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Parameters influencing ectopic gene expression in *Aplysia* neurons

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Abstract

DNA microinjection for expressing exogenous genes in *Aplysia* neurons has been very useful for analyzing not only functions of encoded proteins, but also regulations of gene expression in the nervous system. Some of the factors that affect expression of foreign genes microinjected into *Aplysia* neurons are described. The effect of the DNA form (supercoiled or linear) and promoter modification in the expression vectors are analyzed as well as buffer composition and DNA concentration in the microinjection solution. The time course of reporter gene expression was also monitored. Reporter gene expression was first detected as early as 1 h and maintained at a high level even until 7 days after microinjection. The presence of AP-1 enhancer in the promoter region of the expression vectors was essential in driving a high-level constitutive expression of reporter genes. Particularly, a pNEX derivative containing eight copies of AP-1 enhancer drove constitutive overexpression more effectively than ones harboring either four or 12 copies of AP-1 enhancer. We also found that a prokaryotic promoter/operator from *E.coli lacZ* gene placed upstream from an eukaryotic enhancer/promoter repressed the expression of the downstream reporter gene in *Aplysia* neurons.

Keywords: *Aplysia* neurons; Ganglion culture; Gene transfer; DNA microinjection; AP-1 enhancer element; Transcription factor; β-Galactosidase; Firefly luciferase

Gene transfer into neurons is considered to be one of the most important steps in molecular analysis of the neuronal function of each cloned gene and its regulatory mechanism. However, difficulties in neuronal gene transfer with conventional transfection protocols have limited molecular approach to the understanding of the function of the nervous system. Although viral techniques [2,10] and particle-mediated gene transfer [11] were recently applied to transfect a number of neurons in brain slices and dissociated neuronal cultures, the microinjection method has proven to be quite useful in directing the gene transfer into a specifically identified neuron [1,5,7]. This point is particularly important in analyzing the altered physiology of a single specific neuron after genes of interest were introduced into it.

Most *Aplysia* neurons have quite large cell somata from 40 to 500 μm in diameter, making it relatively easy to introduce cDNAs into them by microinjection. The relative simplicity of the neuronal circuitary has made *Aplysia* useful as a model to study the cellular mechanisms of behavior such as learning and memory [9]. Using direct microinjection as a tool of gene transfer, we have been able to study gene regulation in sensory neurons involved in learning and memory [6,7] as well as to examine the function of gene products such as ion channels [8,14], synaptotagmin [12], and transcription factors [7] in identified neurons. However, factors that affect efficiency with microinjection method are poorly assessed. In the experiments described here, we have investigated potential parameters which affect expression of the genes microinjected into the *Aplysia* neurons. These parameters include DNA form, DNA amount, and buffer ingredients in microinjection solution, and the modifications around the promoter region of the expression vectors including the number of AP-1 enhancers. Finally the duration and level of gene expression were tested.

Aplysia kurodai weighing 50–250 g were purchased from professional sea-divers in Pusan or Sokcho, South Korea and maintained in recirculating sea water tanks at

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Table 1
Effect of DNA concentration on expression in *Aplysia* neurons

Concentration (μg/ml)	Normalized β-gal expression[a]
10	0.01 ± 0.01 (3)[b]
50	0.37 ± 0.19 (3)
100	0.23 ± 0.06 (4)
300	9.11 ± 6.43 (3)
500	2.80 ± 1.21 (3)
700	8.80 ± 2.81 (11)

[a]Ganglion extracts were made 20 h after microinjection as previously described [7] using 30 μl of 1 × Reporter Lysis buffer (Promega). β-gal assay was done by mixing 5 μl of extract and 50 μl of Galacton-Light substrate (Tropix) for 65 min at room temperature. Accelerator (100 μl; Tropix) was added to the reaction mixture at the time of luminescent measurement. Luciferase activity was measured by mixing 5 μl of extract with 50 μl of luciferase substrate (Promega). Emitted light was quantified in Turner Designs model 20/e luminometer (Promega) using a 5 s delay followed by a 10 s integration of light output. Ganglion extract was discarded when it had relatively low level of luciferase activity (less than 10.00 RLU), an indication of poor microinjection. Normalized β-gal expression is represented as β-galactosidase/luciferase activity; [b]parenthesis indicates the number of trials. One trial consisted of microinjections into 20 neurons in one ganglion. Results are expressed as the mean ± SEM. As an internal control for expression, firefly luciferase gene was allowed to express from a weak expression vector pNEX2. pNEX2 was generated by the following procedure. Three copies of AP-1 enhancer and deformed sequences of *E.coli lacZ,Y* (bases 261–548 in pNEXδ) and *Ori* sequence of phage f1 (bases 683–806 in pNEXδ) originating from 4X AP-1 RSV-lacZ were deleted out of pNEX by carrying out two rounds of two-step recombinant polymerase chain reactions (PCR) [4]. DNA modification by this method was verified by performing DNA sequencing using a T7 sequenase system (USB). The coding region of firefly luciferase was excised out from pGL2-Control (Promega) by cutting with *Hin*dIII and *Dra*I. The 1.9 kb fragment was ligated with *Hin*dIII/*Sma*I-cut pNEX2 to generate pNEX2-luc. Microinjection solutions contained 10–700 μg/ml pNEXδ-lacZ, 300 μg/ml pNEX2-luc,10 mM Tris-Cl (pH 7.3), 100 mM NaCl, and 0.1% fast green. pNEX-lacZΔD3 plasmid was added to keep the final DNA concentration 1 mg/ml in all microinjection solutions. pNEX-lacZΔD3 was derived from pNEX-lacZ [8] by eliminating the 188 bp *Hin*dIII fragment containing four copies of the AP-1 enhancer and RSV minimal promoter.

14°C before use. We found that the nervous system of *A. kurodai* is very similar in anatomy as well as in electrophysiological properties of identified neurons to that of *A. californica*, which has been a popular experimental animal. All plasmid DNAs used in microinjection were prepared by a standard midi-prep procedure using Qiagen-tip 100 (Qiagen). Animal dissection, desheathing of ganglia, preparation of ganglion cultures, and microinjection were done as previously described [7,8].

First, we found that reporter gene activity correlated with the amount of DNA microinjected (Table 1). A higher concentration of DNA could yield a higher expression level. However, high viscosity in the microcapillary of an injection solution containing more than 1 mg/ml DNA caused a technical problem in the microinjection procedure, setting the practical limitation in using injection solutions containing more than 1 mg/ml DNA.

Next, we investigated the consequence of buffer components in the microinjection solutions. The injection solutions tested were as follows: 10 mM Tris-Cl (pH 7.3), 100 mM NaCl; 50 mM HEPES (pH 7.3), 40 mM NaCl; 10 mM Tris-Cl (pH 7.5), 0.5 mM EDTA; 10 mM Tris-Cl (pH 7.5), 100 mM KCl; distilled water (no buffer and no salt); 10 mM Tris-Cl (pH 7.5), 500 mM K-acetate; 25% glycerol; 1 M KCl. DNA dissolved simply in distilled water or in buffer solutions containing 10 mM Tris-Cl or 10 mM HEPES (pH 7.3–7.5) with 0.5 mM EDTA, 40–100 mM NaCl, or 100 mM KCl seemed to give rise to somewhat better results in gene expression than other types of solutions containing 25% glycerol, 500 mM K-acetate, or 1 M KCl (data not shown). This suggests that buffer and salt components in the microinjection solution could be flex-

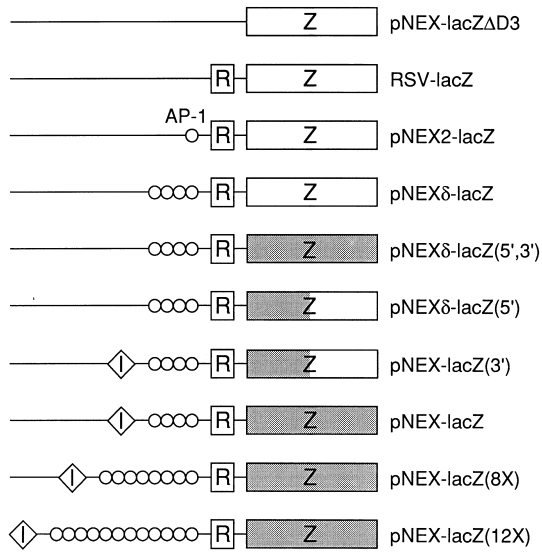

Fig. 1. Structure of the DNA constructs used in Table 2. Either RSV minimal promoter with four copies of the AP-1 enhancer or RSV minimal promoter were subcloned into the multicloning sites of pBluescript II (Stratagene) at the upstream region of 3.8 kb *E.coli lacZ* gene cassettes to generate 4X AP-1 RSV-lacZ and RSV-lacZ, respectively. Derivation of pNEX-lacZ [8] and pNEXδ-lacZ [7] from 4X AP-1 RSV-lacZ was previously described [6]. The *Hin*dIII-*Dra*I fragment containing the *lacZ* gene cassette from 4X AP-1 RSV-lacZ was inserted into the *Hin*dIII/*Sma*I-cut pNEX2 to construct pNEX2-lacZ. The number of AP-1 enhancer elements in the expression vectors was increased by the following steps. An 84 bp *Eco*RV-*Pst*I fragment containing four copies of AP-1 enhancer elements was excised out of pNEX and subject to mung bean nuclease reaction to create blunt ends. This fragment was ligated to *Eco*RV-digested and calf intestinal phosphatase-treated pNEX. The recombinant expression vectors containing eight or 12 copies of enhancer elements were selected by colony PCR. The number and the orientation of AP-1 enhancer cluster in vectors were determined by performing DNA sequencing using a T7 sequenase system (USB). They proved to have two or three direct repeats of the 84 bp fragment harboring four copies of the AP-1 site. I, the intact *lacZ* promoter/operator sequence residing in the 187 bp *Eco*RV-*Pvu*II fragment originated from pNEX; AP-1, the binding site (TGA(C/G)TCA) for the transcription factor AP-1; R, RSV minimal promoter; Z, *Escherichia coli lacZ* gene cassettes. Structures of *lacZ* gene cassettes are different from each other at 5′- and 3′-flanking sequences of the cassettes. A hybrid between these two cassettes was used in pNEXδ-lacZ (5′) and pNEX-lacZ (3′).

Table 2
Expression efficacy of various DNA constructs in *Aplysia* neurons

DNA	Promoter (no. AP-1)	Normalized β-gal expression[a]
pNEX-lacZΔD3	– (0)	0.00 ± 0.00 (4)[b]
RSV-lacZ	RSV (0)	0.08 ± 0.06 (5)
pNEX2-lacZ	RSV (1)	0.08 ± 0.02 (10)
pNEXδ-lacZ	RSV (4)	8.77 ± 2.56 (12)
pNEXδ-lacZ (5′,3′)	RSV (4)	8.76 ± 4.33 (3)
pNEXδ-lacZ (5′)	RSV (4)	5.47 ± 4.20 (2)
pNEX-lacZ (3′)	RSV (4)	0.31 ± 0.03 (2)
pNEX-lacZ	RSV (4)	0.39 ± 0.16 (10)
pNEX-lacZ (8 ×)	RSV (8)	192.04 ± 97.81 (7)
pNEX-lacZ (12 ×)	RSV (12)	10.54 ± 5.04 (5)

[a,b]See footnotes in Table 1. Microinjection solution composed of 0.7 mg/ml each DNA construct, 0.3 mg/ml pNEX2-luc,10 mM Tris-Cl (pH 7.3), 100 mM NaCl, and 0.1% fast green. Results are expressed as the mean ± SEM.

ibly chosen depending on the purpose of injection experiments.

We also tested the effect of the DNA form on the expression of a reporter gene. A DNA construct pNEXδ-lacZ [7] containing *lacZ* encoding *E.coli* β-galactosidase (β-gal) was used to make two linear forms of DNA, flanking with either cohesive ends or blunt ends by cutting it with *Bsm*I or *Xmn*I, respectively. Linear forms of DNA construct having either cohesive or blunt ends seemed to have a similar efficacy to the supercoiled form in driving the reporter gene expression irrespective of incubation time (8–35 h) after microinjection (data not shown). The fact that microinjected supercoiled DNA undergoes the linearization within the neurons [1] may concur with our results showing that initial conformations of DNA in the microinjection solution was not the factor influencing ectopic gene expression.

We also compared β-gal expression from the various expression vectors in a quantitative manner. We found that pNEXδ-lacZ expressed β-gal 22.5-fold more than pNEX-lacZ did (Fig. 1; Table 2). pNEXδ, which is derived from pNEX, is lacking an 187 bp *Eco*RV-*Pvu*II fragment present in pNEX. This short fragment contains the intact *lacZ* promoter/operator sequence located upstream from four copies of the AP-1 enhancer and the Rous sarcoma virus long terminal repeat (RSV) minimal promoter [3]. Structures of *lacZ* gene cassettes subcloned in pNEX and pNEXδ are somewhat different from each other, particularly at 5′- and 3′-flanking sequences of cassettes. It is likely that *lacZ* promoter/operator sequence may negatively affect transcription of a reporter gene controlled by juxtaposing the AP-1/RSV enhancer/promoter region. It is also possible that inequality in expression of β-gal is due to the structural difference in two *lacZ* cassettes. To see whether two forms of *lacZ* gene cassettes affect gene expression differently, *lacZ* cassettes were exchanged between pNEX-lacZ and pNEXδ-lacZ. pNEXδ-lacZ (5′) and pNEXδ-lacZ (5′,3′) were generated by replacing a 1.1 kb *Hin*dIII-*Cla*I fragment containing the 5′ portion of *lacZ* and a 5 kb *Hin*dIII-*Bsa*I fragment containing the whole *lacZ* cassette in pNEXδ-lacZ by those in pNEX-lacZ, respectively. pNEX-lacZ (3′) was constructed by replacing a 3′ portion of *lacZ* (2.2 kb *Cla*I-*Kpn*I fragment) in pNEX-lacZ by that in pNEXδ-lacZ. The data from this *lacZ* swapping experiment clearly showed that the expression level of β-gal was determined by the types of expression vector used, not by the modifications or exchanges in *lacZ* gene cassettes (Fig. 1; Table 2). Difference in expression efficiency between pNEX-lacZ and pNEXδ-lacZ is likely due to structural discrepancy between two expression vectors, not to a difference in *lacZ* gene cassettes. It, however, is not clear whether this transcriptional inhibition occurred through some *Aplysia* factors binding to this exogenous prokaryotic sequence. Nonetheless, it is intriguing that a prokaryotic promoter/operator sequence does have an inhibitory effect on the eukaryotic transcription system.

The role of the AP-1 enhancer and RSV minimal promoter in the expression vectors was also examined. Table 2 illustrates that the plasmid pNEX-lacZΔD3 lacking the whole enhancer/promoter sequence failed to express β-gal. This enhancer/promoter appeared to be critical in driving the reporter gene expression in *Aplysia* neurons. Moreover, the level of gene expression seemed to depend on the number of AP-1 enhancers located upstream from the RSV minimal promoter (Fig. 1; Table 2). The presence of four copies of AP-1 enhancers in pNEX-lacZ was able to increase expression level by about 5-fold compared to vectors containing one (pNEX2-lacZ) or no AP-1 enhancer (RSV-lacZ) at the promoter. A vector pNEX (8 ×), a

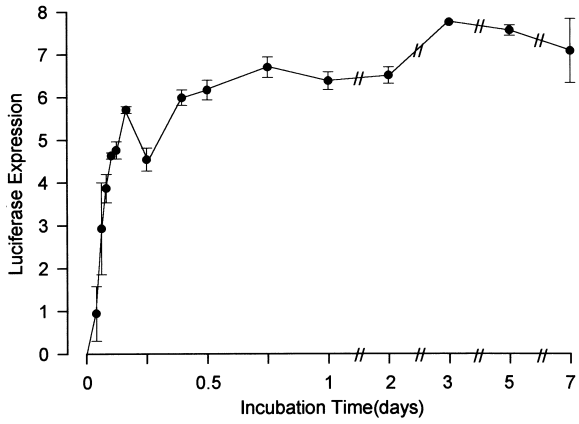

Fig. 2. Time course of luciferase expression in the *Aplysia* giant neurons. Luciferase expression is represented as $\log_{10}$(luciferase luminescence × 1000). In order to minimize variations in size of neurons and in expression efficiency among trials, only LUQ, L7, L11, and R15 in the abdominal ganglion were chosen for microinjection from the animals similar in body weight and only two of them were microinjected in each trial with DNA solution containing 1 mg/ml pNEX2-luc, 10 mM Tris-Cl (pH 7.3), 100 mM NaCl, and 0.1% fast green. Microinjected neurons were processed for luciferase assay at different times after injection. The uninjected neurons exhibited no endogenous luciferase activity.

synonym of pNEX3 containing eight copies of AP-1 enhancers, appeared to be most efficient in driving β-gal expression. On the contrary, a further increase in the number of AP-1 sequences in a vector, pNEX ($12\times$), decreased by about 18-fold the gene expression driven by pNEX ($8\times$). The excessive number of the enhancer sequences may squelch the basal transcription machinery, thereby unbalancing the stoichiometry between the enhancers and the nuclear transcription machinery.

Finally, the time course of expression of reporter genes was examined to see how fast expression occurs or how long it sustains. The expression of β-gal judged by X-gal (GIBCO BRL) staining was first detected 2 h after microinjection (data not shown). On the other hand, the luciferase activity which is known to be a more sensitive reporter than either β-gal or CAT was detectable as early as 1 h after microinjecting DNA (Fig. 2). The luciferase activity increased rapidly up to several hours and was sustained at least up to 7 days after microinjection (Fig. 2). Luciferase proves to be a powerful reporter in the measurement of temporal changes in transcription because it turns over rapidly, having a half-life of 2–4 h [13]. Therefore, the luciferase activity detected days after microinjection may be attributed to steady-state mRNA synthesis from microinjected DNA copies which exist in the concatemerized episome-like state or in the integrated form in the chromosome.

In this study, we tried to address the effect of some parameters involved in gene transfer by microinjection on expression of exogenous genes. A vector, pNEX3, could be particularly suited for overexpressing a foreign gene product that turns over rapidly or for knocking out an endogenous protein by overexpressing its anti-sense sequence or transdominant negative mutant sequence. Ectopic expression by microinjection seemed to be quite fast, occurring as early as 1 h, growing rapidly for several hours, and also continuing for at least as long as a week. Therefore, this gene transfer system for the nervous system of *Aplysia* can provide a reliable way to analyze the function of many genes and DNA sequences within the intact neurons, thus enabling the molecular explanation of diverse neurobiological issues as functions of specific genes or gene products.

The nucleotide sequences of pNEX2 and pNEX3 are deposited in the GenBank data base under accession numbers U67090 and U67091, respectively. I thank Dr. Eric R. Kandel and Dr. Sunyoung Kim for critical reading of the manuscript. This work was supported by the Genetic Engineering Research Fund and the Equipment Purchase Program of the Priority Support Project for Graduate School from the Ministry of Education, South Korea and by a grant from South Korea Science and Engineering Foundation (94-0401-02-01-3). RSV-lacZ and 4X AP-1 RSV-lacZ were obtained from Drs. Karl T. Riabowol, J. Stephen Fink and Richard H. Goodman.

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