A Herpes Simplex Virus Vector System for Expression of Complex Cellular cDNA Libraries[▽]

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Viral vector-based gene expression libraries from normal or diseased tissues offer opportunities to interrogate cellular functions that influence or participate directly in specific biological processes. Here we report the creation and characterization of a herpes simplex virus (HSV)-based expression library consisting of cDNAs derived from PC12 pheochromocytoma cells. A replication-defective HSV vector backbone was engineered to contain both a bacterial artificial chromosome (BAC) and the Invitrogen *in vitro* Gateway recombination system, creating DBAC-GW. A cDNA library was produced and transferred into the DBAC-GW genome by *in vitro* recombination and selection in bacteria to produce DBAC-L. DBAC-L contained at least 15,000 unique cDNAs, as shown by DNA array analysis of PCR-amplified cDNA inserts, representing a wide range of cancer- and neuron-related cellular functions. Transfection of the recombinant DBAC-L DNA into complementing animal cells produced more than 1 million DBAC-L virus particles representing the library genes. By microarray analysis of vector-infected cells, we observed that individual members of this vector population expressed unique PC12 cDNA-derived mRNA, demonstrating the power of this system to transfer and express a variety of gene activities. We discuss the potential utility of this and similarly derived expression libraries for genome-wide approaches to identify cellular functions that participate in complex host-pathogen interactions or processes related to disease and to cell growth and development.

It is well recognized that genome-wide analysis of functions that influence cellular processes is needed to fully appreciate the complexity of tissue development, the establishment and progression of malignancy, and the transcriptional and translational control of cell-cell interactions, cell signaling, and cell growth. The development of unbiased probes coupled to genetic screening methods is essential for the interrogation of cellular environments where multiple biological events are occurring simultaneously. For example, we previously reported the use of replication-defective herpes simplex virus type 1 (HSV-1) vectors that express the TRPV1 capsaicin-sensitive calcium channel as a means to search for genes that modulate channel activity (19). Similar selection schemes can be designed for the identification of other cellular gene functions, and HSV vectors that express complex libraries of cellular genes would provide a key tool to implement these strategies.

A vector suitable for expression libraries must be flexible and able to accommodate a wide array of genes without significant bias, including large sequences, and its gene transfer and expression must be efficient and robust in a broad range of cell types and tissues. A gene expression library that maintains complexity must be generated, and a screen must be developed in order to rapidly and easily identify genes with specific bio-

logical activities. The identification of such products can be a daunting task if standard methods such as transfection of cDNA plasmids or retrovirus infection are used since, in the first case, limiting dilution experiments are required to identify genes from pools and, in the second case, cell lines must be isolated and the transgene characterized by PCR analysis or additional cloning (1, 10, 11, 22). Several lentivirus-based libraries have been generated and used for the identification of genes that prevent HIV-induced cytopathic effects (9) or induce hematopoiesis in embryonic stem (ES) cells (14). In the first case, the lentivirus library was generated with minimal loss of library complexity, but in the latter, only 60% of the clones contained full-length genes. As with retrovirus-based libraries, cDNAs with desired functions must be isolated from host chromosomal DNA. DNA viruses, such as adenovirus (8) or adeno-associated virus (AAV) vectors, demonstrate low transduction efficiencies, as these viruses often require high multiplicities to achieve efficient transduction rates and some systems require helper virus, which may hinder downstream selection schemes. AAV and retroviruses also have a more limited transgene capacity, eliminating larger genes from the pool and preventing the use of multiple transgene systems.

HSV possesses a large number of accessory genes that can be replaced with foreign DNA, has a very broad host range, infects cells efficiently, and is capable of vigorous transgene expression which can be prolonged in the nervous system (6, 7). There are many advantages to using HSV as a gene delivery vector, including the ability to generate high-titer purified stocks in the absence of helper/packaging virus, and the HSV genome does not integrate into the host chromosomal DNA, thereby reducing the risk of altered endogenous gene expression, activation of oncogenes, or host gene silencing, including tumor suppressor genes. The use of HSV vectors for the

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screening stage has a number of advantages, including the ability to create HSV vector cDNA libraries for testing in high-throughput screens where virus plaquing will facilitate the capture of relevant genes. These attributes, combined with the recent availability of methods to rapidly and accurately modify large sequences, such as the 152-kb HSV-1 genome, and the ability to recover these engineered sequences as functional units in animal cells, make the HSV vector a promising new library expression system.

Here we describe the construction of a Gateway-compatible HSV destination vector and its use to rapidly create a viral library expressing a complex array of cellular gene functions. We first recombined a bacterial artificial chromosome (BAC) cassette into an ICP4/ICP27-deleted mutant HSV vector, yielding a recombinant termed DBAC, and recovered the recombinant genome in bacteria. We found that the standard bacterial strain provided by Invitrogen to generate and propagate *ccdB*-containing destination plasmids was inadequate for BAC manipulation, prompting us to derive a *ccdB*-resistant host strain, HerpesHogs, from Invitrogen's BAC-compatible GeneHogs bacteria. HerpesHogs was then used in combination with Red/ET recombineering (GeneBridges) to introduce a Gateway destination cassette flanked by mammalian expression control sequences into the ICP27 locus of DBAC, producing DBAC-GW. We next created a plasmid-based Gateway entry cDNA library derived from a mixture of undifferentiated and differentiated PC12 cells and recombined this library into DBAC-GW to generate a viral genome-based cDNA expression library referred to as DBAC-L. DNA array analysis of en masse-amplified cDNA inserts indicated that DBAC-L contained sequences representing a minimum of 15,000 unique genes, including many of known functions related to a broad array of cellular processes. Recombinant virus particles carrying the cDNA library were produced by DBAC-L transfection of a mammalian cell line engineered to complement the essential ICP4 and ICP27 genes deleted from the vector backbone. The virus particles were capable of efficient delivery and robust expression of the gene library in tissue culture cells. HSV-based cDNA expression libraries are adaptable to a variety of selection protocols on a wide range of cell lines.

MATERIALS AND METHODS

Plasmids and vector construction. The HSV vector D2 was created by genetic cross between the ICP4 deletion mutant virus d120 (5) and an ICP27-deleted virus, 5dl1.2 (16). D2 was identified based on its plaquing dependence on both ICP4 and ICP27 complementation and was confirmed by Southern blot analysis (not shown). BAC elements were engineered into the D2 genome at the thymidine kinase (TK) (*U_L23*) locus by homologous recombination in 7b cells and selection for ganciclovir resistance. The targeting plasmid for recombination was generated by insertion of the bacterial origin of replication and Cm resistance gene from pBeloBACII (New England Biolabs, Ipswich, MA) into the TK coding sequence of a *U_L23* plasmid. The genome of a purified D2/BAC recombinant was circularized by infecting U2OS cells at a multiplicity of infection (MOI) of 5 for 3 h, and DNA was isolated by proteinase K digestion, phenol-chloroform extraction using PhaseLock gel (5Prime, Gaithersburg, MD), and isopropanol precipitation. The DNA was electroporated into GeneHogs bacteria (Invitrogen, Carlsbad, CA) at 2.0 kV, 200 Ω , and 25 μ F in a 0.2-cm cuvette, and BAC DNA (DBAC) was purified from Cm-resistant bacteria using a large-construct DNA purification kit (Qiagen, Valencia, CA) with exonuclease digestion.

The Gateway cassette from Gateway conversion plasmid A (Invitrogen) was modified for this work in the following manner. First, the EcoRV fragment from plasmid A was cloned into the EcoRV site of pSP72 to generate p72GateA. p72GateA was modified to replace the Cm resistance gene in the Gateway

cassette with phleomycin D (Zeocin [Zeo]) resistance by isolating and end filling an XhoI-EcoRI fragment containing the Zeo coding sequence from pEM7/Zeo (Invitrogen) and subcloning between the blunted NotI and MluI sites of p72GateA. Recombinants were screened for insertion in the opposite orientation to that of the *ccdB* gene to obtain plasmid p72GateAZ1. The NheI site between the *attR1* and Zeo^r sequences was converted to a PmeI site by linker ligation to create plasmid pBZPme3. The modified Gateway cassette was isolated from pBZPme3 as a 1.6-kb EcoRV fragment containing *attR1*, a PmeI site, Zeo^r and *ccdB* genes, and *attR2*.

An ICP27-targeting plasmid containing the modified Gateway cassette was constructed in several steps. First, plasmid pHGatePme was created by cloning the EcoRV fragment from plasmid pBZPme3 into the PmeI site of plasmid pPme2 between the HCMV promoter and the simian virus 40 (SV40) poly(A) region. Plasmid pPme2 was derived from pEGFP-N1 (Clontech, Palo Alto, CA) by BamHI-BglII collapse, conversion of SspI to BglII and AseI to BglII by linker insertions, and replacement of the AgeI-NotI fragment with a PmeI linker. The modified Gateway cassette, along with the upstream promoter and downstream poly(A) region, was isolated from pHGatePme as a 2.3-kb BglII fragment and inserted into the BamHI site of plasmid pPXE to create the ICP27-targeting plasmid pPXE-HGate. Plasmid pPXE is pBluescript containing *U_L54* (ICP27) flanks between EcoRI and XbaI restriction sites (18). Functionality of pPXE-HGate with respect to the Gateway cloning system was confirmed using plasmid pENTR-gus and methods provided by Invitrogen.

The Gateway expression cassette from pPXE-HGate was introduced at the DBAC ICP27 locus by Red/ET recombination methods (GeneBridges GmbH, Heidelberg, Germany), as instructed by the manufacturer, in *ccdB*-resistant HerpesHogs bacteria. Recombinants were identified by bacterial selection for zeocin resistance and confirmed by field inversion gel electrophoresis (FIGE) analysis. A representative recombinant was designated DBAC-GW.

Generation of HerpesHogs. In order to generate a *ccdB*-resistant bacterial strain with optimal characteristics for BAC DNA propagation and purification, we initially introduced the *ccdB*-containing plasmid pENTR-1A (Invitrogen) into GeneHogs bacteria (Invitrogen). Transformation of GeneHogs with pENTR-1A resulted in low numbers of kanamycin (Kan)-resistant colonies. Restriction analysis of plasmids from these colonies revealed three types of plasmids: those that were increased in size, those that were similar in size to pENTR-1A but displayed rearrangements, and approximately 10% that were similar to pENTR-1A. Further analysis of the plasmids of increased size identified a hot spot for transposon insertion within the *ccdB* gene inactivating the gene and thus allowing the plasmid to confer Kan resistance without a functional *ccdB* gene. The rearranged plasmids purified from pENTR-1A-transformed GeneHogs revealed a loss of *ccdB* function in each of 24 cases, as demonstrated by their ability to efficiently transform *ccdB*-sensitive DH5 α bacteria to Kan^r. A total of 48 plasmids that were indistinguishable from the parental plasmids were similarly assayed and yielded two that retained *ccdB* activity. Extensive attempts to cure these two *ccdB*-resistant bacterial strains of the pENTR-1A plasmid were unsuccessful, and we therefore developed the alternative strategy described below.

Plasmid pETRS was created by inserting the Cm and *sacB* genes into pENTR-1A, providing additional markers for positive (Cm^r) and negative (5% sucrose sensitivity) selection, respectively. We mutagenized GeneHogs bacteria overnight with 10 μ g/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Sigma-Aldrich, St. Louis, MO), transformed the cells by electroporation with pETRS, and selected the bacteria for Cm resistance. Mutagenesis increased the transformation efficiency, presumably due to increased *ccdB* resistance frequency, by approximately 500-fold (D. Wolfe, unpublished observation). Colonies that tested positive for Kan resistance were then cured of the plasmid by selection on 5% sucrose. These cured strains were confirmed for sensitivity to Cm and Kan and then tested for *ccdB* resistance by transformation with pENTR-1A. The majority of the sucrose-selected colonies yielded abundant Kan-resistant transformants indicative of the acquisition of bacterial genome-borne *ccdB* resistance.

Bacterial growth and selection. Yeast tryptone (YT) medium (2 $\times$) was used for growth of bacterial strains in liquid culture. Luria-Bertani (LB) broth with 1.3% agar was used for growth on solid support. Selective agents included ampicillin (200 μ g/ml), chloramphenicol (12.4 μ g/ml), kanamycin (40 μ g/ml), zeocin (25 μ g/ml), sucrose (5% [wt/vol]), and tetracycline (3 μ g/ml). L-Arabinose (10% [wt/vol]) was used for induction of the pBAD promoter in the pRedET plasmid (GeneBridges). All bacterial strains were grown at 37°C except while performing GeneBridges recombineering, when 30°C was used to maintain the Red/ET temperature-sensitive Ori plasmid.

cDNA library construction in entry clones. mRNA isolated from equal numbers of undifferentiated and nerve growth factor (NGF)-differentiated PC12 cells was converted to a cDNA library in a Gateway-compatible plasmid using the CloneMiner cDNA library construction kit (Invitrogen) as follows. Purified

mRNA was reverse transcribed using SuperScriptII and a 5'biotin-*attB2*-oligo(dT) primer. After second-strand cDNA synthesis with *Escherichia coli* DNA polymerase I, T4 DNA polymerase was used to fill in the cDNA ends, and the products were collected by phenol-chloroform extraction and ethanol precipitation. A double-stranded *attB1* adaptor was ligated to the 5' end of the double-stranded cDNA with T4 DNA ligase at 16°C overnight. To favor the cloning of large inserts, the cDNA pool was size fractionated using a Sephacryl S-500 HR column. Adjacent fractions were combined and precipitated with ethanol. This final cDNA pool contained 5' *attB1* and 3' *attB2* linkers to allow for directional transfer into pDONR222 (Invitrogen), a plasmid containing Cm^r and *ccdB* selectable marker genes between *attP1* and *attP2* sites and Kan^r in the plasmid backbone.

Transfer of the cDNA into the donor plasmid was performed by incubating 250 ng pDONR222 and 150 ng of *attB*-flanked cDNA with BP Clonase enzyme mix (Invitrogen) at 25°C for 16 to 20 h. Following proteinase K-mediated inactivation of the enzyme mix, DNA was ethanol precipitated and electroporated into *ccdB*-sensitive *E. coli* DH10B. Electroporation parameters were 2.0 kV, 200 Ω, and 25 μF. The BP Clonase reaction promotes specific recombination between *attB1* and *attP1* sites and between *attB2* and *attP2* sites to create plasmid-based cDNA libraries flanked by 5' *attL1* and 3' *attL2* sites for subsequent LR Clonase-mediated transfer into the DBAC-GW destination vector. Plasmid DNA was isolated from 22 random Kan-resistant colonies and digested with BsrGI, which cuts in *attL* sites, to examine the efficiency of the BP Clonase reaction and the sizes of resulting plasmid inserts. Pooled transformants were grown in Kan-containing liquid media to an optical density at 600 nm (OD₆₀₀) of 1.0.

Library introduction into HSV. The cDNA library was transferred from its plasmid base into the DBAC-GW genome by LR Clonase (Invitrogen)-catalyzed recombination between the *attL* sites flanking the cDNA library and the *attR* sites flanking the Gateway cassette in the DBAC-GW destination vector, recreating *attB* sites. The products of the LR recombination reaction were used to transform *ccdB*-sensitive DH10B cells, providing selection in the presence of Cm for loss of the *ccdB* gene from DBAC-GW in favor of library cDNAs. DBAC-L library DNA was purified from amplified transformants using a Qiagen large construct kit. To generate infectious virus, purified DBAC-L DNA was transfected into complementing 7b cells and the infection was allowed to progress to 100% cytopathic effect. Cells and supernatant were treated with NaCl (0.45 M final concentration), and the liquid fraction was aliquoted and stored at -80°C.

PCR. cDNA inserts were amplified from the plasmid-based entry library or the DBAC-L library using primers specific for the human cytomegalovirus (HCMV) promoter (GCGTGTACGGTGGGAGGTCTAT) and the SV40 polyadenylation region (GGGGAGGTGTGGGAGGTTTT) using Accuprime *Taq* polymerase (Invitrogen). The reactions were performed by a Bio-Rad iCycler IQ programmed as follows: 94°C for 3 min, 35 cycles of 94°C for 30 s, 61.7°C for 30 s, and 68°C for 1 or 5 min, and a final incubation at 68°C for 5 min. Approximately 100 ng of template DNA was used for each reaction. PCR products were purified using Qiaquick PCR purification kit (Qiagen) with an additional 80% ethanol wash prior to elution.

Microarray analysis of library inserts. PCR products from each of four individual 1-min extension reactions were combined with PCR products generated by four 5-min extension reactions (see Fig. 3A) to create four pseudo-biological replicates. Approximately 10 μg of DNA was chemically labeled at guanine residues with Cy3 using the ULS labeling kit for Agilent gene expression arrays (catalog EA-023; Kretech Biotechnology, Netherlands) according to the manufacturer's instructions with the following modifications: 10 μg of DNA was combined with 10 μl of Cy3 in a 50-μl reaction volume. Following the labeling reaction, each 50-μl reaction was purified on one Kreapure column according to the provided protocol. The labeled cDNAs were stored at -20°C in the dark prior to shipment to Cogenics, Inc. (Morrisville, NC), where the cDNA was fragmented and 3.3 μg of each labeled product was hybridized to an Agilent whole-rat genome oligonucleotide microarray in 4 × 44K format array. Hybridization, washing, staining, and scanning were conducted using established procedures at Cogenics.

A given feature (probe) on the microarray was determined to be "well above background" if the measured mean signal intensity for the given feature was significantly greater than the value for the corresponding background based on a two-sided *t* test and the background-subtracted signal for the feature was greater than 2.6 times the standard deviation of the measured background level. This approach enabled an array-by-array as well as a feature-by-feature determination of whether a given transcript was measured as "detected" in each sample for all of the noncontrol probes present on the array. The results were tabulated in order to determine which transcripts were identified as "detected"

in all four samples, three out of four samples, two out of four samples, and one out of four samples, as well as not detected in any sample.

Gene expression microarray analysis. Vero cells (2 × 10⁶) were infected in duplicate with either the parental DBAC-GW vector, a characterized mix of 5 DBAC-L vectors (Mix5), or a mix of 100 randomly chosen DBAC-L viruses (Mix100) at a total MOI of 10. One day postinfection, total RNA was isolated using the Qiagen RNeasy kit and shipped to Cogenics for analysis.

Total RNA (500 ng) was converted into labeled cRNA using Cy3-coupled nucleotides and the low-RNA-input linear amplification kit (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instructions. Labeled cRNA samples (1.65 ng) were hybridized to Agilent whole-rat genome oligonucleotide microarrays (Cogenics). Hybridized arrays were washed and scanned, and the data were extracted using Feature Extraction software, version 9.1 (Agilent).

Pearson correlation values were calculated for all four pairs of biological replicate samples using all noncontrol features present on the microarray. A Pearson correlation value of 1.0 signifies perfect correlation, while 0 means no correlation and -1.0 denotes perfect anticorrelation. The replicates for each sample were then combined using an error-weighted average, and these combined profiles were compared to that of DBAC-GW-infected Vero cells in order to identify differentially expressed transcripts (threshold of 1.5-fold difference).

Western blot analysis. Cells of the 7b line were infected at MOIs of 2 or 20 with viral vectors encoding tyrosine hydroxylase (TH) or succinate dehydrogenase subunit D (SDHD) isolated from the DBAC-L pool (unpublished results). Cell lysates from infected cells and PC12 control cells were harvested at 24 h postinfection (hpi) in 1× NuPage LDS buffer (Invitrogen), separated on a 4 to 12% SDS-PAGE gel (NuPage; Invitrogen), and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were reacted with antibodies to TH or SDHD (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase (HRP)-conjugated secondary antibodies.

RESULTS

Generation of HSV BAC. A recombinant HSV-1 vector deleted for ICP4 and ICP27, designated D2, was created by genetic cross between the ICP4 deletion mutant virus d120 (5) and an ICP27-deleted virus, 5dl1.2 (16). In the absence of these viral functions, early and late viral genes are not expressed and the virus fails to replicate its DNA or produce virus particles (13). The recombinant vector was therefore propagated in a cell line, 7b, that complements these functions in *trans* (15). To propagate the viral genome in *E. coli*, a BAC cassette (bacterial origin of replication and chloramphenicol [Cm] resistance gene) was introduced at the thymidine kinase (TK) locus of D2 by cotransfection of 7b cells with viral DNA and a TK-targeting plasmid followed by selection in the presence of ganciclovir; recombinants were confirmed by PCR. The HSV:BAC recombinant virus was used to infect noncomplementing U2OS cells at high MOI to produce circularized HSV genomes. This viral DNA was purified and transferred to BAC-compatible GeneHogs bacteria by electroporation and selection for Cm resistance. DNA preparations from selected colonies were assessed for their ability to produce virus after transfection of complementing cells, and one of these BAC clones, designated DBAC, was used for subsequent engineering steps.

Development of HerpesHogs, a *ccdB*-resistant, BAC-compatible bacterial strain. While Invitrogen's GeneHogs bacteria are suitable for standard BAC transformation and propagation, they are sensitive to the *ccdB* gene present in Gateway destination vectors. Therefore, to introduce a Gateway destination cassette into DBAC, a BAC-compatible, *ccdB*-resistant bacterial strain was needed. Following unsuccessful attempts to generate *ccdB*-resistant GeneHogs bacteria by standard methods (see Materials and Methods), we developed an alternative procedure allowing the rapid and efficient conversion of

FIG. 1. Strategy for isolation of *ccdB*-resistant bacterial strains. Illustration of the strategy utilized to derive the *ccdB*-resistant (*ccdB^R*) strain HerpesHogs from the *ccdB*-sensitive (*ccdB^S*) GeneHogs strain (Invitrogen). The starting population of bacteria was mutagenized overnight with MNNG and electroporated with the pETRS plasmid containing the *ccdB*, *Kan^r*, *Cm^r*, and *sacB* genes. *ccdB*-resistant colonies were identified by selection for resistance to kanamycin and chloramphenicol. The negative selectable marker *sacB* enabled subsequent selection for loss of the pETRS plasmid with 5% sucrose. *ccdB* resistance was confirmed by transformation of pENTR-1A (Invitrogen) carrying the *ccdB* and *Kan^r* genes.

any bacterial strain into a Gateway-compatible strain. We engineered a plasmid, pETRS, for double-positive (resistance to kanamycin [Kan] and Cm), double-negative (sensitivity to sucrose [*sacB*] and *ccdB*) selection and introduced this plasmid into MNNG-mutagenized GeneHogs to obtain *ccdB*-resistant colonies that were subsequently cured of the plasmid by selection on 5% sucrose (Fig. 1). The strain obtained in this manner, termed HerpesHogs, allows for efficient transformation and propagation of large BAC DNAs, is Cm and Kan sensitive, and is Gateway compatible by virtue of its resistance to *ccdB*.

Introduction of a modified Gateway recombination/selection cassette into DBAC. Because the BAC cassette of DBAC contained a Cm resistance gene, we first replaced the Cm gene of Invitrogen's Gateway destination cassette with the zeocin resistance (Zeo) gene. The product was placed between the human cytomegalovirus IE promoter (HCMVp) and the SV40 late polyadenylation region (SV40pA), and the resulting Gateway expression cassette was cloned into the ICP27 deletion plasmid pPXE and transferred into the ICP27 locus of the DBAC genome by arabinose-inducible, Red/ET-mediated recombination (GeneBridges) in HerpesHogs. Following bacterial selection for coresistance to zeocin and chloramphenicol, recombinants were confirmed by restriction enzyme digestion

FIG. 2. Structure of replication-defective Gateway vector DBAC-GW. The schematic depicts the structure of the HSV vector DBAC-GW. Open/shaded boxes represent the terminal and internal repeats of the HSV genome; horizontal lines represent the unique long (*U_L*) and unique short (*U_S*) segments. Black boxes depict functional deletions in the ICP4, ICP27, and *U_L*23 (thymidine kinase [TK]) genes. Bacterial artificial chromosome sequences, including an origin of replication (*Ori*) and chloramphenicol-resistance gene (*Cm*), were recombined into the *U_L*23 locus, and the ICP27 locus was replaced with a Gateway expression cassette consisting of the HCMV promoter (HCMVp), Gateway recombination site *attR1*, zeocin resistance (*Zeo*) and *ccdB* genes, Gateway recombination site *attR2*, and the SV40 polyadenylation region (SV40pA). Arrows illustrate the location of PCR primers used to amplify the cDNAs contained within the Gateway cassette following recombination between the DBAC-GW vector and the plasmid-based cDNA library.

and field inversion gel electrophoresis (FIGE) in comparison with the parental DBAC genome, by sequencing across the recombination junctions, and by plaque formation following transfection into 7b cells. A schematic representing a recombinant vector in the correct orientation, designated DBAC-GW, is shown in Fig. 2.

Functionality of the Gateway cassette was determined by performing *in vitro* recombination reactions between the DBAC-GW destination vector or DBAC DNA as a control and either a Gateway entry plasmid containing actin cDNA or no plasmid. Using optimized conditions for LR Clonase-mediated recombination and bacterial electroporation (see Materials and Methods), control reactions with the Gateway-negative DBAC DNA yielded comparable numbers of colonies on *ccdB*-resistant HerpesHogs (~200,000) and *ccdB*-sensitive GeneHogs (~350,000), regardless of the presence or absence of an entry plasmid. The DBAC-GW reaction with or without an entry plasmid generated approximately 250,000 colonies on HerpesHogs. Without the entry plasmid, the reaction produced 7 colonies on GeneHogs, while inclusion of the entry plasmid produced 18,000 GeneHogs colonies. These data indicated a specific recombination frequency of approximately 4% with very low background (<0.002%), demonstrating the utility of this system for the rapid and efficient introduction of foreign sequences into large BACs.

Generation of a PC12 cDNA library in DBAC-GW. We selected PC12 cells to generate an initial plasmid-based cDNA library because this cell line has attributes of both transformed and neuronal cells and can be differentiated with neurotrophic factors. Using the CloneMiner kit (Invitrogen), we produced a PC12 cDNA library flanked by *attL* recombination sites suitable for recombination into the DBAC-GW vector. First, we combined mRNAs from a mixture of undifferentiated and nerve growth factor (NGF)-differentiated PC12 cells to produce a diverse population of *attB*-flanked cDNAs that was transferred into a "donor" plasmid by *attB/attP* recombination and selection of kanamycin-resistant colonies following trans-

TABLE 1. Insert size and sequence analysis of entry clones

Clone no.	BsrGI size (kb)	Sequence accession no. ^a	Expected size	Full-length status ^b	Gene product; source
1	1.0	BC087725	1.0	Y	Histone H3; 3B rat
2	1.5	NM_212496	3.3	N	DEAH box RNA helicase (3' half); rat
3	1.2	BC098059	1.2	Y	Meiotic recombination homologue REC14; rat
4	1.6	NM_031595	1.6	Y	Proteasome 26S subunit Psmc3; rat
5	2.8	XM_215405	2.1	Y	5' nucleotidase, cyto II Nt5c211; rat
6	2.1	NM_175843	2.1	Y	Binds PKC ζ and GABAc receptor; mouse sequestrome (93%)
7	3.5	BC018491	3.6	Y	Transmembrane protein 30A; mammary tumor
8	2.5	NM_053642	2.2	Y	Sterol-C5-desaturase (3' half); rat
9 ^c	0.3				
10	2.5	BC055809	1.9	Y	Chromatin modifying protein 2B; mouse (93%)
11	1.3	NM_177613	1.3	Y	Cell division cycle cdc34 variant; mouse (96%)
12	3.4	NM_198052	5.0	N	T-box 3 variant 2 Tbx3- cardiac differentiation; mouse (93%); unknown full-length
13	1.7	NM_012545	5.1	Y	Dopa decarboxylase Dcd; rat (5' mRNA variant)
14	3.0	AK087802	1.8	Y	Ubiquitin conjugating enzyme binding/d10 cerebellum; mouse (94%)
15	1.6	X05341	1.6	Y	3-Oxoacyl-CoA thiolase; rat
16	2.4	NM_001007625	2.2	Y	Ependymin related protein Epr2/elevated in colorectal cancer; rat
17	0.9	XM_344286	1.3	Y	DnaJ subfamily C (Hsp40); rat
18	2.4	XM_576107	2.1	Y	Hematopoietic progenitor protein homologue; rat
19	4.7	NM_001011909	1.4	Y	Gpatch protein (Gpatch2); rat
20	2.4	BC083796	2.2	Y	Protein kinase C binding protein Precbp1; rat
21	3.9	XM_342595	1.5	Y	Syntaxin 16 (imprint control) deletion in autosomal dominant pseudohypoparathyroidism
22	6.5	NM_019827	8.3	N	Glycogen synthase Gsk3b (3' half); mouse (92%)
23	2.5	<i>E. coli</i> Tet ^r	2.5	Y	Positive control

^a Data banks correspond to accession numbers as follows: BC, GenBank; NM, RefSeq; XM, RefSeq; AK, DNA Data Base of Japan; RN, GenBank.

^b Y, full-length; N, not full-length.

^c Reaction failed.

formation of *ccdB*-sensitive DH5 α bacteria. Approximately 20 million initial plasmid (entry) clones were produced, as assessed by limiting dilution analysis of transformants. The cDNA inserts, now flanked by *attL* sites, from 22 randomly picked plasmids were examined by DNA sequence analysis, revealing the presence of 21 unique cDNAs with a size range of 0.9 to 6.5 kb (Table 1), all in the same orientation, as expected from this directional cloning strategy; one sequencing reaction failed. The majority of these cDNAs (18/21) were full-length, demonstrating the creation of an exceptionally high-quality cDNA entry library.

In order to introduce the plasmid-based entry library into the DBAC-GW backbone, the host cells carrying the plasmid library were grown en masse in liquid culture and harvested after 8 h at 37°C to minimize size-based bias due to differential rates of plasmid amplification, and total plasmid DNA was purified using a Qiagen MaxiPrep kit. Following optimization of BAC DNA preparation and Gateway *in vitro* recombination conditions (see Materials and Methods), the library was transferred into DBAC-GW by LR Clonase (Invitrogen)-mediated *attL/attR* directional recombination. The reaction products were introduced into *ccdB*-sensitive DH10B bacteria by electroporation, cultures were grown without selection for 1 h and then in Cm-containing media for 14 h, and total BAC DNA was isolated to obtain a pure DBAC-GW-based cDNA library designated DBAC-L.

The quality of the DBAC-L library was initially assessed by direct sequencing of the Gateway inserts in five randomly chosen DBAC-L clones using primers specific for the flanking HCMVp and SV40pA regions (see Fig. 2). These clones were found to contain full-length cDNAs for prefoldin-5, brix

domain containing 2 (Bxdc2), dynactin 2a, a coiled-coil containing protein similar to myosin (RGD1306908), and an unknown sequence similar to amino acid aminotransferases (LOC363056, XM_343384).

The number of unique genes contained in the DBAC-L cDNA library was estimated by whole-rat genome microarray analysis of en masse PCR-amplified cDNA inserts (size range, 200 bp to >5 kb [Fig. 3A]). A total of 17,671 probes were detected in each of four replicate samples, and an additional 1,989 probes were detected in three out of four replicate samples (Table 2). Analysis of the 17,671 probes using Ingenuity Pathways Analysis software (Ingenuity Systems) showed representation in the DBAC-L library of a diverse range of cellular functions (Fig. 4).

The five DBAC-L clones with known cDNA inserts were used to assess the ability of the vector backbone to express cDNAs at elevated levels compared to those of endogenous cellular genes. First, the purified BAC DNAs were transfected into complementing 7b cells to produce infectious virus particles. Noncomplementing Vero cells were then infected with a mixture of equal amounts of the five viruses (Mix5) at a total MOI of 10 such that each cell would be infected with all five replication-defective vectors. At 24 hpi, mRNA was harvested from infected cells and converted to Cy3-labeled cRNA to probe a whole-rat genome oligonucleotide microarray. Compared to the expression profile of cells infected with the parental DBAC-GW, cells infected with Mix5 demonstrated increased levels of the transcripts specified by the five vectors (4- to 100-fold upregulation) (Table 3 and Fig. 5A). Analysis of duplicate samples demonstrated >96% correlation (Table 4), validating the use of microarrays to examine insert expression

FIG. 3. PCR amplification of DBAC-L cDNA inserts demonstrates library diversity. (A) cDNA inserts were amplified from DBAC-L genomic DNA, purified from *E. coli*, using primers specific for the HCMV promoter and SV40 poly(A) sequences. Extension times for PCRs varied from 1 min (lanes 1 to 4) to 5 min (lanes 6 to 9). The reaction products were separated on a 1% agarose gel, revealing cDNAs of various sizes. Lane 11, size markers; arrows indicate approximate fragment sizes in kilobases (kb). (B) Distinct cDNAs were amplified from total DNA harvested from 2×10^6 7b cells infected with the DBAC-L vector stock at an MOI of 0.05. Viral and cellular DNA was harvested at 24 hpi and eluted in 250 μ l TE. Serial 10-fold dilutions of total DNA (lanes 1 to 5) were used as template DNA for PCR, such that one microliter (lane 1) represented approximately 400 input PFU. Lane 6, water control; lane 7, size markers. (C) Ten individual PCRs each using 10^{-4} μ l DBAC-L viral DNA demonstrated the presence of unique cDNA insert sizes.

from mixed library vectors. In addition, we used Western blot analysis to examine vector-directed expression of cDNA inserts identified in two separately characterized DBAC-L isolates, DBAC-TH and DBAC-SDHD, encoding tyrosine hydroxylase (TH) and succinate dehydrogenase subunit D (SDHD), respectively. As shown in Fig. 5C, Vero cells infected at an MOI of 2 or 20 with either DBAC-TH or DBAC-SDHD showed abundant expression of the corresponding proteins compared to that of the control vector. Together, these data demonstrated the ability of the vector backbone to express library-derived cDNA inserts in the absence of virus replication at levels clearly above those of cellular messages.

Generation and characterization of an infectious viral library. To generate a stock of infectious library virus particles, DBAC-L DNA was transfected en masse into complementing 7b cells to produce approximately 1 million plaques. Virus was collected from the media and amplified on 7b cells to yield a total of 1×10^8 PFU. The pooled virus stock was examined for cDNA insert size diversity by infection of 7b cells at low multiplicity (MOI of 0.05, $\sim 1 \times 10^5$ PFU), collection of cellular and viral DNA at 24 hpi, and PCR analysis of serial 10-fold dilutions of the DNA preparation using HCMVp and SV40pA primers. Individual insert sizes were distinguishable at a dilution corresponding to a virus input of approximately 0.4 PFU (Fig. 3B). A similar PCR analysis of multiple individual 10-fold-higher dilutions showed a variety of distinct band sizes (Fig. 3C). These analyses demonstrated the presence of unique

cDNA inserts after conversion of the DBAC-L DNA into infectious virus particles.

To explore the ability of the library vectors to express a variety of cDNAs following high-MOI infection, a subpopulation of DBAC-L virus particles, designated Mix100, was generated by expanding 100 random isolates from the original DBAC-L virus stock on 7b cells. Vero cells were then infected with the Mix100 stock at a total MOI of 10 (individual vector MOI of 0.1), and mRNA extracted the following day was converted to labeled cRNA for oligonucleotide array analysis, as before. A log-ratio plot comparing the Mix100 infection and the control vector infection showed a greater number of up-regulated genes than seen previously for the Mix5 infection (295 versus 16) (Table 4 and Fig. 5B). Notably, TH and SDHD transcripts were upregulated more than 5-fold (Table 3), consistent with the subsequent isolation of DBAC-TH and DBAC-SDHD from the Mix100 vector pool. A similar analysis on a mixture of 1,000 random DBAC-L PFU (Mix1000) yielded comparable results, with a greater number of upregulated genes but a lower level of upregulation of each individual gene (data not shown). These analyses suggested that the viral library efficiently delivered and expressed a pool of random, unknown cDNAs whose transcripts can be detected. Moreover, since certain genes were downregulated and the number of upregulated genes exceeded the number of different cDNAs introduced in each infection (Table 4), it is clear that vector-derived cDNAs affected the expression of cellular genes as early as the 24-h time point of these analyses, suggesting the production of biologically active proteins from the library cDNAs.

DISCUSSION

Herpes simplex virus has been extensively engineered as a delivery vehicle for introducing genes into various cells and tissues. HSV has a large transgene capacity (12), transduces most cell types with high efficiency (4, 13), and can be retargeted for selective infection of cells bearing specific receptors (17, 23). The availability of large expression libraries in vectors capable of efficient gene delivery and expression in a broad range of cell types provides an opportunity to develop methods to select or screen for functions that affect a variety of cellular processes. The use of HSV vectors has a number of advantages, including the ability to discover gene functions in high-throughput selections where conditional virus plaquing enables

TABLE 2. Number of probes detected on the Agilent whole-rat gene expression array

No. of samples in which probe was detected ^a	No of probes measured as detected ^b	Intensity range ^c
4	17,671	8,116.8–658,704.5
3	1,989	6,110.5–45,379.1
2	837	4,887.6–18,926.3
1	791	2,845.8–10,051.6
0	22,091	1,506.1–8,181.0

^a Of 4 total samples.

^b Of a total of 43,379 noncontrol probes.

^c Intensity equals background minus subtracted mean fluorescent intensity for a given feature (probe) on the microarray.

FIG. 4. Functions of top 1,500 genes. Ingenuity Pathways Analysis software was used to classify the cDNAs identified by rat whole-genome analysis of the DBAC-L library inserts. Genes from the data set that met the intensity cutoff of 100,000 and were associated with biological functions, pathways, or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Those that were most significant to the data set are shown. (A) The top 30 functional categories with the lowest *P* values containing at least 100 molecules (*P* < 0.0003) and the number of associated genes are depicted; some molecules belong to more than one functional category. (B) Functional analysis of 322 total molecules related to cellular growth. (C) Functional analysis of 609 total molecules related to cellular signaling.

the capture of relevant genes. Additionally, high-throughput screens of a more traditional nature can be combined with these virus-delivered cDNA libraries to increase efficiency, and the variety of drug targets can be expanded by the simultaneous expression of a specific receptor from the library vector.

Vectors identified by either method can be directly used for downstream experimentation, including *in vivo* gene transfer.

HSV provides a particularly attractive system for modifying the behavior of the nervous system since it is within neurons that the virus naturally establishes long-term persistence in the form of latency. Replication-defective derivatives are uniquely capable of establishing persistent infection and can be engineered for long-term expression in the nervous system (7, 21). Moreover, we have reported the development of vectors that can express transgenes in highly sensitive cells, such as embryonic stem cells, without altering cell viability or interfering with cell differentiation processes (4), and we have developed a conditional plaquing strategy to select for genes that may reduce the initiation of pain pathways (19, 20).

Here we describe the development of a replication-defective HSV vector genome containing a BAC sequence and engineered to harbor a library of cDNAs derived from neuron-like PC12 cells. BACs provide an ideal means for manipulation of the vector genome, since any change can be rapidly introduced

TABLE 3. Fold increase in transcripts expressed from identified DBAC-L vectors in Mix5- and Mix100-infected Vero cells		
DBAC-L cDNA	Fold increase in transcripts ^a	
	Mix5	Mix100
Prefoldin-5	100	1.94
Brix domain containing 2 (Bxdc2)	100	N/A
LOC363056, XM_343384	3.80	N/A
Coiled-coil-containing protein RGD1306908	100	N/A
Dynactin 2a	9.48	N/A
Tyrosine hydroxylase	N/A	8.75
Succinate dehydrogenase D	N/A	40.9

^a N/A, not applicable.

FIG. 5. Expression analysis of DBAC-L at the RNA and protein levels. (A and B) Microarray analysis was used to assess gene expression in Vero cells infected with DBAC-L vectors. Log-ratio plots depict differential gene expression in cells infected with a mix of 5 known (A) or 100 unknown (B) DBAC-L vectors compared to cells infected with the parental DBAC-GW vector. The *x* axis depicts the combined fluorescent intensity measured on the microarray, while the *y* axis depicts the log ratio from the comparison. Each transcript is represented as a plus sign. The colors are based on the log-ratio *P* value. Red denotes transcripts that are significantly upregulated by at least 1.5-fold compared to the control (significance is defined as a log-ratio *P* value below 0.001), green indicates transcripts that are significantly downregulated by 1.5-fold or more compared to the control, and blue shows transcripts that are not differentially expressed. In panel A, transcripts derived from three of the five Mix5 vectors (prefoldin, Bxdc2, and RGD1306908) are readily identified (circled) by their high level of upregulation (100-fold) (Table 3). (C) Complementing 7b cells were infected with either DBAC-TH (top) or DBAC-SDHD (bottom) at MOIs of 2 or 20 or with control vector (C) at an MOI of 1. Uninfected PC12 cells (P) were used as a control for endogenous gene expression. Western blot analyses with antibodies specific for TH (top panel) or SDHD (bottom panel) were used to examine protein expression at 24 hpi.

using recombineering methods independently of requirements for virus replication. For example, the generation of recombinant vectors using either the Invitrogen Gateway recombination system to add a cDNA or the GeneBridges system to manipulate the viral backbone, both of which are utilized in this report, avoids the more difficult task of generating recombinants by classical marker transfer methods in animal cells. These classical methods are inefficient and therefore not suitable for the generation of complex expression libraries. The BAC recombinant libraries generated by bacterial selection can be readily converted to virus particles at various scales to yield infectious library stocks of different complexities. We were able to demonstrate efficient incorporation of full-length cDNAs into our vectors using this system, and the number of different genes detected by microarray analysis suggests substantial diversity among the incorporated cDNAs. In addition to the generation and use of viral cDNA libraries, this system can be used to rapidly incorporate known cDNAs into the vector. Many human genes are now available in a variety of Gateway formats, and their insertion into the vector can be completed within 1 week.

Rat pheochromocytoma PC12 cells are commonly used as a model for neuronal differentiation, since they are derived from

the rat adrenal medulla and terminally differentiate in response to nerve growth factor. The ability of HSV to persist in these cells in a latent-like state may provide a model for evaluation of cellular genes that affect vector persistence. For example, we reported that PC12 differentiation with NGF alters the cellular localization and stability of ICP0 (3). This viral IE protein is believed to play a role in virus reactivation from latency, and its absence as a functional molecule may help to establish the latent state (2). Thus, a cDNA library from differentiated PC12 cells may be used to identify downstream genes in the NGF signaling pathway that are responsible for ICP0 destabilization or mislocalization and can thereby promote latency when expressed in the absence of NGF. Conversely, deregulated expression of genes that are normally turned off in response to NGF signaling may prevent latency and promote plaque formation. The combination of undifferentiated and differentiated PC12 cells as a source of mRNA was therefore used to increase the diversity and utility of the cDNA library. Indeed, microarray analysis demonstrated that the incorporated cDNAs represented a wide variety of neuronal processes and cell signaling pathways.

Other potential applications of this system relate to the discovery of genes that improve the antitumor activities of attenuated replication-competent vectors. Oncolytic vectors are promising tools for cancer therapy, but current vectors do not replicate efficiently in all tumor cells and cellular genes responsible for this difference are largely unknown. Accordingly, cellular genes derived from tumor cells that support efficient oncolytic vector replication may be selectable on the basis of increased vector growth (plaque formation and size, virus yield) in nonsupportive tumor cells. As we described previously, cellular genes that inhibit ion channels whose activity can result in osmotic shock can be discovered by their ability to prevent cell death and thereby rescue virus replication (19). Thus, ion channel antagonist genes can be selected

TABLE 4. Numbers of differentially expressed genes in Vero cells infected with library subpools versus empty DBAC-GW vector

Vector	Pearson's correlation	No. of genes differentially expressed ^a			
		Up 1.5-fold	Up 5-fold	Down 1.5-fold	Down 5-fold
DBAC-GW	0.99481	N/A	N/A	N/A	N/A
Vero Mix5	0.99583	91	16	85	22
Vero Mix100	0.96544	428	295	96	19

^a Numbers are in relation to expression of the DBAC-GW vector. N/A, not applicable.

by plaque formation in the presence of an agonist and such genes are of potential interest for the treatment of pain by gene therapy. Finally, the selection of genes that activate specific signaling pathways tied to virus replication may provide a means to identify new factors that enhance the generation of inducible pluripotent stem cells or encourage stem cell differentiation along a particular lineage. In this scenario, a vector-expressed cDNA gene product that activates a particular promoter associated with an expressed gene in a defined lineage can be linked to expression of an essential virus gene (e.g., ICP4), thereby tying the activity of the cDNA product to virus replication.

In summary, we report the construction of a novel genomic tool suitable for the implementation of powerful selection protocols based on different aspects of HSV biology. The broad host range and high infection efficiency of HSV, along with the availability of vector backbones that minimally affect the host cell, support the wide applicability of this system. Experiments are ongoing in our laboratory to develop selection methods for genomic studies of stem cell differentiation, virus host-cell interactions, innate immune responses, and nerve cell biology.

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