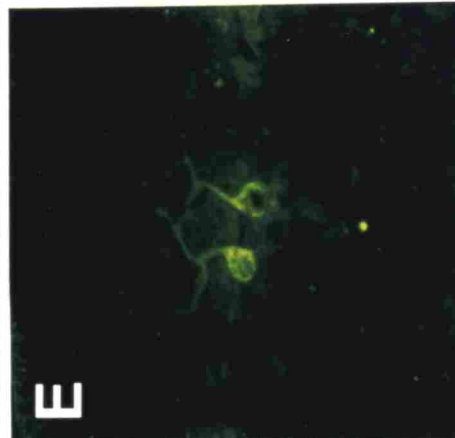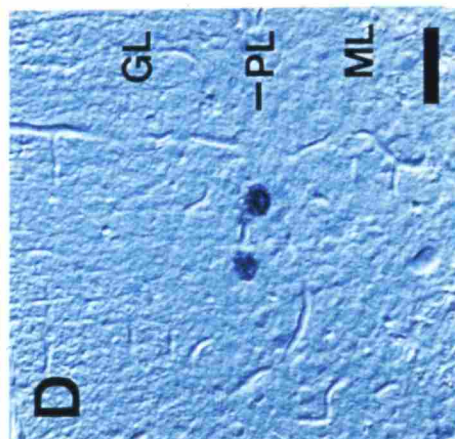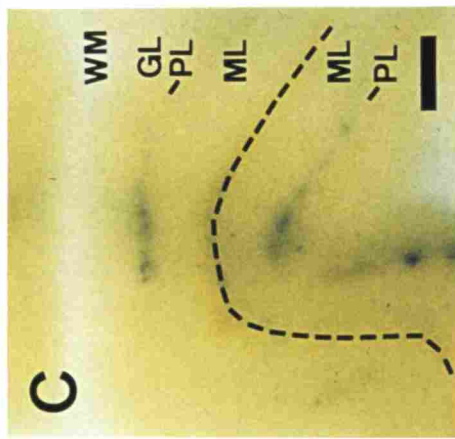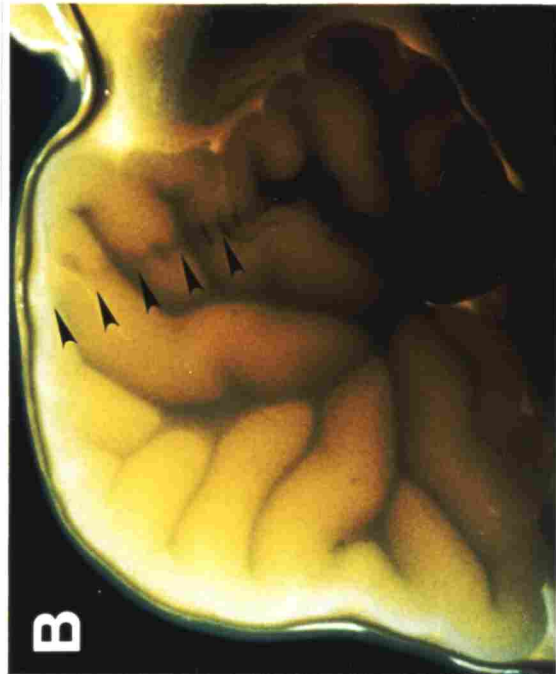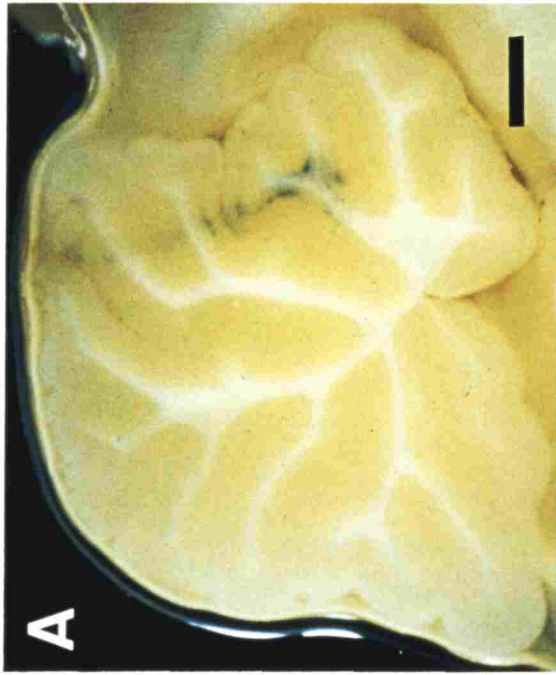
Fig. III-6. Oligodendrocyte-specific gene expression using AdexMBP-NL-LacZ. **A,** The injection route is indicated by white dotted lines. One microliter of AdexMBP-NL-LacZ (1 x 10^{11} p.f.u./ml) was injected into the rat cerebellum. The brain was stained for β-galactosidase 7 days later. The white matter (WM) is β-galactosidase positive along the needle track. **B** and **C,** The 10 μm sagittal cryosection near the injected site includes nuclear targeted β-galactosidase-positive cells, which were also stained with anti-MBP antibody (C) in the white matter. The arrowhead in (A) indicates nonspecific staining (probably due to the leakage of enzymatic activity from lysed cells; we did not detect stained nuclei in this area of the cryosections). The scale bar in A indicates 1 mm. The scale bar in B indicates 50 μm for B and C.
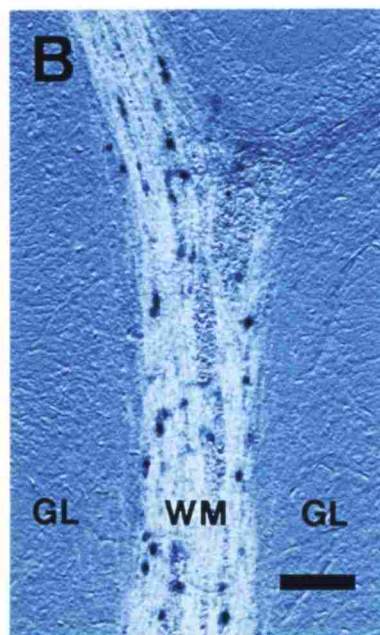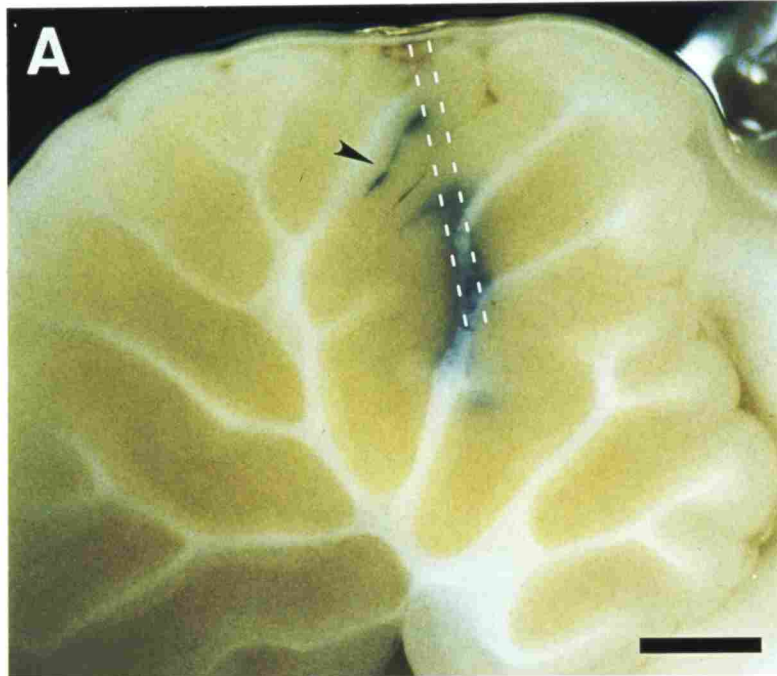
(Fig. III-6)

CHAPTER IV

CONSTRUCTION OF GLIAL CELL-TYPE-SPECIFIC ADENOVIRUS VECTORS AND GENETICAL MODIFICATION OF PRIMARY ASTROCYTES TO EXPRESS TYROSINE HYDROXYLASE

INTRODUCTION

In Parkinson disease, there is a selective loss of dopaminergic neurons in the substantia nigra and a corresponding decrease of dopamine in the target area, the striatum (Hornykiewicz and Kish, 1986), resulting in a movement disorder of tremors, rigidity, and akinesia. In the short term, oral administration of dihydroxyphenylalanine (L-DOPA), which is the immediate precursor of dopamine, or other dopamine agonists attenuated many of the motor symptoms. However, long term treatment of Parkinson disease with L-DOPA is frequently complicated by loss of response, dyskinetic movements and psychosis (Marsden and Parkes, 1977; Sweet et al., 1988). Then, systems to continuously and locally deliver L-DOPA or dopamine to striatum have been explored for genetical therapy to Parkinson

disease. Adrenal medullar chromaffin cells were autologously transplanted in experimental animals (Freed et al., 1990) and in human patients (Hirsh et al., 1990). However, transplanted chromaffin cells has showed poor viability. The transplantation of genetically engineered nonneuronal cells, primary fibrobrasts (Fisher et al., 1991) or myoblasts (Jiao et al., 1993) which have stably transduced with the rate-limiting dopamine synthesizing enzyme, tyrosine hydroxylase, which catalyzes the conversion of L-tyrosine to L-DOPA (Fig. IV-1) has been shown to be effective in rodent model of Parkinson disease. However, that grafted efficiency in rodent model of Parkinson disease appeared to diminish in a several week after grafting, though the grafted fibrobrasts survived within the brain up to a month (Fisher et al., 1991). In other studies, it has been attempted to transplant genetically engineered neural cells which are already differentiated and not dividing. However, it is difficult to transfer a foreign gene into them with currently available techniques including retrovirus vectors (Cepko et al., 1993; Morgan and Anderson, 1993) and DNA-liposome complexes (Ono et al., 1990). Because retrovirus vectors do not infect non-dividing cells and DNA-liposome complexes show cytotoxicity (Neve 1993). Use of adenovirus vectors that does not require active cell division may overcome some of the technical difficulties of gene transfer into neural cells. Furthermore, adenovirus vectors could directly, locally and high-efficiently introduce a foreign

gene into a mature brain (Le Gal La Salle et al., 1993; Davidson et al., 1993; Akli et al., 1993; Bajocchi et al., 1993; Caillaud et al., 1993; Hashimoto et al., 1996). In this study, replication-defective adenovirus vectors, AdexGFAP-NL-LacZ and AdexGFAPhTH1 were constructed. AdexGFAP-NL-LacZ have 256 bp promoter region of glial fibrillary acidic protein (GFAP) gene linked to a nuclear targeted β-galactosidase (β-Gal) as a reporter. AdexGFAP-NL-LacZ high-efficiently express β-Gal *in vitro* and *in vivo* with glial cell-specific manner without cytotoxicity. AdexGFAPhTH1 includes GFAP promoter linked to human tyrosine hydroxylase type-1 (hTH-1) cDNA. C6 rat glioma cells and primary astrocytes from rat striatum infected with AdexGFAPhTH1 high-efficiently expressed hTH-1. This is the first report that glial cell-specific expression system with replication-defective adenovirus vectors. Especially, it becomes possible to modify genetically primary astrocytes from rat striatum to express hTH-1 with AdexGFAPhTH1. This system maybe allow the development of a novel therapy for Parkinson disease.

RESULTS

Viral constructs

In this study, two recombinant adenovirus, AdexGFAP-NL-LacZ and AdexGFAPhTH1, were constructed (Fig. IV-2). AdexGFAP-NL-LacZ and AdexGFAPhTH1 included 256 bp 5'-franking region of mouse glial fibrillary acidic protein (GFAP) gene linked β-Gal gene and hTH-1 cDNA, respectively. This promoter region of GFAP gene conferred astrocyte-specific expression (Miura et al., 1990). AdexGFAP-NL-LacZ included a nuclear localization signal (*NL1*) from glucocorticoid receptor (Picad and Yamamoto 1987), thus the expression of β-Gal was localized in nuclei of host cells. The hTH-1 enzymatically produced L-DOPA from L-tyrosine (Fig. IV-1). Recombinant adenoviruses were purified and concentrated to 1 x 10^{11} p.f.u./ml by double cesium step gradient (Kanegae et al., 1994). For each vector, a single batch of viral stock was carefully investigated by restriction analyses, Southern blot analyses and PCR analysis for E1A region in order to avoid contamination of wild-type Ad (data not shown).

Glial cell-specific expression of AdexGFAP-NL-LacZ

To test glial cell-specific expression with AdexGFAP-NL-LacZ, C6 glioma (Fig. IV-3 A) cells which produced GFAP and primary astrocytes from rat striatum (Fig. IV-3 C) were infected with AdexGFAP-NL-LacZ at an m.o.i. of 20. Two days after infection, the C6 cells and the primary astrocytes were stained for β-Gal

and then the primary astrocytes were stained with anti-GFAP antibody (Fig. IV-3 D). About 70 % of C6 cells were observed β-gal-positive at their nuclei (Fig. IV-3 A). Almost all primary astrocytes which showed β-Gal-positive (Fig. IV-3 C) were also stained with anti-GFAP antibody (Fig. IV-3 D). In contrast, C6 cells (Fig. IV-3 B) and primary astrocytes (Fig. IV-3 E) infected with AdexΔP-NL-LacZ (described in CHAPTER II; m.o.i. 20) were completely β-Gal-negative in spite of the GFAP-positive (Fig. IV-3 F).

To examine the cell-type specificity of AdexGFAP-NL-LacZ *in vivo*, one miclolitter of AdexGFAP-NL-LacZ (10^{11} p.f.u./ml) was injected into the cerebellum of the adult rat using a stereotactic apparatus (Fig. IV-4). Seven days after injection, the tissue was fixed and stained for β-Gal. Many β-Gal-positive cells existed in white matter and along Purkinje cell layer (Fig. IV-4 A and B) of the cerebellum. Sagittal cryosections (10 μm) were prepared near the injection site and stained with anti-GFAP monoclonal antibody (Fig. IV-4 D and F). β-Gal positive cells in Purkinje layer (Fig. IV-4 C) and in white matter (Fig. IV-4 E) were also positive for anti-GFAP antibody and mimicked morphology of Bergman glia cells (Fig. IV-4 D) and the astrocytes (Fig. IV-4 F), respectively. In morphology of Bergman glia cells, the cell bodies of Bergman glia cells existed in Purkinje cell

layer and their processes were reached toward cerebellar fissure through the molecular layer. β-Gal-positive astrocytes extended their processed into cerebellar white matter (Fig. IV-4 F).

Glial cell-specific expression of human tyrosine hydroxylase type-1 with AdexGFAPhTH1

C6 cells were infected with AdexGFAPhTH1 at an m.o.i. of 20. Two days after infection, C6 cells were fixed and stained with anti-tyrosine hydroxylase monoclonal antibody (Fig. IV-5 A and B). C6 cells infected with AdexGFAPhTH1 could express tyrosine hydroxylase and did not show morphological changes. In contrast, C6 cells infected with Adex1w (described in CHAPTER II) at an m.o.i. of 20 did not stain with anti-tyrosine hydroxylase antibody (Fig. IV-5 C and D). To test whether it was possible or not to modify primary astrocytes from rat striatums to express hTH-1 with AdexGFAPhTH1, primary astrocytes were infected with AdexGFAPhTH1 at an m.o.i. of 20 (Fig. IV-6 A). Two days after infection, the cells were fixed and stained with anti-tyrosine hydroxylase monoclonal antibody (Fig. IV-6 B). The expression of hTH-1 was observed in primary astrocytes infected with AdexGFAPhTH1 (arrow head in Fig. IV-6 A and B). The primary astrocytes infected with Adex1w (m.o.i. 20) (Fig. IV-6 C) were almost negative for hTH-1 (Fig. IV-6 D). Non-infected primary astrocytes were also stained with anti-tyrosine hydroxylase antibody, but no astrocytes were stained (data not shown).

(IV-6)

AdexGFAPhTh1 could transfer hTH-1 gene into C6 glioma cells and

primary astrocytes.

DISCUSSION

Glial fibrillary acidic protein (GFAP) is a marker protein for astrocytes and is one of the intermediate filament proteins. The expression of intermediate filament proteins exhibits a high degree of cell-type specificity, such as keratins in epethelial cells, neurofilaments in nerve cells, desmin in muscle cells, vimentin in various mesenchymal cells, and GFAP in astrocytes (Steinert and Roop 1988). In studying *in vitro* promoter analysis, the promoter region required for glial cell-specific expression was located within only the 256 bp 5'-flanking region of GFAP gene (Miura et al., 1990). Glial cell-specific expression was also observed by means of an analysis using a retrovirus vector which included the same promoter region (Ikenaka et al., 1992). Moreover, transgenic mice which had 2.2 kbp 5'-flanking region of the human GFAP gene linked β-Gal were established (Brenner et al., 1994). Transgene expression was distinguished in astrocytes of white and gray matter, and Bergmann glia cells in the cerebella of transgenic mice. The same observation was obtained in the *in vivo* infection with AdexGFAP-NL-LacZ (Fig. IV-4). Previous studies (CHAPTER III) also shows that the activity of cell-type-specific promoters in recombinant adenovirus is maintained. Adenovirus-mediated gene transfer would make it possible to do *in vivo* promoter analysis independently of transgenic system. Thus, adenovirus vectors would provide an alternative to the transgenic mice. Moreover,

it would be possible to do *in vivo* promoter analysis in many mammalian species because of the broad host range of adenovirus vectors.

Dopamine is synthesized from L-tyrosine in a series of enzymatic steps (Fig. IV-1). The first step is that L-tyrosine was catalyzed to L-DOPA by tyrosine hydroxylase, which activity is critically dependent on tetrahydrobiopterin (BH_4). The second step involves the decarboxylation of L-DOPA by aromatic L-amino acid decarboxylase (AADC), so that L-DOPA converted to dopamine. If AADC and BH_4 did not exist in cells which were genetically modified to express tyrosine hydroxylase, dopamine could not be provided. To avoid this problem, primary astrocytes are selected, because AADC message, protein (Li et al., 1992) and the enzymatic activity of AADC (Juorio et al., 1993), and BH_4 (Sakai et al., 1995) are presented in rat astrocytes. In brain, the highest activity of AADC existed in striatum (McCaman et al., 1965; Sims et al., 1973; Bouchard et al., 1981), so that astrocytes were prepared from striatum. Moreover, grafted primary astrocytes which had genetically modified to express β-Gal with an adenovirus vector survived up to five months and continuously expressed the transgene in the rat brain (Ridoux et al., 1994b). Fisher et al. (1991) transplanted the primary fibroblast cells, which were genetically modified to express tyrosine hydroxylase gene with a retrovirus vector, into rodent model of Parkinson disease, but enzymatic efficiency of the tyrosine hydroxylase

appeared to diminish in a several week after grafting, though the grafted fibrobrasts survived within the brain up to a month. One reason for it is following that the tyrosine hydroxylase was noted to be much more stable in PC-12 cells than in fibroblast cells (Wu and Cepko,1994). The stability of tyrosine hydroxylase may be regulated in cell-type-specific manner. Based on these reasons, the transplantation of genetically modified primary astrocytes with adenovirus vectors could be most adapted to gene therapy in Parkinson disease.

Unilateral 6-hydroxydopamine (6-OHDA) lesions of the substantial nigra have been used to generate an established rodent model of Parkinson disease. In this model of Parkinson disease, the asymmetry caused by differing postsynaptic receptor sensitivities between the denervated and intact striatum results in rotational behaviour (Hefti et al., 1980). In previous studies, the tyrosine hydroxylase gene was directly transferred into a striatum of rodent model of Parkinson disease with an adenovirus vector (Horellou et al, 1994), an adeno-associated virus (AAV) vector (Kaplitt et al, 1994) or a herpes simplex virus type 1 (HSV-1) vector (During et al, 1994). Behavioral recovery was observed after *in vivo* infection of them, with all three vectors. Using recombinant HSV-1, however, pathogenicity and reversion to wild-type virus were observed (Johnson et al., 1992; Isacson 1995; Geller et al., 1995). These virus vectors employed a viral strong and ubiquitous promoter, such as the human cytomegalovirus

(HCMV) major immediate early (IE) promoter, for the over-expression of tyrosine hydroxylase gene. However, previous studies (Scharfmann et al., 1991; Kaplitt et al., 1994) have indicated that long-term expression may be limited when using HCMV-IE promoter and the replacement of it with a promoter for an endogenous cellular gene resulted in long-term expression. Therefore, endogenous cell-type specific promoters may provide more uniform long-term expression. In this study, the GFAP promoter (256 bp) is chosen in order to express tyrosine hydroxylase gene in primary astrocytes for long-term. I reported that the glial cell-specificity of the GFAP promoter in adenovirus vectors is maintained *in vitro*, in C6 or primary astrocyte from rat striatums (Fig. IV-3), and *in vivo* (Fig. IV-4) and it is possible to genetically modify primary astrocytes with AdexGFAPhTh1. The test of the release of catecolamines, L-DOPA and dopamine from genetically modified primary astrocytes with AdexGFAPhTH1 are in progress. AdexGFAPhTH1 and genetically modified primary astrocytes with AdexGFAPhTH1 are now to be evaluated in a rodent model of Parkinson disease, by means of the direct injection or the transplantation of genetically modified primary astrocytes, respectively. This is the first report that functional protein was expressed specifically in neural primary cells with replication-defective adenovirus vectors. The significance of neural cell-type-specific expression seems to be increasing for studying a neural function

(IV-11)

in vitro and *in vivo*, moreover gene therapy in neural disorders.

ACKNOWLEDGMENT

Fig. **IV-1** Dopamine biosynthetic pathway. BH_4, tetrahydrobiopterin; PLP, pyridoxal phosphate. BH_4 and PLP are cofactors.

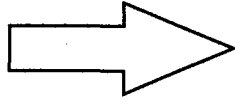(Fig. IV-1)

L-tyrosine
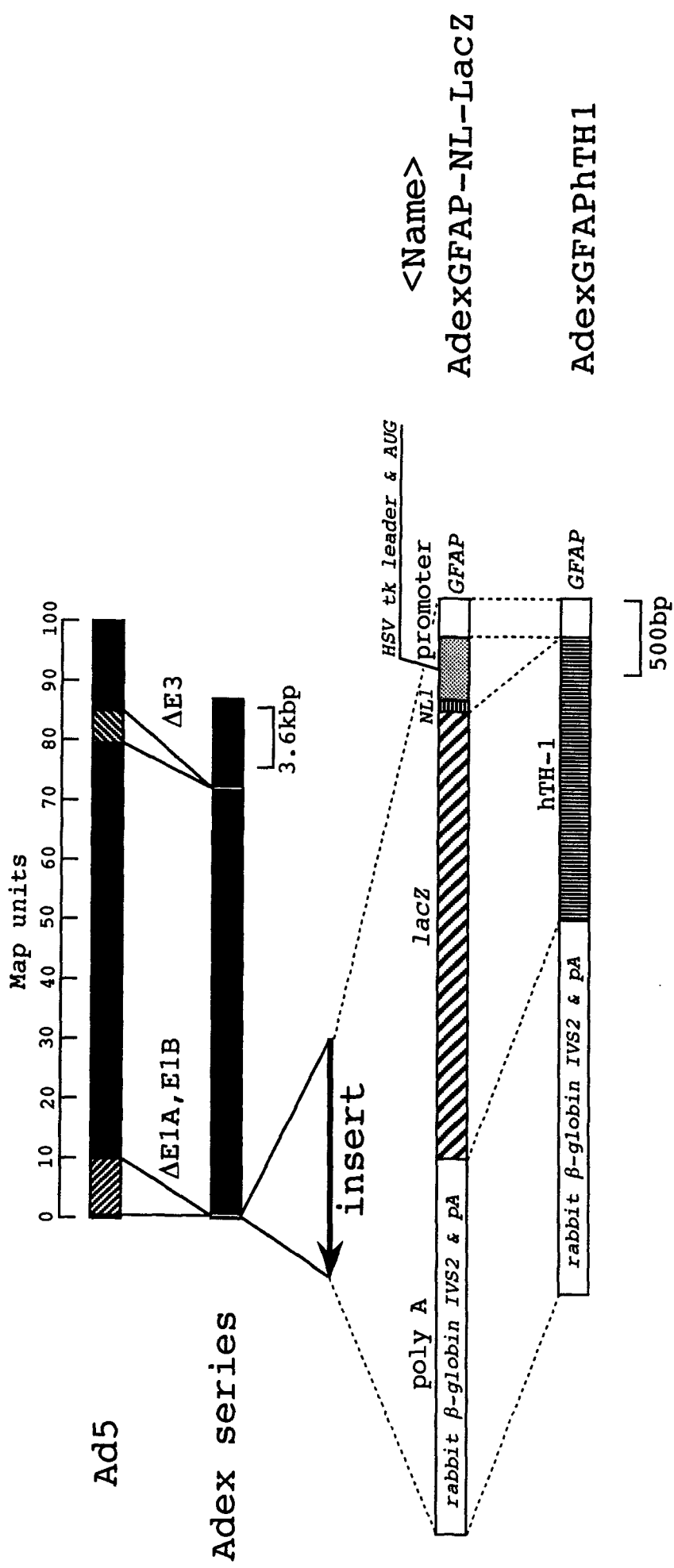
→
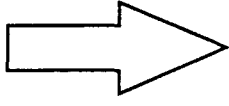
tyrosine hydroxyrase (TH)

BH4

L-DOPA

→

aromatic L-amino acid
decarboxylase (AADC)

PLP

dopamine

Fig. IV-2 Constructs of AdexGFAP-NL-LacZ and AdexGFAPhTH1.

The expression cassette for AdexGFAP-NL-LacZ contains GFAP

promoter (256 bp) (*GFAP*) upstream of the NLlacZIVSpA unit (See

CHAPTER II). AdexGFAPhTH1 contains human tyrosine hydroxylase

type 1 cDNA (hTH-1; 1.9 kbp) between GFAP promoter and rabbit

β-globin IVS2 & pA (See CHAPTER II).

(Fig. IV-2)

Map units

Ad5

Adex series

ΔE1A,E1B

ΔE3

insert

3.6kbp

<Name>

AdexGFAP-NL-LacZ

AdexGFAPhTH1

HSV tk leader & AUG

promoter

GFAP

NLi

GFAP

lacZ

hTH-1

500bp

poly A

rabbit β-globin IVS2 & pA

rabbit β-globin IVS2 & pA

Fig. IV-3 *In vitro* glial cell-specific expression with AdexGFAP-NL-LacZ. A and B show C6 glioma cells. C to F show primary astrocytes from rat striatums. C6 glioma cells and primary astrocytes were infected with AdexGFAP-NL-LacZ (A and C) or AdexΔP-NL-LacZ (B and E) at an m.o.i. of 20. Two days after infection, cultures were stained for β-Gal, following that, C and E were stained with anti-GFAP monoclonal antibody and FITC-coupled secondary antibody (D and F, respectively). **A**, About 70 % of C6 glioma cells in this culture were positive for β-Gal. **B** and **E**, C6 cells (B) and primary astroytes (E) were almost negative for β-Gal. **C** and **D**, Almost all primary astrocytes were nuclear-targeted β-Gal-positive (C) and also stained with anti-GFAP antibody (D). **E** and **F**, Primary astrocytes were negative for β-Gal, but positive for GFAP. Scale bar: F, 50 μm for A to E.
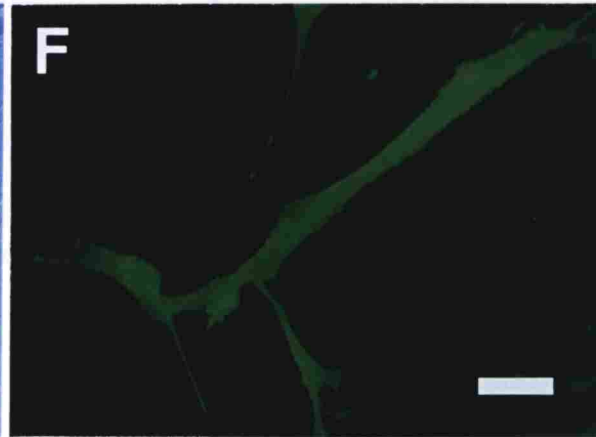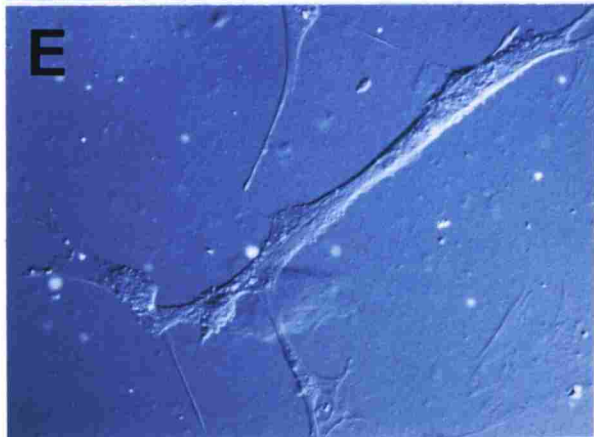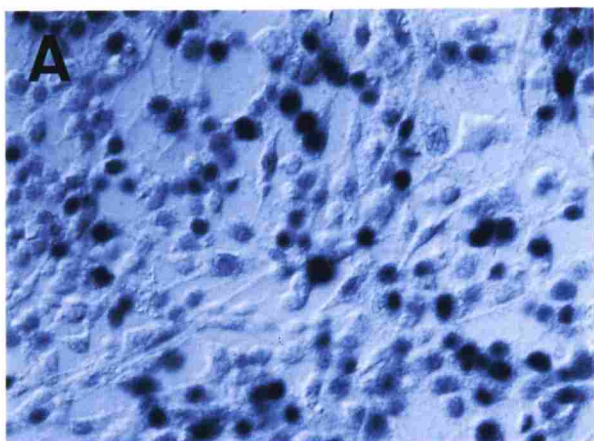
(Fig. IV-3)

Fig. IV-4 *In vivo* glial cell-specific gene expression using AdexGFAP-NL-LacZ. One microliter of AdexGFAP-NL-LacZ (1×10^{11} p.f.u./ml) was injected into the rat cerebellum. Seven days after infection, the brain was stained for β-galactosidase. **A** and **B**, White matter (WM) and Purkinje cell layers (PL) were β-galactosidase-positive. B is higher magnification of A. **C-H**, 10 μm sagittal cryosections near the injected site includes nuclear targeted β-galactosidase-positive cells, which were also stained with anti-GFAP antibody (D and F). **C** to **F**, The β-galactosidase-positive cells along the PL (C and D) should be Bergman glial cells, and in WM (E and F) might be astrocytes. GL, granule cell layer; The scale bar in C indicates 20 μm for D to F.
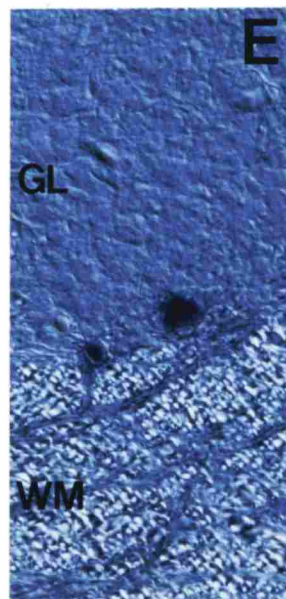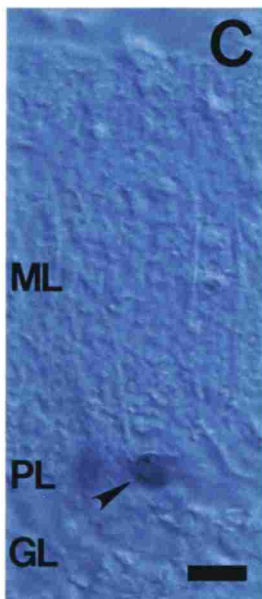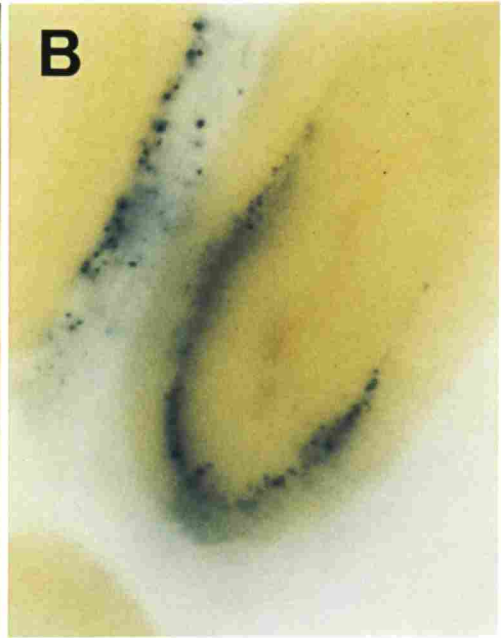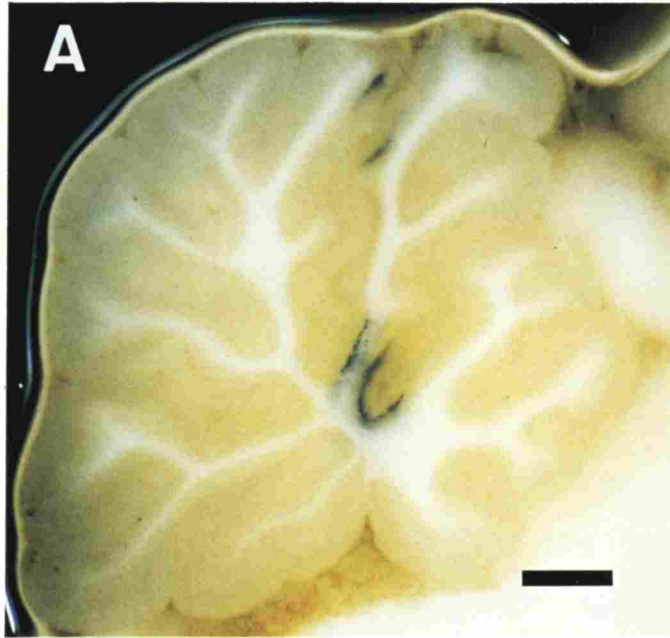
(Fig. IV-4)

Fig. IV-5 Expression of human tyrosine hydroxylase type-1 in C6

glioma cells infected with AdexGFAPhTH1. C6 were infected with

AdexGFAPhTH1 at an m.o.i. of 100 (A). C6 were infected with Adex1w

at an m.o.i. of 100 (C). Two days after infection, the cells were

fixed and stained with TH-17 monoclomal antibody against tyrosine

hydroxylase and FITC-coupled secondary antibody (B and D). B is

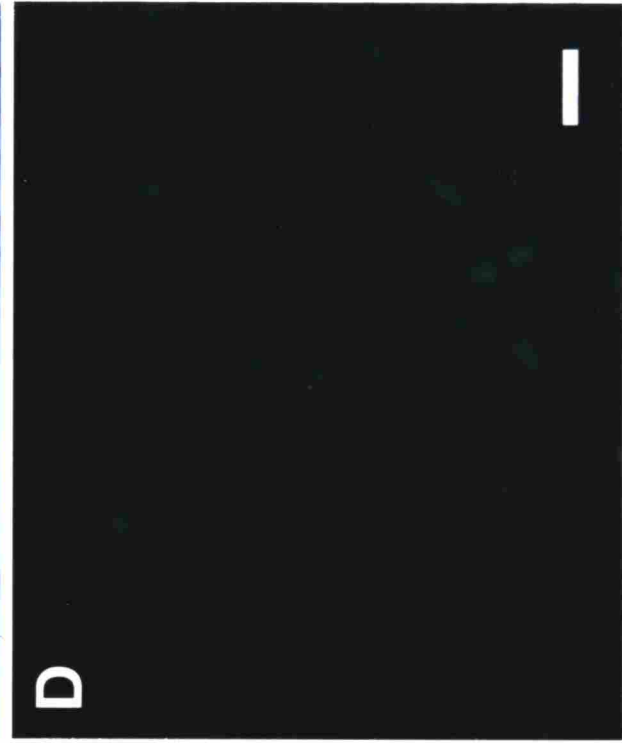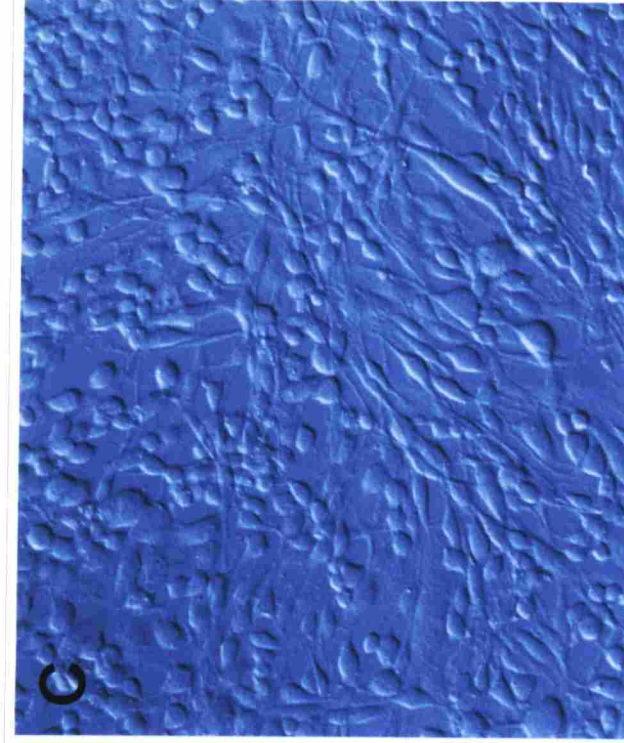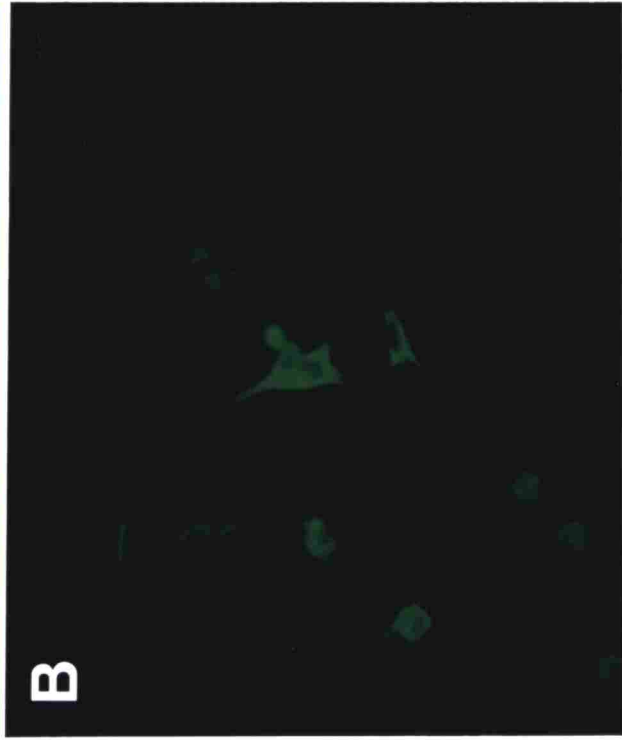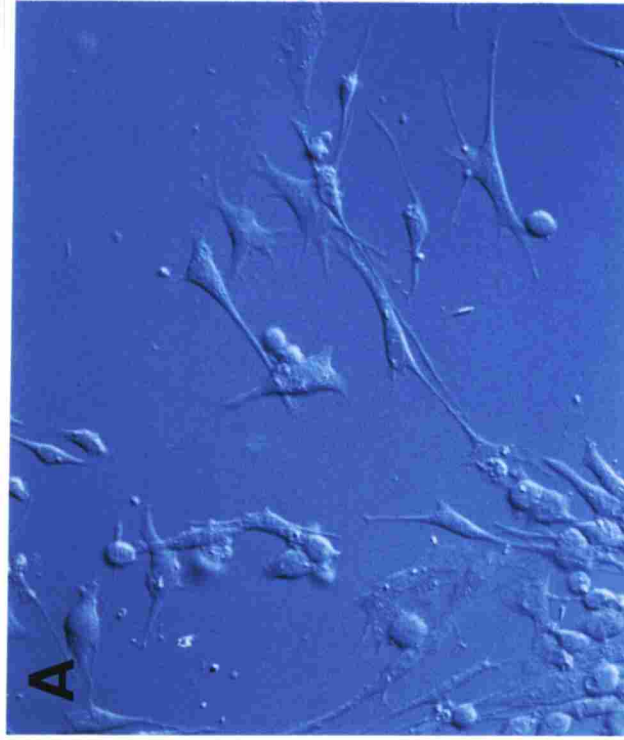same view of A. D is same view of C. The scale bar in D indicates

50 μm for A to C.

(Fig. IV-5)

Fig. IV-6 Expression of human tyrosine hydroxylase type-1 (hTH-1) in a primary culture of astrocytes with AdexGFAPhTH1. Primary astrocytes from rat striatums were infected with AdexGFAPhTH1 (A and B) or Adex1w (C and D) at an m.o.i. of 20. Two days after infection, primary astrocytes were stained with anti-tyrosine hydroxylase monoclonal antibody and FITC-coupled secondary antibody (B and D). **A** and **B**, Arrow head shows a tyrosine hydroxylase-positive primary astrocyte, which could be genetically modified to express TH with AdexGFAPhTH1. **C** and **D**, Primary astrocytes infected with Adex1w were almost negative for tyrosine hydroxylase. Scale bar: D, 50 μm for A to C.

(Fig. IV-6)
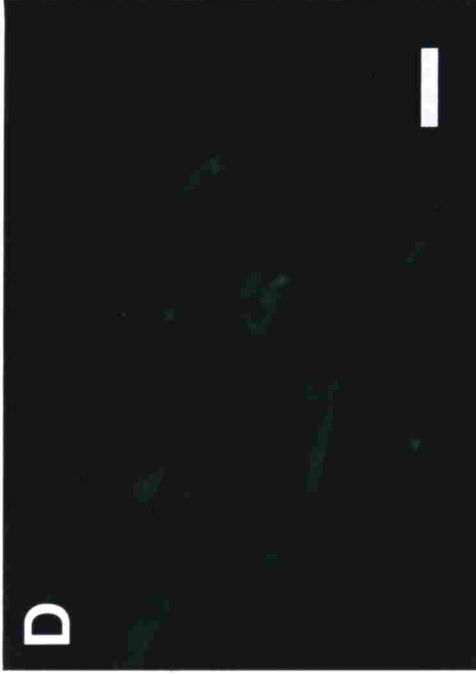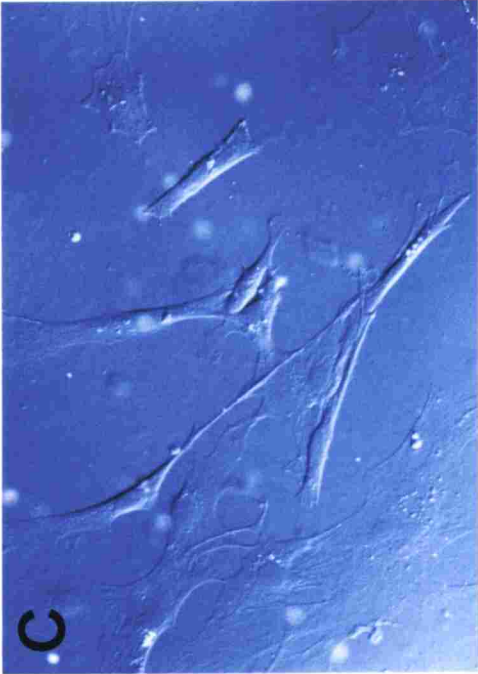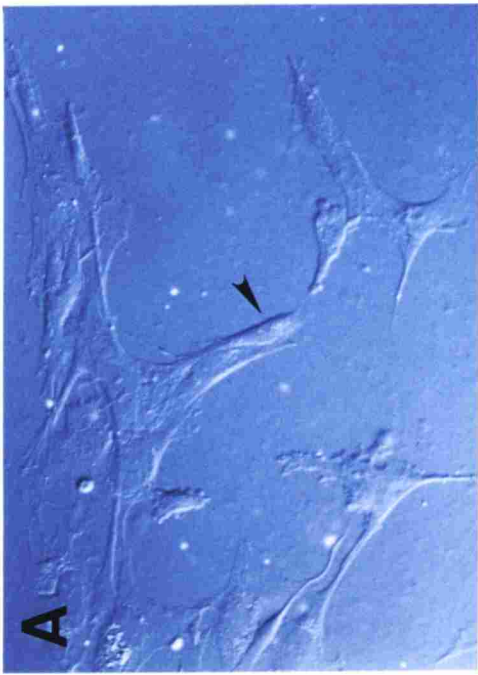
CHAPTER V

ADENOVIRUS-MEDIATED GENE EXPRESSION IN THE ORGANOTYPIC SLICE CULTURE OF RAT CEREBELLUM

INTRODUCTION

Brain slice cultures (Stoppini et al., 1991) offer a powerful system for studying synaptic transmission and plasticity because slices maintains intact synaptic circuitry and function. Cerebellar slice preparations were useful for studying the long-term depression (LTD) (Hartell, 1994), but it is still difficult to apply molecular approaches to cerebellar slice preparations except for particle-mediated gene transfer (Arnold et al., 1994).

In this study, I report that adenovirus-mediated gene transfer is an effective method to transfer a foreign gene into mature neural cells in rodent cerebellar slices. This system might make it possible to study synaptic transmission and plasticity with molecular strategies.

RESULTS

*Adenovirus-mediated gene transfer into the organotypic slice culture of rat cerebellum.*

Cerebellar slices were obtained from 17-days Wister rats. The slices were incubated for three days, then they were infected with AdexCAG-NL-LacZ (Fig. V-1 A and C) or AdexΔP-NL-LacZ (Fig. V-1 B and D) (total of viruses: 2 x 10^9 p.f.u.). Two days after infection, slices were fixed and stained for β-Gal. β-Gal-positive cells were more densely located in the granule cell layers (GL) than the molecular layers (ML) (Fig. V-1 A and C). The efficiency of infection is very high. The adenovirus vectors infiltrated into cerebellar slice culture and expressed nuclear-targeted β-Gal in host cells (data not shown). In contrast, slices infected with AdexΔP-NL-LacZ were almost negative for β-Gal (Fig. V-1 B and D). In Fig. V-1 B and D background was saw through the ML of the slices. Morphological changes were not observed.

DISCUSSION

In cerebellar slice cultures infected with AdexCAG-NL-

LacZ, the β-Gal-positive cells were located in GL (Fig.V-1 A and

C), while ML was relatively negative for β-Gal (Fig. V-1 B and

D). AdexCAG-NL-LacZ included a nuclear localization signal, so

that nuclei of infected cells were stained for β-Gal. GL contains

a vast number of densely packed small neurons, mostly small

granule cells, but ML contains almost all fibers or dendrites.

Thus, that observation might reflect the locarization of cells

in cerebellum. The injection of purified adenoviral solutions

(10^{11} p.f.u./ml) is in progress, but reasonable observations have

not yet obtained. Then, further development of this system seems

to be required.

This is a first report to transfer a foreign gene into the

organotypic slice culture of rat cerebellum with

replication-defective adenovirus vectors. This system might make

it possible to address many questions regarding the role of a

gene product in synaptic transmission and plasticity.

Fig. V-1 Adenovirus-mediated gene expression in the organotypic

slice culture of rat cerebellum. Cerebella were dissected from

17-days Wister rats. The slices were mounted on a collagen-coated,

porous (2μm) membrane which was floated at the interface between

air and a culture medium in a petri dish. The organotypic slice

culture was infected with AdexΔP-NL-LacZ (B and D) or

AdexCAG-NL-LacZ (A and C). C and D are higher magnifications of

A and B, respectively. Two days after infection, these slices

were stained for β-galactosidase. A and C, The granule cell layers

(GL) were β-galactosidase-positive. The molecular layers (ML)

were nearly negative for β-galactosidase.

(Fig. V-1)
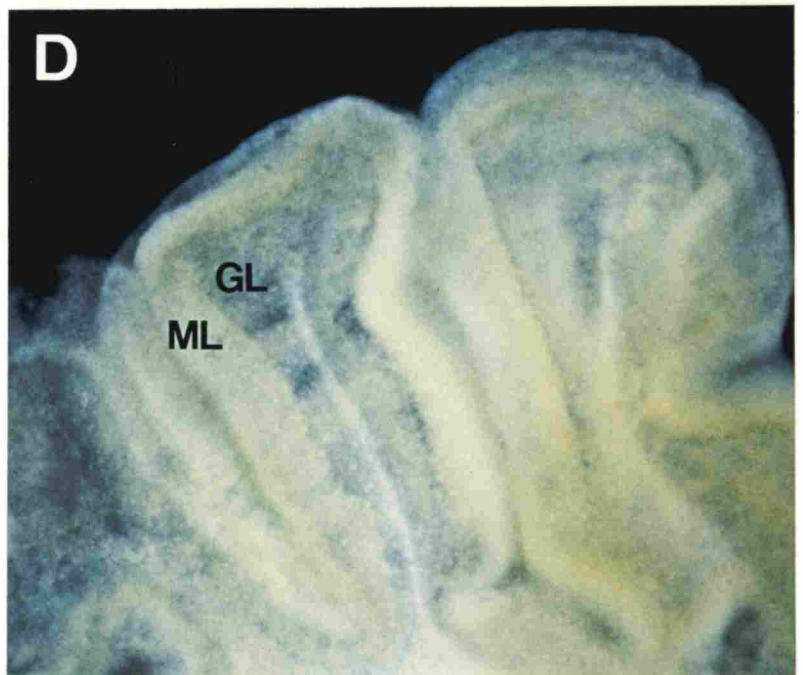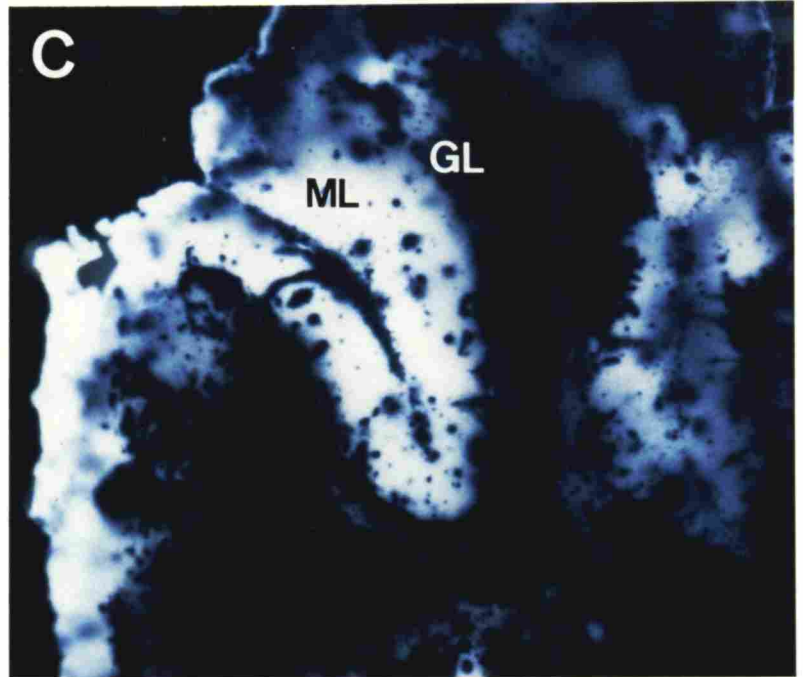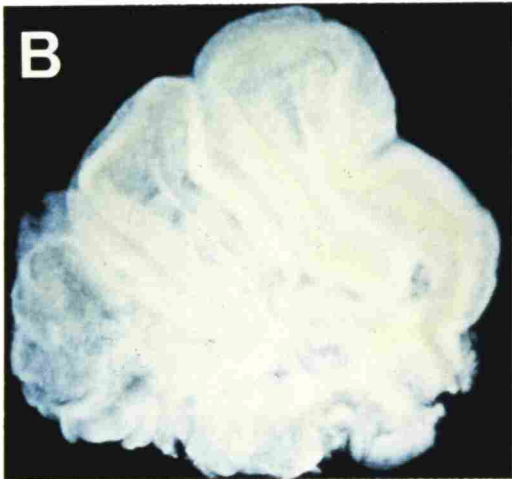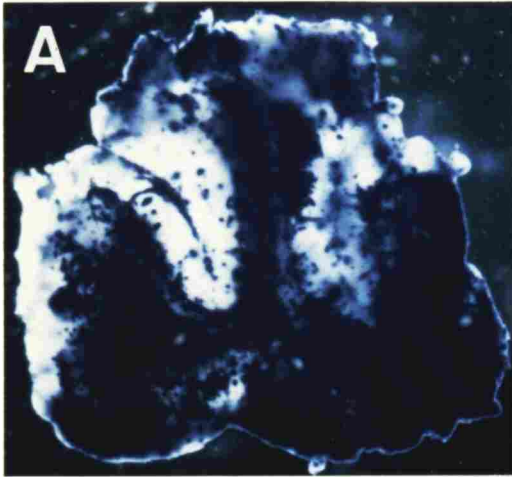
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