Retinoblastoma Protein Purification and Transduction of Retina and Retinoblastoma Cells Using Improved Alphavirus Vectors

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Purpose. To develop and use improved Semliki Forest vectors (SFVs) for functional and structural analyses of the retinoblastoma protein (RB) in developing retina and retinoblastoma cells.

Methods. Virus was harvested from cells transfected with replicon and helper plasmids. Combinations of producer and target cells were tested for optimal virus production and protein expression. The replicon was improved by adding an expanded multiple cloning site, translational enhancer, and FLAG and HIS_{10} epitope and affinity tags. Affinity chromatography was used to purify β-galactosidase or RB. RB function was assessed through interaction with E2F1. The efficacy of SFV as a retinal delivery system was tested on mouse explants and cultured human retinoblastoma cells.

Results. The optimal producer and target cell lines were an HEK-293 derivative (Phoenix Eco) and BHK-21, respectively. Stable expression of structural proteins in the BHK-21 helper line simplified virus production and amplified virus yield 100-fold. The translational enhancer improved expression three- to eightfold. Full-length, functional RB was produced without the truncated products characteristic of bacterially produced RB and was purified using the affinity tags. SFVs efficiently transduced mouse retinal explants and multiple hard-to-transduce retinoblastoma tumor lines.

Conclusions. This study describes a simple, rapid, SFV vector system to produce recombinant proteins, such as RB, in mammalian cells. These SFV vectors represent an efficient approach to purification of proteins and protein complexes and transduction of retinal or retinoblastoma cells for the purpose of in vivo analysis of protein functions. (*Invest Ophthalmol Vis Sci.* 2004;45:3320–3329) DOI:10.1167/iovs.04-0140

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Gene transduction and expression systems are fundamental tools in basic research, gene therapy, vaccine development, and the generation of purified protein for biochemical studies, clinical applications, and structural analyses. For purification of a protein of interest, or its partners in vivo, the vector system should target the cell of interest efficiently and express the target protein at high levels. Our goal was to develop an efficient system for the purification of RB protein (RB) and for the delivery of proteins to the retina and retinoblastoma cell lines.

The RB gene was identified because of its critical role in suppressing retinoblastoma, but is the key component of a pathway disrupted in most human cancers (reviewed by DiCiommo et al.[1]). RB interacts with more than 100 proteins,[2] but members of the E2F family are among the most critical targets.[3] RB binds in stable complexes with key corepressors such as histone deacetylase and heterochromatin protein 1 to repress E2F activity and silence gene activity.[4]

Several methods have been used to purify RB for biochemical studies. Bacterial vectors have usually included only a portion of the RB cDNA.[5] When the full-length RB cDNA is expressed in bacteria, a significant fraction of RB produced is truncated, and much of the full-length RB is insoluble. In addition, bacteria cannot reproduce mammalian posttranslational modifications, such as phosphorylation and acetylation, which are essential for the activity of RB.[6] Baculovirus-generated RB[7,8] generated in insect cells also may not produce correctly modified protein. The production of recombinant baculovirus, adenovirus, or vaccinia vectors can be time consuming, limiting the number of mutants that can be studied. To date, the expression and purification of RB from mammalian cells has not been described.

Several viruses can be used to target a variety of mammalian cell types, including the intact retina, such as adenoviruses, adeno-associated viruses (AAV), lentiviruses, and retroviruses. Many studies have been published describing the utility of these vectors in gene therapy, and their advantages and disadvantages for this application have been comprehensively reviewed elsewhere.[9,10] Retroviruses have been used to express RB in retinoblastoma cell lines,[11] and adenoviruses can also transduce these cells,[12,13] although RB studies with adenoviral vectors have focused on other cell types.[14–16] Transfection of retinoblastoma cell lines with plasmid DNA is typically very inefficient,[13,17] although DNA uptake can be improved by a technique known as adenofection in which the plasmid is noncovalently attached to the adenovirus.[13]

It is important to note that these systems are not ideal for purifying large amounts of recombinant protein, because production of adenoviral vectors can be time consuming, and expression levels are constrained by the limited capacity of the enhancers that drive transcription. However, optimized en-

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hancers have been incorporated into adenovirus vectors and used to express levels of polypeptide that can reach 10% to 20% of total cellular protein.[18,19]

SFV, a member of the Togaviridae family and the *Alphavirus* genus, is also well suited for high-level protein expression, as well as for the transduction of the retina or retinoblastoma cells. Recombinant SFV (rSFV) can be produced rapidly in culture, transduces a broad range of mammalian cell types, including nondividing cells,[20] and the self-amplifying genome ensures high levels of protein expression that can reach 25% of total cellular protein.[21] Furthermore, rSFV transduces neurons more efficiently than adeno-, adeno associated-, or retroviral vectors,[22] making it an attractive choice for targeting retinal cells. Although wild-type and recombinant alphaviruses induce p53-independent apoptosis in many immortalized cell lines,[23,24] hippocampal slice cultures transduced with recombinant SFV retain morphology and synaptic connections and support study of protein function for at least 5 days.[25,26] Thus, SFV is well suited for biochemical or short-term cell biology experiments.

SFV codes for two polyproteins: One contains the nonstructural proteins (NSP1–NSP4) that code for protease, RNA capping, and polymerase activities. The other, containing the structural proteins C, E3, E2, 6K, and E1, is synthesized from an mRNA derived from a subgenomic promoter. The recombinant SFV (rSFV) systems consist of a "replicon" plasmid, in which the gene of interest replaces the structural proteins and a "helper" plasmid that encodes the structural proteins necessary for viral production. Originally, replicon and helper plasmids were transcribed in vitro, and RNA was transfected into mammalian cells for virus production.[27] We[28] and others[29–31] have developed DNA-based SFV systems in which transcription from DNA is driven by a eukaryotic promoter, eliminating the need to generate RNA in vitro.

We now describe the development and characterization of pSMART (*S*FV, *M*ammalian RNA-polymerase II dependent promoter, *A*ffinity tags, *R*estriction site expansion, *T*ranslation enhancer), a derivative of the SFV vector pSCA1,[28] for production of recombinant viruses that express large amounts of soluble protein in many cell types. The pSMART vector includes a translation enhancer that improves protein yield severalfold, epitope affinity tags for protein purification, and an expanded multiple cloning site. A packaging cell line stably expressing the SFV structural protein genes was used to amplify viral yield 100-fold. The cell type used to generate virus influenced the efficiency of transduction. We exploited the improved system to express and affinity purify full-length functional RB from mammalian cells. We demonstrate that SFV vectors efficiently transduce both embryonic retinal explants and multiple retinoblastoma tumor cell lines. Thus, SFV vectors will be useful tools for biochemical and in vivo analysis of retinal proteins.

Materials and Methods

Plasmids

The complete sequence of pSCA and pSMART vectors is available at http://vsrp.uhnres.utoronto.ca/Bremner.html (hosted in the public domain by the University of Toronto). pSMART2a was built in three stages: (1) The SFV capsid structural protein gene (amino acids 1-267) was amplified by polymerase chain reaction (PCR) using pSCAHelper (Fig. 1A) as a template, Vent DNA polymerase, and the following primers: O-Help1, 5′-attcatagtttttgacagcgtcac-3′, and O-Help2 (*XmaI*), 5′-gcg*cccgggaggatcccactcttcgga*-3′. The 1.2-kb product was cut with *Eco*RI and *Xma*I, and an 833-bp fragment ligated into the *Eco*RI/*Xma*I-cut vector (pBluescript KS+; Stratagene, La Jolla, CA). The capsid gene in the resultant plasmid pBS-capsid was sequenced and found to have two silent PCR-generated errors: c→t (bp 7485; capsid amino acid [aa] 22) and g→t (bp 8103; capsid amino acid 228). (2) An *Xma*I-*Eco*RI fragment containing the SFV capsid gene was isolated from pBS-capsid and, along with a 676-bp fragment isolated by *Bgl*II/*Eco*RI/*Acc*I digestion of pSCAHelper, was ligated to a 10.8-kb *Bgl*II/*Xma*I fragment from pSCA1 (Fig. 1B).[28] (3) The oligonucleotides, O-SFVM1 (*Bgl*II), 5′-agcg*agatct*tgactacaaggacgacgatgacaagcaccaccatcatcaccaccatcaccatcacagca-gcggcctggttccgcgtgggtctggatccgctaagcgcgcttcgaatcgatgcatcctagggccccggg-3′, and O-SFVM2, 5′-gcgcccgggccctaggatgcatcga-3′, were annealed, filled using Klenow (New England Biolabs, Beverly, MA), digested with *Xma*I/*Bgl*II, and ligated to a 12.3-kb *Bam*HI/*Xma*I pSCAE7 fragment to generate pSMART2a (Fig. 1C). pSMART2b, in which the reading frame downstream from the capsid gene is altered, was built by ligating *Bam*HI/*Blp*I cleaved pSMART2a to a linker generated by annealing the oligonucleotides O-SFVM5, 5′-gatcggatccgc-3′, and O-SFVM6, 5′-ttagcggatcc-3′ (Fig. 1C). pSMART-RB was constructed by a three piece ligation of (1) a 12.4-kb fragment from *Bam*HI/*Apa*I-digested pSMART2a; (2) a 2.6-kb RB1 fragment from *Eag*I/*Apa*I-digested pIndhRb(wt)-puro (Bremner R, unpublished vector, 1998); and (3) a linker with *Bam*HI and *Eag*I overhangs generated by annealing ORbSFV1, 5′-gatccatgccgcccaaaacccccccgaaaaa-3′, and O-RbSFV2, 5′-ggcgtttttcgggggttttgggcggcatg-3′. pSCA-RB was built by inserting a 2.9-kb *Apa*I/blunt+*Bam*HI fragment from pIndhRb(wt)-puro into *Bam*HI/*Sma*I-digested pSCA1. pSMARTLacZ was built by inserting a 3.5-kb *Bam*H1/*Apa*I LacZ fragment from *Bam*HI/*Apa*I/*Sca*I-digested pAdloxΔDCMVLacZ (Bremner R, unpublished vector, 2002) into *Bam*HI/*Apa*I-digested pSMART2b.

Cell Culture

BHK-21 infant hamster kidney cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES buffer, and L-glutamine. HEK-293; 293T, a derivative of HEK-293 (Phoenix-Eco, kind gift of Garry Nolan); and NG108 cells[32] were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS. Retinoblastoma tumor cells were grown in suspension at high density (1–5 × 10^5 /mL) in IMDM with 15% Fetal Clone III (Hyclone Laboratories, Inc., Logan, UT), 10 μg/mL insulin, and 55 μM β-mercaptoethanol. *RB1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured in DMEM and 10% FBS. Cells were incubated in a humidified atmosphere of 5% CO_2 in air.

Transfection

HEK-293, 293T, Phoenix-Eco, and NG108 cells were grown to 60% to 80% confluence and transfected by the calcium phosphate method as described.[33] BHK-21 cells were transfected by electroporation, as described previously.[28]

Generation of SFV Particles

A complete and detailed protocol for viral production and titering is available at http://vsrp.uhnres.utoronto.ca/Bremner.html.

Stable Packaging Cell Line Selection and Screening

BHK-21 cells were electroporated with pSCAHelper[28] and pBABE-puro.[34] Transfected clones were grown in IMDM supplemented with 8 μg/mL puromycin (Roche Diagnostics, Indianapolis, IN). HEK-293 packaging clones were isolated by calcium phosphate transfection of pSCAHelper. Packaging activity of the resultant helper clones was assessed by determining the production of viral particles from clones transfected with pSCAβ.

β-Galactosidase Assays

β-Galactosidase activity was measured by an ONPG (*O*-nitrophenyl-β-D-galactopyranoside, Amresco, Solon, OH) assay as described previously.[35] Known β-galactosidase standards (Roche Diagnostics) were

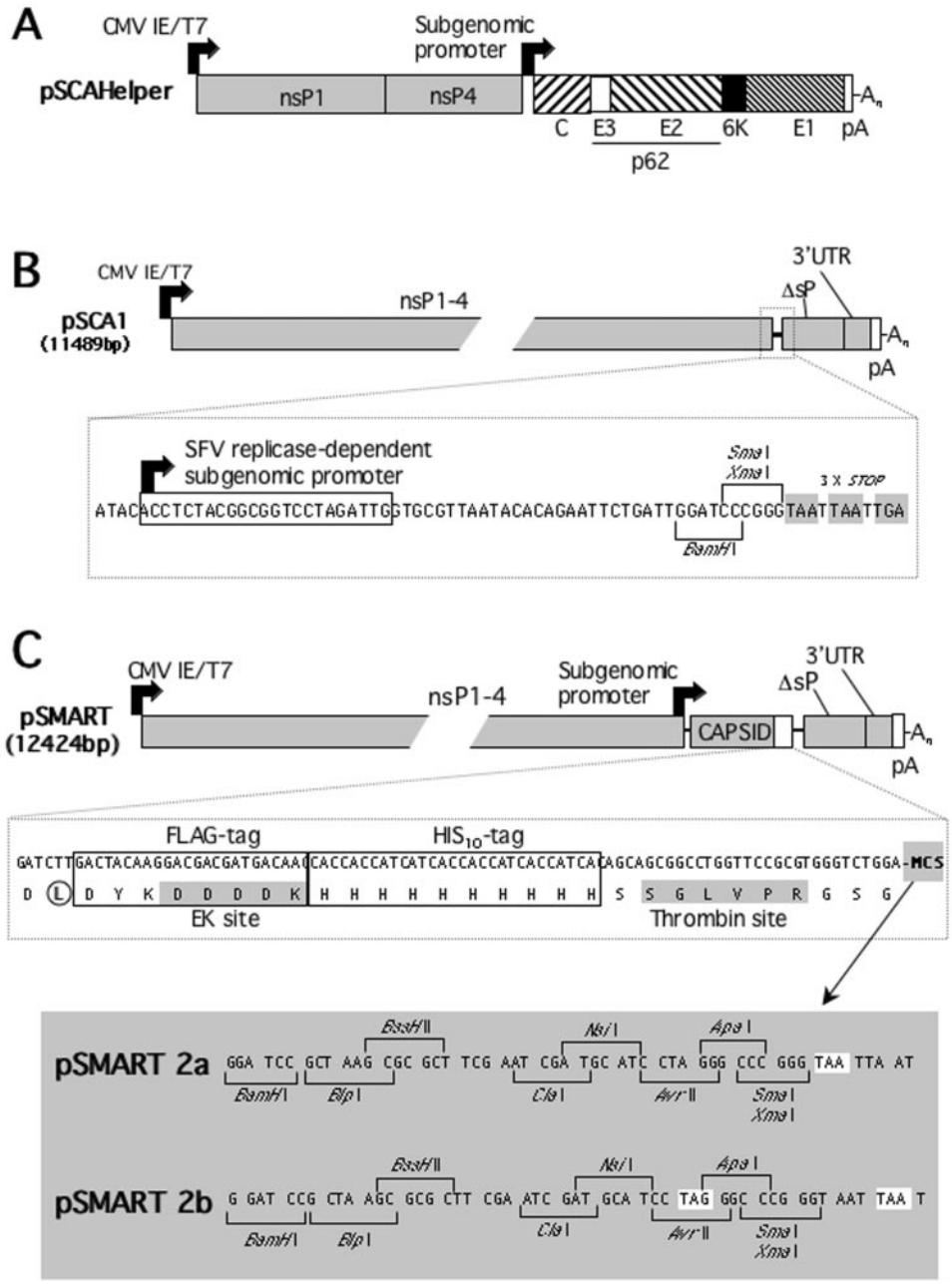

FIGURE 1. SFV vectors. (**A**) Helper vector used to generate a DNA-based packaging cell line that synthesizes SFV structural proteins. Starting from the 5′-end, the pSCAHelper[28] contains the CMV IE promoter/enhancer, nsP1 and 4, structural protein genes (C, p62 [E3/E2] and E1) and an SV40 polyadenylation signal (pA). (**B**, **C**) Schematic representation of pSCA1 and pSMART replicons. Both vectors contain the CMV IE/T7 promoter and SV40 polyadenylation signal (pA). pSMART was further modified to include the entire SFV capsid gene, which functions as a self-cleaving translation enhancer. The leucine at +2 (*bold*, *circled*) ensures effective capsid removal.[40] In addition, the vector was modified to include two affinity tags (FLAG and 10 histidine residues [HIS_{10}]) two proteolytic cleavage sites (EK, enterokinase, and thrombin) and an expanded multiple cloning site (MCS) in two reading frames (*shaded box*). The MCS restriction enzyme sites are expanded to show detail. STOP codons are on an *unshaded* background.

used to convert optic density (420 nm) activity readings into nanograms of protein. The number of transfected cells was determined by counting X-Gal–stained cells.

Immunohistochemistry

Protein expression was detected in cell cultures with antibodies using 3,3′-diaminobenzidine tetrahydrochloride (DAB Sigma Fast; Sigma-Aldrich, St. Louis, MO) staining according to the manufacturer's directions.

Immunoprecipitation of Flag-HIS_{10}-LacZ

The FLAG-HIS_{10}-LacZ protein from BHK-21 cells transduced by the SMART-LacZ virus was immunoprecipitated with 0.5 μg of α-FLAG M2 antibody, using published methods.[6]

Protein Purification by Ni^{2+}-NTA Ion Chelating Chromatography

Cells were lysed in buffer L (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole [pH 8.0]) and pulse sonicated twice for 15 seconds on ice. Lysates were centrifuged at 14,000*g* at 4°C for 1 hour. Supernatants were mixed with pre-equilibrated Ni^{2+}-NTA resin (Qiagen, Valencia, CA) and rocked for 1 hour at 4°C. After three 10-minute washes in 15 resin volumes of buffer W (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole [pH 8.0]), samples were eluted with buffer E (50 mM NaH_2PO_4, 300 mM NaCl, and 500 mM imidazole). All protein preparations were performed at 4°C.

GST Pull-down Assay

One microgram each of GST-E2F1 (amino acids [aa] 89-437) or glutathione *S*-transferase (GST) was incubated at 4°C for 1 hour with affinity-purified RB (0.5 μg) from pSMART-RB–transfected HEK-293

cells or HEK-293 lysates. Beads were washed four times with cold TEE buffer (200 mM Tris [pH 8.0], 5 mM EDTA, and 5 mM EGTA) and resuspended in SDS-loading buffer. Bands were visualized by Western blot with anti-RB monoclonal antibody (model 14001a; BD-Pharmingen, San Diego, CA).

Determination of RB Yield

Bradford assay was used to determine the total amount of RB (full-length and truncated products) purified from TRC-hRB eluted Ni^{2+}-NTA resin. The proportion of full-length TRC-hRB was visualized on a Coomassie-stained 12% SDS-polyacrylamide gel and quantified against BSA standards with results consistent with the Bradford assay.

Mammalian cell lysates transduced by SMART-RB virus were applied to 20 μL Ni^{2+}-NTA slurry, washed three times with buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole [pH 8.0]), and boiled in SDS loading buffer, and the amount of RB estimated by comparison to the intensity of full-length TRC-hRB on Western blot. The fraction of lysed cells that were transduced or transfected was determined by α-RB immunostaining to calculate the amount of RB produced per cell.

Retinal Explants

Mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the University Health Network Animal Care Committee. Timed pregnancies of B-6 mice (Ontario Cancer Institute) were determined by vaginal plug, with midday of plug observation counted as embryonic day (E)0.5. Cervical dislocation and decapitation was used to euthanatize pregnant females and pups, respectively. Eyes were isolated from E13.5 and postnatal day (P)0 mouse embryos and transferred to PBS solution. The retinas were removed with lens intact, without the pigment epithelium, and placed in chamber filters (0.4 μm pore diameter; Millicell-CM; Millipore, Bedford, MA) chamber filters. The chamber was placed in a six-well culture plate containing 0.8 mL explant culture medium (DMEM/F-12 supplemented with 5% FBS, insulin [5 μg/mL], pyruvate, and glutamate). A cutoff 200 μL pipette tip formed a minichamber around the explant to restrict spread of viral media away from the explant. The explants were cultured at 37°C in 5% CO_2.

X-Gal Staining

X-Gal stain was prepared and used as described.[28] For staining of retinal explants, tissue was fixed for 30 minutes in 4% paraformaldehyde and washed three times for 15 minutes each with PBS. Explants were submerged in X-Gal stain at 37°C for 24 hours and washed in PBS. After fixing with 70% ethanol for 24 hours, explants were embedded in paraffin. Retinal sections were counterstained with 1% neutral red.

Results

Generation of Recombinant SFV from DNA-Based Vectors

Usually rSFV was generated by electroporation of vector RNA/DNA into BHK-21 cells, which necessitated reoptimization of capacitance and voltage conditions for each new construct. Transfection of BHK-21 cells using calcium phosphate or various cationic lipids was inefficient (data not shown). We produced virus efficiently in HEK-293 cells after calcium phosphate transfection of pSCAHelper,[28] encoding viral structural proteins (Fig. 1A), and the β-galactosidase expression vector pSCAβ (Fig. 1B). Titers of virus produced in transfected HEK-293 and electroporated BHK-21 cells were similar (10^6 IU/mL) when assayed on BHK-21 cells. Recombinant virus production was optimal when cells were transfected with plasmid DNA at a molar ratio of 1:1 vector to helper (data not shown).

Although alphaviruses have an extensive host range, recombinant SFV and Sindbis virus generated in BHK-21 cells transduce different cell lines with varying efficiency.[24] To identify the optimal producer–target cell line combination, we transfected four different cell types with pSCAβ and pSCAHelper and measured viral titers on BHK-21, HEK-293, and/or NG108 cells (Fig. 2A). Production cell lines included BHK-21; HEK-293, a derivative of HEK-293 (Phoenix-Eco); and NG108 cells. Virus produced in Phoenix-Eco, HEK-293, or BHK-21 cells transduced BHK-21 cells best, suggesting that this target line may be optimal for protein production and/or the construction of helper lines. The highest-titer virus was produced in Phoenix-Eco cells, despite the fact that transfection was equally efficient in this line and NG108 (data not shown). We did not measure the number of viral particles, and so we are unable to say whether the higher titers were due to the presence of more total particles or to a greater proportion of active particles. Factors that may influence the efficiency with which a cell may take up and express virus include posttranslational modifications of the viral E1 and E2 spike glycoproteins, which are critical determinants of target cell specificity,[36,37] and/or the lipid component of the viral coat, derived from the cell membrane in which the virus is produced.[38]

SFV Packaging Cell Line for Stable Structural Protein and Viral Production

An alphavirus packaging cell line that expressed Sindbis structural proteins has been shown to simplify the process of generating recombinant Sindbis virus and SFV, since production of recombinant virus necessitates only transfection of the replicon.[39] Because structural protein gene transcription is under the control of a subgenomic promoter that is activated only in the presence of the replicon, toxicity due to structural proteins is not an issue. To generate DNA-based packaging cell lines, BHK-21, and HEK-293 cells were cotransfected with pSCA-Helper (Fig. 1A)[28] and a selectable puromycin resistance marker. Of 11 BHK-21 clones, clone 1/10 exhibited the highest packaging activity (4.5×10^5 IU/mL) when transfected with the pSCA replicon. No virus was generated from control pSCA-transfected BHK-21 cells. Clone 1/10 showed no cytopathology or reduction in packaging ability over 35 serial passages. Two positive HEK-293 clones were isolated that were 20 times less efficient than clone 1/10 at generating virus (titer of 2.4×10^4 IU/mL). In addition, clone 1/10 could also be used to amplify existing viral stocks 100-fold, greatly reducing the dependency on transfection of helper plasmid for virus production. From transduction of clone 1/10 with 1.1×10^4 SCA virions at a multiplicity of infection (MOI) of 0.1 (within the linear range of transduction, data not shown), 1.2×10^6 virions were recovered at 24 hours. Transduction beyond the linear range resulted in lower multiples of amplification. We were unable to quantitate the levels of structural proteins provided by clone 1/10, because we did not have a good capsid antibody for Western blot.

pSMART, Enhanced Version of the DNA-Based Replicon pSCA

To improve cloning, protein production, and recovery, we made a series of structural modifications to the pSCA1 vector (Fig. 1B). First, to increase protein production, the SFV capsid gene, which contains a translation enhancer,[40,41] was added downstream of the SFV replicase-dependent subgenomic promoter. Second, an octapeptide FLAG tag and a 10-histidine tag flanked by enterokinase and thrombin cleavage sites were added in frame with the capsid sequence for immunohistochemical detection and affinity purification of recombinant proteins. Third, a synthetic polylinker containing nine unique restriction enzyme sites was inserted in two reading frames. The enhanced vector, pSMART (Fig. 1C), can accommodate

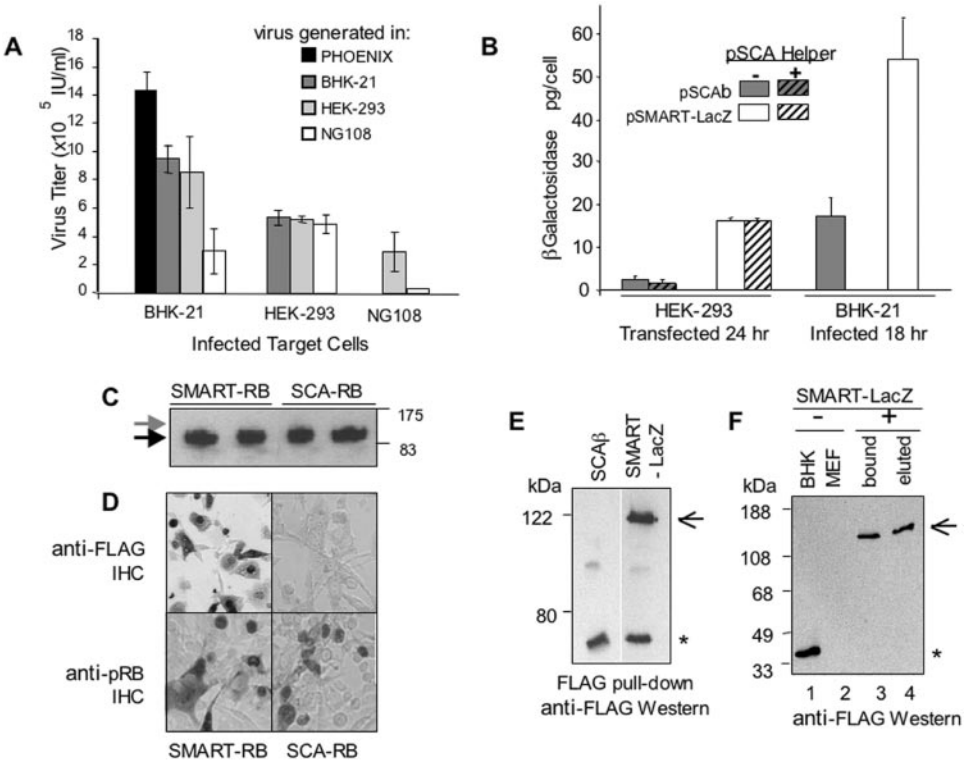

FIGURE 2. pSMART is an efficient replicon. (**A**) Assessment of different producer–target cell lines. Virus was collected 24 hours after cotransfection of HEK-293 derivative (Phoenix-Eco), BHK-21, HEK-293, and NG108 cells with pSCAβ and pSCAHelper. The highest titer was obtained on BHK-21 cells except for virus generated in NG108 cells, which had the highest titer in HEK-293 cells. (**B**) The SFV capsid protein in pSMART functions as a translation enhancer. Twenty-four hours after transfection into HEK-293 cells pSMART-LacZ, containing the capsid, produced approximately eight times more β-galactosidase per cell than pSCAβ, which lacks the capsid. In replicon-transfected cells, the presence of pSCAHelper did not impact translation enhancement (*batched boxes*). In virus-transduced cells, SMART-LacZ virus produced approximately three times more protein than SCAβ virus per BHK-21 cell 18 hours after transduction. (**C**) The translation enhancer self-cleaves. SMART-RB. SCA-RB–transduced BHK-21 cells were analyzed by SDS-PAGE gel and Western blot. RB produced with each vector migrated at the same position (*black arrow*), indicating self-cleavage of the 30-kDa capsid. No uncleaved capsid-RB was evident (*gray arrow*). Lanes were loaded with equal amounts of RB to compensate for differences in intensities due to translation enhancement from pSMART. (**D**) The FLAG epitope tag can be used to detect recombinant protein. In BHK-21 cells transduced with SMART-RB virus (10^6 IU/mL; MOI, 0.5), RB was detected predominantly in the nucleus after immunohistochemical staining with anti-FLAG or anti-RB antibodies (*left*). In BHK-21 cells transduced with SCA-RB, RB could only be detected with anti-RB antibodies (*right*). (**E**, **F**) The FLAG and HIS tags are functional after self-cleavage of capsid. (**E**) FLAG-HIS_{10}-LacZ (*arrow*) was detected by immunoprecipitation and Western blot with anti-FLAG antibody from BHK-21 cells transduced with SMART-LacZ virus (*lane 2*) but not SCAβ virus (*lane 1*). (**F**) Lysates from BHK-21 cells transduced with SMART-LacZ virus were applied to Ni^{2+}-NTA resin. The bound and eluted fractions contained β-galactosidase (*arrow*) visualized by anti-FLAG Western blot (amount loaded per lane adjusted to equal intensity). A nonspecific band (*) is recognized by anti-FLAG antibody in BHK-21 (**E**, **F**, *lane 1*) that is not present in MEFs (**F**, *lane 2*).

3.5 kb of recombinant gene insert without breaching its nucleocapsid packaging limit.

In BHK-21 transduced or HEK-293 transfected cells, the SMART-lacZ virus or pSMART-lacZ plasmid produced three or eight times more β-galactosidase than SCAβ virus or pSCAβ plasmid, respectively, which lack the enhancer (Fig. 2B and Table 1). Cotransfection of pSCAHelper did not affect the amount of β-galactosidase produced per HEK-293 cell (Fig. 2B). β-Galactosidase was produced by SMART-lacZ virus at an average of 55 pg per transduced BHK-21 cell, comparable with that obtained with RNA-based vectors containing the translational enhancer.[40] Because we did not optimize the time for maximum protein expression, the results in BHK-21 cells could be an underestimate of the enhancer's full capabilities.

Although only the first 102 of 801 nucleotides of the capsid gene are necessary to enhance translation, including the complete gene in replicon vectors ensures that the capsid is self-cleaved from the recombinant protein soon after translation of the structural proteins.[40] Because autoproteolysis is inhibited when the second amino acid after the cleavage site is a proline,[40] we designed pSMART with leucine at +2 (Fig. 1C), ensuring that the capsid is removed regardless of the N-terminal sequence of the inserted gene. Self-cleavage of the capsid was confirmed by the single bands at ~110 kDa of FLAG-HIS_{10}-RB expressed from SMART-RB virus with capsid and RB expressed from SCA-RB virus without capsid, after transduction of BHK-21 cells (Fig. 2C), indicating that the ~30-kDa capsid had been cleaved from the FLAG-HIS_{10}-RB protein. FLAG-HIS_{10}-RB was detected in BHK-21 cells transduced with SMART-RB virus with both anti-FLAG M2 and anti-RB antibodies in comparison with SCA-RB–transduced BHK-21 cells, which stained only with anti-RB antibodies (Fig. 2D). The presence of SFV nonstructural proteins did not impair nuclear localization of RB (Fig. 2D).

The FLAG and HIS_{10} epitopes could be used for affinity purification after capsid autoproteolysis. After immunoprecipitation and Western blot analysis with anti-FLAG M2 antibody on lysates from BHK-21 cells transduced with SMART-lacZ, a band at ~120 kDa was detected corresponding to FLAG-HIS_{10}-βGal (Fig. 2E). A similar band was observed after lysates from transduced cells were passed through an Ni^{2+}-NTA column (immobilized metal affinity chromatography, IMAC), and bound and eluted fractions were analyzed by Western blot

TABLE 1. Protein Produced

Vector	Transfected Cells*	Transduced BHK Cells	Protein	Protein (μg)
pSCAβ	3.8×10^5	2.8×10^6	β-Gal	50
pSMART-lacZ	4.0×10^5	3.1×10^6	β-Gal	167
pSMART-RB	4.5×10^5	4.2×10^6	pRB	38

* Transfection efficiency 15% (6-cm plate, calcium phosphate transfection, HEK-293 cells).

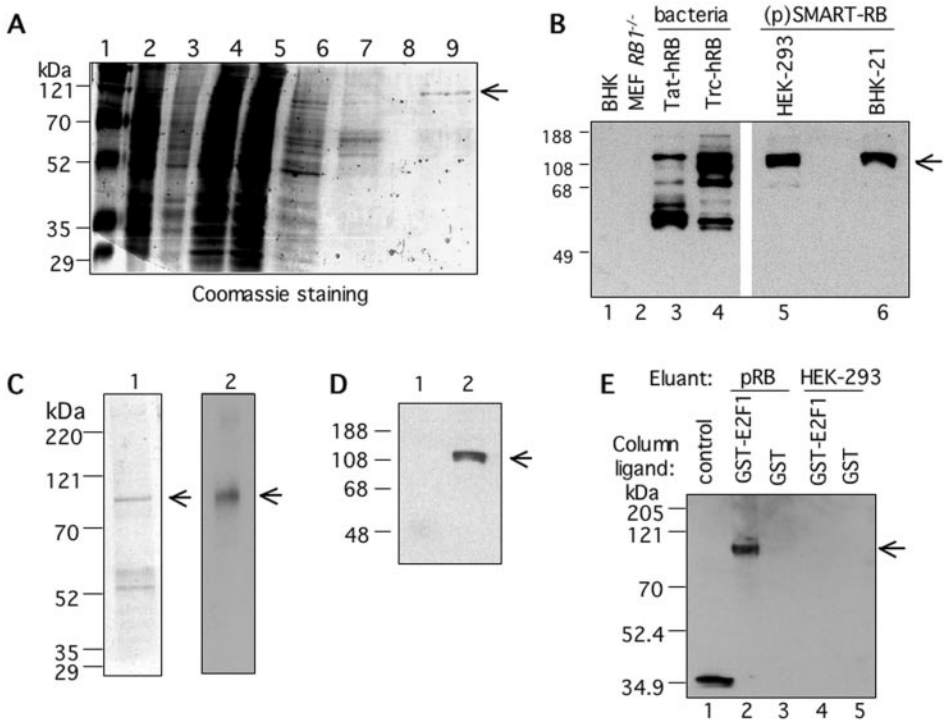

FIGURE 3. Purification of full-length proteins. (**A**) β-Galactosidase was purified from 4×10^5 SMART-LacZ virus-transduced cells by IMAC, and eluted protein was resolved by 12% SDS-PAGE and visualized with Coomassie brilliant blue R-250. *Lane 1*: pre-stained molecular weight markers; *lane 2*: 3% of total control untransduced BHK-21 cell lysate; *lane 3*: 5% of insoluble fraction; *lane 4*: 3% of soluble fraction; *lane 5*: flow-through; *lanes 6* and 7, washes 1 and 2, respectively, of Ni^{2+}-NTA slurry with buffer W; *lane 8*: empty; *lane 9*: 3% of total β-galactosidase eluate. (**B**) Full-length RB produced in mammalian cells transfected with pSMART-RB plasmid or transduced with SMART-RB virus. The quantity and quality of RB from bacteria and mammalian cells was assessed after purification by IMAC. Protein bound to Ni^{2+}-NTA beads was boiled in sample buffer, resolved by 10% SDS-PAGE, and detected by Western blot with an anti-RB monoclonal antibody (14001a). *Lanes 1* and *2*: untransduced controls; *lanes 3* and *4*: HIS-tagged RB purified from *Escherichia coli* transformed with either pTat-HIS-hRB (*lane 3*) or pTRC-HIS-hRB (*lane 4*) expression vectors, and from HEK-293 cells transfected with pSMART-RB (*lane 5*) or BHK-21 cells transduced with SMART-RB virus (*lane 6*). (**C**) Human RB was isolated from pSMART-RB–transfected HEK-293 cells by IMAC. Protein was eluted and visualized by 10% SDS-PAGE. *Lane 1*: Coomassie stain of 200 ng and *lane 2*: Western blot of 10 ng of total RB purified from 7.35×10^5 transfected cells. (**D**) Anti-RB Western blot analysis of lysate from untransduced BHK-21 cells (*lane 1*) or human RB purified on an anti-FLAG antibody column from BHK-21 cells transduced with SMART-RB virus (*lane 2*). (**E**) RB isolated from pSMART-RB–transfected cells bound E2F1. Affinity-purified RB from HEK-293 cells transfected with pSMART-RB was incubated with GST-E2F1 (*lane 2*) or GST (*lane 3*) and bound protein was visualized by Western blot with anti-RB monoclonal antibody. Negative controls: HEK-293 lysates incubated with GST-E2F1 (*lane 4*) or GST (*lane 5*). Positive control: truncated GST-RB (aa 298-396, *lane 1*).

analysis with anti-FLAG detection blot used to examine both bound and eluted fractions (Fig. 2F).

Protein Purification by IMAC from SMART-Transduced Cells

To assess the purity and yield of protein using the new virus vector, BHK-21 cells were transduced with SMART-lacZ virus, and 24 hours later FLAG-HIS_{10}-LacZ protein was purified using IMAC and eluted protein analyzed by SDS-PAGE and staining with Coomassie blue dye. Whereas most of the mammalian proteins did not bind to the Ni^{2+}-NTA column or were removed in the washes (Fig. 3A, lanes 4–7), a single ~120-kDa band was detected in the eluate corresponding to FLAG-HIS_{10}-βGal (Fig. 3A, lane 9). No other bands were seen when the gel was silver stained (data not shown). The yield of affinity purified, eluted β-galactosidase after elution was 3.3 μg from 4×10^5 transduced BHK-21 cells or 8.25 pg per cell (Table 2). The total β-galactosidase activity before purification was 55 pg/cell (Fig. 2B and Table 2). Therefore, without optimization, recovery of the recombinant protein was 15%.

pSMART-RB Generates Full-Length RB in Mammalian Cells

Mammalian proteins expressed in bacteria may fold improperly, leading to decreased solubility.[42] Different codon usage in bacteria can also lead to prematurely truncated protein products. Thus, we compared the quality of RB made by the mammalian pSMART expression vector with that made by two bacterial expression vectors. The full-length RB gene was cloned into both the TAT fusion vector[43] and pTRC-HIS vector (Invitrogen, Inc., Carlsbad, CA), expressed in bacteria and purified by IMAC. Western blot using anti-RB antibody to the N terminus showed only 10% to 30% of RB to be full length (Fig. 3B, lanes 3, 4). In contrast, all the RB similarly recovered from HEK-293 cells transfected with pSMART-RB plasmid and BHK-21 cells transduced with SMART-RB virus was full-length (Fig. 3B, lanes 5, 6).

Next, we assessed the purity and yield of RB obtained using the pSMART system. In the first instance, we purified protein directly from HEK-293 cells transfected with pSMART-RB. This

TABLE 2. Purified Recombinant Protein

Mode	Method	Protein	Total Protein (pg per cell)	Protein Purified (pg per cell)	Recovery (%)
Replicon	pSMART-RB transfected HEK-293 cells	pRB	8.0	1.36	17
Virus	SMART-lacZ infected BHK-21 cells	β-Gal	55.5	8.25	15

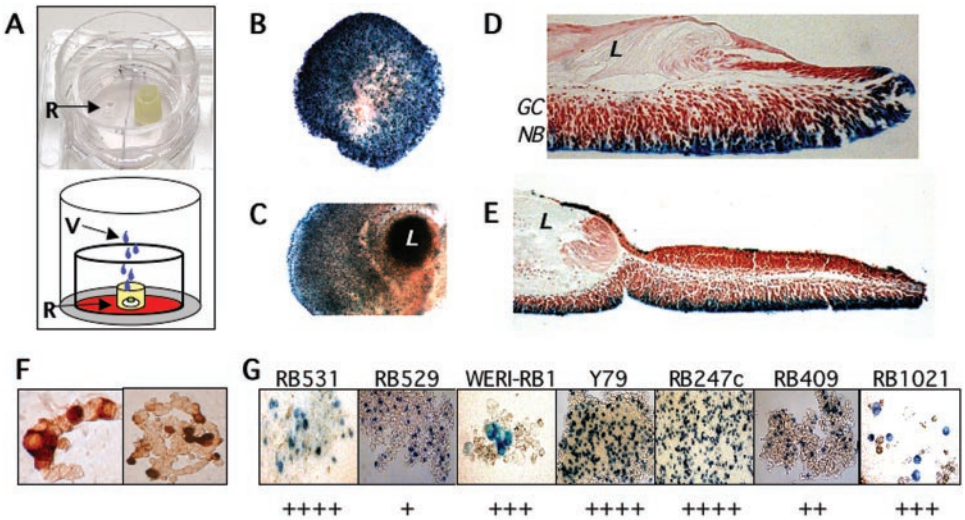

Figure 4. Transduction of retinal explants and retinoblastoma. (**A**) Retinal explants were placed on 0.4-μm membranes (*red*) and surrounded by a barrier constructed from a 1-mL plastic pipette tip (*yellow*) to minimize diffusion of virus (*blue* droplets) away from the explant. E13.5 (**B**, **D**) and P0 (**C**, **E**) murine retinas were transduced with 100 μL of 10^6 IU/mL SCAβ virus. After 24 hours, positive cells were detected in whole E13.5 (**B**) and P0 (**C**) retinas by X-Gal staining. Sectioned retinal explants show transduction of the neuroblastic layer (NB) in E13.5 (**C**) and P0 retina (**E**) 24 hours after transduction. GC, ganglion cell layer; L, lens. (**F**) Positive nuclear staining for RB in WERI-RB1 cells 18-hours after transduction by SMART-RB virus (10^6 IU/mL; MOI, 1.0). No loss of tumor cell morphology was detected up to this time point. (**G**) The efficiency of transduction of retinoblastoma cell lines by SCAβ virus was determined by X-Gal staining. Transduction of tumor relative to control cells (BHK-21) was estimated by comparing the fraction of blue cells (positive) in tumor and BHK-21 cells; the relative transduction efficiency is denoted by ++++, 1:1; +++, 1:2; ++, 1:5; and +, 1:10.

approach is useful for rapid production of small amounts of protein. After IMAC and Coomassie staining, the eluate (Fig. 3C, lane 1) contained two unidentified bands at 50 to 60 kDa, and a ~110-kDa band that was confirmed to be FLAG-HIS$_{10}$-RB by anti-RB Western blot (Fig. 3C, lane 2). The total yield of RB was 8 pg per transfected HEK-293 cell with recovery of 1.36 pg per cell after purification (17%; Table 2).

In a second approach, we transduced BHK-21 cells with SMART-RB virus and affinity-purified FLAG-HIS$_{10}$-RB on an anti-FLAG antibody column (Fig 3D). The yield of protein per cell was similar whether RB was purified from transfected HEK-293 cells using IMAC (as described earlier) or transduced BHK-21 cells, using anti-FLAG affinity chromatography (data not shown).

Binding of E2F1 to RB Isolated from pSMART-RB–Transfected Cells

A properly folded A/B pocket is essential for interaction of RB with many of its partners, such as the transcription factor, E2F1. Untransfected or pSMART-RB–transfected Phoenix-Eco cells were lysed and subject to IMAC. A pull-down assay showed that purified RB interacted with GST-E2F1 (Fig. 3E, lane 2), but not GST (Fig. 3E, lane 3). As expected, there was no signal with purified protein from untransfected cells (Fig. 3E, lanes 4, 5). These results demonstrate that purified RB assembled into a complex with E2F1, indicating that correct folding had occurred.[44]

Transduction of Explanted Murine Retina and Retinoblastoma with Recombinant SFV

To determine whether embryonic murine retinal cells were permissive of SFV entry and RNA replication, we exposed retinal explants to virus (Fig. 4A). Retinal explants retain the architectural characteristic of in situ retina for up to 3 weeks.[45,46] Retinas explanted at embryonic day 13.5 (E13.5; $n = 10$) or at birth (P0; $n = 11$) were transduced (Figs. 4B, 4C) with 100 μL of 10^4, 10^5, or 10^6 IU/mL SCAβ virus produced in the clone 1/10 BHK-21 packaging cell line. β-Galactosidase activity was detected as early as 16 hours and is shown at 24 hours after transduction (Figs. 4B, 4C). The highest transduction rates for E13.5 and P0 retinas were obtained at the highest viral titer, 10^6 IU/mL. There were no noticeable histologic abnormalities of the transduced retinas at 24 hours (Figs. 4D, 4E), in comparison with untransduced explants (not shown). Both E13.5- and P0-transduced retinas showed recombinant protein production in the outer layer, which was in direct contact with the virus, facilitating efficient transduction (Figs. 4D, 4E). Inner retinal layers were not transduced, perhaps due to either the inability of virus to penetrate the retina or through depletion of virions by cells nearer the membrane. P0 retinas appeared morphologically intact for 5 days after transduction (data not shown).

We assessed the ability of SFV to transduce a broad range of retinoblastoma tumor cell lines. To assess the impact on cell morphology during the early stage of SFV transduction in RB tumor cells, we transduced WERI-RB1 cells with SMART-RB virus and detected RB at 18 hours, with no detectable change in cell morphology (Fig. 4F). Transduction of seven retinoblastoma cell lines (Fig. 4G) with SCAβ virus (MOI 1.0) resulted in an up to 50% transduction efficiency and 2 to 10 pg of β-galactosidase per cell at 24 hours (Fig. 5A). These results were comparable to protein production in SFV-transduced BHK-21 cells.[28]

To assess the effect of SCAβ virus on viability, three retinoblastoma cell lines (RB529, RB247c, and RB1021) were transduced at an MOI of 1.0, and cells were harvested after 3, 6, 12, and 24 hours and assessed for β-galactosidase activity and viability by trypan blue exclusion. The maximum β-galactosidase activity occurred at 12 hours (Fig. 5B). By 24 hours, there was a ninefold drop in the number of viable cells (Fig. 5C).

Discussion

We describe the first purification of full-length functional RB from mammalian cells and a novel method of transducing retina and retinoblastoma cells. Purification of RB was accomplished using an improved SFV vector system (pSMART) that simplified vector construction, enhanced expression, and exploited an optimal producer/target cell line combination. There are several advantages of using SFV to purify a protein of interest. First, virus production is rapid, especially with a DNA-based system, which facilitates the production of many versions of mutated proteins for functional studies. Second, the transduced viral genome is RNA, and expression is therefore rapid and does not necessitate nuclear targeting or integration

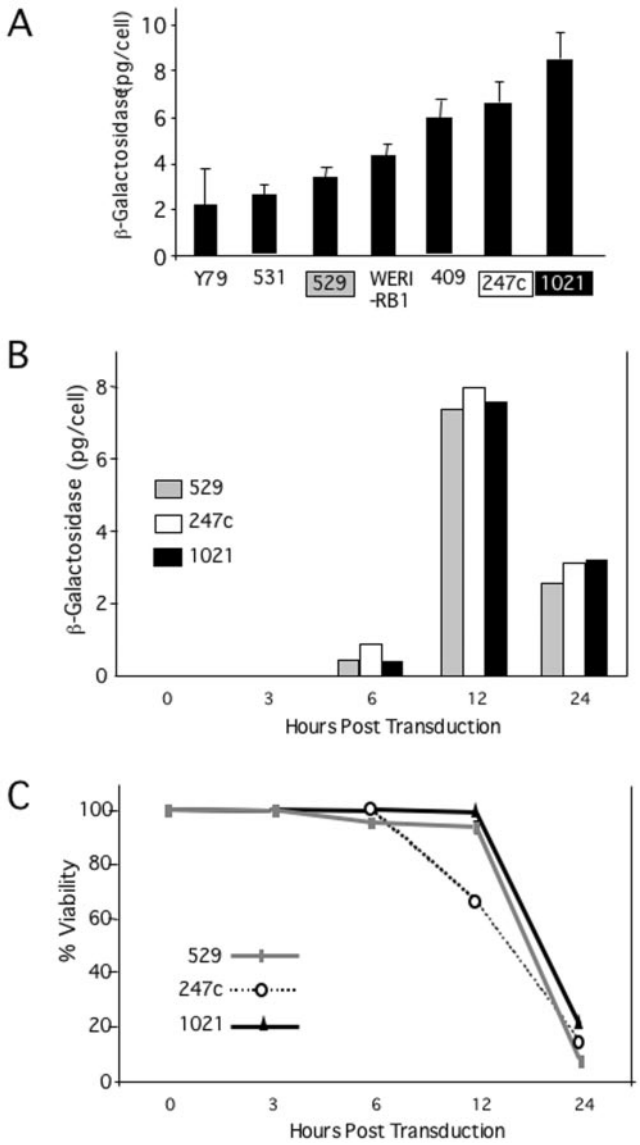

FIGURE 5. SFV transduction decreased retinoblastoma tumor cell viability. (**A**) SCAβ virus (10^5 IU; MOI, 1.0) transduced retinoblastoma cell lines. β-Galactosidase per cell was assayed at 18 hours with an ONPG assay. (**B**) Plates of three retinoblastoma cell lines containing 5×10^5 cells each were transduced with SCAβ virus (5×10^5 IU; MOI, 1.0) in triplicate and the total β-galactosidase produced per cell was determined. (**C**) retinoblastoma cell viability was determined by counting live cells that excluded trypan blue, normalized against the untransduced control.

into chromosomal DNA. Third, the self-amplifying viral genome ensures efficient protein production. Typically, we obtained 3×10^6 IU of virus from one 6-cm transfected plate of HEK-293 cells. By passaging the virus through the helper line (clone 1/10) we could amplify viral yield 100-fold, which would be sufficient to produce approximately 2.5 mg of purified recombinant β-galactosidase or 0.4 mg of purified RB. The high-level expression and the multiple epitope tags facilitates the purification and identification of protein complexes from transduced cells. Fourth, significant amounts of protein can be produced simply by transfecting the DNA-based vectors without the need to generate virus. We generated ~1 μg (9 micromoles) affinity-purified RB from a single transfected 10-cm plate of Phoenix-Eco cells, which is sufficient for multiple in vitro assays of RB transcriptional activity.[47]

Our purpose in building the SMART-RB vector was to produce large amounts of RB for biochemical studies and to study the function of RB in retinal development. Because SMART virus–transduced retina and retinoblastoma cells, the vector could be used to target RB and other proteins to the retina to study biological function and to purify binding partners in these cell types. RB-associated proteins have been identified in many cell types, but there have been no attempts to determine RB partners in retinal cells

Several groups have used virus generated from our original vector, pSCA1, for short-term studies in neurons.[48–50] Although a more thorough analysis is needed, we did not note any obvious toxicity several days after transduction of developing retina, and similar results have been reported in the adult hippocampus.[25,26] Less cytotoxic alphavirus variants could be exploited if the unmodified viral genome impaired retinal cell survival and/or function.[51–53] Cytotoxicity would not be an issue in short-term assays, such as those designed for purification of recombinant protein or identification of binding partners.

SFV induces apoptosis in cultured cell lines,[23,24] a finding we verified in retinoblastoma cell lines. Typical of other cell types, apoptosis was observed within 24 hours of transduction. The toxicity of SFV in tumor or immortalized lines, but not in normal cells, suggests that it might have value as a cancer therapy. Moreover, Tseng et al.[54,55] recently showed that another alphavirus can clear a variety of primary and metastatic tumors in vivo.

We do not anticipate that the vectors we developed will be used in gene therapy for intraocular retinoblastoma in humans. First, it is generally not considered safe to enter an eye with active retinoblastoma to inject any therapeutic agent. Rescue of the child in the event of dissemination of tumor is not yet certain. Second, the object of therapy is to kill the tumor cells. It is extremely unlikely that the RB gene would succeed in controlling a tumor that has already accumulated many subsequent mutations.[56–59] The speed, simplicity, and high level of expression of abundant, soluble, well-folded protein in a diverse range of mammalian cells, make the SMART system particularly suitable for functional and structural analyses.

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