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NOTES

Transduction of Terminally Differentiated Neurons by Avian Sarcoma Virus

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Recent studies have demonstrated that avian sarcoma virus (ASV) can transduce cycle-arrested cells. Here, we have assessed quantitatively the transduction efficiency of an ASV vector in naturally arrested mouse hippocampal neurons. This efficiency was determined by comparing the number of transduced cells after infection of differentiated neurons versus dividing progenitor cells. The results indicate that ASV is able to transduce these differentiated neurons efficiently and that this activity is not the result of infection of residual dividing cells. The transduction efficiency of the ASV vector was found to be intermediate between the relatively high and low efficiencies obtained with human immunodeficiency virus type 1 and murine leukemia virus vectors, respectively.

Human immunodeficiency virus type 1 (HIV-1)-based vectors have been shown to transduce cycle-arrested cells (2, 17, 25). This capability has been attributed to (i) an active nuclear import mechanism mediated by nuclear localization signals (NLSs) on viral proteins and (ii) a DNA flap, which promote the entry of lentivirus DNA into the nuclei of nondividing cells (reviewed in reference 20). In contrast, it has been reported that murine leukemia virus (MLV, a gammaretrovirus) requires passage of the host cell through mitosis for efficient integration and subsequent expression of viral or foreign transduced genes (15, 18). It has been proposed that this requirement reflects the need for nuclear membrane disassembly to allow MLV DNA to access and integrate into the host genome (14, 18). Consistent with this notion, MLV appears to lack a discernible NLS.

Previous studies in our laboratory identified an NLS in the integrase of avian sarcoma virus (ASV, an alpharetrovirus) (11, 12). This suggested that, like lentiviruses, ASV might possess an active import mechanism that would allow transduction of nondividing cells. Furthermore, recent studies in our laboratory (9) and others (8) have demonstrated that ASV can transduce cells arrested with chemical cell cycle inhibitors. We have therefore extended our investigations (9) to determine the efficiency with which ASV can transduce naturally arrested neurons.

Many groups have examined the ability of HIV-1-based vectors to transduce neurons in vivo and ex vivo as model systems for gene delivery into terminally differentiated, postmitotic cells (reviewed in reference 5). To determine if ASV has a similar ability, we used primary mouse hippocampal neurons isolated from embryos between days 14.5 and 16.5 of gestation (Fig. 1) (13). The isolated neural progenitors (Fig. 1A) undergo a single division event immediately after isolation and differentiate into neurons within approximately 4 days (Fig. 1B). The resultant cultures contain greater than 90% neurons, as indicated by morphology and immunostaining with a neuron-specific marker, microtubule-associated protein 2 (MAP2) (Fig. 1C). This limited cell division allows the comparison of retroviral transduction with dividing and nondividing cells in the same culture system.

For these experiments, we used an ASV vector (9) derived from RCASBP(A), which was designed by Barsov and Hughes (1). This ASV vector is replication competent in avian cells and encodes a murine amphotropic envelope gene, which allows efficient entry and integration (but not propagation) in mammalian cells (1). To identify cells transduced by this ASV vector, it has been engineered to express the reporter gene for enhanced green fluorescent protein (GFP) under control of the cytomegalovirus (CMV) immediate-early promoter. Examination for GFP expression 3 days postinfection showed that this ASV vector could transduce the neuronal progenitors infected within 1 day of explantation, when cell division should occur (Fig. 2A), as well as differentiated neurons that were infected after either 5 days (Fig. 2A) or 2 weeks in culture (data not shown). The majority of the GFP-positive cells, whether derived from infection at 1 day or 5 days after isolation, exhibited neuronal morphology, with dendrites extending from a large cell body, as the cultures have differentiated by the time GFP expression is observed.

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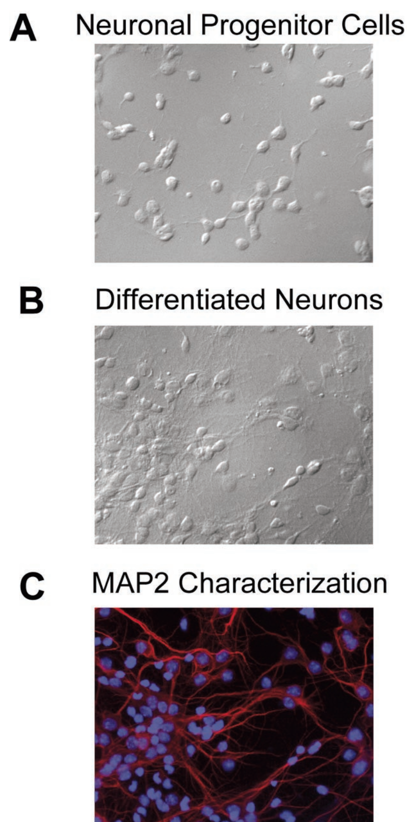

FIG. 1. Mouse embryonic hippocampal neuron explants. The hippocampus was isolated from BL/6 mouse embryos between days 14.5 and 16.5 of gestation. The hippocampus was stripped of meninges, and a single-cell suspension was plated. The neuronal progenitors divide once and become fully differentiated neurons after approximately 4 days in culture. Representative differential interference contrast micrographs are shown. Panels: A, neuronal progenitors 1 day after isolation; B, differentiated neurons after 5 days in culture; C, MAP2 staining of the same differentiated neurons that were fixed and incubated with an anti-MAP2 antibody (red). 4′,6′-Diamidino-2-phenylindole (DAPI) (DNA)-stained nuclei are shown in blue.

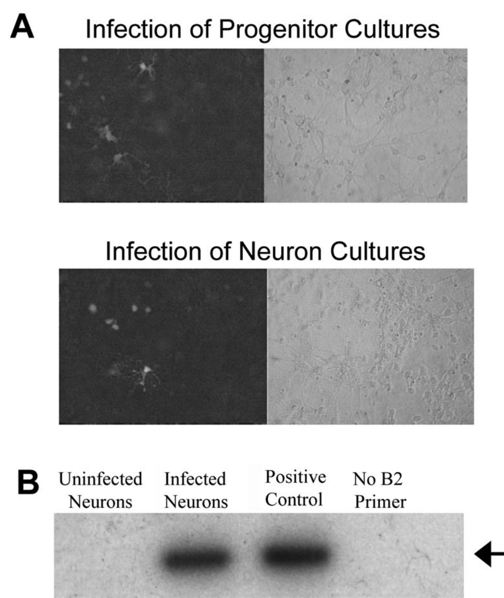

FIG. 2. Transduction of neurons with an ASV-GFP vector. (A) Neuronal cultures were infected with the same virus stock, and GFP reporter expression was examined 3 days postinfection by microscopy. Representative fluorescent fields (GFP expression, left) and phase-contrast fields (right) are shown. Cultures of neural progenitors infected 1 day after isolation (top) and differentiated neurons infected 5 days after isolation (bottom) are shown. (B) ASV transduction correlates with integration detected by B2-PCR. Neurons were infected 5 days after isolation. Total cellular DNA was isolated 3 days postinfection. B2-PCRs of uninfected neurons, infected neurons, infected murine fibroblasts, and infected neuron DNA amplified without B2 primers are shown.

We previously reported that the ASV vector with an inactive integrase does not express detectable GFP (9). To confirm that GFP expression from the ASV vector corresponded to stable transduction, we performed B2-PCR, a modified Alu-PCR method (23, 24), to detect the covalent joining of viral DNA to cellular DNA. Chromosomal DNA isolated from differentiated neurons infected after 5 days in culture was used in this assay with a primer for mouse B2 genomic repeats (5′-TTCACAA CTCTCGGTGGATGGTGG-3′) (7) and a primer for the ASV long terminal repeat (5′-GGCTTCGGTTGTACGCGG TTAGGAGT-3′). The samples were subsequently diluted, and a nested PCR and Southern blotting procedure (4) was performed to generate a unique PCR product for quantitation. A strong signal was detected in the infected neuron cultures but not in the uninfected cultures or in a reaction mixture that did not include the B2 primer (Fig. 2B). B2-PCR demonstrated that the GFP expression from the ASV reporter virus correlates with integration of the retroviral DNA into the genome of the neurons.

To confirm that the GFP-positive cells that were transduced after the culture had differentiated were indeed neurons, we asked if the GFP signal colocalized with antibody staining against the neuronal marker, MAP2. Examination of MAP2 expression in the neurons after 5 days in culture verified that this protein was expressed in the dendrites of greater than 90% of the cells (Fig. 1C). In contrast, antibody staining revealed that very few cell bodies (less than 5% of the culture) expressed glial fibrillary acidic protein (antibody from DAKO), an astrocyte-specific marker. We infected these differentiated neuronal cultures with the ASV vector, and 3 days after infection, the cells were fixed in paraformaldehyde and stained with the MAP2 antibody (Chemicon). Microscopic counting revealed that 68% of the GFP-positive cells (n = 113) were also positive for MAP2 (Fig. 3). It was unclear if the remaining GFP-positive cells (without detectable MAP2 staining) were neurons that did not stain with this antibody or were cells that lacked MAP2, such as astrocytes. We note that the milder permeabilization conditions that were used to favor retention of GFP may have resulted in incomplete MAP2 staining. From these results, we conclude that the majority of the cells transduced with the ASV reporter were differentiated neurons.

To address our concern that the differentiated neuronal cultures may contain a small percentage of dividing cells that would complicate our analyses, we performed a bromodeoxyuridine (BrdU) pulse to label dividing cells as they proceeded through S phase (22). Both progenitor (1 day after isolation) and differentiated (5 days after isolation) neuron

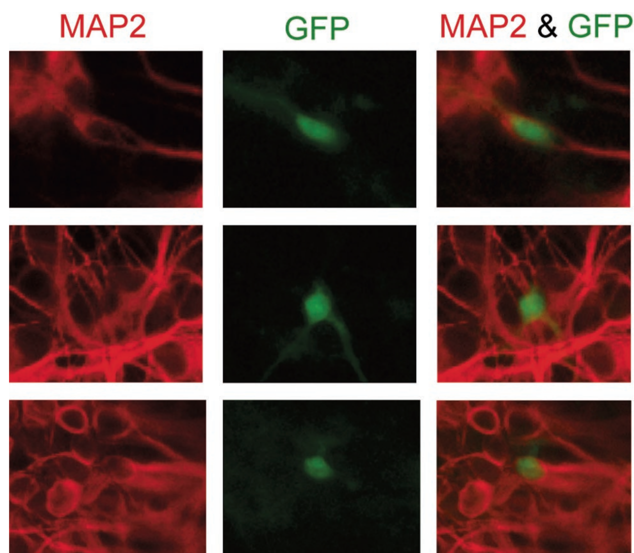

FIG. 3. MAP2-positive neurons are transduced by the ASV reporter virus. Neurons were infected 5 days after isolation, and at 3 days postinfection they were fixed and stained for MAP2. MAP2 is localized to the dendrites and around the cell body, while GFP is localized throughout the cell. GFP-expressing cells were frequently MAP-2 positive. Representative fields of fluorescent staining by MAP2 (left) and GFP expression (center) and their overlay (right) are shown.

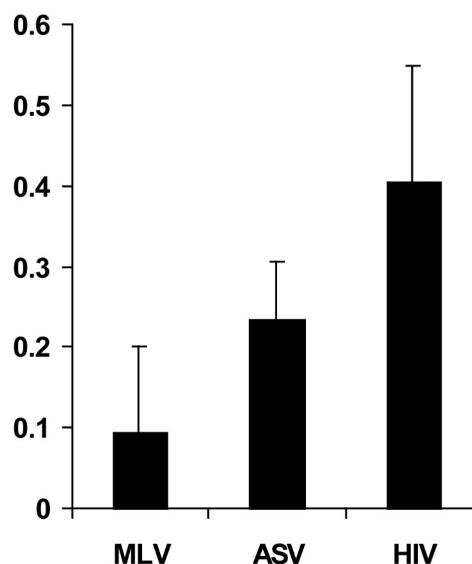

FIG. 5. Transduction efficiencies of ASV, HIV-1, and MLV. Dividing progenitor cells on the day of isolation and differentiated neurons after 5 days in culture were infected with the same stock of virus. The number of GFP-positive cells was determined by fluorescence-activated cell sorter analysis approximately 3 days postinfection. Transduction efficiency was determined by the ratio of GFP-expressing cells in the dividing progenitors to differentiated neurons. The mean transduction efficiency from at least two parallel experiments carried out in triplicate is shown with the standard deviation between the experiments.

cultures were treated with 50 μM BrdU (Sigma) for 30 min at 37°C. Forty-eight hours after the BrdU pulse, we examined BrdU incorporation by staining the cells with an anti-BrdU antibody (BD Bioscience) after fixation and treatment with DNase I (Promega). As expected, microscopic examination showed that a significant number of the progenitor cells (Fig. 4A) incorporated BrdU, whereas no incorporation (fewer than 1 cell per 10,000) was detected in the differentiated neuronal culture (Fig. 4B). We also examined the accumulation of proliferating-cell nuclear antigen (PCNA) in these cells. This protein accumulates in cells as they enter S phase, but it is rapidly degraded in other phases of the cell cycle (22). Similar to the results obtained with BrdU incorporation, a large percentage of the progenitor cells (Fig. 4C) stained with an anti-PCNA antibody (DAKO) while cells in the differentiated cultures (Fig. 4D) contained no detectable PCNA (fewer than 1 cell per 10,000). The results of the BrdU incorporation and PCNA staining experiments demonstrate that, unlike the progenitors, terminally differentiated neuron cultures do not contain cells that are undergoing significant DNA replication or cell division. From these results, we conclude that contamination of differentiated cells with dividing cells cannot account for the observed transduction of differentiated neuronal cultures.

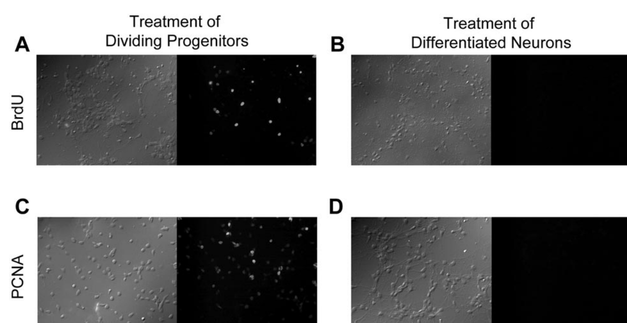

FIG. 4. Assays for cell cycling. (A) BrdU pulse of dividing progenitor cells that were stained for BrdU after 2 days. Representative differential interference contrast (left) and fluorescence (right) fields are shown. (B) BrdU pulse of differentiated neurons. Differentiated neurons after 5 days in culture were treated as described above. (C) PCNA levels in dividing progenitor cells. Dividing progenitor cells cultured for 1 day were fixed and stained with an anti-PCNA antibody. (D) PCNA levels in differentiated neurons. Differentiated neurons were cultured for 5 days before they were fixed and stained with an anti-PCNA antibody.

The mouse neuron system provided us with a unique opportunity to compare the relative efficiency with which pseudotyped ASV transduces nondividing versus dividing cells. Similar analyses were carried out with an HIV-1-based vector (3) pseudotyped with the vesicular stomatitis virus (VSV) G protein (17) and an MLV vector (pLEGFP-C1; Clontech). The MLV vector production system yields infectious particles after transient transfection of a vector plasmid into the AmphoPack-293 packaging cell line expressing Gag-Pol and the amphotropic Env. As with the ASV vector, expression of enhanced GFP encoded in the HIV-1 and MLV vectors is under control of the CMV immediate-early promoter. In these assays, GFP-positive cells were quantitated by flow cytometry. Relative transduction efficiencies were measured by analysis of dividing neuronal progenitors infected on the day of isolation and differentiated neurons infected after 5 days in culture with the same virus stock. To compare these vectors, the results are expressed as a transduction ratio, the ratio of the number of

GFP-positive cells after infection of differentiated neurons compared to the infection of dividing progenitor cells.

As summarized in Fig. 5, with the ASV vector, there were four times more GFP-positive cells when the cultures were infected prior to differentiation, yielding a transduction ratio of ca. 0.25. This value was approximately one-half of the 0.4 ratio observed with the HIV-1 vector. This difference is consistent with the titer of the ASV vector being half that of the HIV-1 vector on the neuronal cultures ($1.57 \times 10^4 \pm 0.9 \times 10^4$ for ASV compared to $2.56 \times 10^4 \pm 0.8 \times 10^4$ for HIV-1), even though the titers of these vectors were similar on dividing progenitors ($6.25 \times 10^4 \pm 2.0 \times 10^4$ and $6.6 \times 10^4 \pm 2.4 \times 10^4$, respectively). Previous studies with differentiated myofibers (19) and unstimulated G_0 hematopoietic stem cells (21) showed similar HIV transduction ratios. In our studies, the GFP promoter had little effect on transduction efficiency because an HIV-1-based vector with the phosphoglycerol kinase promoter (6) gave results similar to those obtained with the vector containing the CMV promoter (data not shown). The MLV vector had the lowest transduction ratio, less than 0.1. Previous investigations have reported similar results following MLV infection of rat neuronal cultures (16). It is generally assumed that MLV is unable to infect noncycling cells and that transduction of such cultures signifies the presence of residual dividing cells. However, the ratio that we obtained with MLV cannot be attributed to contamination with dividing cells, as there is no detectable background in these differentiated cultures (Fig. 4). We suspect that the cellular environment provided in the neuronal cultures, which recently exited the cell cycle, may provide favorable conditions for completion of early events and thereby allow more efficient detection of viral DNA nuclear import and integration.

Although the vectors used in this study encode similar reporter cassettes, we note that the vectors were not completely matched. In the MLV and HIV-1 expression vectors, essential viral genes are replaced with the reporter cassette, whereas the ASV vector encodes a full complement of viral genes in addition to the reporter cassette and is replication competent in avian cells. The HIV-1 vector contains the VSV G protein for entry, whereas both the ASV and MLV vectors enter cells via the amphotropic envelope receptor. Nevertheless, any differences in titers caused by vector differences should be irrelevant as our comparison relies on the relative transduction efficiency of each vector on dividing versus nondividing cultures (transduction ratio), rather than on absolute titers. However, it is possible that as the cultures differentiate, the surface expression of the amphotropic receptor, Pit-2, could be altered. This possibility is less likely for the ubiquitous VSV G receptor. Despite these differences between vectors, it is clear that ASV can transduce nondividing neuron cultures with reasonable efficiency.

We (9) and others (8) have shown that ASV can transduce nondividing cells. The results presented here confirm this finding, although the transduction ratio of ASV is less than that of HIV-1. The viral sequences that account for these differences have not been mapped and may include, but not be limited to, nuclear import signals. Studies with HIV-1 vectors that lack all accessory proteins, Vpr, Vpu, Vif, and Nef, have shown reduced transduction efficiencies with cycle-arrested cells (10). This suggests that these accessory proteins may play a role in enhancing the ability of HIV-1 to transduce nondividing cells but are not required for this function. As ASV shares this ability with HIV-1, some determinants may be shared as well.

Here, we have confirmed that ASV can transduce naturally arrested, terminally differentiated neurons. We show that the terminally differentiated neuronal cultures do not contain detectable dividing cells and that therefore such cells cannot account for the observed transduction activities of the ASV, HIV-1, and MLV vectors. MLV was the most restricted, and ASV transduction efficiency was reduced compared to that of HIV-1. These results demonstrate the ability of a non-lentivirus vector to transduce postmitotic cells and indicate that the determinants for this ability are not lentivirus specific but may be shared with other retroviruses.

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