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Baculovirus-Mediated Gene Delivery into Mammalian Cells Does Not Alter Their Transcriptional and Differentiating Potential but Is Accompanied by Early Viral Gene Expression

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Gene delivery to neural cells is central to the development of transplantation therapies for neurological diseases. In this study, we used a baculovirus derived from the domesticated silk moth, *Bombyx mori*, as vector for transducing a human cell line (HEK293) and primary cultures of rat Schwann cells. Under optimal conditions of infection with a recombinant baculovirus containing the reporter green fluorescent protein gene under mammalian promoter control, the infected cells express the transgene with high efficiency. Toxicity assays and transcriptome analyses suggest that baculovirus infection is not cytotoxic and does not induce differential transcriptional responses in HEK293 cells. Infected Schwann cells retain their characteristic morphological and molecular phenotype as determined by immunocytochemistry for the marker proteins S-100, glial fibrillary acidic protein, and p75 nerve growth factor receptor. Moreover, baculovirus-infected Schwann cells are capable of differentiating in vitro and express the P0 myelination marker. However, transcripts for several immediate-early viral genes also accumulate in readily detectable levels in the transduced cells. This transcriptional activity raises concerns regarding the long-term safety of baculovirus vectors for gene therapy applications. Potential approaches for overcoming the identified problem are discussed.

During the last decade, it has become apparent that baculoviruses not only represent a powerful expression system for production of recombinant proteins in insect cells but also can be used for transduction of dividing and nondividing mammalian cells and tissues in vitro, ex vivo, and in vivo (49). Advantages of the use of baculoviruses as gene delivery agents include their inability to replicate in mammalian cells, apparent lack of cytotoxicity, capacity to sustain large insertions of foreign DNA, ability to target many different cell types, and superior safety features relative to mammalian virus-based transduction systems (25, 37, 38, 48). Thus, increasing interest for the development of recombinant baculoviruses as gene delivery vectors for use in human gene therapy exists.

All baculovirus vectors for gene delivery in mammalian cells reported thus far have been based on the *Autographa californica* nuclear polyhedrosis virus (AcNPV). This is primarily due to the relatively wide host range of the virus and the multiplicity of lepidopteran cell lines in which it can be grown and propagated effectively as well as the availability of integrated methodologies allowing the rapid generation of recombinant viruses (55, 80). In contrast, there is a paucity of information regarding the suitability of other baculovirus species, particularly species with narrow host ranges to function as transduction vectors for mammalian cells.

In this regard, it is also known that AcNPV has a propensity to express endogenous viral genes in nonhost insect species (12, 13). Moreover, studies involving transfection of mammalian cells with gene constructs employing baculovirus gene promoter elements have shown that certain early baculovirus promoters are marginally functional in mammalian cells and can also be activated by mammalian virus regulators (46, 66). These findings raise safety concerns related to the potential for low-level baculovirus gene expression in mammalian cells and the triggering of cellular immune responses against the transduced cells by the recipient host (61).

In this work, we have explored the capacity of *Bombyx mori* NPV (BmNPV), the second best characterized baculovirus species after AcNPV (2, 20, 21, 27, 40, 56, 57, 65), which has a very limited host range (58), to function as a transducing vector for mammalian cell lines and primary Schwann cells. The choice for the latter rests with the results of experimental transplantation in rodent and primate models, which has provided substantial evidence that Schwann cells are good candidates for cell therapy in human central nervous system (CNS) demyelinating diseases, such as multiple sclerosis, and trauma (5, 6, 9, 19, 26). Schwann cells provide trophic support and remyelinate demyelinated CNS axons. However, their integration in the CNS is limited. Therefore, modifying Schwann cells to express "therapeutic" factors enhancing axonal regeneration and remyelination by ex vivo gene transduction is a promising strategy to improve their capacity to repair the injured or demyelinated nervous system (26, 51). Despite their obvious potential for ex vivo gene therapy in demyelinating diseases of the central nervous system, Schwann cells were never before

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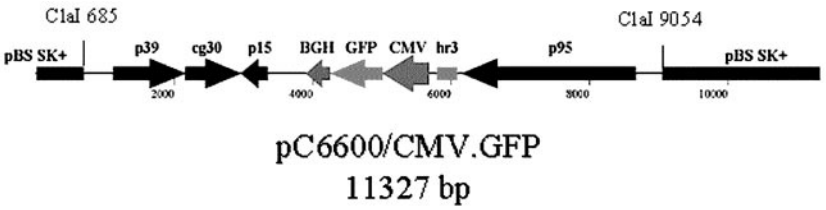

FIG. 1. Transfer vector pC6600/CMV.GFP, used for the generation of recombinant BmNPV/CMV.GFP. The insertion site of the CMV-GFP cassette is in the *p95* region of the BmNPV genome, next to the *hr3* enhancer (at map unit 52.5) (54). Indicated are the ORFs of the *p95*, *p15*, *p39*, and *cg30* genes and the position of the *hr3* enhancer. BGH, bovine growth hormone polyadenylation region.

examined for their amenability to baculovirus-mediated transduction.

Our results demonstrate that BmNPV rivals AcNPV in its transduction efficiency for mammalian cells and can be used as an efficient vector for transduction of Schwann cells. Cytotoxicity assays and validated microarray analyses revealed that the infection process is not associated with cytotoxicity and does not affect the transcriptome profile of the transduced HEK293 cells to any appreciable degree, even at very high multiplicities of infection (MOI). Importantly, infected Schwann cells retain their characteristic morphological and molecular phenotype as well as their ability to differentiate in vitro towards a myelinating phenotype. However, our analysis also revealed a potentially important problem that may hinder the widespread use of baculoviruses as vectors for gene therapeutic applications. Specifically, as has been previously suggested indirectly for several AcNPV gene promoters (46, 66), BmNPV immediate-early viral genes were found to be expressed at low but readily detectable levels in the transduced mammalian cells. This residual transcriptional activity raises concerns regarding the long-term safety of baculovirus vectors for gene therapy applications. We discuss these concerns and suggest potential approaches for overcoming the identified problem.

MATERIALS AND METHODS

Insect cell cultures and viruses. Bm5 culture cells (28) were maintained in IPL-41 medium (Life Technologies) containing 10% fetal calf serum (Life Technologies) at 28°C. Infection of Bm5 cells with BmNPV (ML1 isolate [54]) and amplification of virus stocks were carried out as described previously (67). Virus 50% tissue culture infectious doses were determined by endpoint dilution (67). Infection was scored by the appearance of occlusion bodies (in insect cells) or green fluorescent protein (GFP) fluorescence (in insect and mammalian cells).

Generation of recombinant baculovirus. To generate the recombinant baculovirus BmNPV/CMV.GFP, transfer vector pC6600/CMV.GFP (Fig. 1) was employed. This transfer vector was generated by inserting a 1.8-kb CMV.GFP expression cassette, which contained the open reading frame (ORF) of the GFP (32) linked to the cytomegalovirus (CMV) promoter and the polyadenylation region of the bovine growth hormone gene, into the unique StuI site of plasmid pC6600 (54). Because the insertion does not interrupt any ORFs or regulatory sequences, BmNPV/CMV.GFP has the same growth properties as wild-type BmNPV and produces polyhedra in the nuclei of the infected cells (C. Kenoutis, R. C. Efrose, L. Swevers, and K. Iatrou, unpublished results). Wild-type BmNPV genomic DNA and pC6600/CMV.GFP transfer vector DNA were cotransfected into silk moth Bm5 cells (43, 44), and the recombinant BmNPV/CMV.GFP baculovirus was purified from wild-type virus by endpoint dilution (67) based on the weak fluorescence it generated in Bm5 cells.

Infection of established cell lines. To optimize the infection protocol, human embryonic kidney 293 (HEK293; American Tissue Culture Collection) cells were used initially. The cells were seeded onto 6-well plates at a density of 2×10^5 cells per well in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and allowed to incubate at 37°C overnight. Prior to infection, the medium was removed, the cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) (free of Ca^{2+} and Mg^{2+}), and 0.5 ml of medium (see below) containing baculovirus at MOI ranging from 1 to 1,000 was added. The tested infection media included IPL-41 (with or without 10% FBS), Dulbecco's PBS (with or without 10% FBS), DMEM (with 10% FBS), and OptiMEM (Life Technologies). For comparative analysis of the transduction efficiencies, the periods of incubation with the virus ranged from 1 to 8 h and incubations were carried out at 4, 28, or 37°C. At the end of each incubation period, the viral inocula were removed, the cells were washed with PBS, and 2 ml of DMEM containing 10% FBS was added prior to continued culture at 37°C. To maximize transgene transcription in the transduced cells, 1 μM trichostatin A (TSA) (Applichem) was added postinfection (p.i.) (73) for periods ranging from 2 to 48 h. Determination of the percentage of cells expressing GFP was carried out with a Zeiss Axiovert 25 inverted microscope equipped with an HBO 50 illuminator for incidental light fluorescence excitation and a Zeiss filter set 09 (450- to 490-nm excitation filter; 510-nm barrier filter). Percentages of viable cells were determined by staining with ethidium bromide as described previously (59).

Preparation and infection of Schwann cell primary cultures. Pure Schwann cell cultures (SC cultures) were prepared as previously described (60). Briefly, sciatic nerves were dissected from 5-day-old Winstar rats, desheathed, and dissociated for 40 min in 0.125% trypsin and 0.2% collagenase in 250 μl DMEM at 37°C in an atmosphere of 5% CO_2. Following trituration, cells were centrifuged at 500 × *g* for 10 min, resuspended in DMEM containing 10% FBS and a combination of 100 IU penicillin and 100 IU streptomycin (P-S), and plated in a 35-mm tissue culture petri dish. After 24 h, the medium was replaced and 10 μM cytosine arabinoside was added for 72 h to eliminate dividing fibroblasts. Following this treatment, the medium was replaced with fresh DMEM containing 10% FBS and P-S. After 24 h, heregulin β (250 ng/ml) and forskolin (2 μM) were added to the SC culture medium to stimulate cell proliferation. Purified SC cultures of approximately half confluence were used for baculovirus transduction after at least 24 h of exposure to the mitotic agents. The baculovirus was added to the cells in sterile PBS at an MOI of 500, and incubation was carried out for 8 h at 28°C. The virus was then washed off and replaced by the supplemented cell culture medium (DMEM containing FBS, P-S, and heregulin-forskolin) containing or not 0.5 μM TSA. After 18 h, the medium was washed off and replaced with fresh culture medium supplemented with the mitotic agents. Infection efficiency was estimated by monitoring GFP fluorescence in live cells with a Leica DMIL inverted microscope equipped with fluorescence optics. Three fields of a 40× lens, each containing 50 to 100 cells, were measured for each of a total of nine experiments.

Southern blot analysis. Genomic DNA isolated from HEK293 cells was digested by BamHI and EcoRI, separated on a 1% agarose gel, and transferred to a Hybond-NX nylon membrane (Amersham Biosciences) as described previously (17). The hybridization probe was a 9-kb EcoRI fragment encompassing the polyhedrin (*pol*) gene region of BmNPV (41), which was labeled by random priming (22). DNA hybridizations and washes were carried out at 65°C as described previously (18).

Cytotoxicity assays. HEK293 cells were seeded into individual wells of a 96-well plate at a density of 20,000 cells/well in a volume of 0.1 ml, incubated overnight at 37°C, and infected with BmNPV/CMV.GFP in PBS at an MOI of 500 or mock infected for 8 h at 28°C followed by incubation for 72 h at 37°C. The first 24 h of the incubation period occurred either in the presence or in the absence of 1 μM TSA. To determine cytotoxicity (16), 100 μl of modified Eagle medium (Life Technologies) containing 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) was added to the cells, followed by incubation for 4 h at 37°C. After 4 h, the MTT mix was removed and replaced with 100 μl of isopropanol. The plate was shaken for 10 min, and the amount of formazan product (index of viability) was determined by measuring absorbance at 550 nm with a FLUOStar Galaxy Unit microplate reader.

Transcriptional activity assays. Total RNA was extracted from infected, mock-infected, or control HEK293 and Schwann cells by use of TRIzol (Life Technologies) per the manufacturer's instructions. For detection of BmNPV gene expression, the RNA was pretreated for 30 min with RQ1 RNase-free DNase (Promega) at 0.1 U/μg RNA in reverse transcription (RT) buffer containing 20 mM dithiothreitol and 2 U/μl of human placenta RNase inhibitor (HT Biotechnology). RT reactions were carried out using 1 to 2 μg of RNase-treated RNA as the template, oligo(dT) as the primer, and SuperscriptII reverse transcriptase (Invitrogen). PCRs using *Taq* DNA polymerase (HyTest) were carried out as described before (18), using as templates 2% to 20% of the RT reactions. To detect specific transcripts, the following forward and reverse primers (FP and RP, respectively) were used in the PCRs.

For the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene (425-bp product), the FP was 5′-CAATGACCCCTTCATTGACC-3′ and the RP was 5′-CATGAGTCCTTCCACGATAC-3′; for the *ie0* gene (484-bp product), the FP was 5′-CCAGCAGTCACGTGCTGAAC-3′ and the RP was 5′-GGCGATG GTTGCTCCGCAAC-3′ (same as the *ie1* RP); for the *ie1* gene (312-bp product), the FP was 5′-CGCGTCGTACACCAGTGCTC-3′ and the RP was 5′-GGCG ATGGTTGCTCCGCAAC-3′ (same as the *ie0* RP); for the *he65* gene (450-bp product), the FP was 5′-GCTGATGACGGTGTCGATGG-3′ and the RP was 5′-GTTGTGGCGAATGTCGGTGC-3′; for the *p39* gene (530-bp product), the FP was 5′-GCCCGACGCGTATCATGACG-3′ and the RP was 5′-GCGCTAC TGCGCGTCGAATC-3′; for the *pol* gene (382-bp product), the FP was 5′-CG CCGGACCAGTGAACAGAG-3′ and the RP was 5′-CGTGTACCTCGTCGC CAACC-3′; and for the *gfp* gene (723-bp product), the FP was 5′-GCCACCA TGGTGAGCAAG-3′ and the RP was 5′-CTTGTACAGCTCGTCCATG-3′.

Amplifications were carried out for 30 (*gapdh* and *gfp*) or 40 (BmNPV genes) cycles at 94°C for 1 min (denaturation), 59°C for 1 min (annealing), and 72°C for 45 s (extension), except for the *gapdh* gene, where an annealing temperature of 54°C was used. For PCRs carried out directly with DNase-treated RNA preparations (controls for the presence of baculovirus genomic DNA contamination), aliquots equivalent to those present in the cDNA preparations used for PCR amplification were used as templates.

DNA microarray hybridization assays. Labeled cDNA was prepared from DNase-treated total RNA obtained from control and baculovirus-transduced HEK293 cells (without TSA stimulation) at 3 days p.i. by use of a Fairplay microarray labeling kit (Stratagene), which reverse transcribes the RNA in the presence of the aminoallyl nucleotide and chemically couples the appropriate fluorescent dye (Cyanine 3 or Cyanine 5). After treatment with base to hydrolyze the RNA and heat inactivation of the enzyme, each dye-coupled cDNA was purified and dissolved in a total of 5 μl of 10 mM Tris, 1 mM EDTA, pH 8.0, combined with 65 μl of hybridization mix (prepared by mixing 90 μl of digoxigenin EasyHyb [Roche Applied Science], 5 μl yeast tRNA at 10 mg/ml, and 5 μl fish sperm DNA at 10 mg/ml); heated at 65°C for 2 min; and cooled to room temperature. Human arrays with 13,972 70-mer oligonucleotides (14k arrays; QIAGEN Operon), spotted in duplicate by the Southern Alberta Microarray Facility (University of Calgary, Canada), were hybridized in duplicate overnight at 37°C with each of the differentially labeled cDNA probes in a Boekel InSlide Out hybridization oven. At the end of the hybridization period, the slides were sequentially washed at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (70) and 0.2% sodium dodecyl sulfate for 5 min, 0.2× SSC for 5 min, and 0.05× SSC for 5 min. After removal of the final wash, the slides were scanned with a Perkin-Elmer ScanArray 5000 by using a green HeNe 543.5-nm laser for excitation of Cy3 and a red HeNe 632.8-nm laser for excitation of Cy5. The scans were saved in TIFF format and imported into the QuantArray version 3.0 (Perkin-Elmer) microarray analysis software for spot identification, quantification, and background estimation. The quantification and image files were then loaded into Gene Traffic Duo (Iobion) for microarray data management and analysis, and the data were filtered to flag spots with intensities less than 100 U, or less than twice the average background. With maximal hybridization signal intensities of 65,000 fluorescent units usually obtained, signals greater than 1,000 U were considered to represent expressed genes. Finally, the data were normalized according to the Lowess method (77) resident in the Gene Traffic software.

Validation of microarray hybridization results. To validate the results obtained from the microarray hybridizations, 0.5 μg of total mRNA isolated from mock-infected cells or cells infected with recombinant BmNVP for 48 h in the absence of TSA was used for real-time RT-PCR amplification after pretreatment with RQ1 RNase-free DNase (Promega). The real-time RT-PCRs for transcripts of two selected genes, *znf141* (zinc finger gene 141, GenBank accession number NM_003441) and *acci1* (apoptotic chromatin condensation inducer 1, GenBank accession number NM_014977), which displayed the widest ranges of expression differences between control and infected cells in the microarray hybridization assays, were performed in parallel with those for the detection of *gapdh* gene transcripts (the internal standard) by use of a LightCycler RNA amplification kit (Roche) in conjunction with gene-specific unlabeled external forward and reverse primers and pairs of differentially labeled forward internal primers (Hybprobes FL and LC, emitting at 530 and 640 nm, respectively; TIB MOLBIOL, Germany) as follows: for *znf141*, the FP was 5′-ACCCAGACCTGGTCACC-3′, the RP was 5′-ATCTTCTATGCCCTGCACTG-3′ (corresponding to regions from bp 302 to bp 318 of exon 3 and from bp 435 to bp 416 of exon 4, respectively), FL was 5′-AGCCCTACAATGTGAAGATACATAAGATCG-3′, and LC was 5′-AGCCAGACCCCCAGCTATGTGT-3′; for *acci1*, the FP was 5′-CCAGAGGTTACAGCCTG-3′, the RP was 5′-GCTGACTTGGTCTGCA A-3′ (corresponding to regions from bp 2487 to bp 2503 and from bp 2718 to bp 2702, respectively), FL was 5′-GACTCAGACCTCTCATCTGCCAG-3′, and LC was 5′-ATCAGAAAGAATTCATCACACTGTTGAGGA-3′; and for human *gapdh*, the FP was 5′-GAAGGTGAAGGTCGGAGTC-3′, the RP was 5′-GAA GATGGTGATGGGATTTC-3′, FL was 5-AGGGGTCATTGATGGCAACAA TATCCA-3′, and LC was 5′-TTTACCAGAGTTAAAAGCAGCCCTGGTG-3′. Rates of amplification for the transcripts of each examined gene relative to the reference gene were calculated from the crossing points of the generated amplification curves.

Immunocytochemistry. Determination of the molecular phenotype of the infected Schwann cells was performed using double immunofluorescence labeling with mouse monoclonal anti-GFP and rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP), S-100 (DAKO, Denmark), or p75 nerve growth factor (NGF) receptor (Santa Cruz, CA), followed by anti-mouse Alexafluor green (Molecular Probes, Eugene, OR) and anti-rabbit tetramethyl rhodamine isothiocyanate (Sigma) as described previously (60). The potential of Schwann cells to express myelin antigens was assessed by stimulation with 4 μM forskolin (63) followed by double immunofluorescence labeling with polyclonal anti-GFP (Santa Cruz, CA) and monoclonal anti-P0 (GFN, Graz, Austria). Preparations were analyzed with a Leica TCS-SP confocal microscope.

RESULTS

Optimization of mammalian cell transduction conditions. To examine the efficiency of BmNPV-based vectors for transduction of mammalian cells, a vector, BmNPV/CMV.GFP, which contained an insertion of a CMV promoter-driven GFP reporter expression cassette into the genome of BmNPV, was constructed. The insertion site for the mammalian reporter cassette was in the *p95* region of the BmNPV genome, immediately adjacent to the *hr3* enhancer (Fig. 1), a position that does not affect viral growth or any other steps of the infection cycle, including production of occlusion bodies in the nuclei of infected host cells at the end of infection (42).

For the initial optimization of the transduction conditions, HEK293 cells were used, because earlier work with AcNPV has shown that these cells have the highest degree of transducibility relative to other widely used mammalian cell lines (73). When HEK293 cells were infected with BmNPV/CMV.GFP, significantly more substantial levels of GFP expression were achieved upon addition of 1 μM of the histone deacetylase inhibitor TSA (78) for 24 h p.i. (Fig. 2A). Because more-prolonged treatments or higher TSA concentrations were toxic to the cells (data not shown), subsequent optimization of transduction conditions were evaluated following a 24-h postinfection treatment of the cells with 1 μM TSA.

To confirm that TSA increases the levels of transgene expression in the infected cells without enhancing the rate of uptake of the BmNPV vector by the cells, genomic DNA obtained from HEK293 cells infected with BmNPV/CMV.GFP and subsequently incubated in the absence or presence of TSA was isolated at 56 h p.i. and analyzed by Southern hybridization for the presence of vector sequences. As may be seen in Fig. 2B, this analysis showed that BmNPV

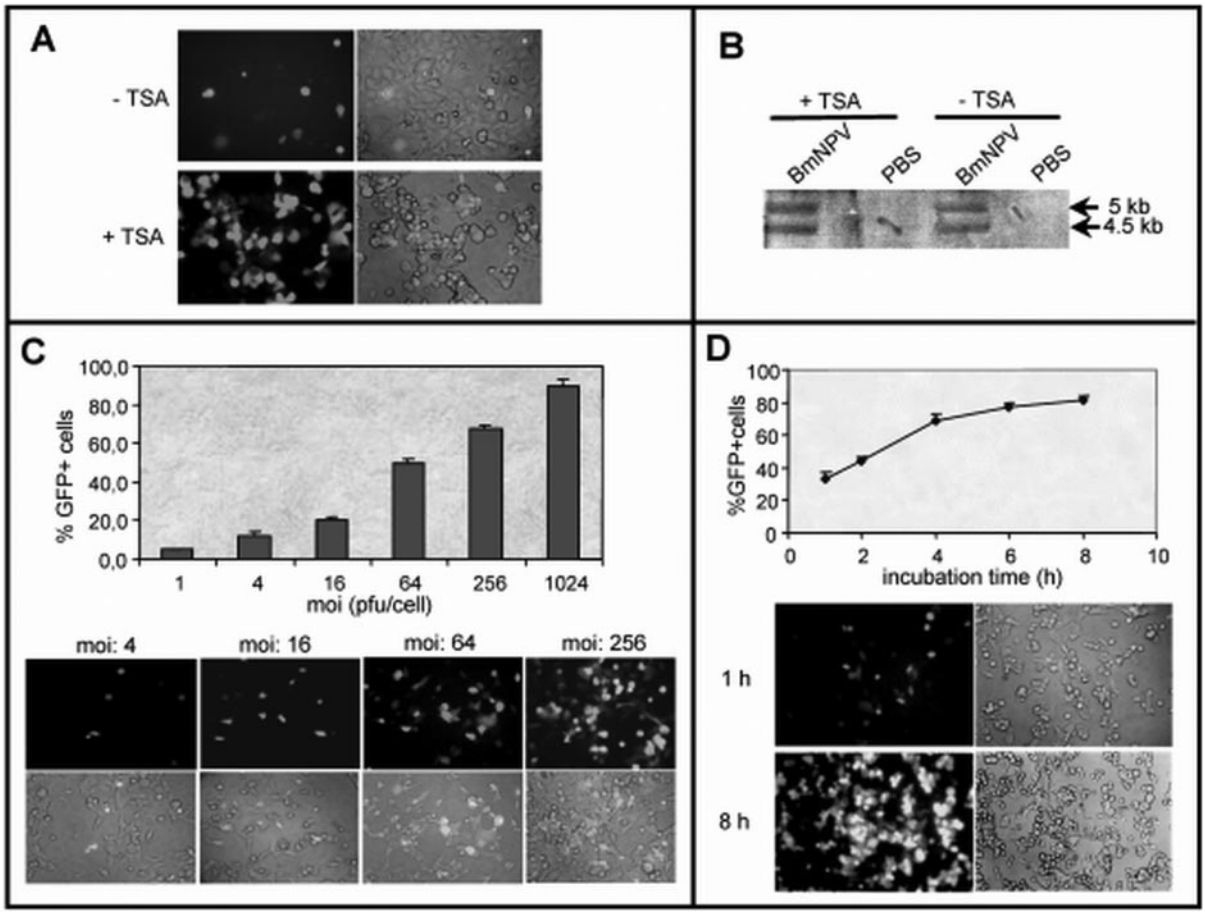

FIG. 2. Transduction efficiency in HEK293 cells by infection with BmNPV/CMV.GFP. (A) Effect of TSA treatment. HEK293 cells were transduced with BmNPV/CMV.GFP at an MOI of 500 for 8 h and incubated in complete medium without TSA (−TSA) or incubated in the presence of 1 μM TSA (+TSA) for 24 h. Transduced cells were photographed at 72 h p.i. with a fluorescence microscope in the absence (left) or presence (right) of phase contrast illumination. (B) Genomic Southern blot analysis of HEK293 cells after infection with BmNPV/CMV.GFP under optimal conditions (8 h at an MOI of 500 at 28°C with PBS as the transduction medium). After infection, cells were treated with 1 μM TSA or left untreated. Genomic DNA was isolated 56 h p.i., digested with BamHI and EcoRI, and probed with a fragment encompassing the polyhedrin region of BmNPV. Sizes of specific hybridization products are shown at the right. The hybridized bands correspond to those expected to arise from an intact polyhedrin region (41). Note that the intensity of the hybridization is similar irrespective of TSA treatment, indicating that TSA does not affect BmNPV uptake. Lanes "PBS" indicate mock-infected HEK293 cells. (C) Effect of MOI on transduction efficiency of HEK293 cells. HEK293 cells were transduced with BmNPV/CMV.GFP at different MOI (1, 4, 16, 64, 259, and 1,024) for 8 h at 28°C in PBS. After virus infection, cells were treated for 24 h with 1 μM TSA. (Top) Quantification of fluorescent cells (percentages of the total) in relation to MOI. (Bottom) Representative photographs showing GFP reporter transduction in HEK293 cells infected at different MOI. Fluorescence photographs in the absence or presence of phase contrast are shown in the top and bottom rows, respectively. (D) Effect of incubation time on transduction efficiency of HEK293 cells. HEK293 cells were transduced with BmNPV/CMV.GFP at 28°C (MOI of 500) for various incubation periods (1, 2, 4, 6, and 8 h) and subsequently treated with 1 μM TSA in complete medium for 24 h. (Top) Graph showing the increase in transduction efficiency following an increase in incubation time with virus. Transduction efficiency was determined as the percentage of GFP^+ cells at 72 h p.i. (Bottom) Photographs of HEK293 cells transduced with BmNPV/CMV.GFP for different periods of time (1 h or 8 h) under optimal conditions (MOI of 500, 28°C, in PBS medium). Transduction efficiencies are approximately 30% for the 1-h incubation and >90% for the 8-h incubation. Fluorescence images in the absence or presence of phase contrast illumination are shown at left and right, respectively.

genomic DNA could indeed be detected with similar intensity in HEK293 cells irrespective of whether or not the cells had been exposed to TSA.

Gene transduction efficiency, as estimated by the appearance of fluorescence in the cells, increased with an increasing MOI (Fig. 2C). Under optimal conditions (see below) at an MOI of 500 or above, 90% (±3.1% standard deviation for six repeat experiments) of the cells displayed green fluorescence. The gene transduction efficiency increased with the duration of infection with BmNPV/CMV.GFP, with plateau values being achieved with an exposure of 8 h (Fig. 2D). As has been previously reported for AcNPV-based vectors (33, 36), maximal efficiency of transduction was achieved by using Dulbecco's PBS as the transduction medium, with higher efficiencies of transduction being achieved at 28°C (data not shown).

The highest numbers of cells expressing GFP were observed at 2 to 3 days p.i. (Fig. 3). Subsequently, the numbers of expressing cells declined gradually, presumably due to losses of the episomal transducing vector from the cells during cell division, but considerable numbers of fluorescent cells were still visible at 7 to 9 days p.i. (Fig. 3A). The persistence of transgene expression in the transduced cells was also assessed by RT-

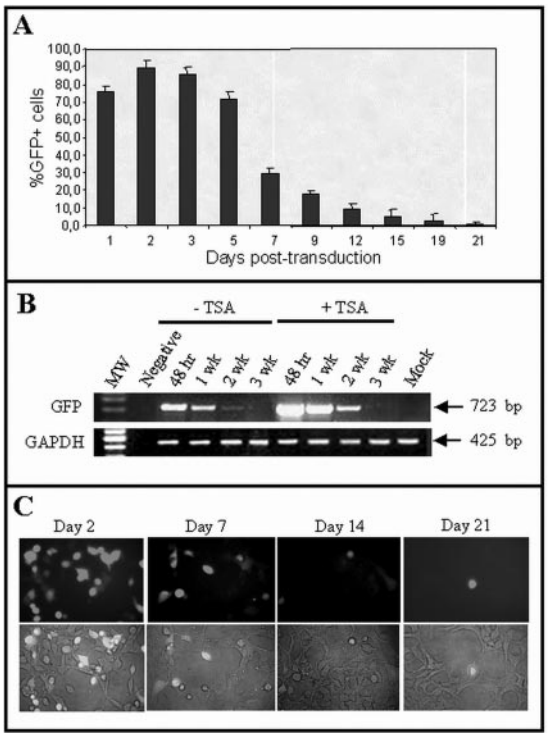

FIG. 3. Persistence of reporter gene expression in transduced HEK293 cells. (A) Quantification of the persistence of fluorescence (percentage of GFP^+ cells) in HEK293 cells transduced by BmNPV/CMV.GFP under optimal conditions (MOI of 500, 28°C, in PBS medium, 8-h incubation with virus, followed by incubation in complete medium containing 1 μM TSA for 24 h). (B) RT-PCR analysis of GFP reporter gene expression in HEK293 cells transduced with BmNPV/CMV.GFP at an MOI of 500 for 8 h in PBS medium. Transduced cells treated with 1 μM TSA are compared with nontreated cells. Samples were taken at 2 days, 1 week (wk), 2 weeks, and 3 weeks p.i. Controls include amplification in the absence of template ("Negative") and mock-infected cells ("Mock"). Amplification of the housekeeping *gapdh* gene serves as an internal control for the efficiency of the reverse transcription reaction. Molecular weight (MW) markers are 100-bp multimers (Gene Ruler DNA ladder), and the sizes of amplified PCR fragments are indicated at the right. (C) Micrographs that illustrate the persistence of GFP reporter expression in the transduced HEK293 cells. Fluorescence images in the absence (top) or presence (bottom) of phase contrast illumination were taken at 2 days, 1 week, 2 weeks, and 3 weeks p.i.

PCR detection of GFP mRNA. Under optimal transduction conditions, GFP mRNA expression was detected, albeit at low levels, up to 2 weeks p.i., even in the absence of TSA (Fig. 3B). GFP fluorescence, on the other hand, could also be observed with rare single cells even at 3 weeks p.i. (Fig. 3C).

Transduction of primary cultures of rat Schwann cells. We tested the potential of the BmNPV/CMV.GFP vector to infect primary cultures of pure Schwann cells obtained from the sciatic nerves of early postnatal rats. The primary cultures of Schwann cells were infected under slightly different conditions than those used for HEK293 cells. Because Schwann cells were found to be more sensitive to TSA treatment (data not shown), TSA was added at a lower concentration (0.5 μM) and for a shorter time period (18 h). It was also necessary to culture the cells continuously in the presence of factors that stimulate cell division, such as heregulin and forskolin. Under optimal conditions, transduction efficiency was in the range of 60 to 80%, with a mean value of 70% ± 5.7% ($n = 9$), as judged by GFP fluorescence (Fig. 4A, top). The transduction efficiency was dependent on cell density at the time of infection and was reduced when the cells reached near confluence (Fig. 4A, bottom). A parallel RT-PCR analysis confirmed the presence of GFP mRNA in the transduced cells at 36 h p.i even in the absence of TSA treatment (Fig. 4B). Notably, no alterations in the morphology of the transduced Schwann cells were noted to occur after baculovirus infection (Fig. 4; also see Fig. 6).

Infection with BmNPV transducing vectors does not alter normal HEK293 cell physiology. Because safety represents a major consideration in gene therapy approaches for correction of human disease, we examined whether baculovirus infection results in cytotoxicity and/or deregulation of the normal physiology of the target cells. First, MTT assays were employed in order to measure the potential cytotoxicity of the overall transduction protocol (infection with BmNPV as well as TSA treatment). Second, global changes occurring in the target cells' transcriptional profiles upon infection with BmNPV in the absence of TSA treatment were assessed by microarray hybridization assays.

To detect possible BmNPV and TSA cytotoxicity effects, MTT assays were performed at different time intervals following infection in the absence or presence of TSA. As may be seen in Table 1, the MTT assays suggested that infection by BmNPV in the absence of postinfection TSA treatment does not affect the normal physiology of the cells. Furthermore, although the assays revealed that treatment with 1 μM TSA for 24 h had an initial mild cytotoxic effect on the cells (examined at 72 h p.i.), the toxicity indices returned to nearly normal values at 1 week p.i. and to normal values by 2 weeks p.i. (Table 1).

To find out whether the infection process affects the cells' transcriptome profile, HEK293 cells were either mock infected or infected at an MOI of 500 for 8 h in the absence of postinfection TSA treatment, and total RNA isolated from them at 3 days p.i. was examined for possible changes in the levels of specific mRNAs by microarray analysis. The data are summarized in Table 2, which shows that 3,915 of the 13,972 potentially expressed human genes represented on the microarray slides were found to be expressed in HEK293 cells irrespective of infection status. Using a 1.4-fold difference in expression level as an arbitrary cutoff value (differences below the cutoff were considered experimental variations of equal levels of expression), a total of 22 genes (0.56% of the total) were found to be differentially expressed, albeit with small differences in their expression levels, in mock- and BmNPV-infected HEK293 cells (Table 2). Fifteen of these genes displayed increased mRNA levels upon infection, while for the remaining seven the mRNA levels were decreased. (Tables 2 and 3) Given the paucity of genes whose expression appeared to be affected by the infection process and the small differences in the amplitudes of change in the mRNA levels (from a minimum of 0.65 to a maximum of 2.3), we have tentatively concluded that infection with BmNPV-based vectors does not appreciably change the gene expression profiles of the target HEK293 cells.

To validate this tentative conclusion, the expression profiles of two genes selected from among those which displayed the largest transcriptional differences in infected cells in the microarray hybridization assays (Table 3), *znf141* (up-regulated

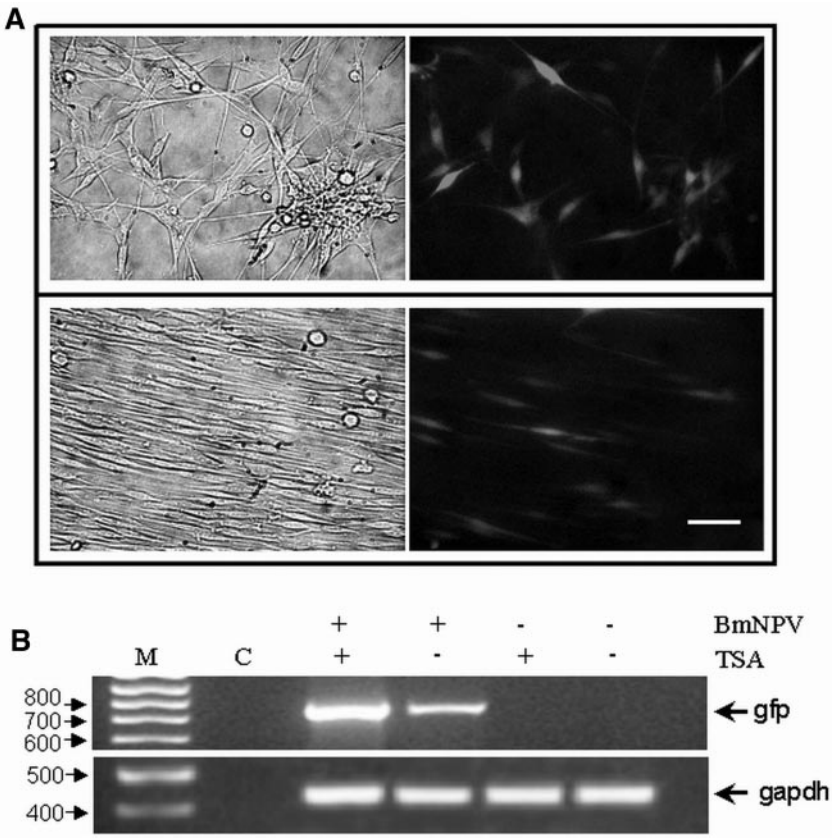

FIG. 4. GFP reporter gene transduction in rat primary Schwann cells after infection with BmNPV/CMV.GFP under optimized conditions. (A) Shown are examples of transduction efficiencies after infection at low (top) or high (bottom) confluence. (Left) Phase contrast/GFP fluorescence overlay. (Right) GFP fluorescence micrographs of the same field from live cell cultures visualized with an inverted microscope. Transduction efficiencies were 60 to 80% at low confluence and 10 to 20% at high confluence. Bar, 40 μm. (B) RT-PCR analysis of GFP gene expression in rat Schwann cell primary cultures after infection with BmNPV/CMV.GFP under optimized conditions (MOI of 500 for 8 h in PBS medium). Transduced cells treated with 0.5 μM TSA are compared with nontreated cells at 36 h p.i. Controls include amplification in the absence of template (lane C) and amplification with cDNA made from RNA of mock-infected cells (−BmNPV). Amplification of the housekeeping *gapdh* gene serves as an internal control for the efficiency of the reverse transcription reaction. Molecular weight markers (lane M) are 100-bp DNA multimers, and the amplified PCR fragments are indicated at right.

by a factor of 2.3) and *acci1* (down-regulated by a factor of 1.52), were further analyzed by real-time RT-PCR in parallel with the housekeeping *gapdh* gene, which did not display transcriptional perturbation in the infected cells (data not shown) and can, therefore, serve as a reference gene. In each case, the relative quantification was expressed as a reference gene/modulated gene expression ratio for mock-infected and BmNPV-infected HEK293 cells at 48 h postinfection. As can be seen in the example shown in Fig. 5 and the compiled results shown in Table 4, the real-time RT-PCR analyses demonstrated that the relative differences in the expression levels of the two selected genes in the control and infected cells were essentially indistinguishable from those observed for the *gapdh* gene and well within the range of experimental variability. Based on the combined results presented above, we conclude that baculovirus infection does not induce any appreciable changes in the transcriptome profiles of the target cells.

TABLE 1. Toxicity effects of BmNPV/CMV.GFP infection on HEK293 cells[a]

Cell treatment	Toxicity index (% [avg ± SD]) at time p.i.		
	72 h	1 wk	2 wk
PBS	100 ± 4	100 ± 9	100 ± 6
BmNPV	106 ± 9	102 ± 8	105 ± 3
PBS plus TSA	72 ± 11	92 ± 7	99 ± 5

[a] HEK293 cells were transduced with BmNPV/CMV.GFP at an MOI of 500 or mock transduced in PBS for 8 h at 28°C in the presence or absence of a 24-h TSA treatment. Cell viabilities were determined by measurements of the absorbance at 550 nm, which reflects the production of formazan dye from the MTT precursor. Results are normalized for the amount of cells and are expressed as percentages of the control (n = 12).

Schwann cell phenotypic marker analysis. Schwann cells infected with BmNPV/CMV.GFP were examined for expression of GFAP, S-100, and p75 NGF receptor and showed normal expression of all three characteristic Schwann cell markers (Fig. 6A to I). It is interesting to note that transduced cells retained the characteristic bi- and tripolar Schwann cell morphology. Furthermore, to assess the ability of baculovirus-transduced Schwann cells to initiate myelination in dissociated cultures, we increased intracellular cyclic AMP to levels that are known to favor differentiation into myelin-forming cells in vitro (63). After exposure to 4 μM forskolin for 4 days, cul-

TABLE 2. Overview of the transcriptome profiles of HEK293 cells[a]

Category	No. of genes in category (characteristic or determinant)
Genes examined	13,972 (unique 70-mer oligonucleotides)
Genes expressed in control cells	3,915 (1,000 intensity level cutoff)
Genes expressed in transduced cells at 3 days posttransduction	3,915 (1,000 intensity cutoff)
Genes with transcriptional changes in transduced cells	22 (>1.4-fold up or down)
Genes up-regulated in transduced cells	15 (range of 1.42- to 2.3-fold over control)
Genes down-regulated in transduced cells	7 (range of 0.65- to 0.7-fold over control)

[a] HEK293 cells were either mock infected or infected with BmNPV/CMV.GFP at an MOI of 500 in the absence of TSA treatment, as determined by oligonucleotide microarray hybridization assays. Indicated are the number of genes analyzed and expressed under both conditions, the numbers of genes found to have differential expression levels, and the quantitative differences between mock-infected and infected cells.

tured Schwann cells were doubly immunostained for GFP and P0, the major peripheral nervous system myelin protein (62). Almost all cells expressed various levels of P0 at 4 days (Fig. 6J to L), with no specific differences between transduced and nontransduced cells. These results show that Schwann cells transduced with the baculovirus to express GFP retain their neurochemical signature and can switch from a non-myelin-forming to a myelin-forming phenotype under the influence of environmental stimuli, just like wild-type Schwann cells do (63).

Baculovirus early genes are expressed in infected mammalian cells. Previous work with AcNPV has suggested that upon transfection into mammalian cells, the promoter elements of two immediate-early genes, *ie1* and *he65*, are marginally functional (66). Because these observations suggest that these genes may also be transcriptionally active in mammalian cells while in the context of a baculovirus genome, we undertook an RT-PCR analysis of the RNA contents of transduced HEK293 and Schwann cells at 2 days p.i. to deduce whether or not transcriptional activation of early (and late) BmNPV genes occurs. The RT-PCR analyses encompassed detection of transcripts originating from three early genes, *ie1* (39), *he65* (8), and *ie0*, the only baculovirus gene whose transcript contains an excisable intron (the splicing event involves the use of the splicing junction of the *ie0* to the *ie1* exon early in baculovirus infection and results in the addition of new N-terminal sequences to the IE1 transactivator [50]), as well as two late genes, *p39* (53, 75) and *pol* (41). Because of the lack of intronic sequences, which could enable an easy distinction between amplification of cDNA and genomic DNA, in all but the *ie0* gene, all RNA preparations were treated with RNase-free DNase prior to PCR. Furthermore, RNA quantities equivalent to those present in the cDNA preparations used for the amplification reactions were also used directly as templates in the

TABLE 3. Overview of the 22 genes whose expression levels were altered in HEK293 cells[a]

GenBank accession no.	Gene product (gene or protein designation)	Function	Change (*n*-fold)
NM_003441	Zinc finger protein 141 (*znf141*)	Transcription factor	2.30
U87460	Putative endothelin type B-like protein	Receptor	1.67
Y18198	One cut domain, family member 2	Transcription factor	1.60
NM_016024	RNA-binding motif protein, X-linked 2	RNA binding	1.57
AF034780	DNA (cytosine-5-)-methyltransferase 1 (DNMT1), endothelial differentiation, sphingolipid G-protein-coupled receptor (EDG5)	DNA methyltransferase, lysosphingolipid receptor	1.57
NM_015894	Stathmin-like 3 (STMN3)	Neurogenesis, signaling	1.55
AF251040	Family with sequence similarity 53, member C	?[b]	1.55
AK000431	Chromosome 14 open reading frame 94	?	1.53
L12141	Forkhead box A3 (FOXA3)	Transcription factor	1.52
AF068744	Double homeodomain protein (DUX2)	Transcription factor	1.49
AK001638	F-box and leucine-rich repeat protein	?	1.45
AF063228	Dynein cytoplasmic intermediate polypeptide 1 (DNCI1)	Motor activity	1.44
AL117606	Hypothetical protein (LOC132241)	Ribosome constituent, protein biosynthesis	1.42
M32110	Nucleolar protein 1 (NOL1)	Methyltransferase	1.42
M35198	β6 integrin (ITGB6)	Cell matrix adhesion, signal transduction	1.42
NM_014206	Chromosome 11 open reading frame 10	?	0.70
AL049539	Sequence from clone RP5–836N17 on chromosome 20q11.1–11.21	?	0.69
AF227137	Candidate taste receptor (T2R13)	Receptor, signaling	0.68
AF000377	Regulator of G-protein signaling 3 (RGS3)	GTPase activator	0.66
NM_014977	Apoptotic chromatin condensation inducer 1 (*acci1*)	Apoptotic chromosome condensation	0.66
AL080059	TSPY-like 5 (TSPYL5)	DNA binding, nucleosome assembly	0.65
AK000264	Hypothetical protein (FLJ20257)	?	0.65

[a] Shown are the genes whose expression levels were altered 3 days following infection with BmNPV/CMV.GFP at an MOI of 500 for 8 h. In the microarray analysis, gene expression was considered to occur if hybridization intensity signals greater than 1,000 fluorescent units were observed (with maximum hybridization signal intensities of 65,000 U). Differential gene expression was defined by using a 1.4-fold difference in expression level as an arbitrary cutoff value.
[b] ?, function unknown.

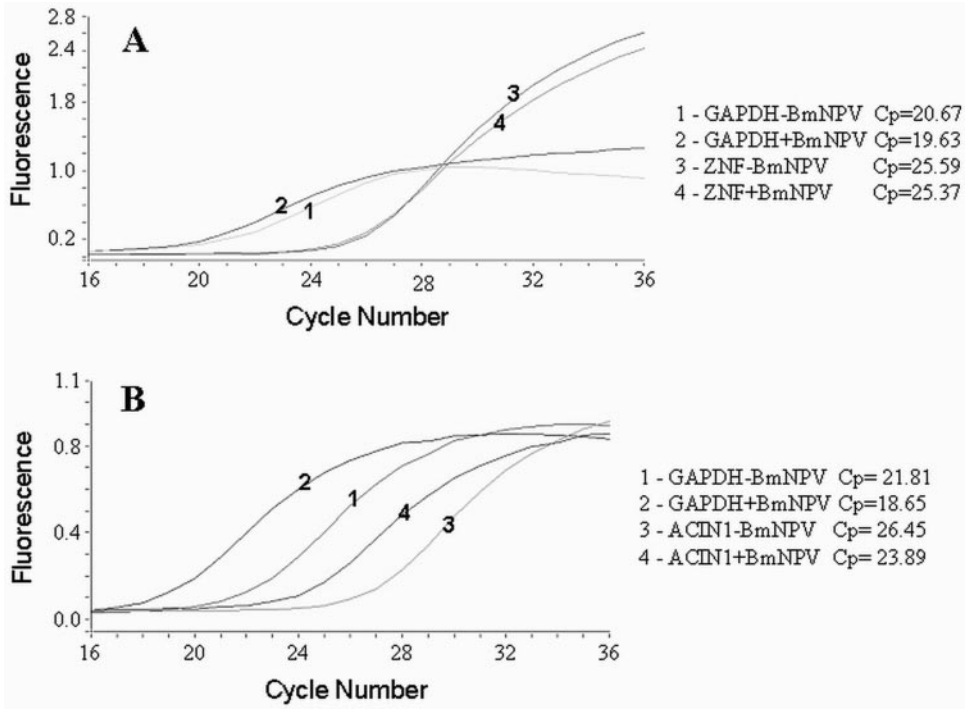

FIG. 5. Validation of gene expression levels by real-time RT-PCR following BmNPV/CMV.GFP infection of HEK293 cells, shown at 48 h p.i. Real-time RT-PCR was performed for two genes, *znf141* and *acci1*, which displayed the largest transcriptional differences between control and BmNPV-infected cells in the microarray hybridization assays (Table 3). Shown are typical experimental graphs obtained for the expression of (A) the *znf141* gene (ZNF) and (B) the *acci1* gene (ACIN1) in control (−BmNPV) and baculovirus-transduced (+BmNPV) cells. The curves in panels A and B show the rates of amplification for the transcripts of each of the two examined genes as well as for *gapdh*, which was used in both cases as a reference gene. Shown are the crossing point (Cp) values for each generated curve. No differences in expression levels of the examined genes, *znf141* and *acci1*, between control and infected HEK293 cells were observed (Table 4).

PCRs to control for the presence of low amounts of undigested baculovirus DNA.

As shown in Fig. 7A, the RT-PCR analysis of the RNA of HEK293 cells that were incubated for 24 h in the presence of TSA demonstrated the presence of transcripts originating from the early genes *ie1*, *he65*, and *ie0* but not from the late genes *p39* and *pol*. Detection of the early gene transcripts was not feasible in the RNA samples in the absence of reverse transcription, suggesting that the observed amplification products did not originate from BmNPV genomic DNA contaminating the preparation. Moreover, the detection of the spliced *ie0* transcripts establishes unequivocally that early gene transcription does occur in the nuclei of baculovirus-infected mammalian cells.

Identical results were obtained from a parallel analysis of Schwann cells infected by the recombinant baculovirus in the absence of TSA treatment (Fig. 7B). These results suggest that the transcription of early viral genes in mammalian cells is neither cell type specific nor an artifact caused by changes in chromatin conformation induced by TSA treatment.

DISCUSSION

Baculovirus-based vectors have previously been reported to be capable of transducing mammalian genes into neural cell lines and primary neuronal cells as well as nervous tissue in vivo (52, 74). Here we have expanded on these findings by

TABLE 4. Summary of real-time RT-PCR experimentation carried out for validation of the microarray hybridization data[a]

Gene(s)	Rate of amplification (crossing point value [avg ± SD])		
	−BmNPV	+BmNPV	Ratio (−BmNPV/+BmNPV)
gapdh	21.23 ± 0.57 (n = 7)	19.49 ± 1.20 (n = 7)	1.09 ± 0.08 (n = 7)
acci1	26.22 ± 1.04 (n = 5)	24.46 ± 1.09 (n = 5)	1.07 ± 0.05 (n = 5)
znf141	24.76 ± 1.17 (n = 2)	24.58 ± 1.12 (n = 2)	1.01 ± 0.002 (n = 2)
gapdh/*acci1*[b]	0.82 ± 0.04	0.78 ± 0.03	1.04 ± 0.05
gapdh/*znf141*[b]	0.85 ± 0.05	0.83 ± 0.08	1.02 ± 0.03

[a] Real-time RT-PCR was performed for two genes, *znf141* and *acci1*, which displayed the largest transcriptional differences between mock-infected and BmNPV-infected cells according to the microarray hybridization data (Table 3). In both cases, *gapdh* was used as a reference gene. Values represent the rates of amplification for the transcripts of each examined gene, as estimated by the crossing points of the generated amplification curves in control (−BmNPV) and baculovirus-transduced (+BmNPV) HEK293 cells 48 h after infection (Fig. 5).

[b] The relative quantification is expressed as a reference gene/modulated gene expression ratio for mock-infected and BmNPV-infected HEK293 cells.

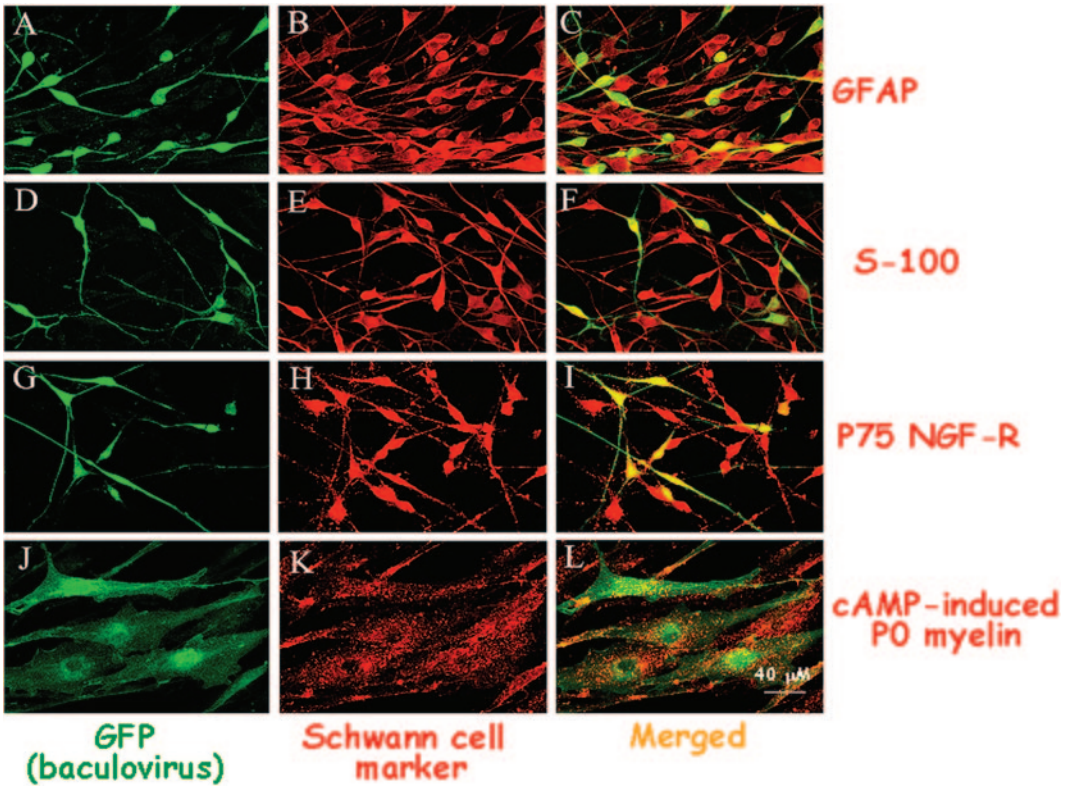

FIG. 6. Baculovirus-transduced Schwann cells retain their normal phenotype. (A to I) Double immunofluorescence labeling of transduced Schwann cells for GFP (green) and the characteristic marker proteins (red) GFAP (A to C), S-100 (D to F), and p75 NGF receptor (G to I), followed by confocal analysis. Transduced cells are identified by GFP immunofluorescence (A, D, and G); all cells, including the transduced cells, are positive for the three characteristic Schwann cell markers (B, E, and H), as shown by the merged (green/red) labeling (C, F, and I). (J to L) In vitro emergence of myelination markers in baculovirus-transduced Schwann cells. Confocal microscopic analysis shows that forskolin treatment to increase intracellular cyclic AMP (cAMP)-induced expression of myelin protein P0 (red) results in the characteristic flattening of Schwann cells indicative of "myelin sheath-like" expansions. (J) GFP (green), (K) P0 (red), and (L) merged GFP/P0 immunofluorescence labeling. Bar, 40 μm.

demonstrating for the first time efficient transduction of primary cultures of rat Schwann cells in vitro (Fig. 4). Thus, we have shown that BmNPV-based vectors are not only capable of achieving high Schwann cell transduction efficiencies but also constitute an efficient alternative to AcNPV-based vectors for gene transduction into mammalian cells in general. The achieved transduction rate for HEK293 cells (greater than 90%) is comparable to the one achieved with mammalian virus-based vectors (34, 72) as well as that achieved with AcNPV-based vectors (33, 36, 73), despite the fact that BmNPV has a much narrower insect host range than AcNPV.

Schwann cells are considered good candidates for cell-based therapies of demyelinating diseases or traumatic lesions in the central and peripheral nervous systems (5, 29). Indeed, experimental transplantation has provided evidence of the repair potential of grafted myelin-forming cells, including Schwann cells, oligodendrocytes, olfactory ensheathing cells, and, more recently, embryonic and neural stem cells (6, 10, 11, 68, 79). So far, each cell type has its own advantages and limitations. However, Schwann cells are the most likely candidate for autologous grafting. They constitute an accessible source of cells, they can be easily expanded ex vivo from adult human and nonhuman primate peripheral biopsy samples (4, 64), and they are not a target of the immune system in most dysmyelinating or demyelinating diseases of the CNS. Their engraftment in various animal models of demyelination has demonstrated their ability to remyelinate CNS lesions (5, 9, 19, 26) and restore axonal conduction (35). However, their integration into the host environment is insufficient. Modifying Schwann cells to express "therapeutic" factors enhancing axonal regeneration and remyelination, such as cell adhesion molecules (14, 15, 51, 69, 71, 76) or trophic factors (26, 45), is a promising strategy to improve their capacity to repair the injured or demyelinated nervous system.

In this context, baculovirus-based vectors have several advantages over mammalian virus-based vectors. Thus, they allow for the use of "therapeutic" genes characterized by large ORFs, which tend to inhibit the generation of high titers of retrovirus- or lentivirus-based vectors (24). In contrast, baculovirus-based vectors have an almost unlimited capacity for insertions of foreign sequences, irrespective of their length (23, 47), and can therefore be used to deliver several different genes, either alone or in combination with other genetic tools, such as transposition systems. These advantages, in combination with the high transduction efficiency of Schwann cells by BmNPV, set the foundations for baculovirus-mediated gene therapy applications aimed at the treatment of various demyelinating diseases and injuries.

Baculoviruses are also considered to be nontoxic to mammalian cells and safer than mammalian virus-based vectors (1,

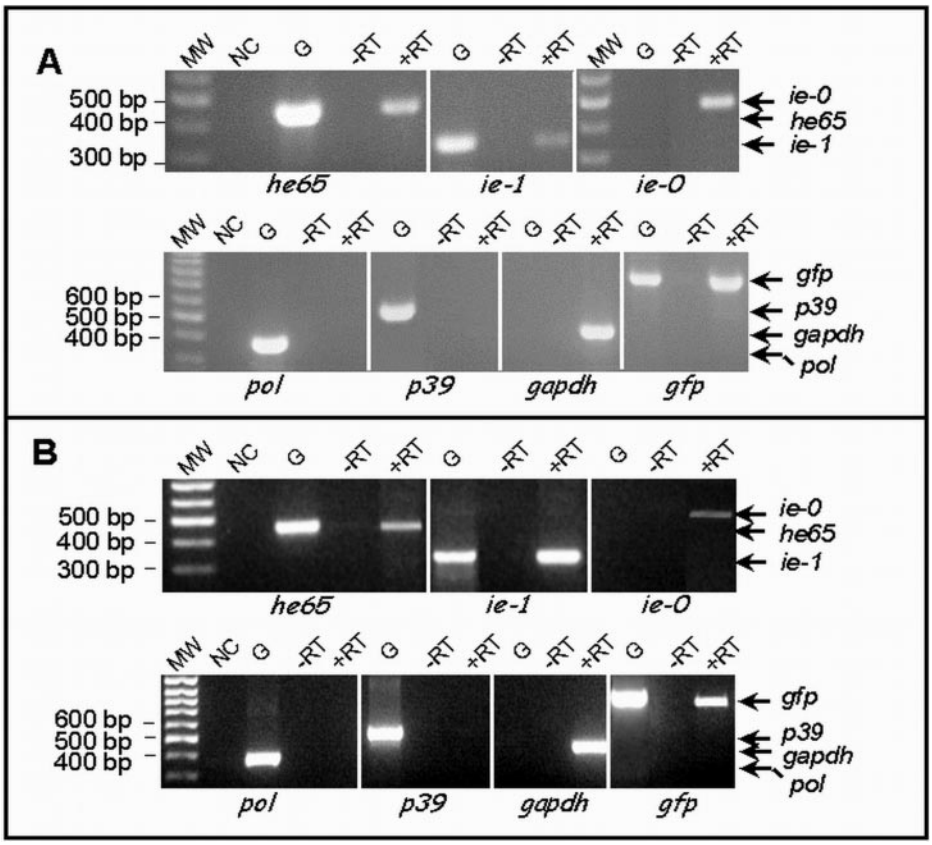

FIG. 7. RT-PCR analysis of endogenous BmNPV gene expression in HEK293 cells (A) and rat primary Schwann cells (B) infected with BmNPV/CMV.GFP under optimal conditions and subcultured for 24 h either in the presence of 1 μM TSA (A) or without TSA addition (B). RNA was collected at 72 h p.i. (HEK293 cells) and 36 h p.i. (Schwann cells) and treated with DNase-free RNase before reverse transcription. White bars separate the different sets of amplification reactions that are specific for each transcript. The names of the transcripts examined by each set of amplification reactions are indicated below the relevant portions of the gel photographs. The sizes of specific PCR fragments are the following: *gapdh*, 425 bp; *ie1*, 312 bp; *ie0*, 484 bp; *he65*, 450 bp; *p39*, 530 bp; *pol*, 382 bp; and *gfp*, 723 bp. Abbreviations: MW, molecular weight markers consisting of synthetic 100-bp DNA multimers; NC, negative control; G, BmNPV genomic DNA; −RT, DNase-treated RNA template; +RT, reverse-transcribed DNase-treated RNA template (cDNA).

7, 30). Indeed, the results of our toxicity assays showed that BmNPV infection does not affect cell viability (Table 1). Moreover, the microarray hybridization analysis of the mRNA expression patterns of HEK293 cells and subsequent data validation have also suggested that no appreciable changes occur in these cells upon infection by BmNPV (Tables 2 to 4 and Fig. 5). It should be noted that, because at 3 days p.i. the numbers of transduced cells that do not carry the transducing vector are fewer than 10% of the total (Fig. 2 and 3), the obtained values are representative (by at least 90%) of the status of the transduced cells. These findings are of major importance because despite the general acceptability of the notion that baculovirus-based vectors are safer than vectors derived from mammalian retroviruses, lentiviruses, or adenoviruses, to our knowledge, the experimental evidence in support of this contention is incomplete. In fact, some reports have suggested that baculovirus infection can induce innate immune responses in mammalian cells (1, 7, 30). Our microarray experiments did not reveal any up-regulation of this pathway (e.g., differential activation of genes regulated by NF-κB or interferons). It is nevertheless possible that the induction of the innate immune response may occur in particular cell types.

Importantly, Schwann cells infected with BmNPV/CMV.GFP retain their characteristic morphology as well as expression of typical markers in culture, such as GFAP, S-100, and p75 NGF receptor (Fig. 6A to I). In addition, upon treatment with forskolin, which increases intracellular cyclic AMP levels, they start to express markers of myelin-forming Schwann cells at the same time as wild-type Schwann cells do. The induction of P0 myelin protein in wild-type and transduced cells (Fig. 6J to L) coincides with the formation by Schwann cells of large membrane expansions that are considered to represent myelin sheath-like structures (63). It is therefore possible to transduce Schwann cells with a baculovirus vector without impinging on their morphological and molecular phenotype or their myelinating ability.

Baculoviruses can be engineered to act as powerful immunogens upon intramuscular and intraperitoneal injection or intranasal administration (1, 3). Furthermore, a well-known side effect of the repeated use of baculovirus injection into diseased sites is the induction of an acquired humoral immune response. An equally important but not yet adequately addressed aspect of the use of baculovirus-based vectors in therapeutic protocols involving transplantation of ex vivo-trans-

duced cells into sites of injury or disease is the possible induction of cellular immune responses into grafted recipients because of the low level of expression of vector-resident genes in the transduced cells (61). This concern arises from the potential for transcription, even at a low level, of at least some of the many vector-resident genes. In agreement with a previous report that had shown two AcNPV early gene promoters to be marginally active upon transfection into mammalian cells (66), our RT-PCR analysis has clearly shown that early, but not late, gene transcription does occur in mammalian cells, both human HEK293 and rat Schwann cells (Fig. 7), even in the absence of TSA treatment. Thus, the risk that small quantities of the polypeptides encoded by the active viral genes may be synthesized in the transduced cells and trigger the mounting of cellular immune responses by the host exists (66). The consequence of such an event would be that baculovirus-transduced cells may become targeted for destruction by the host's immune surveillance system.

A complete answer to the safety concerns arising from our findings in relation to the usage of baculovirus-transduced cells for therapeutic transplantation applications will require additional detailed studies. These should address issues related to the numbers of early baculovirus gene transcript that are produced in each transduced cell, the duration of persistence of viral gene expression, the stability and translatability of the viral transcripts, the persistence of the respective proteins (if the mRNAs are translated) within the cells, and, finally, the possible triggering of long-term immune responses in recipient animals. If our predictions are substantiated, further engineering of baculovirus-based vectors will be required. Although deletion of all early genes would require a major engineering effort, which is not very likely to succeed, it is also known that transcription of many early genes is dependent on the IE1 transactivator (31, 39). Accordingly, inactivation or deletion of the *ie1* gene from the baculovirus genome may result in the functional silencing of early gene expression in both insect and mammalian cells. Construction of a BmNPV deficient for the IE1 function will require the construction of transformed insect host cell lines that will produce constitutive or inducible levels of rescuing IE1 from relevant transgenes. This work is currently in progress.

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