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Cytoplasmic Gene Expression System Enhances the Efficiency of Cationic Liposome-Mediated *in Vivo* Gene Transfer into Mouse Brain

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Development of methodologies for gene transfer into the central nervous system (CNS) is important for fundamental research as well as clinical studies for gene therapy. Cationic liposomes (CL) are attractive vectors because of their safety and ease of use. However, to date only low rates of success have been reported. We succeeded in obtaining high transfection efficiencies into the newborn mouse brain in vivo by CL and a cytoplasmic gene expression system based on T7 RNA polymerase and T7 RNA polymerase- and the luciferase-gene with the T7 promoter sequence. This system showed an efficiency rate 2 orders of magnitude higher than the standard system, which used CL and luciferase genes with a Rous sarcoma virus promoter, pRSVL. In addition, in vitro experiments using LLCMK$_2$ cells showed that cytoplasmic gene expression occurred rapidly (within 6 h) after transfection. In contrast, pRSVL required 24-48 h for induction of luciferase expression. Our results suggest that the cytoplasmic gene expression system is useful for gene delivery into the CNS.

Introduction of a gene of interest into the central nervous system (CNS) offers the prospect of manipulating gene expression for the investigation and treatment of neurological disorders. The most important research problem for gene transfer into the CNS is the development of effective vectors that optimize gene expression and minimize toxicity. To date successful virus vector-mediated gene transfer has been reported (1-3). However, there are many limitations of virus vector systems, the most significant of which are safety considerations.

Non-viral vectors are an attractive alternative because of their safety and lack of toxicity. Several investigators have reported gene delivery into the CNS using cationic liposome (CL)-DNA complexes as non-viral vectors (4,5). However, gene transfer efficiencies of CL-DNA complexes are lower than those of viral vectors. In addition, transfection efficiency into stationary cells by CL-DNA complexes is much lower than that of viral vectors (6,7). This may be because DNA traffic into the nucleus may occur preferentially in those cells that are entering mitosis, as a result of breakdown of the nuclear membrane. Cellular proliferation augments the efficiency of CL-mediated gene transfer (7). Therefore, CL-mediated gene transfer into non-proliferating cells in vivo including the CNS is limited.

One method to overcome this problem is to carry DNA into the nucleus. However, no successful results about this strategy have been reported. It is difficult to condense DNA within the size to pass the nuclear pore (outer diameter of the nuclear pore complex is about 80 nm.). And it has not yet been clarified whether there is any signal capable of carrying DNA into the nucleus.

Another method is to express transgenes in the cytoplasm after the genes are introduced into the cells. Gene expression in the cytoplasm can be expected to achieve higher transfection efficiency in non-dividing cells as well as dividing cells, because it does not re-

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Abbreviations: CNS, central nervous system; CL, cationic liposome; IRES, internal ribosomal entry site; RSV, Rous sarcoma virus.

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quire transport of DNA into the nucleus, which is one of the most significant limiting steps in gene transfer using non-viral vectors. In this study, we demonstrated that a cytoplasmic gene expression system (8,9), based on T7 RNA polymerase and a reporter gene with the T7 promoter sequence, enhanced the efficiency of CL-mediated in vivo gene transfer into the newborn mouse brain.

MATERIALS AND METHODS

Plasmid construction. A cytoplasmic gene expression plasmid, pT7-IRES-L, containing the T7 promoter and terminator, the firefly luciferase gene, and internal ribosomal entry site (IRES) derived from the 5′ nontranslated regions of the encephalomyocarditis virus genome (10) was constructed as described below. (1) The EcoRI fragment of pBS-ECAT (11) was cloned into the EcoRI site of pET3b (12), and the fragment between EcoRV and BamHI of the plasmid was deleted. Then, the AatII-BglII fragment of this plasmid was cloned into the AatII-BglII site of pT7-ø (13), resulting in pT7-IRES. (2) The HindIII-SmaI fragment of pRSVL (14), a luciferase expression plasmid, was cloned into the HindIII-SmaI fragment of pGEM-7Zf(−) (Promega Corp., Madison, USA), resulting in pGEM-L. (3) The fragment in IRES was amplified by polymerase chain reaction (PCR) using two primers, TTGGAATAAGGCCGGTGTGCG and CGCCAAGCCCATGGTATTATCG, to generate a NcoI site. The antisense primer contained a mis-matched base (underlined). Then, 4-piece ligation was performed using the ApaI-BamHI fragment of pT7-IRES, the SphI-BamHI fragment of pGEM-L, the ApaI-NcoI fragment of PCR product, and the NcoI-SphI fragment of pCAL2 (15), resulting in pT7-IRES-L. The ATG codon of the luciferase gene was adjusted to that just after the IRES sequence to obtain maximal efficiency of translocation.

pT7AUTO-2 (16), a T7 RNA polymerase expression plasmid with the T7 promoter, was kindly provided by Dr. F. W. Studier (Brookheven National Lab., Uptown, NY). pT7AUTO-2 was amplified using HMS174 cells containing pLysS, a T7 lysozyme (inhibitor of T7 RNA polymerase) expression plasmid. pLysS did not influence luciferase expression from pT7-IRES-L.

Cells. $LLCMK_2$ cells which expressed T7 RNA polymerase were prepared by co-transfection with pCAT7,[3] which contained the cytomegalovirus enhancer/chicken β-actin hybrid promoter, T7 RNA polymerase gene and SV40 early gene poly(A) signal, and pSV2neo (13) and selection with G418. T7 RNA polymerase was expressed at 3 ng/10^6 cells.

Gene transfer into cultured cells. CL (Lipofectin) was used for gene transfer. pT7-IRES-L (0.33 μg), pT7AUTO-2 (0.67 μg, including pLysS) and T7 RNA polymerase (125 units) were mixed with 100 μl of serum-free MEM. Then, they were incubated with CL (5.0 μg) suspended with 100 μl of serum-free MEM at room temperature for 15 min. After addition of 0.8 ml of MEM, they were incubated with $LLCMK_2$ cells at 37 °C for 3 h. The cells were washed twice with chilled medium, then given fresh medium.

In vivo gene transfer into the newborn mouse brain. Direct gene transfer into the mouse brain (2-3 day) was performed by the method of Schwartz et al. (5) with some modifications. Briefly, 5 μl of glucose (0.25 mg/ml)-BSS (150 mM NaCl, 10 mM Tris, pH 7.6) containing CL, DNA, and T7 RNA polymerase (CL, 250 ng; pT7-IRES-L, 7.0 ng; pT7AUTO-2 (including pLysS), 43 ng; T7 RNA polymerase, 2.5 units) was injected into the newborn mouse brain using a 30-gauge needle. As a control, complexes of CL and pRSVL, which contained the Rous sarcoma virus (RSV) promoter, (CL, 35 ng; pRSVL, 7.0 ng) were injected similarly. For the measurement of luciferase activity, the brain was recovered and treated with the reagent to dissolve the cells and tissues (PicaGene, Toyo Inki Co. Ltd, Tokyo, Japan).

[3] T. Kondo et al., unpublished observation.

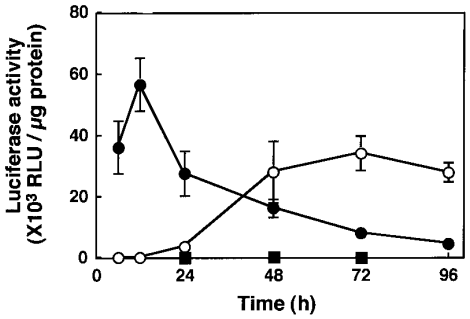

FIG. 1. Time course of luciferase expression in T7 RNA polymerase-expressing $LLCMK_2$ cells transfected with pT7-IRES-L. $LLCMK_2$ cells which expressed T7 RNA polymerase were incubated with CL-pT7-IRES-L complex or CL-pRSVL complex (CL 5.0 μg/ml; DNA 1.0 μg/ml) at 37 °C for 3 h. Normal $LLCMK_2$ cells were also incubated with CL-pT7-IRES-L complex. At the indicated times, the cells were harvested and luciferase activity was determined as described in Materials and Methods. (●) T7 RNA polymerase-expressing $LLCMK_2$ cells were transfected with pT7-IRES-L. (○) T7 RNA polymerase-expressing $LLCMK_2$ cells were transfected with pRSVL. (■) Normal $LLCMK_2$ cells were transfected with pT7-IRES-L. Each point represents the mean ± S.D. of four experiments.

Luciferase assay. Luciferase activity was measured using a luciferase assay system (PicaGene, Toyo Inki Co. Ltd, Tokyo, Japan) and a luminometer (Lumat LB9501, EG & G Berthold, Bad Wildbad, Germany). The activity was indicated as relative light units (RLU) per μg protein, RLU per 35-mm dish or RLU per mouse.

RESULTS AND DISCUSSION

Cytoplasmic Gene Expression in $LLCMK_2$ Cells Which Express T7 RNA Polymerase

To determine the transfection efficiency and luciferase expression pattern of pT7-IRES-L, which expresses luciferase in the cytoplasm under the control of the T7 promoter, in $LLCMK_2$ cells with the T7 RNA polymerase, pT7-IRES-L was first transfected into these cells by CL (Fig.1). The IRES sequence derived from the 5′ nontranslated regions of the encephalomyocarditis virus genome (10) was inserted between the T7 promoter and terminator to facilitate efficient translocation. pRSVL, which contains the RSV promoter and requires to be transferred into the nucleus for luciferase expression, was also transfected as a control.

The cells treated with pT7-IRES-L showed rapid luciferase expression, and maximal activity was observed 12 h after transfection. In contrast, luciferase activity in the cells treated with pRSVL was low within 24 h after transfection and maximal at 48 h. Moreover, the cells transfected with pT7-IRES-L expressed more luciferase than those with pRSVL. $LLCMK_2$ cells, which did not express T7 RNA polymerase, showed no luciferase activity when transfected with pT7-IRES-L. These

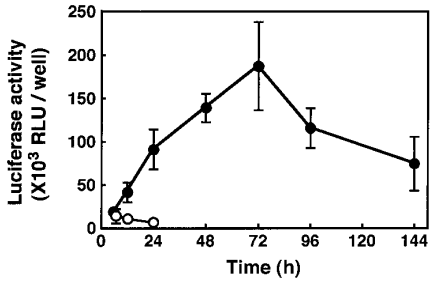

FIG. 2. Luciferase expression in normal $LLCMK_2$ cells co-transfected with pT7-IRES-L, pT7AUTO-2 and T7 RNA polymerase. Normal $LLCMK_2$ cells were incubated with complexes of CL (5.0 μg/ml), pT7-IRES-L (0.33 μg/ml), pT7AUTO-2 (0.67 μg/ml), and T7 RNA polymerase (125 units/ml) at 37 °C for 3 h. As a control, the cells were also treated with complexes of CL (5.0 μg/ml), pT7-IRES-L (1.0 μg/ml), and T7 RNA polymerase (125 units/ml). At the indicated times, the cells were harvested and luciferase activity was determined as described in Materials and Methods. (●) Complex of CL, pT7-IRES-L, pT7AUTO-2, and T7 RNA polymerase (○) Complex of CL, pT7-IRES-L, and T7 RNA polymerase Each point represents the mean ± S.D. of four experiments.

results suggest that pT7-IRES-L shows rapid and efficient gene expression in the presence of sufficient levels of T7 RNA polymerase.

Cytoplasmic Gene Expression in Normal $LLCMK_2$ Cells

To apply this system commonly, we next examined the luciferase expression in normal $LLCMK_2$ cells transfected with pT7-IRES-L (Fig.2). These cells co-transfected with pT7-IRES-L and T7 RNA polymerase showed weak and short-term luciferase activity. This weak expression may have resulted from the failure of active T7 RNA polymerase in the cytoplasm, because the T7 RNA polymerase-expressing cells transfected with pT7-IRES-L showed stronger and longer-term luciferase expression as described in Fig.1. Therefore, we co-transfected cells with pT7-IRES-L, T7 RNA polymerase, and pT7AUTO-2 (16), which expressed T7 RNA polymerase under the control of the T7 promoter. Luciferase activity in $LLCMK_2$ cells co-transfected with pT7-IRES-L, pT7AUTO-2, and T7 RNA polymerase was stronger and lasted until 96 h. This higher luciferase expression was also observed in primary cultured rat astrocytes and bovine aortic endothelial cells (data not shown). These results suggest that this cytoplasmic gene expression system can work in many cells with the addition of pT7AUTO-2.

In Vivo Gene Transfer Using Cytoplasmic Gene Expression System

The CNS is one of the most important target tissues for gene therapy. CL have been reported to be applicable for direct introduction of genes into the CNS (4,5). However, the efficiency of this method is very low, and one of the main reasons for this is that the cells in the CNS are in the stationary phase of the cell cycle. In general, cell growth is required for efficient CL-mediated gene expression (7). As the cytoplasmic gene expression system does not need transport of DNA into the nucleus or cell proliferation, it might improve the efficiency. We introduced the complexes of CL, pT7-IRES-L, pT7AUTO-2, and T7 RNA polymerase directly into the newborn mouse brain (Fig.3). The newborn mouse brain was used as a model system because of the ease of operation, although some cells in the ventricular and subventricular zones, which will give rise to different glial cell populations (17) and some neurons and interneurons destined to migrate to the olfactory bulb (18), are mitotic. A pT7-IRES-L : pT7AUTO-2 ratio of 1 : 6 (w) was used for gene transfer, which was optimal (data not shown). Similar dose (as the luciferase expression plasmid) of CL-pRSVL complexes was injected as a control.

Injection of the cytoplasmic gene expression unit into the mouse brain showed much higher luciferase activity in the brain than the CL-pRSVL complexes, and the transfection efficiency was increased by over 300-fold. Luciferase activity in the brain treated with the cytoplasmic gene expression unit was maximal at 2 days, and then decreased thereafter. The activity in the CL-pRSVL complex-treated brain was lower than that reported by Schwartz et al. (5). This might have been due to differences in the plasmid used, CL, and dose injected. These results suggest that for gene transfer into the newborn mouse brain, the cytoplasmic gene expression system works more efficiently than conventional plasmid vector systems, and that this system may overcome the less efficiency of gene transfer using non-viral vectors. Cytoplasmic gene expression systems might work more effectively in the mature brain.

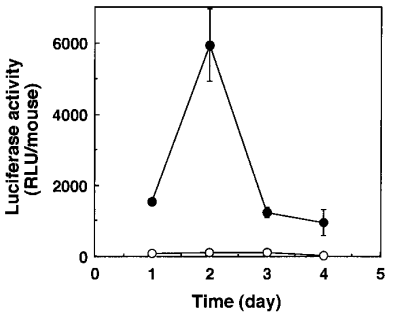

FIG. 3. Time course of luciferase expression in newborn mouse brain. Complexes of CL, pT7-IRES-L, pT7AUTO-2 and T7 RNA polymerase or CL-pRSVL complexes were injected into the newborn mouse brain as described in Materials and Methods. At the indicated times, the brain was recovered and luciferase activity was determined as described in Materials and Methods. (●) Complex of CL, pT7-IRES-L, pT7AUTO-2, and T7 RNA polymerase. (○) Complex of CL and pRSVL. Each point represents the mean ± S.E. of four-eight experiments.

In addition, as transgene expression using this system depends on only T7 promoter sequence and T7 RNA polymerase, this system would work in the in vivo transfection to other tissues efficiently.

We are currently engaged in studies to increase the efficiency of this system to express transgenes in the cytoplasm. One approach is to use fusogenic liposomes based on Sendai virus (15,19,20) as the vector for gene transfer instead of CL. Fusogenic liposomes introduce genes and proteins into the cytoplasm much more efficiently than CL (15). Another approach is to construct plasmids capable of expressing T7 RNA polymerase at higher levels. The level of production of T7 RNA polymerase would be increased by insertion of an IRES sequence between the T7 promoter and the sequence encoding T7 RNA polymerase. Placement of the IRES sequence just downstream of the T7 promoter was found to increase the expression by 5- to 10-fold (10). These combinations could result in more efficient expression of transgenes even into stationary cells in vivo.

In summary, this study suggests that transgenes can be expressed in the CNS efficiently by the direct injection of CL and a cytoplasmic gene expression unit. Our results indicate the potential usefulness of the cytoplasmic gene expression system in gene therapy of neurological disorders.

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