Preface

Ligand-gated ion channels in the brain are a family of membrane receptor molecules critical to the chemistry of synaptic transmission. Since the synthesis of *N*-methyl-D-aspartate by J. C. Watkins in the 1960s, and subsequent pharmacological studies by a number of groups, NMDA receptors have emerged as key molecules involved in neuronal excitation and toxicity. The molecular studies of NMDA receptors began in the late 1980s, thanks to molecular cloning of the genes encoding various receptor subunits. The ability to express, purify, and analyze various forms of recombinant NMDA receptor proteins in a variety of heterologous systems has offered us enormous opportunities for both basic research of the NMDA receptor structure and function, as well as for clinical investigation and high-throughput drug screens for bioactive compounds.

Ion channels have been studied extensively using pharmacological and electrophysiological approaches. *NMDA Receptor Protocols* differs from other books covering various aspects of techniques involved in conducting those experiments in that it details experimental protocols for studying NMDA receptors, with a strong emphasis on state-of-the-art molecular techniques. The wide range of topics includes molecular cloning of NMDA receptor subunits, expression and functional characterization of cloned genes, investigation of NMDA receptor properties using in vivo and in vitro preparations, and design and construction of expression systems suitable for special purposes, such as high-throughput drug screens. Although *NMDA Receptor Protocols* does focus on NMDA receptors, the described methods are applicable to related ligand-gated ion channels. With some modifications, one may extend the techniques to other membrane receptors or signaling systems.

I want to thank many who have contributed to this book. I am particularly grateful to Stephen Desiderio, Jeremy Nathans, Lily Jan, and Yuh Nung Jan, who have played a profound role in shaping my view and approaches to science. William Dower at Affymax Research Institute was instrumental in this project. John Walker has read through every chapter and provided helpful editorial comments. Many thanks also go to Ms. Robyne Butzner for her secretarial participation in all stages of this project. Last, I thank Lu for her continuing encouragement and support.

Min Li

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Expression of NMDA Receptor Channel Subunit Proteins Using Baculovirus and Herpesvirus Vectors

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1. Introduction

Foreign gene transfer and expression in the cells are among the most important techniques for determining the characteristics and function of cloned genes. Glutamate receptor genes have been expressed by RNA injection of *Xenopus* oocytes or transfection of mammalian cells, such as HEK, CHO, and COS cells, and bacterial *Escherichia coli* cells. Viral vectors, such as baculovirus (*Autographa californica* nuclear polyhedrosis virus, AcNPV) or herpesvirus (herpes simplex virus-1, HSV-1), have been also used because of high efficiency of gene transfer. Existing viral clones expressing recombinant glutamate receptor proteins ***(1–25)*** are summarized in **Table 1**.

Although the baculovirus system is useful for some molecular and functional studies, the system is limited to insect cells and mammalian liver cells ***(26–28)***. **Figure 1** illustrates a general construction scheme for baculovirus expression vectors. Since the baculovirus DNA (130 kb) is too long to be manipulated by using standard genetic engineering techniques, a so-called transfer vector is used to insert a foreign gene into the virus, replacing the polyhedrin gene. The recombinant transfer vector containing glutamate receptor (GluR) cDNA is transfected into the insect cell, together with wild-type baculovirus DNA. Since the two DNA molecules have common sequences flanking the genes of interest, a recombinant baculovirus is obtained by homologous recombination. Wild-type baculovirus produces the polyhedrin protein, whereas the recombinant baculovirus produces the foreign gene product under the control of very strong polyhedrin promoter.

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Table 1
Summary of Viral Vectors
Expressing Glutamate Receptor Proteins

Viral vector, mouseGluR/ratGluR	Reference
Baculovirus vector	
α1	***1–5***
α2/GluRB	***3,6–9***
(α4)/GluRD	***9–11***
(β2)/GluR6	***12–14***
ζ1/NMDAR1	***15–17***
ε1	(***18*** and this study)
ε2	(***18*** and this study)
δ2	(***18*** and this study)
mGluR1 (rat)	***13***
Herpesvirus vector	
(α1)/GluR1	***19***
(β2)/GluR6	***20–22***
ζ1	This study
Adenovirus vector	
(α1)/GluR1	***23***
(α2)/GluR2	***23***
(ζ1)/NMDAR1 (antisense)	***24***
Adenoassociated virus vector	
(ζ1)/NMDAR1 (antisense)	***25***

Here we have used the transfer vectors pJVP10Z and pBlueBacIII. Since the vectors contain the *lac*Z gene, selection of recombinant virus clones was performed by β-galactosidase assay with X-gal as a substrate. The cDNA of interest is inserted downstream from the polyhedrin promoter in the transfer vectors. The recombinant baculovirus produced in vivo by homologous recombination was plaque-purified by screening using both β-galactosidase assay and polyhedra-negative morphology as criteria. Dot-blot hybridization analysis of DNA produced in insect cells infected with the purified recombinant virus revealed the presence of foreign gene, GluR subunit cDNA.

Molecular neurobiological approaches by which foreign genes can be transferred to or expressed in cultured neurons and in brain would greatly facilitate research and therapeutics. Viral vectors, such as herpesvirus, adenovirus, or adeno-associated virus, but not retrovirus, are good candidates, because they can transfer or express genes in the nondividing neurons. Recent reviews ***(29–30)*** have focused on the characteristics of these three viral vectors for gene expression in neurons. The wide host range of HSV-1, both in vivo and in vitro, and the

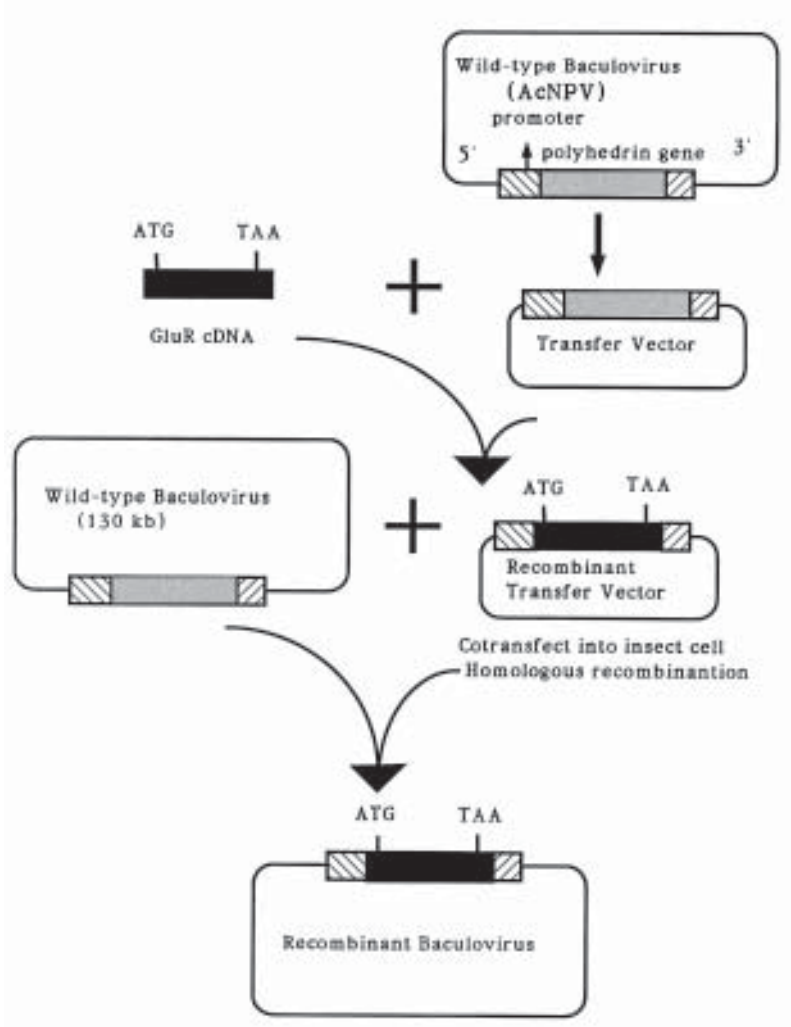

Fig. 1. General construction scheme for baculovirus expression vector. *See* details in the text.

relative ease of its genetic manipulation have made it an attractive candidate as a tool for gene transfer. In particular, the natural propensity of the virus to infect and establish lifelong latent infection in postmitotic neurons has prompted much recent effort in developed HSV-1 vectors.

Two different types of HSV-1 vectors have been developed. For the first type, the recombinant virus vector, the gene of interest is inserted into the backbone of the viral genome by genetic recombination. The second type of HSV-1 vector is "ampicon"-based and has been termed a "defective" virus vector

because of its inability to replicate in the absence of the parental virus as helper. The amplicon plasmid includes prokaryotic sequences that allow propagation and drug selection in bacteria, an origin of replication and packaging signal from HSV-1, and a transcriptional unit for expressing the gene of interest. The latter type of HSV-1 vector has been used in our laboratory, and the protocols about that have been described here.

We ***(1–7,15,17,18)*** have characterized or would like to characterize glutamate receptor proteins by using the baculovirus vector system in vitro, and their function in vitro and in vivo with a herpesvirus vector system. This chapter describes the detailed protocols to isolate these viruses. Several protocols for baculovirus ***(31–33)*** or herpesvirus ***(34,35)*** expression systems have already been published (*see* **Notes 1** and **2**).

2. Materials

2.1. Baculovirus Vector System

1. *Spodoptera frugiperda* insect Sf9 (Sf21) cells: Sf cells can be maintained at 27°C without CO_2 (*see* **Note 1**).
2. TNM-FH insect medium (Sigma #T-1032) (Sigma Chemical Co., St. Louis, MO) or EX-CELL 400 serum-free medium (JRH Biosciences [Lenexa, KS] #14400), containing 50 µg/mL gentamicin sulfate.
3. AcNPV DNA (circular or linear) from Invitrogen (San Diego, CA).
4. Transfer vector (*see* **Note 2**): the pJVP10Z vector was provided by Palmer Taylor (University of California, San Diego), and pBlueBacIII was purchased from Invitrogen. They contain β-galactosidase gene, and color selection can be done for virus purification or virus titer check.
5. Lipofectin reagent (Gibco-BRL #18292) (Gibco-BRL, Gaithersburg, MD).
6. Fetal bovine serum (FBS).
7. SeaPlaque agarose (FMC Bioproducts [Rockland, ME] #50101) was dissolved in water (3% [w/v]) and autoclaved.
8. X-gal stock solution (20 mg/mL dimethylsulfoxide).
9. TE: 10 m*M* Tris-HCl, pH 8.0, and 0.1 m*M* EDTA.

2.2. Herpesvirus Vector System

1. Rabbit skin cells provided from Michael G. Kaplitt (Cornell University Medical College and The Rockefeller University).
2. Vero cells.
3. DMEM medium (Gibco-BRL #12800-017).
4. MEM medium (Nissui #05901, Nissui Pharmaceutical Co., Tokyo, Japan).
5. Opti-MEM medium (Gibco-BRL #31985-021).
6. FBS.
7. Trypsin-EDTA solution (Sigma #T-3924).
8. Phosphate-buffered saline (PBS) (Dulbecco's PBS [-] "Nissui," Nissui #05913).

9. TE: 10 m*M* Tris-HCl, pH 8.0, and 0.1 m*M* EDTA.
10. 0.1 *N* Na_2Co_3.
11. Suspension buffer A: 20 m*M* Tris HCl, pH 7.5, and 150 m*M* NaCl.
12. Suspension buffer B: 50 m*M* Tris-HCl, pH 7.4, 0.3 m*M* EDTA, 1 m*M* dithiothreitol, and 1 m*M* phenylmetylsulfonyl fluoride.

3. Methods

3.1. Expression System Using Baculovirus Vectors

3.1.1. Cloning of NMDA Receptor Channel Subunit cDNAs into the Transfer Vectors

The NMDA receptor channels contain two classes of subunits in hetero-oligomeric association, the core ζ1 (NMDAR1, NR1) subunit, and the regulatory ε1-ε4 (NMDAR2A-2D, NR2A-2D) subunits.

1. The 3020-bp *Nru*I-*Nae*I fragment, containing 53 bp of 5'-untranslated region (*see* **Note 3**), the complete coding region (2814 bp), and 153 bp of 3'-untranslated region, was prepared from the mouse NMDA receptor channel ζ1 subunit cDNA ***(36)***. The fragment was inserted into the unique *Nhe*I site downstream of the polyhedrin promoter in the transfer vector pJVP10Z using blunt-end ligation.
2. The cDNAs encoding the mouse NMDA receptor channel ε1- and ε2-subunits were inserted into the unique *Nhe*I site downstream of the polyhedrin promoter in the transfer vectors pBlueBacIII (Invitrogen) (for GluRε1 cDNA) or pJVP10Z (for GluRε2 cDNA) as follows. For the ε1-subunit, the 4395-bp fragment, containing the complete coding region, was subcloned to the *Nco*I site of pBlueBacIII. For the ε2-subunit, the 4855-bp fragment, containing 10 bp of 5'-untranslated region (*see* **Note 3**), the complete coding region (4446 bp) and 399 bp of 3'-untranslated region, was subcloned to the unique *Nhe*I site of pJVP10Z.
3. Recombinant plasmids, pJVP10Z/GluRζ1, pBlueBacIII/GluRε1, and pJVP10Z/GluRε2, carrying the respective inserts in the correct orientation were identified by DNA sequencing using an automatic DNA sequencer 373A (Applied Biosystems).

3.1.2. Cotransfection of Viral and Transfer Vector DNAs Using Lipofectin

1. Seed six-well dishes with 2.5×10^6 Sf9 (or Sf21) cells/well. Incubate at 27°C for 1 h to allow the cells to attach.
2. Wash the cells twice with 1 mL of serum-free TNM-FH insect medium.
3. Add 1 mL of serum-free TNM-FH insect medium.
4. Mix 1.0 μg recombinant transfer vector and 0.2 μg AcNPV DNA in 6 μL TE, and 4 μg lipofectin in a polystyrene tube (total 12 μL). Incubate at room temperature for 15 min.
5. Add the lipofectin/DNA mixture to the cells.

6. Incubate for 24 h at 27°C.
7. Add 1 mL serum-supplemented (10% [v/v] FBS) TNM-FH insect medium.
8. Incubate for 48 h at 27°C. Transfer the medium to the tube, centrifuge at 1000*g* for 15 min at 4°C. Store the supernatant (recombinant virus solution) at 4°C.

3.1.3. Purification of Recombinant Virus by Plaque Assay

1. Seed 10-cm dishes with 4×10^6 Sf cells per dish, and incubate at 27°C for 1 h to allow the cells to attach.
2. Dilute the recombinant virus solution to 10^3- to 10^4-fold.
3. Remove media, and add 1.6 mL diluted virus solution to each plate.
4. Incubate plates for 1 h at 27°C.
5. Incubate 2 mL serum-supplemented TNM-FH insect medium and 2 mL 3% (w/v) SeaPlaque agarose at 42°C in polypropylene tubes.
6. Add 60 µL X-gal stock solution and 4 mL TNM-FH insect medium.
7. At the end of the 1-h incubation period, remove all inoculum by using Pasteur pipets. Slowly add 8 mL of overlay. Leave the overlay to solidify for 1 h.
8. Seal the plates with Parafilm.
9. Incubate plates for several days at 27°C.
10. To pick a blue plaque, place the top of a sterilized Pasteur pipet directly onto the plaque. Carefully apply gentle suction until a small plug of agarose is drawn into the pipet.
11. Place the agarose plug in 1 mL medium. Vortex well, and store at 4°C.
12. Several rounds of plaque purification are necessary to isolate recombinant viruses.

3.1.4. Amplification of Recombinant Virus

1. Seed 75-cm^2 flask with 9×10^6 Sf cells/flask. Incubate at 27°C for 1 h to allow the cells to attach.
2. The medium is removed, and the cells are inoculated with recombinant AcNPV at a multiplicity of infection (MOI) of 0.01 in a volume of 2 mL.
3. After incubation for 1 h at 27°C, add 8 mL TNM-FH insect medium supplemental with 10% (v/v) FBS.
4. After incubation for 5–7 d, the medium is harvested. Transfer the medium to the tube, and centrifuge at 1000*g* for 15 min at 4°C. Store the supernatant (recombinant virus solution) at 4°C (*see* **Note 4**).

3.1.5. Expression of NMDA Receptor Channel Subunit Proteins Using Recombinant Baculovirus

Both monolayer culture and suspension culture can be used for expression of recombinant NMDA receptor channel proteins. Multiwell plates, dishes, or flasks can be used for monolayer culture and spinners for suspension culture.

Sf21 cells were infected with recombinant baculovirus at an MOI of 5–10. Several (usually three) days postinfection, the sufficient expression level of the recombinant proteins can be obtained (*see* **Fig. 2** and **Note 5**). Character-

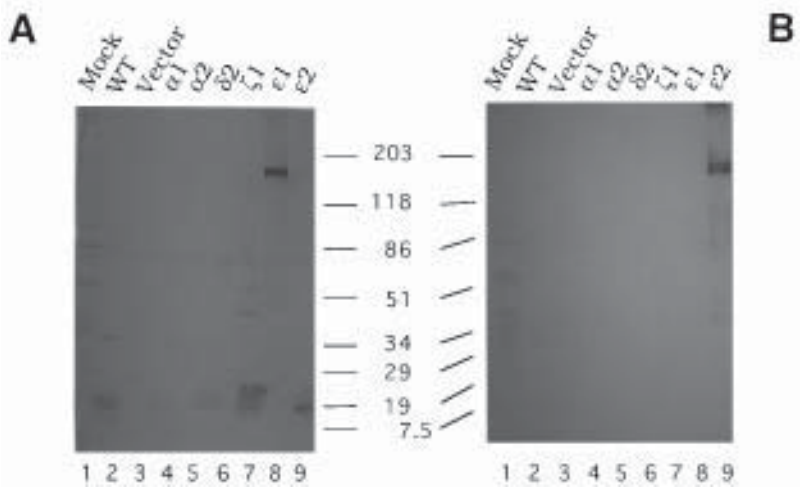

Fig. 2. Western blotting of baculovirus-infected Sf21 cells. Sf21 cells were placed in 12-well dishes and infected with AcNPV (lane 2), AcNPV/pJVP10Z **(A)** (or AcNPV/pBlueBacIII) **(B)** (vector only; without GluR cDNA insert) (lane 3), AcNPV/GluRα1 (lane 4), AcNPV/GluRα2 (lane 5), AcNPV/GluRδ2 (lane 6), AcNPV/GluRζ1 (lane 7), AcNPV/GluRε1 (lane 8), AcNPV/GluRε2 (lane 9), or mock-infected (lane 1). At 3 d postinfection, the particulate fractions (12,000*g* pellet; 2.6 μg of protein) of cell lysates were analyzed on a continuous 4–20% gradient SDS-PAGE gel and subjected to immunodetection using anti-GluRε1 (A) or anti-GluRε2 (B) antibodies. The positions of molecular-size markers (kDa) are indicated. Our experiments using tunicamycin or peptide: *N*-glycosides F (EC 3.5.1.52) indicate that the large- and small-sized bands are glycosylated and non-*N*-glycosylated species, respectively (data not shown).

ization of the baculovirus-expressed mouse ζ1-subunit has been already described in detail ***(15–17)***, and that of ε1- and ε2-subunits will be described elsewhere in detail.

3.2. Expression System Using Herpesvirus Vectors

In order to obtain HSV-1 vector, amplicon plasmid (**Fig. 3**) is first transfected to the Vero cells or rabbit skin cells, and superinfected with helper virus for packaging to recombinant defective virus.

3.2.1. Construction of Plasmid pHCMV/GluRζ1

The amplicon plasmid, which is a vector for producing defective HSV-1 vector, essentially contains a eukaryotic transcription unit and *cis*-acting HSV sequences, encoding an origin of DNA replication and a cleavage/packaging signal ***(37)***. The plasmid pHCL contains the bacterial *lac*Z gene under the control of the human cytomegalovirus (CMV) immediate early promoter ***(38)***.

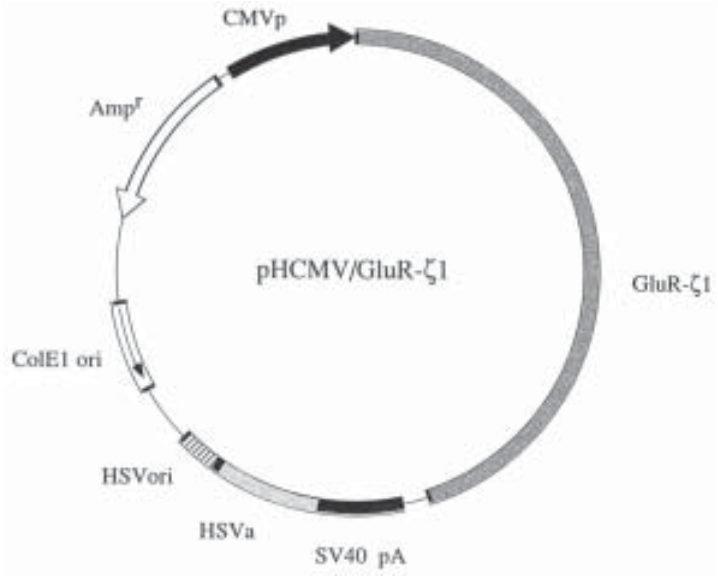

Fig. 3. Construction of HSV amplicon plasmid. The mouse NMDA receptor GluRζ1 subunit cDNA was inserted into the multiple cloning site of the vector. Key elements of the vector for packaging and expression of recombinant genes in eukaryotic cells are the HSV-1 cleavage/packaging signal "a" sequence (HSVa), the HSV-2 Oris origin of replication (HSVori), the CMV immediate early gene 1 promoter, and the simian virus 40 (SV40) polyadenylation signal.

The amplicon pHCMV/GluRζ1 was generated as follows. pHCL was digested with *Hin*dIII and *Sal*I to remove the bacterial *lac*Z gene and ligated to simian virus 40 polyadenylation signal, which was released from pRep4 (Invitrogen) digested with *Hin*dIII and *Xho*I. The resultant plasmid, pHCMV-pA, has several cloning sites for expression of a foreign gene. A 3.3-kb *Nru*I-*Nsi*I fragment from the pBSKζ1 ***(36)*** was inserted into *Hin*dIII site downstream from the CMV promoter by using blunt-end ligation.

3.2.2. Transfection

1. Seed 75-cm^2 flask with 5×10^6 rabbit skin cells/flask.
2. After 24–48 h (70–90% confluency), cells were detached by trypsin-EDTA solution and washed twice with PBS.
3. Resuspend the cells with Opti-MEM (1×10^7 cells/400 µL), add amplicon plasmid (30 µg/30 µL TE), mix well by pipeting, and transfer to a cuvet.
4. By using Gene Pulser (Bio-Rad) charge the pulse (960 µF, 220 V).
5. Leave at room temperature for 5 min, then resuspend the cells with 12 mL 10% FBS-DMEM, and mix well by pipeting.

6. Aliquot 4 mL each to three filter-capped 25-cm^2 flasks.
7. Incubate overnight at 37°C in 5% CO_2 incubator.

3.2.3. Superinfection

1. Wash the cells once with 4 mL 1% FBS-PBS.
2. Inoculate with 200 µL helper virus (tsK strain) (*see* **Note 6**) solution (1% FBS-PBS) at an MOI of 0.1.
3. Rock at room temperature for 5 min.
4. Leave at 31°C for 1 h in 5% CO_2 incubator.
5. Add 4 mL 1% FBS-DMEM, and incubate at 31°C in 5% CO_2 incubator.
6. When most cells are infected, harvest the virus solution (2–4 d).

3.2.4. Preparation of Stocks

1. Peel off the infected cells by cell scraper, and transfer to 15-mL centrifuge tube.
2. Freeze/thaw three times in liquid N_2 or dry ice/ethanol.
3. Centrifuge at 800*g* for 8 min at 4°C.
4. Use the supernatant as the virus solution (P0).

3.2.5. Amplification of Recombinant Defective HSV-1 Vector

1. Seed 25-cm^2 filter-capped flasks with Vero cells, and incubate the cells to 90–100% confluency.
2. Wash the cells with 4 mL of 1% FBS-PBS.
3. Add 1 mL of P0 virus solution.
4. Rock at room temperature for 5 min.
5. Leave at 31°C for 1 h in 5% CO_2 incubator.
6. Remove the virus solution.
7. Add 4 mL of 1% FBS-DMEM, and incubate at 31°C in 5% CO_2 incubator.
8. When all the cells are infected (24–48 h), recover the virus solution (P1).

3.2.6. Preparation of Defective HSV-1 Vector Solution for Experiments

1. Inoculate with the virus solution at the optimal (usually the highest) ratio of defective/helper virus.
2. Freeze/thaw three times.
3. Centrifuge at 800*g* for 8 min at 4°C to remove cell debris.
4. Transfer the supernatant to the tube, and centrifuge at 14,500*g* for 1 h at 4°C.
5. Remove supernatant.
6. Resuspend the pellet in suspension buffer A, aliquot, and store at –70°C.

3.2.7. Expression of NMDA Receptor Channel Subunit Protein Using Defective HSV-1 Vector

Defective HSV-1- or mock-infected Vero cells were lysed in 0.1 *N* Na_2CO_3 and then pelleted by centrifugation at 12,000*g* for 30 min. The pellets were

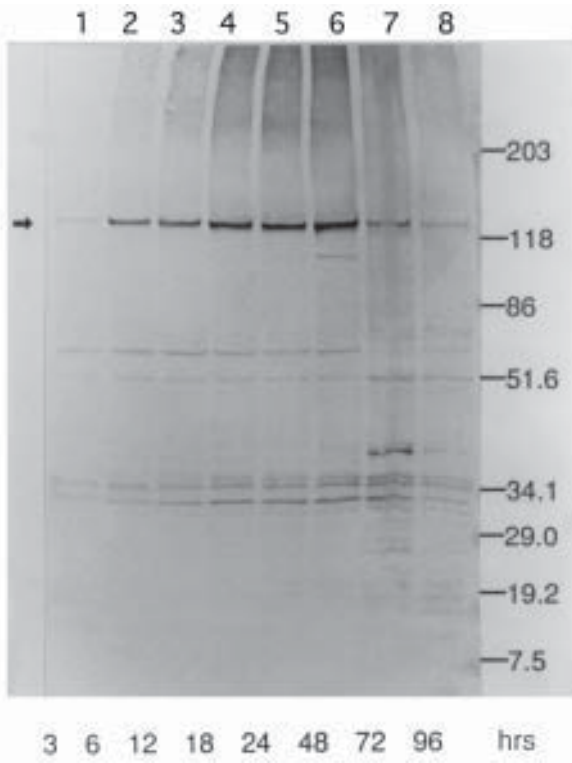

Fig. 4. Time-course of NMDA receptor channel GluRζ1-subunit expression in dvGluRζ1-infected Vero cells. At 3, 6, 12, 18, 24, 72, and 96 h postinfection, the particulate fractions (12,000*g* pellet; 2.6 μg of protein) of cell lysates were subjected to Western analysis using anti-GluRζ1 antibody as in **Fig. 2**. The positions of molecular-size markers (kDa) and recombinant GluRζ1 protein (arrow) are indicated.

suspended in suspension buffer B. In the experiment with immunoblotting, proteins were separated by SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane (Millipore). To detect GluRζ1 protein, incubation was done sequentially with the rabbit anti-GluRζ1 antibody and goat anti-rabbit IgG conjugated to horseradish peroxidase with 3,3'-diaminobenzidine as substrate.

In the initial characterization of GluRζ1 expressed in mammalian cells, the total particulate fraction of the cell lysate was analyzed by immunoblotting using rabbit anti-GluRζ1 antibody. As shown in **Fig. 4**, a predominant band was observed in Vero cells infected with defective HSV vector containing GluRζ1 cDNA (dvGluRζ1). No predominant band was present in the cells mock-infected or infected with *Lac*Z recombinant defective HSV-1.

Time-course analysis of GluRζ1 expression in Vero cells was performed. Vero cells were infected with dvGluRζ1 at an MOI of 1. **Figure 4** shows the time-course of immunodetected proteins expressed in dvGluRζ1-infected Vero cells. The size of the recombinant GluRζ1 protein expressed in dvGluRζ1-infected cells is ca. 120 kDa and is slightly greater than the calculated value (M_r of 103,477 after the removal of an amino-terminal signal peptide), suggesting that the apparent size difference may be owing to *N*-glycosylation of the protein, as is the case with AcNPV/GluRζ1-infected Sf21 cells ***(15–17)***. Expressed GluRζ1 was detectable 3 h postinfection and reached a maximum between 6 and 48 h post-infection (lanes 2–6).

4. Notes

1. Conventional manipulations of insect cells and baculoviruses were described in **refs. *31–33.***
2. Convenient kits for baculovirus expression system are available from Invitrogen, Pharmingen, Clontech, Novagen, or Gibco-BRL.
3. It is believed that 5'-untranslated region should be <100 bp for efficient expression.
4. It is believed that expression efficiency of recombinant viruses after several generations often decreases possibly because of the appearance of mutant viruses.
5. Our attempts to express the ε4-subunit of the NMDA receptor channel with the baculovirus system were unsuccessful. The reason for this is unknown at present. Failure to express this subunit in *Xenopus* oocyte was also reported ***(39,40)***, and a likely reason, as Monyer et al. ***(40)*** suggested, may be the high GC content of ε4 mRNA, particularly around and downstream of the translation initiation site.
6. We use temperature-sensitive tsK strain for helper virus to generate recombinant defective HSV-1 vector. The strain can amplify at 31°C, but not at 37°C. Therefore, when experiments are done at over 37°C, the effects of helper virus amplification can be neglected. Infection of cells by the tsK strain can be detected as the cytopathic effects (CPE) of cell rounding and cell fusion. Most of the tsK strains remain within the cells. Plaque assay of the resulting stock at a non-permissive temperature (37°C) demonstrated that the reversion of the temperature-sensitive helper virus tsK to wild-type virus was at a rate of $<10^{-7}$/mL, consistent with previous observations. Defective virus titer was estimated from the ratio of amplicon/helper virus DNA.

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