

Short Communication

Interruption of *env* Gene Expression Depending on the Length of the SV40 Early Region Used for the PolyA Signal

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SUMMARY: In order to invent a screening system to check in vivo gene function and the efficiency of gene transfer mediated by a retroviral vector system, we established a novel packaging cell, PacNIH/A8, based on the neuropathogenic retrovirus A8-V. To construct the expression vector, pA8(Ψ^-), which expresses the genes *gag*, *pol* and *env* derived from A8-V, the SV40 early region was used for the polyadenylation signal (polyA signal). When a 0.85 kbp fragment in the SV40 early region was employed for the expression vector (pA8(Ψ^-) β), *env* expression was abolished. This abolition was rescued by shortening the SV40 early region to 0.14 kbp (pA8(Ψ^-) δ). The NHI3T3 cells transfected with pA8(Ψ^-) δ showed expressions of both *env* and *gag* genes.

A8-V is a molecular clone of the neuropathogenic variant FrC6-V (1,2), isolated from Friend murine leukemia virus (Fr-MLV). FrC6-V and A8-V induce spongeform degeneration mainly in the grey matter of the central nervous system (CNS), including the brain cortex, thalamus and brain stem of infected rats (3). Studies using chimeras constructed from the A8 virus and non-neuropathogenic Fr-MLV clone 57 (57-V) revealed that the *env* gene of A8 is a primary determining factor for the induction of neurodegeneration, and the LTR and/or 5' leader sequence of A8 is also necessary for neuropathogenicity (2,4,5). The PVC211 virus, which is also a neuropathogenic variant of Fr-MLV, can infect rat brain capillary endothelial cells in vivo and in vitro (3,6,7), and the tropism of the virus for rat brain capillary endothelial cells causes its neuropathogenicity. The efficient entry of these neuropathological viruses into the CNS of rats is mediated by an interaction between the viral surface protein (Env), a product of the *env* gene, and F10-ecoR, which is a receptor for MLV infection isolated from the rat glial cell line F10. The efficiency of the F10-ecoR-mediated entry of the pseudotyped virus carrying the Env protein of A8 was one order of magnitude greater than that of the pseudotyped virus carrying Env derived from the 57-V gene (8). These findings prompted us to construct a retroviral vector system to transfer genes of interest into the rat CNS using the genes of A8-V.

An expression vector for Gag, Pol and the Env protein of A8-V was constructed. The vector contained LTR, *gag*, *pol* and the *env* gene of A8-V and the SV40 early polyA signal, but did not have a packaging signal (pA8(Ψ^-)), as shown in Fig. 1. In order to select stable transformants, the blastocidin deaminase (BSD) gene was introduced into the vector. This construct was transfected into the NIH3T3 cell line by calcium phosphate co-precipitation as reported elsewhere (2). First, we obtained pA8(Ψ^-) β cells, which employed a 0.85 kbp fragment in the SV40 early region for the polyA signal (Fig. 1). We did not detect transient *env* expression in pA8(Ψ^-) β -transfected cells (data not shown). Next, we

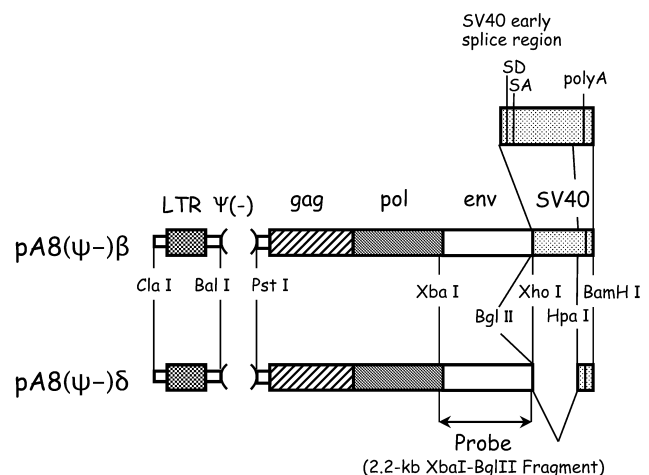


Fig. 1. Structures of pA8(Ψ^-) β and pA8(Ψ^-) δ . 0.35 kb of the BalI-PstI fragment of the 5' leader sequence of A8 (accession no. D88386) was deleted in pA8(Ψ^-) β and pA8(Ψ^-) δ in order to remove the packaging signal (9). 0.61 kb of the Sau3AI (4710)-Sau3AI (4100) fragment containing an SV40 small T antigen intron (accession no. V01380) was ligated to 0.24 kb of the BclI (2770) - BamHI (2533) fragment of the SV40 early region, and used as a polyA signal of pA8(Ψ^-) β . 0.14 kb of the HpaI (2666)-BamHI (2533) fragment of the SV40 early region (accession no. V01380) was used as a polyA signal of pA8(Ψ^-) δ . SD and SA: splicing acceptor and donor sites, respectively. Probe: region of a ³²P-labeled fragment used for the Northern blot hybridization shown in Fig. 3.

employed the shortened SV40 early region (0.14 kbp) for the polyA signal, and pA8(Ψ^-) δ was constructed (Fig. 1). The drug-resistant cells were selected in medium containing 1.25 μ g/ml of blastocidin (Invitrogen, Carlsbad, Calif., USA) and cloned. Thirty clones from the cells transfected with pA8(Ψ^-) β (PacNIH/A8 β) and 24 clones from pA8(Ψ^-) δ transfected cells (PacNIH/A8 δ) were established (9). The cloned cells were seeded on glass slides, cultured overnight, and then fixed in 100% ethanol for 2 min. Each clone was examined by immuno-histochemistry to determine whether it expressed Env or Gag proteins (Fig. 2).

In PacNIH/A8 β cells, most of the clones expressed the Gag protein (Table 1), whereas only 3 clones expressed the Env protein. The ratio of the clones that expressed both Env and Gag proteins (Env⁺, Gag⁺) to the clones that expressed

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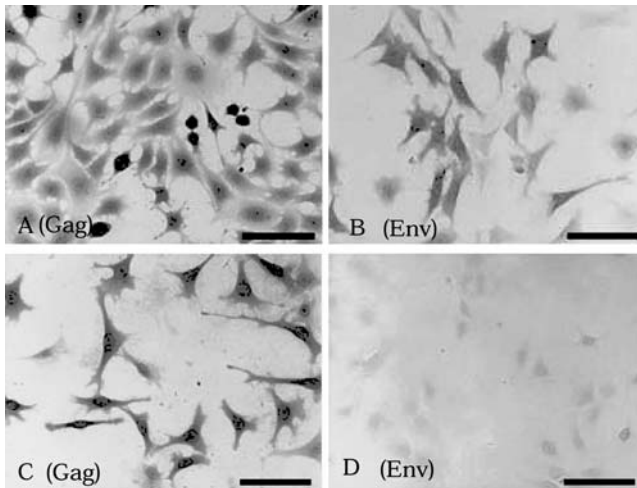


Fig. 2. Expressions of Gag and Env proteins. Immunohistochemistry was performed on glass slides seeded with PacNIH/A8 β (C and D) and PacNIH/A8 δ cell clones (A and B) using goat anti-Rauscher MLV gp70 (Quality Biotech, Incorporated Resource Laboratory) to detect Env protein (B and D) and using anti-AKR p30^{Gag} (Quality Biotech, Incorporated Resource Laboratory) to detect Gag protein (A and B), as described elsewhere (9). The bars indicate 30 μ m.

Table 1. Expression of Gag and Env proteins in the cloned cells

Packaging cells	Clone no.	Gag+ Env+	Gag+ Env-	Gag- Env+	Gag- Env-
PacNIH/A8 β	30	3	21	0	6
PacNIH/A8 δ	24	13	0	0	11

Packaging cells PacNIH/A8 β and PacNIH/A8 δ were cloned under selection by blasticidin. Each clone was examined by immunohistochemistry to determine whether it expressed Gag or Env proteins. Clone no. indicates the established numbers of cell clones. + and - indicate whether the examined proteins are expressed (+) or not (-).

Gag protein alone (Gag+) was 3/24 (in other words, 21/24 of the PacNIH/A8 β cell clones expressed the Gag protein only, without expressing the Env protein [Env-, Gag+]). No clone expressed the Env protein only (Env+, Gag-). In contrast, all of the 13 Gag-protein-expressing clones of PacNIH/A8 δ cells expressed the Env protein as well (13/13). Also, in the PacNIH/A8 δ cells, no clone was found to be grouped into the (Env+, Gag-) expression type. These results suggested that Env protein expression is less stable than Gag protein expression. In order to elucidate whether this labile Env expression is due to incomplete DNA insertion, we performed Southern blotting for the PacNIH/A8 β cell clones with the (Env-, Gag+) type expression, and all of the 5 clones tested had the expected size of the transfected DNA (data not shown). On the other hand, a shortened mRNA was detected after Northern blotting of 5 PacNIH/A8 β cell clones with the (Env-, Gag+) type expression. The mRNA that encodes the Env protein (mRNA_{env}) is 3.1 kb in length and is yielded after splicing out 5.2 kb of the *gag* and *pol* region from the full-length transcripts (8.4 kb) of the A8-V genome. The mRNA_{env} extracted from the (Env-, Gag+) type PacNIH/A8 β cell clones was shorter by 1 kb than the expected size, whereas the full length mRNA, which encodes the *gag* and *pol* gene products, retained the expected size (Fig. 3). Thus, the (Env-, Gag+) type PacNIH/A8 β cell clones did express the Gag protein, but failed in Env expression. This phenomenon was observed only in PacNIH/A8 β cells and not in

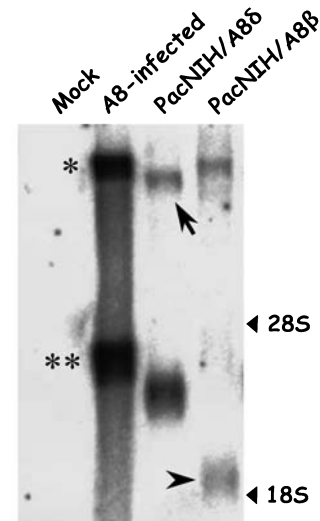


Fig. 3. Northern blot hybridization of PacNIH/A8 δ and PacNIH/A8 β cell clones. PacNIH/A8 δ and PacNIH/A8 β cell clones were introduced by pA8(Ψ -) δ . DNA and pA8(Ψ -) β DNA, respectively. RNA extracted from the cell clones was electrophoresed and transferred to a membrane. RNA quality was confirmed by visualization of ribosomal RNA on the membrane using a UV lamp. A ³²P-labeled 2.2-kb XbaI-BglII fragment (Fig. 1) containing the entire *env* region was used as a hybridization probe. In A8-infected NIH3T3 cells, full-length viral transcripts (*) and spliced viral transcripts (**) were detected. The size of the spliced transcripts in PacNIH/A8 β (indicated by arrowhead) was smaller than that in PacNIH/A8 δ . The size of the full-length transcripts in PacNIH/A8 δ (indicated by arrow) was smaller than that in PacNIH/A8 β because a part of the SV40-derived sequences was deleted in the pA8(Ψ -) δ construct (see Fig. 1).

PacNIH/A8 δ cells (Fig. 3). Therefore, the unstable expression of the Env protein observed in the PacNIH/A8 β cells was induced by the 0.85 kb fragment of the SV40 early region, which was used for the polyA signal of pA8(Ψ -) β (Fig. 1). This vulnerable Env expression was rescued by removing 5' five-sixths of the region derived from SV40 in the expression vector. The sequences that can be used as splicing donor and acceptor sites were located in the removed sequence of the SV40 early region (Fig. 1). Furthermore, candidates for splicing donor and acceptor sites in the *env* region of the A8-V gene were found at nucleotide positions 6402-6429 and 7716-7730 (accession no. D88386), respectively. These sequences might have induced abnormal splicing for the expression of the *env* gene. Recently, microRNAs (miRNAs) encoded by SV40 were identified (10). miRNAs are small RNAs that serve important regulatory roles in development and gene expression in lower organisms. Although the encoding regions for the miRNAs were not mapped to the region we used for the polyA signal, it remains possible that miRNAs expressed by the SV40 gene regulate *env* gene expression, because the gene encoding one of the candidates for a miRNA, which was predicted by computer analysis and was not expressed by the full-length SV40 gene, was located in the 0.85 kb fragment of the SV40 early region (10). We are now sequencing the shortened mRNA_{env} extracted from the PacNIH/A8 β cells. The results of this sequence analysis should help to clarify the possibility that the unstable *env* expression described in this report is due to an abnormal splicing event.

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