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Hexamethylene bisacetamide leads to reduced helper virus-free HSV-1 amplicon expression titers via suppression of ICP0

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Abstract

The Herpes simplex virus (HSV)-derived amplicon vector has evolved into a promising gene transfer platform for widespread DNA delivery in gene replacement strategies and vaccine development given its ease of molecular manipulation, large transgene capacity, and transduction efficiencies of numerous cell types *in vivo*. The recent development of helper virus-free packaging methodologies bodes well for this vector system in its eventual implementation as a clinically viable therapeutic modality. For realization of clinical application, efforts have been made to enhance yields and quality of helper-free amplicon stocks. Hexamethylene bisacetamide (HMBA), a hybrid polar compound that exhibits stimulatory activity of HSV-1 immediate-early gene expression, has been employed as a standard reagent in helper virus-free packaging given its purported mode of action on virus gene expression kinetics. Unexpectedly, we have found that HMBA exhibits no titer-enhancing activity; in contrast, the compound enhances the proportion of amplicon virions that are non-expressive. Omission of HMBA during vector packaging led to a marked reduction in the ratios of vector genome-transducing to transgene-expressing virions. This effect was neither packaging cell-specific nor amplicon promoter-dependent. Analysis of resultant vector stocks indicated amplicon genome replication/concatenation was unaffected, but the level of particle-associated ICP0 was reduced in stocks packaged in the presence of HMBA. Inclusion of a co-transfected, ICP0-expressing plasmid into the packaging process led to significant rescue of amplicon expression titers, indicating that regulation of ICP0 concentrations is critical for maintenance of the amplicon genome expressive state.

Keywords

Gene transfer; HSV amplicon; ICP0; helper virus-free packaging; bacterial artificial chromosome; HMBA

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Introduction

The utilization of the Herpes simplex virus (HSV) amplicon for somatic gene transfer into peripheral organs and to cells comprising the central nervous system has revealed its significant preclinical potential and versatility for becoming a viable treatment option for a number of acute and chronic diseases. The utility of the amplicon platform lies in its inherent ability to harbor multiple transcription units up to 130 kb in size [1] and in its ease of molecular manipulation [2,3]. The use of different promoters with varying transcriptional regulatory abilities enhances the precision and potential safety of gene transfer via the HSV amplicon [4-6]. Moreover, the development of new virion packaging strategies has significantly improved the safety profile of the vector platform.

Amplicon plasmids are dependent upon helper virus function to provide the replication machinery and structural proteins necessary for packaging amplicon vector DNA into viral particles. A replication-defective virus that lacks an essential viral regulatory gene was initially employed to provide helper packaging function [7]. The final product of helper virus-based packaging contains a mixture of varying proportions of helper and amplicon virions. More recently, helper virus-free amplicon packaging methods were developed by providing a packaging-deficient helper virus genome via a set of five overlapping cosmids, or by using a bacterial artificial chromosome (BAC) that encodes for the entire HSV genome minus its cognate cleavage/packaging signals [8-11]. The titers obtained from amplicon packaging range from 10^7 to 10^9 transducing particles/ml following concentration. The absence of viral genes, especially in virions propagated using helper virus-free methods, results in significantly reduced cytotoxicity and immunogenicity profiles [12,13].

Propagation of amplicon virions via helper virus-free methodologies for clinical applications poses a number of varying but related challenges. First, scale-up of helper virus-free packaging approaches is difficult as presently available methods involve co-transfection of DNA constructs encoding HSV helper function. Because no helper virus is co-propagated, the ability to re-passage stocks on fresh cell monolayers to incrementally increase titers is not possible. Secondly, co-transfection of cosmid- or BAC-encoded HSV genomic sequences inherently differs from helper virus-based transfer of the HSV genome to packaging cells. Intact HSV virions deliver numerous preformed viral proteins harbored within the viral tegument that modulate target cell metabolism and a subset of which activate viral immediate-early (IE) gene expression. Optimal initiation of HSV gene expression and control of host cell machinery, in turn, leads to efficient amplicon genome replication and virion generation. The co-transfection of helper cosmids/BACs for helper virus-free amplicon packaging results in delivery of the HSV-1 genome without tegument proteins, which may be partially responsible for the modest titers derived from such methods. Hence, efforts have been made to include activators of IE gene expression at the time of helper cosmid/BAC HSV introduction to efficiently initiate the highly orchestrated HSV gene expression cascade.

Hexamethylene bisacetamide (HMBA) represents one such IE gene transcription inducer that has been employed in helper virus-free amplicon packaging [8,11,14]. HMBA is a polar compound shown previously to enhance replication of HSV-1 via enhancing IE gene transcription and can promote the propagation of VP16-deficient HSV-1 clones [15]. The structure of HMBA resembles that of HDAC inhibitors but the compound itself does not inhibit histone deacetylase (HDAC) activity or induce histone hyperacetylation [16]. The exact mechanism by which HMBA exerts its activity on HSV gene transcription is unknown, while its overall effects on patterns of HSV gene expression have been deemed significant [17].

In the present study, we sought to better define the role of HMBA in modulating helper virus-free amplicon packaging. Unexpectedly, we found HMBA addition during packaging led to

the generation of amplicon stocks with higher ratios of non-expressive virions and imparted no significant effect on overall yield of propagated amplicon virions. Conversely, omission of HMBA resulted in enhanced numbers of expression-competent virions, an effect that was neither vector promoter- nor packaging cell type-specific. The increased “functionality” of amplicon stocks correlated with increased HSV infected cell protein-0 (ICP0) transcript expression in packaging cells and a concomitant enhancement of ICP0 incorporation within the resultant virions. ICP0, an HSV α protein also termed Vmw110K, has been shown previously to block histone deacetylation of the HSV genome to enable gene expression by interacting with the complex CoREST/REST/HDAC1/2 and dissociating the HDACs from the complex within the host cell nucleus [18-20]. ICP0 has been shown to function as a transcriptional activator of the HSV ribonucleotide reductase large subunit (RR1) gene promoter [21]. In addition, ICP0 helps to subvert antiviral host responses by blocking interferon signaling via the targeted degradation of the promyelocytic leukemia protein (PML) and dispersion of ND10 nuclear structures [22]. The suppressive effect of HMBA on amplicon expression titers could be overcome by tuned expression of ICP0 during packaging. In aggregate, our findings indicate that optimal ICP0 incorporation within amplicon virions and/or co-packaged light particles is critical for preserving the expressive state of the amplicon genome upon delivery to target cells, and moreover, these data will lead to the development of novel strategies centered on extending HSV amplicon transgene expression duration *in vivo* for protracted treatment of neurodegenerative diseases.

Materials and Methods

Cell culture

Baby hamster kidney (BHK) and Vero cell lines were maintained as previously described [6]. The NIH 3T3 mouse fibroblast cell line was originally obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle medium plus 10% fetal bovine serum.

Helper virus-free amplicon packaging

Amplicon stocks were also prepared using a modified helper virus-free packaging method [8]. Vectors were packaged in the absence or presence of 1-2 mM HMBA as noted. Subsets of packaging runs were conducted to examine the effect of ectopically expressed HSV-1 ICP0 protein on resultant amplicon titers. In those instances, titration of pCI-110 plasmid DNA (50, 200, 400, and 700 pmol; kindly provided by Dr. R. Everett) was performed along with companion packaging runs using pBSIIS (Stratagene, Inc.) in equivalent molar quantities as a negative control to account for co-transfected DNA input during transfection. Resultant viral preparations were stored at -80°C until analyzed.

Virus Titering

Amplicon titers were determined using previously published methods [23]. Amplicon expression titers ('blue-forming' units (bfu)/ml), and amplicon transduced vector genome titers (TVG/ml) obtained from these methods were used to calculate amplicon titer. For expression titers, 5 μ l of concentrated amplicon stock was incubated with confluent monolayers of NIH 3T3 cells. Following a 24-h incubation for HSVlac or 48-h incubation for HSV-CMVlac, cells were fixed with 1% glutaraldehyde for 15 min. at room temperature (~22°C) and processed for X-gal histochemistry to detect the *lacZ* transgene product. Positively stained blue cells were counted on a light microscope using a reticle, and expression titer was calculated and represented as blue-forming units per ml (bfu/ml). For transduced vector genome titers, infection of confluent monolayers was carried out as described above, but following the overnight incubation, monolayers were processed for total DNA. Briefly, monolayers were rinsed thrice with 1X phosphate-buffered saline (PBS). Cells were lysed in 100 mM potassium phosphate pH 7.8 and 0.2% Triton X-100. An equal volume of 2X Digestion Buffer (0.2 M

NaCl, 20 mM Tris-Cl pH 8, 50 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K) was added to the remainder of the lysates and the samples were incubated at 56°C overnight. Samples were processed further by one phenol:chloroform, one chloroform extraction, and a final ethanol precipitation. Total DNA isolated from amplicon-transduced NIH 3T3 cells was quantified using a spectrophotometer and 25 ng of purified DNA was analyzed in a PE7300 quantitative PCR reaction using a designed *lacZ*-specific primer/probe combination multiplexed with an 18S rRNA-specific primer/probe set. The ICP0 probe sequence was 5'-CCCCGTCTCCATGTCCAGGATGG-3'; The ICP0 sense primer was 5'-ATGTTTCCCGTCTGGTCCAC-3'; and the ICP0 antisense primer was 5'-CCCTGTCGCCTTACGTGAA-3'. The *lacZ* probe sequence was 5'-FAM-ACCCGTACGTCTTCCCGAGCG-TAMRA-3'; the *lacZ* sense primer sequence was 5'-GGGATCTGCCATTGTCAGACAT-3'; and the *lacZ* antisense primer sequence was 5'-TGGTGTGGGCCATAATTCAA-3'. The 18S rRNA probe sequence was 5'-FAM-TGCTGGCACCAGACTTGCCCTC-TAMRA-3'; the 18S sense primer sequence was 5'-CGGCTACCACATCCAAGGAA-3'; and the 18S antisense primer sequence was 5'-GCTGGAATTACCGCGGCT-3'.

SDS-PAGE and Western Blot Analysis

Aliquots of helper virus-free amplicon virions packaged in the presence or absence of 2 mM HMBA were thawed and processed consecutively through 20- and 26-gauge needles to shear contaminating genomic DNA. A positive control for HSV-1 ICP0 was prepared in parallel from lysates of BHK cells transiently transfected with an ICP0-expressing plasmid (pCI-110, kindly provided by Dr. R. Everett). Protein concentrations were determined using the Bio-Rad DC Protein Assay (Temecula, CA). Prior to loading, 40 µg of viral lysate (2 µg for the positive control) was mixed with 6X sample buffer (350 mM Tris-HCl pH 6.8, 30% glycerol, 10% SDS, 1% bromophenol blue, 30% 2-mercaptoethanol) and boiled for 90 sec. Each sample was loaded onto an 8% SDS-PAGE gel, and following electrophoretic separation, proteins were transferred to a PVDF membrane (Polyscreen, Perkin-Elmer, MA). The resultant blots were initially incubated with a primary antibody specific for HSV-1 ICP0, and following chemiluminescent-based detection, blots were stripped and incubated with a primary antibody directed against HSV-1 VP16. To detect the HSV-1 ICP0 protein, the blot was first blocked with 5% nonfat dry milk in 1X TBST (Tris-buffered saline + 0.1% Tween 20). Primary antibody incubation was conducted overnight at 4°C with mouse monoclonal anti-ICP0 (Mab 11060) at a dilution of 1:5000 [24]. An antibody specific for HSV-1 VP16 (rabbit polyclonal anti-HSV-1 VP16, Sigma, 1:1000 dilution) was used to verify equal loading of viral protein samples. Secondary antibody incubation was performed for 2 h at 25°C using an HRP-conjugated rabbit anti-mouse IgG/IgM antibody (Jackson ImmunoResearch, West Grove, PA) or HRP-conjugated goat anti-rabbit IgG antibody at a dilution of 1:2000. Antibody dilutions were prepared in 5% nonfat dry milk suspended in 1X TBST. Immunocomplexes were detected using ECL (Western Dura SuperSignal, Pierce) with a 1-min. incubation prior to film exposure (Hyperfilm, GE Healthcare Amersham, Piscataway, NJ). Densitometry was performed using the LabWorks software package (UVP, Inc., Upland CA).

Immunocytochemistry

Confluent NIH/3T3 cell monolayers were grown on glass coverslips and transduced with designated preparations of HSVlac at an MOI of 0.25. Twenty-four hours later, monolayers were fixed with 4% paraformaldehyde for 15 min. and cells were permeabilized using a 0.5% Triton X-100 solution diluted in DPBS without Ca²⁺ or Mg²⁺. Following room temperature incubation for 10 min., the permeabilizing solution was removed and replaced with a 5% goat serum-containing blocking solution. After blocking for one hour at 4°C, the monolayers underwent two 5-min. washes. An antibody (mouse monoclonal; Chemicon) specific for promyelocytic leukemia protein (PML) protein was added at a dilution of 1:1000. Concurrent

application of an anti- β -galactosidase (LacZ) antibody (rabbit polyclonal; BioDesign, Saco, ME) was performed at a dilution of 1:4000. Following incubation at room temperature for 1 h, the monolayers were washed with DPBS twice for 5 min. each. The appropriate secondary antibodies (Alexa 488 for LacZ and Alexa 568 for PML; Invitrogen), diluted 1:2000, were then applied for 1 h at room temperature. Monolayers were subsequently washed twice with DPBS for 5 min. each. The cover slips were then mounted on glass slides using Mowiol. Images of LacZ-immunopositive cells and PML bodies were obtained using an Olympus FluoView BX50WI confocal microscope using identical aperture and PMT settings.

Quantitation of PML bodies

Five confocal images of transduced monolayers that had undergone processing of PML/LacZ co-immunocytochemistry were obtained for each experimental group. Images were analyzed for numbers of PML bodies falling into three arbitrarily assigned area ranges using MCID Elite Version 6.0 software. PML bodies with an area less than or equal to $0.22 \mu\text{m}^2$ were defined as “Small”, while those falling into area range of greater than $0.22 \mu\text{m}^2$ but less than or equal to $0.86 \mu\text{m}^2$ were considered “Medium”. PML bodies larger than $0.86 \mu\text{m}^2$ but less than or equal to $1.94 \mu\text{m}^2$ were classified as “Large”.

Statistical Analyses

Experiments were conducted using a minimal of N=3 per condition. Statistical analyses were carried out using one-way analyses of variance (ANOVA) or Student T-test, as indicated, with HMBA treatment or ICP0 plasmid co-transfection as the between-group variable.

Results

Given its documented positive effects on the titers of wild-type HSV and certain deletion mutant derivatives as well as its postulated mechanism of action, hexamethylene bisacetamide (HMBA) has been employed as a means to potentially enhance modest amplicon titers produced by helper virus-free amplicon packaging methodologies. The immediate-early (IE) gene transcription-activating function of HMBA was deemed advantageous for vigorous and orchestrated initiation of the HSV gene expression cascade from a cosmid- or bacterial artificial chromosome-harbored, packaging signal-deficient HSV genome. In fact, Sun and colleagues demonstrated that addition of HMBA at the time of co-transfecting amplicon plasmid DNA and an HSV genome-containing cosmid set into a permissive cell line results in a minimal, but measurable, amplicon titer enhancement [14]. We had previously determined that helper virus-free amplicon stocks prepared in the presence of HMBA possessed a high proportion of virions, which harbored intact and concatenated amplicon genomes that were unable to express the vectored transgene [23]. We reasoned that, although it appears to enhance IE gene transcription overall, HMBA may impart deleterious effects on the transcription of a subset of HSV or cellular genes, ultimately leading to the propagation of an appreciable proportion of amplicon virions that are non-expressive. Hence, in the present study, we sought to better define the effects of HMBA on helper virus-free amplicon packaging and determine whether or not this compound contributes to the generation of expression-defective viral particles.

The amplicon plasmid pHSVlac [3] that expresses the *E. coli* β -galactosidase (*lacZ*) gene under the transcriptional control of the HSV immediate-early (IE) 4/5 gene promoter was packaged using a previously published helper virus-free method [8] in the presence (final concentration of 1 or 2 mM) or absence of HMBA using either baby hamster kidney (BHK) or Vero cells. The co-transfected pBAC-V2 DNA construct, which harbors a packaging signal (‘a’ sequence)-deficient HSV genome, was used to provide HSV gene products *in trans* for replication and packaging of the HSVlac amplicon genome [11]. A plasmid (pBSKSVhs) harboring the genomic locus of the virion host shutoff (vhs) gene and its cognate 5' and 3'

transcriptional regulatory sequences, was also included in the packaging strategy, as our group showed previously that ectopic vhs expression during helper virus-free packaging results in a 10-fold enhancement of viral titers [8]. Helper virus-free packaging for our studies was performed using both BHK and Vero cells, which are they themselves or their derivatives commonly employed in amplicon propagation, to assess any cell type-specific effect that may be exerted by HMBA.

Resultant stocks were incubated with monolayers of NIH 3T3 cells and 24 h later, cells were either washed and processed for determination of transduced amplicon genomes (transduced vector genome titers; Fig. 1A and D) or were fixed with 1% glutaraldehyde and histochemically stained with X-gal (expression titers; Fig. 1B and E). Transduced vector genome titers are expressed as transduced vector genomes (TVG) per ml, and expression titers are represented as “blue-forming” units (bfu) per ml. Ratios of transduced vector genome titers to expression titers were calculated for each packaged amplicon stock (Fig. 1C and F). The final concentration of HMBA provided in the culture medium during the packaging process did not affect the overall numbers of packaged amplicon genomes derived from either BHK or Vero cell lines. However, we observed a concentration-dependent effect of HMBA when we assessed amplicon expression titers by X-gal histochemistry. Reducing the typically used HMBA concentration of 2 mM to 1 mM, or completely removing HMBA from the procedure, resulted in 57% and 175% increases, respectively, in expression titers of HSVlac packaged in BHK cells (Fig. 1B). The inverse relationship of HMBA concentration and expression titers of HSVlac packaged in Vero cells was also significant, albeit slightly more modest in magnitude (Fig. 1E). Given the lack of an effect imparted by HMBA inclusion on overall amplicon transducing units, the alteration in expression titers as a reverse function of HMBA concentration translated into a significant improvement in the proportion of transgene-expressive amplicon particles (Fig. 1C and F). Performing helper virus-free packaging in the presence of 5 mM HMBA, a concentration originally reported previously to modestly enhance titers [14], resulted in transduced vector genome and expression titers similar to stocks prepared in the presence of 2 mM HMBA (data not shown). The differences in total transduced vector genome and expression titers obtained from BHK cells as compared to Vero cells are likely attributable to the inherent transfection efficiency of each of the packaging cell lines (Bowers and Federoff, unpublished observations).

To determine whether the suppressive effect of HMBA was a promoter-dependent phenomenon, we separately packaged HSV-CMVlac, which harbors the *E. coli* β -galactosidase gene under the transcriptional control of the cytomegalovirus (CMV) immediate-early promoter, in BHK cells in the presence (2 mM) or absence of HMBA. Transduced vector genome and expression titers were determined at 48 h post-transduction using NIH 3T3 cells, when the CMV promoter in the context of an amplicon vector is maximally expressed (Bowers and Federoff, unpublished observations, and [25]). Removal of HMBA from the amplicon packaging procedure resulted in a marked increase in expression titers, while transduced vector genome titers remained unchanged (Fig. 2). This led to a reduction in the ratio of transducing particles to expressive particles in stocks packaged in the absence of HMBA, a result similar to that observed for the IE4/5 promoter-containing HSVlac amplicon vector.

The enhancement of transgene-expressing to transduced vector genome ratios when amplicons are packaged in the absence of HMBA suggests addition of this compound leads to the modulation of key cellular and/or viral gene products that are involved in maintaining the expressive state of amplicon genomes. A leading candidate viral gene product that could be affected directly or indirectly by HMBA treatment is infected cell protein-0 (ICP0). ICP0, an HSV immediate-early protein also termed Vmw110K, has been shown previously to block histone deacetylation of the HSV genome to enable viral gene expression by interacting with the complex CoREST/REST/HDAC1/2 and dissociating HDACs from the complex within the

host cell nucleus [18-20]. In fact, HSV-1 mutants that lack ICP0 function are significantly crippled in escaping latency and in initiating the lytic gene expression cascade [26]. Moreover, Dargan and Subak-Sharpe found ICP0 to be a major component of HSV-1 light-particles (L-particles) and demonstrated that these smooth-membrane bound inclusion vesicles enhance viral infectivity and gene expression [27], whereas L-particles generated from ICP0 null HSV-1 mutants were unable to enhance viral gene expression and plaquing efficiency.

To determine the effects of HMBA treatment on ICP0 gene expression from pBAC-V2, we packaged HSVlac using BHK or Vero cells in the absence or presence (2 mM) of HMBA. A subset of packaging cultures were processed for quantitative real-time RT-PCR analyses of ICP0 mRNA levels at 24 h post-transfection, while the remaining cultures were incubated to term (3 days) and helper virus-free amplicon particles were harvested and analyzed for levels of virion-associated ICP0 protein (N=3). Quantitative transcript analysis showed a marked increase in ICP0 transcript levels in BHK and Vero cultures not incubated with 2 mM HMBA immediately following the co-transfection step (Fig. 3A). Assessment of virion-associated ICP0 protein by Western blotting (Fig. 3B) and comparing band intensities to that of HSV-1 VP16, as a control for viral particle loading, demonstrated that amplicon stocks packaged in the absence of 2 mM HMBA exhibited 3-4 fold higher levels of ICP0 than amplicon stocks prepared in the presence of the compound (Fig. 3C). These data suggest that ICP0 protein is incorporated at higher concentrations within amplicon genome-containing virions and/or co-propagated light (L) particles when HMBA is omitted from the packaging process.

Given this observation, we predicted the repressive effect of HMBA on expression titers could be overcome by ectopically overexpressing ICP0 during helper virus-free packaging. We performed a titration study by adding progressively increasing doses of ICP0-expressing plasmid (pCI-110; kindly provided by Dr. R. Everett) at the time of the co-transfection step. To control for increased plasmid DNA levels during lipofection, parallel packaging runs were performed in the presence of a titrated control pBSIIKS plasmid added at molar equivalents to pCI-110. All BHK packaging cultures were treated with 2 mM HMBA immediately following co-transfection, and viral particles were harvested for determination of expression and transduced vector genome titers as described above.

Addition of 200 or 400 pmol of pCI-110 at the time of co-transfection led to a significant increase in HSVlac expression titers even when packaged in the presence of 2 mM HMBA as compared to particles packaged under the control conditions (Fig. 4A). Interestingly, use of more pCI-110 (700 pmol) during helper virus-free packaging did not result in an enhancement of expressive HSVlac particle numbers, suggesting higher ICP0 levels are potentially toxic to either the packaging cells or target cells [28-31], thus subverting the observed expression titer-enhancing effect. Moreover, ICP0 addition led to a consistent increase in amplicon transducing units per ml as compared to control stocks when virions were analyzed by quantitative real time PCR-based transduced vector genome titering on NIH 3T3 cells (Fig. 4B). This result indicates ectopic ICP0 expression during packaging enhanced viral gene expression from pBAC-V2 and/or replication of the HSVlac amplicon DNA. Hence, amplicon stocks prepared in the presence of progressively titrated ICP0 and 2 mM HMBA exhibited augmented transduced vector genome to expression ratios, as compared to HSVlac stocks packaged in the presence of a titrated control plasmid and 2 mM HMBA or those propagated using HMBA alone (Fig. 4C).

Given the reported activity of ICP0, increased incorporation of this key HSV regulatory protein into packaged amplicon virions and/or co-propagated L particles would be predicted to influence the status of promyelocytic leukemia (PML) nuclear bodies (also called ND10) in transduced cells. PML bodies have been implicated in numerous cellular processes, including defense against viral infections (reviewed by [32]). ICP0 expressed via wild type HSV-1 during

the lytic life cycle initially associates with ND10, then leads to progressive dispersal of these structures within the nuclei of infected cells (reviewed by [33]). HSVlac stocks packaged in the presence or absence of 2 mM HMBA were applied to NIH 3T3 monolayers and co-immunocytochemistry for β -galactosidase and mouse PML was performed at 24 h post-transduction (Fig. 5A-C). Cultures transduced with HSVlac prepared in the absence of HMBA exhibited qualitative differences in the size distribution of PML bodies (Fig. 5C). Quantitation of the numbers of PML-positive structures falling into three arbitrarily defined area ranges showed that indeed in conditions where ICP0 levels were higher in HSVlac stocks, transduced and non-transgene expressing cells harbored higher proportions of smaller and more dispersed PML bodies (Fig. 5D). No quantitative differences were observed in PML body size distribution between LacZ-expressing and non-expressing cells within a given experimental group (data not shown). These findings, in aggregate, indicate that ICP0 is incorporated at higher levels within genome containing as well as non-genome containing L particles and that this viral protein can markedly influence the expressivity of the amplicon vectored transgene.

Discussion

HSV replication and persistence is dependent on its capacity to subvert host innate responses and maintain its intricate viral gene expression cascade [34]. Several HSV encoded proteins have been suggested to interfere with anti-viral immunity including the RNase virion host shutoff (VHS) protein, which suppresses both innate and adaptive response through degradation of host mRNA, US11 [35] and γ 34.5 [36] via inhibition of the serine/threonine kinase PKR, viral glycoproteins by subverting host antibody and complement activity [37, 38], and ICP47 through inhibition of major histocompatibility complex (MHC)-I antigenic peptide loading [39,40]. ICP0 represents another such candidate due to its capacity to suppress interferon responses and to inhibit transcription of interferon-stimulated genes (ISG) [30]. Moreover, ICP0 has been shown to participate in suppression of host cell-mediated viral gene silencing via interacting with the complex CoREST/REST/HDAC1/2 and dissociating the HDACs from the complex within the host cell nucleus [18-20], presumably leaving the viral genome free to be actively transcribed within the infected cell nucleus.

The present study may provide insight into the transient nature of transgene expression derived from HSV amplicon vectors *in vitro* and *in vivo*. The promoters used in these experiments (HSV IE4/5 and CMV) are known to be active at times shortly following transduction, leading to optimal transgene expression at 24-48 h, with vector-mediated transcription being progressively down-regulated thereafter [41]. The HSV-1 derived immediate-early (IE) 4/5 gene promoter was employed to facilitate rapid assessment of vector titer modulation in response to changes in helper virus-free packaging. The IE 4/5 gene promoter, as well as other non-mammalian transcriptional control elements, may possess sequence motifs that activate cellular silencing mechanisms. Moreover, proteins derived from the packaging cells themselves may co-package within the amplicon virion. These so-called “passenger proteins” consist of not only HSV-1 tegument proteins, but may include packaging cell-derived DNA-binding proteins, deacetylases, and histone proteins. Co-packaged ICP0 may function to subvert the amplicon-silencing activities of these proteins by acting at ND10 foci in transduced cells [42,43]. The use of cellular promoters in HSV amplicons to direct transgene expression appears to be a successful strategy for prolonging transgene expression, as these promoters tend to yield longer term, cell-specific expression and may contain sequence motifs that promote euchromatic histone assembly and perhaps co-packaging of a different virion-associated passenger protein profile [5,41]. Moreover, non-promoter element DNA sequences found within the amplicon backbone may influence transgene expression duration. Recent evidence from Suzuki and colleagues demonstrates that the removal of bacterially derived DNA sequences within the amplicon plasmid backbone results in measurable extensions of transgene expression, suggesting that such sequences serve as signals to activate host cell gene

silencing machinery [44]. However, it is of note that these altered amplicon vectors still succumbed to progressive transgene expression extinction.

The enhancement of ICP0 levels during helper virus-free amplicon packaging via omission of HMBA or co-transfection of an ICP0-expressing plasmid likely influences HSV gene expression derived from the co-transfected HSV-BAC and packaging cell transcription programs. Packaging cells treated with 2 mM HMBA expressed lower levels of ICP0 transcript as determined by quantitative RT-PCR analysis and concomitant diminution of ICP0 protein associated with packaged viral particles (Fig. 3), suggesting that HMBA negatively impacts transcription of the ICP0 gene harbored within the co-transfected pBAC-V2 construct. HMBA has been shown previously to arrest eukaryotic cells in G1 phase, thus preventing transition into the DNA synthesis phase of the cell cycle and inducing a series of metabolic changes reminiscent of cellular differentiation (reviewed by [45]). These observations led to the clinical evaluation of HMBA and its hybrid polar derivatives as potential therapeutics for myelodysplastic syndrome and acute myelogenous leukemia [46,47]. The differentiation-related effect of HMBA has not been demonstrated directly in BHK- or Vero-derived cell lines, which are commonly used to package HSV amplicon vectors, but such a mechanism of action could significantly alter viral and cellular protein profiles and metabolism, thereby influencing expression and transduced vector genome titers. In fact, HMBA has been shown to impart an inhibitory effect on human cytomegalovirus (CMV) replication via blocking the accumulation of the immediate-early 2 (IE2) protein [48]. Our data suggest that HMBA does not impinge on the replication of the HSV-1 amplicon plasmid in packaging cells, as genome-containing amplicon particle numbers (transduced vector genome titers) were unaffected whether stocks were packaged in the presence or absence of 2 mM HMBA (Fig. 1 and 2). However, the levels and/or kinetics of expression of other HSV gene products may be impacted by HMBA. For example, Elliot and colleagues demonstrated that deletion of the UL49 gene, which encodes VP22, from the HSV-1 genome leads to reduced ICP0 expression in infected cells and diminished virion-associated ICP0 once the newly replicated viral genomes are packaged [49]. The expression profiles of other HSV-derived gene products, such as VP22, may be influenced by HMBA, but this question requires further experimentation.

Higher nuclear concentrations of ICP0 within the packaging cell would be expected to suppress cellular response to quench the expression of and/or sequester transfected plasmid DNAs, hence manifesting as higher amplicon expression and transduced vector genome titers. Recently, Block and colleagues reported that transfected plasmid DNAs target to PML nuclear bodies and ectopic expression of HSV ICP0 enhances transcriptional activation of plasmid DNAs tethered to those subnuclear domains [50]. An oversized HSV BAC described by Saeki et al., which contains two copies of the ICP0 gene within an ICP27-deleted HSV genome, has been reported to yield consistently higher amplicon expression titers as compared to HSV BACs possessing a single copy of ICP0 [10]. When an ICP0 expression plasmid was titrated into our helper virus-free packaging process performed in the presence of 2 mM HMBA, a dose-dependent effect on expression titers was observed (Fig. 4), suggesting that optimal amplicon virion-associated ICP0 plays a key role in the expression competence of amplicon stocks. The expression titer enhancing effect was similar to when HSVlac was packaged in the absence of HMBA (Fig. 1 and 2). However, transduced vector genome titers were significantly enhanced when the ICP0 expression plasmid was added to packaging, whereas they were unaffected when HMBA was omitted from the process. The overall enhancement of transduced vector genome titers, even at the lowest level of co-transfected ICP0 expression plasmid, indicates that the level of ICP0 and/or kinetics of expression from the plasmid harboring ICP0 are favorable (up to 400 pmol plasmid DNA) for amplicon genome replication and concomitant virion incorporation, while ICP0 levels derived solely from pBAC-V2, which harbors a single copy of the ICP0 gene locus, are sufficient for derivation of expression competent amplicon virions.

In aggregate, our data speak to the importance of achieving an optimal HSV gene expression cascade within transfected packaging cells and passenger protein profile within the packaged amplicon virion. Unique to a majority of helper virus-free packaging strategies is the transfection of a “naked” HSV-1 genome, which is devoid of co-packaged regulatory proteins present in the tegument of intact HSV-1 virions. It is therefore, imperative to create an environment within the packaging cell that is homologous to that created by transduction with wild type or recombinant HSV-1 virions to efficiently produce amplicon stocks that are of optimal titer and expression competence. The analysis of co-propagating L particles may provide insight into the supportive role of ICP0 and other virion-associated proteins during HSV-1 virion generation. L particles consist of viral envelope and tegument proteins, but lack the virus genome and components of the nucleocapsid. Dargan and Subak-Sharpe have dissected the functional significance of several HSV-1 proteins found within L particles. ICP0 and VP16, when harbored within L particles that are added at the time of transfecting naked HSV-1 genomes, exhibit the most significant titer-enhancing effects, while VP11/12, VP13/14, and vhs show lesser impact on titers [27]. Our work further implicates ICP0 as a key component of helper virus-free HSV-1 amplicon particles, but does not rule out the importance of other passenger proteins in virion stability and expression competence, whether they are derived from an HSV BAC or the packaging cell line. Future studies will focus on multifaceted proteomic analyses of the proteinaceous components of amplicon particles and co-propagated L particles to further refine and define the HSV amplicon vector platform.

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Figure 1. Inclusion of HMBA in helper virus-free HSV amplicon packaging results in a dose-dependent reduction in “expression-competent” amplicon virions, but no change in genome-containing virions

The amplicon plasmid pHSVlac, which harbors the *E. coli* β -galactosidase (*lacZ*) gene under the transcriptional control of the HSV immediate-early (IE) 4/5 gene promoter, was packaged using a previously published helper virus-free method [8] in the presence (final concentration of 1 or 2 mM) or absence of HMBA using either BHK or Vero cells. Resultant stocks were incubated with monolayers of NIH 3T3 cells and 24 h later, cells were either washed and processed for determination of transduced amplicon genomes (transduced vector genome titers; panels A and D) or were fixed with 1% glutaraldehyde and histochemically stained with X-gal (expression titers: panels B and E). Transduced vector genome titers are expressed as transduced vector genomes (TVG) per ml, and expression titers are represented as “blue-forming” units (bfu) per ml. Ratios of transduced vector genome titers to expression titers were calculated for each packaged amplicon stock (panels C and F). Error bars indicate standard deviation and “*” indicates $p < 0.05$.

Figure 2. The suppressive effect of HMBA on amplicon expression titers is not specific to the amplicon-resident promoter

The cytomegalovirus immediate-early promoter-containing pHSV-CMVlac amplicon plasmid was packaged using our helper virus-free method [8] in the presence (final concentration of 2 mM) or absence of HMBA using BHK cells. Resultant stocks were incubated with monolayers of NIH 3T3 cells and 24 h later, cells were either washed and processed for determination of transduced amplicon genomes (transduced vector genome titers; panel A) or were fixed with 1% glutaraldehyde and histochemically stained with X-gal (expression titers; panel B). Transduced vector genome titers are expressed as transduced vector genomes (TVG) per ml, and expression titers are represented as “blue-forming” units (bfu) per ml. (C) Ratios of

transduced vector genome titers to expression titers were calculated for each packaged amplicon stock. Error bars indicate standard deviation and “*” indicates $p < 0.05$.

A. ICP0 Transcript Levels in Packaging Cells

B. Amplicon Stock-associated ICP0 Protein

C. Relative Levels of Virion-associated ICP0 Protein

Figure 3. HMBA addition during helper virus-free amplicon packaging leads to reduced ICP0 transcription in packaging cells and a concomitant decrease in virion-associated ICP0 protein (A) BHK cell monolayers were transfected with pHSVlac amplicon, pBSKSvhs, and pBAC-V2 DNAs as normally performed for helper virus-free packaging in the presence (2 mM) or absence of HMBA. Twenty-four hours post-transfection, total RNA was prepared using the Trizol method. Complementary DNA was subsequently synthesized and analyzed by quantitative real-time PCR for levels of HSV-1 ICP0 transcript and 18S RNA, which served as an internal normalization control. Analysis of HMBA-treated monolayers indicated a significant reduction in ICP0 mRNA levels. (B) Separate aliquots of these packaged amplicon virions were analyzed by Western blotting using an HSV-1 ICP0-specific monoclonal antibody

(kindly provided by Dr. R. Everett) to determine the levels of ICP0 associated with the viral particles. Subsequent to visualizing and quantifying ICP0-specific signals by densitometry, blots were stripped and incubated with an HSV-1 glycoprotein D (gD)-specific antibody to control for viral protein loading. (C) Bands corresponding to ICP0 (110 kDa) and gD (50 kDa) were analyzed by densitometry to determine pixel intensity. Relative pixel intensity for ICP0 was calculated by dividing pixel intensity for the ICP0 band by pixel intensity of the corresponding gD immunopositive species. HSV1ac virions packaged in the absence of HMBA showed enhanced levels of immunodetectable ICP0 as compared to vector particles packaged in the presence of 2 mM HMBA. Error bars indicate standard deviation, “*” indicates $p < 0.001$, while “**” indicates $p < 0.05$.

Figure 4. Expression of HSV ICP0 via a co-transfected plasmid during amplicon packaging in the presence of HMBA leads to a dose-dependent rescue of amplicon expression titers

The IE4/5 promoter-containing pHSVlac amplicon plasmid was packaged in BHK cells in the presence of 2 mM HMBA and a dose titration of either a co-transfected plasmid encoding HSV-1 ICP0 (pCI-110) or empty vector control plasmid (pBSKSII) matched by molar quantities. Resultant stocks were incubated with monolayers of NIH 3T3 cells and 24 h later, cells were either washed and processed for determination of transduced amplicon genomes (transduced vector genome titers; panel B) or were fixed with 1% glutaraldehyde and histochemically stained with X-gal (expression titers; panel A). Transduced vector genome titers are expressed as transduced vector genomes (TVG) per ml, and expression titers are

represented as “blue-forming” units (bfu) per ml. (C) Ratios of transduced vector genome titers to expression titers were calculated for each packaged amplicon stock. Error bars indicate standard deviation and “*” indicates $p < 0.05$.

Figure 5. Increased particle-associated ICP0 within helper virus-free amplicon stocks results in alteration of PML staining patterns in transduced NIH 3T3 cultures

The IE4/5 promoter-containing pHSVlac amplicon plasmid was packaged in BHK cells in the presence (B) or absence (C) of 2 mM HMBA. Resultant stocks were incubated with monolayers of NIH 3T3 cells and 24 h later, cells were processed by co-immunocytochemistry for PML staining and β -galactosidase reporter gene expression. Subsets of cultures served as non-transduced controls (A). Images were obtained by confocal microscopy and were subsequently analyzed using the MCID system to determine the size distribution of PML bodies. Scale bar = 20 μ m. Three area ranges of PML sizes were arbitrarily assigned and numbers of stained PML bodies were quantified: "Large" included areas $> 0.86 \mu\text{m}^2 \leq 1.94 \mu\text{m}^2$, "Medium" included areas $> 0.22 \mu\text{m}^2 \leq 0.86 \mu\text{m}^2$, and "Small" included areas $\leq 0.22 \mu\text{m}^2$ (D). Cultures transduced with HSVlac packaged in the presence of 2 mM HMBA (w/HMBA) exhibited changes in the distribution of PML body sizes in each area range as compared to non-transduced cultures ($P < 0.001$ indicated by '#'). Similarly, HSVlac stocks prepared in the absence of HMBA (w/o HMBA) showed more pronounced changes in all PML size ranges as compared to control cultures ($P < 0.001$ indicated by '*'). Analysis of numbers of PML bodies falling into each size range in cultures transduced with amplicon prepared in the absence of HMBA

revealed significant reduction in “Large” PML bodies ($P < 0.001$ indicated by ‘+’), and significant enhancement of “Medium” ($P < 0.001$ indicated by ‘+’) and “Small” ($P < 0.05$ indicated by ‘&’) PML bodies as compared to HSVlac packaged in the presence of 2 mM HMBA. Error bars indicate standard deviation.