PRODUCTION AND PURIFICATION OF HIGHLY REPLICATION DEFECTIVE HSV-1 BASED GENE THERAPY VECTORS

by

Kyle Grant

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This dissertation was presented

by

Kyle Grant

It was defended on

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and approved by

Mohammad Ataai, Professor, Department of Chemical and Petroleum Engineering

Joseph Glorioso III, Professor, Department of Microbiology and Molecular Genetics

Richard Koepsel, Research Associate Professor, Department of Chemical and Petroleum Engineering

William Wagner, Professor, Surgery, Department of Bioengineering, and Chemical and Petroleum Engineering

Dissertation Director: Mohammad Ataai, Professor, Department of Chemical and Petroleum Engineering

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Herpes Simplex Virus Type 1 (HSV-1) represents an attractive vehicle for a variety of gene therapy applications. To render this virus safe for clinical use, its cytotoxic genes must be removed without losing its ability to express transgenes efficiently. Therefore, complementing cell lines that can provide high titer virus are essential for clinical application. Manufacturing methods that can purify these vectors of host cell DNA and protein are also necessary for translation of this gene therapy strategy into clinic.

In this thesis, I have developed complementing cell lines that allow propagation to up to 1E6 PFU/ml routinely for triple as well as quadruple deleted vectors which are among the most difficult to culture to high titer. Replacement of the ICP4 promoter with the VP16 enhancer element enriched ICP0 IE gene promoter resulted in higher induction levels and faster kinetics of ICP4 expression and a 10 fold increase in vector yield.

Along these lines, I investigated the repressive nature of Vero cells to a quadruple IE mutant. A high throughput cell based chemical screen revealed a metal chelator, 1 10 phenanthroline, that was able to derepress EGFP expression not only from the quadruple backbone but of other ICP0 mutant viruses suggesting a unique cell state that was permissive for HSV-1 transgene expression. Interestingly, proteosome inhibition studies reveal that this pathway is essential for this effect not only in Vero cells but also normally permissive U2OS cells.

Finally, a systematic study into ion exchange chromatography for purification of these vectors reveals dramatic differences in infectious yield depending on the matrix chosen. Anion exchange resins bound the virus with high affinity and require high salt concentrations to recover adequate titers. Cation exchange chromatography was able to purify HSV-1 to moderate titers while removing a majority of the host contaminating DNA and protein in accordance with FDA standards for clinical grade viral based vaccines.

DESCRIPTORS

Complementing Cell lines	High-throughput Cell Based Screen
Gene Therapy	HSV-1
Genomic Repression	Ion Exchange Chromatography

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PREFACE

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1.0 INTRODUCTION

Viral based gene therapy vectors have long been an active area of research owing to their different tissue tropisms and efficient method of gene delivery. One caveat that has complicated this technique is the issue of safety. Since most viruses are pathogenic they need to be made safe for clinical use and this is done through the construction of replication defective viruses. Replication defective viruses are constructed in a way that the essential gene functions are missing so as to block replication and therefore a major pathogenic effect once inside the host. Another barrier to clinic is the issue of scale of manufacture. Large scale production of replication defective vectors remains challenge on both the upstream and downstream sides. Historically, these replication defective vectors, which also include vaccines, are manufactured through the use of packaging cell lines that provide missing functions *in trans*. Generation of stable complementing cell lines remains a challenge since the products they conditionally express are normally quite toxic to the cell. Additionally, these products must be expressed with adequate kinetics and levels that mirror a normal wild type infection. Compounded with these issues are the stringent requirements imposed by the FDA for product purity (contaminating cellular DNA and protein) and lot to lot variability. Purification of viral based products is a relatively new field, but recent advances have revealed major breakthroughs in downstream processing of these vectors which include optimized size exclusion and ion exchange chromatography processes for different viral strains.

1.1 BACKGROUND

1.1.1 Gene Therapy

With the advent of the human genome project and recent advances in stem cell technology, the field of gene therapy is destined to become a frontrunner for new treatments of life threatening diseases. Gene therapy is traditionally defined as the transfer of genetic material either from a virus, naked DNA or RNA, or liposomes to treat a certain disease state. Gene therapy has the advantage of delivering the precise gene product to the particular disease state while taking advantage of the viral immune evasion mechanisms *in vivo*. Viruses are naturally derived vehicles that offer the advantage of tissue tropism as well as large genetic payload. Typically, gene therapy works well for providing a single missing gene function as is the case with parkinson's, cystic fibrosis, or muscular dystrophy. However, recent work has shown that other avenues can be explored such as chronic pain, bone regeneration, oxidative injury, and cancer therapy. The incorporation of gene therapy as an adjunct therapy is becoming more common place and may serve more utility than as an alternative to current approaches (4, 70, 145).

For gene therapy to be successful, a few criteria must be met. (1) There must be a suitable disease model, (74) specific delivery to the disease state, (2) efficient gene transduction with suitable kinetics, and (3) limited toxicity *in vivo*. Commonly the rate limiting step for these

criteria is effectively targeting the specific malady within a myriad of cellular receptors while minimizing toxic effects to normal tissue. Recent efforts have shown that retargeting of viral vectors is possible through genetic modification of the nucleocapsid and glycoprotein coat of different viruses mainly for cancer therapy (94, 119, 190). Removal of toxicity is commonly carried out by deleting essential gene functions that allow viral replication and therefore halting any pathogenic effect at the level of entry. These vectors, termed replication defective, exhibit reduced toxicity and enhanced gene transduction in a wide range of tissue types (15, 31, 77, 93). In addition to the aforementioned limitations to gene therapy is the issue of efficient manufacturing processes for well defined gene therapy products that are suitable for clinical trials. There have been many recent advances in the field of gene therapy vector production (133, 134). Once these processes have been optimized for efficient large scale manufacture the full potential of gene therapy can be realized.

1.1.2 Gene Therapy Vectors

Gene transduction is the method of expressing an ectopic gene in a particular tissue. The success of this process relies on efficient delivery and stable expression. The vector itself serves to (i) deliver the genetic payload to the desired tissue and (ii) protect the genes from degradation by host immune surveillance. An ideal vector should be able to efficiently deliver the genetic material as well as avoid any pathogenicity. Additionally, these vectors must be able to be produced at high concentration easily. With the wide variety of disease models available it is clear that there is no universal method of delivery that is suitable for all applications. Vector mediated gene transfer can be carried out by one of two ways either viral or non-viral.

Most non-viral methods are composed of synthetic carriers such as liposomes, polyplexes, nanoparticles, or alternatively naked DNA injection. Naked DNA injection has worked well in

the case of muscle transduction since there are low levels of nuclease activity (5). However, systemic injection suffers from the presence of serum nucleases that can clear the foreign DNA within minutes (78). Synthetic carrier methods are attractive from the standpoint of toxicity since there is little immune response to most of the cationic polymers used to house DNA. These polymers consist of positively charged repeating monomers that are tailored to bind to cell surface receptors *in vivo*. In addition to protection of the genetic material, the polymers chosen can interact with multiple biologic interfaces within the cell to alter the functionality of the carrier. Some examples of the systems chosen for liposomal delivery are poly-L-lysine and polyethylene glycol that have attractive biocompatibility profiles and suitable clearance rates (35). An active area of research is polymer design to complex foreign DNA that can be targeted to specific tissue types. Lipoplexes with DNA offer the advantage of higher order interactions through multi lamellar complexes (79). As the DNA condenses around positively charged head groups different lipid layers can be formed and tightly controlled to specifically interact with different subcellular components. Some of the major hurdles this technology still faces are endosomal escape and nuclear transport. One other limitation to these synthetic systems is the lack of an ideal cationic lipid since there are no general correlations with structure and function *in vivo*. In addition, correlations between *in vitro* and *in vivo* work remains unclear complicating development in this delivery method due to the presence of multiple interaction possibilities within the bloodstream (161). Even with these limitations, this delivery method will no doubt with experience and understanding rival that of the viral based gene therapy due to the tunable nature of synthetic polymers and their attractive biocompatibility profiles.

Viral based gene delivery methods benefit from natural tissue tropisms and exploitation of the host cell machinery to amplify the exogenous DNA. Gene therapy based on a broad range of viruses has been the focus of many institutions and each vector system has their own intrinsic

limitations and advantages. Table 1 shows the current state of the art in regard to virus based gene therapies and their use in clinical trials dating from 1989 to the present.

Table 1. Virus Based Gene Therapy Clinical Trials (102)

Virus	Genetic Material	Genetic Payload Capacity	Percentage of Clinical Trials
Adenovirus	dsDNA	< 5kb	(n=331) 36.65%
Retrovirus	RNA	< 8 kb	(n=305) 33.77%
Vaccinia	dsDNA	25 kb	(n=91) 10.07%
Pox Virus	dsDNA	25 kb	(n=86) 9.52%
Adeno Associated Virus	ssDNA	5 kb	(n=47) 5.2%
Herpes Simplex Virus	dsDNA	40 kb	(n=43) 4.76%

All of these viruses are rendered therapeutic by insertion of foreign DNA into the viral genome in place of a toxic viral gene while leaving the cis acting elements intact such as origins of replication and packaging signals. These deleted genes usually comprise immediate early genes, replication machinery, or nonessential structural components.

1.1.2.1 Retrovirus

Most retroviral vectors are based on the betaretrovirus Murine Moloney Leukemia Virus (MMLV). The MMLV genome encodes for three essential genes: *gag*, *pol*, and *env*. The *env* gene is cleaved within the *trans*-Golgi network to produce transmembrane and surface unit subunits interacts with cell surface receptors to allow binding of the virus on the target cell. Once inside the cell, the virus is uncoated and the RNA is reverse-transcribed into proviral double stranded DNA by the *pol* gene and subsequently transported to the nucleus for integration. All that is required for viral replication in *cis* are the 3' and 5' long terminal repeats

(LTRs), which house the sites of DNA transcription initiation and polyadenylation. These sites are essential for production of progeny viral genomes and viral mRNA. A packaging signal termed psi is also located near the 5' repeat sequence which directs incorporation of viral DNA into the capsid. As the viral DNA is packaged, the *gag* gene directly binds to the viral RNA to promote packaging into the virion. Retroviral vectors are typically propagated on murine based cell lines that provide the missing essential genes *in trans*. These cell lines have been recently developed to contain the env, gag, and pol genes on separate plasmids to reduce the possibility of generating replication competent retroviral vectors (107, 115). Sequence homology limitation is another strategy used to minimize recombination events between the gene supplied *in trans* and the deleted virus. In addition, suspension cell lines are being developed for the use of large scale manufacture of recombinant retroviral vectors (143). Downstream processing and concentration of retroviral particles is often difficult due to the fragile nature of the virus (32). Step-wise filtration can be used to purify these vectors but can be clone specific resulting in titer improvements from 2-10 fold (141). Recently, ion exchange chromatography through the use of a weak anion exchange matrix allowed superior recovery while minimizing contaminating cellular debris and DNA (146).

For most types of retroviruses, mitosis is required to integrate into the host genome which remains a limitation to the development of this as a system for gene therapy. This stable integration contrasts with other viral DNA vectors such as herpes simplex virus and adenovirus where the genome remains episomal. The main drawback to this system of gene transfer is the issue of insertional activation. A recent French trial for severe combined immunodeficiency (SCID) resulted in a fatality due to induced expression of lmo2 which is abnormally expressed in childhood acute lymphoblastic leukemia (73). These safety concerns have severely limited the number of funded studies using retroviral systems and resulted in a recommendation from the NIH that this vector system be used in extreme cases (54).

1.1.2.2 Adenovirus

Adenovirus is a double stranded DNA virus that is the focus of a wide variety of gene therapy strategies ranging from oncolytic treatment of glioma to cystic fibrosis. Structurally, the virus is around 200 nm in diameter with the viral capsid extended by fibers with distal knobs that interact with specific host cell receptors. Currently there are approximately 50 serotypes of adenovirus with group C being the most studied which contains serotypes 1, 2, 5, and 6. Adenovirus serotype 5 is the prototypical vector used for gene therapy. The 36 kb genome is flanked at the termini by inverted repeats that each contains an origin of replication and a terminal protein binding site. Unique to this virus and hepadnaviruses is the mechanism of protein primed viral DNA replication in which the viral encoded proteins preterminal protein and DNA polymerase attach at the 3' end in association with other cellular factors (Nf-1 and Oct-1) to form a preinitiation complex. The genomic replication occurs continuously through strand displacement. Much like other DNA viruses, the gene expression of adenovirus occurs in a temporally regulated cascade with the immediate early gene, E1A, serving as the major transactivator. This gene is commonly removed to generate replication defective vectors and supplied *in trans* in complementing cell lines to propagate viral vectors. As with other replication defective vectors, generation of recombinant replication competent vectors during large scale production is a major source of concern. Further deletion of the E2 region of the genome, which contains both the preterminal protein and DNA polymerase, attenuates the toxicity of these vectors but efforts to complement *in trans* have shown that this gene product may be limiting for efficient replication defective adenoviral production (208, 209). Using this production strategy viral titers up to 10^9 pfu/ml to 10^{12} transducing units/ml have been reported making this virus a popular choice on the basis of scaleable production (167).

Since transgene expression from adenoviral vectors is temporary, the host immune response is a major limitation for application into clinic (200, 201). Another limitation is the generation of a humoral response against this vector which may limit repeated administration (104). However, the knob region of the fiber rods of adenovirus can be modified to pseudotype gene therapy vectors such that retargeting to different tissue types and possible circumvention of the immune response can occur (188). Since long term transgene expression is not feasible with this vector type, the future of adenovirus gene therapy may be more amenable to diseases that require transient gene expression and vaccine generation.

1.1.2.3 AdenoAssociated Virus

Adeno associated virus is a parvovirus that requires the aid of helper virus such as adenovirus or herpes simplex virus for replication. This virus is a single stranded DNA virus that is able to infect nondividing human cells and stably integrate into a specific locus on chromosome 19 (153). Structurally, these viruses are the smallest among gene therapy vectors at 25 nm in diameter. Like adenoviruses, the AAV genome contains inverted terminal repeats which upon uncoating within the host nucleus fold to form palindromic structures that serve as primers for DNA synthesis. The viral encoded machinery is derived from overlapping sequences that take advantage of alternative splicing to generate multiple genes from the same transcript (177). Two of these genes, Rep78 and Rep68, comprise the regulatory elements that are necessary for DNA replication and gene expression regulation (183).

Therapeutic versions of AAV are gutted vectors that contain only ITR sequences with the transgene in place of the capsid genes (154, 155). Propagation of AAV vectors is commonly carried out in 293 cells. These cells are co transfected with a gutless infectious rAAV vector and a plasmid that provides the missing rep and cap genes followed by infection with an E1A deleted adenovirus. Alternatively, stable cell lines can be generated that express the rep and cap genes or

HSV-1 and/or Adenoviruses can be engineered to provide these functions *in trans*. Recently, a cell line has been generated by lentiviral transduction that provides rep, cap, E2, and E4 (125). Development of cell lines for production of AAV has been hindered by the toxicity of the essential rep gene and elaborate schemes have been devised to complement this gene through inducible systems (118, 130, 137, 199). For clinical applications, however, the helper virus must be removed from stocks to avoid toxicity associated with the helper virus and concentrate the rAAV.

Purification of rAAV is traditionally carried out through density gradient centrifugation which is not amenable to large scale production. However, recent developments in the field of viral purification using ion exchange chromatography have yielded improvements in AAV production (36, 138, 168). Size exclusion chromatography has also been successfully implemented due to the small size of the virus (168). Even with these advances in AAV purification the scalability remains an issue. Another limitation to AAV based gene therapy is the need to co- transfect the helper functions which severely limits the economy of scale for this vector. Complicating development of AAV as a gene therapy vector is the lack of correlation between in vitro and in vivo functional gene transduction (66, 103). Furthermore, random integration in the absence of helper functions *in vivo* needs to be investigated in greater detail before this vector strategy is implemented as this can lead to insertional mutagenesis. The risk associated with AAV based gene therapy is exemplified by the recent death in a arthritis trial where a fatalilty halted the study (87).

1.1.2.4 Pox Virus

Poxviruses are among the most complex viruses studied to date. The double stranded DNA genome is on the order of 190 kb and they encode approximately 185 open reading frames and contains inverted repeats of about 10 kb at each terminus. The double enveloped virion has

an inner core which houses viral DNA dependent RNA and DNA polymerases and is on the order of 350 nm in diameter (41). Once the virus has entered the cell through uknown mechanisms, the viral genome remains episomal due to its large size and the virion associated proteins direct gene expression in a temporal cascade. The early genes are synthesized first which include DNA processing and metabolism functions. A subset of these genes have sequence similarity to cellular growth factors and when secreted can induce proliferation to neighboring cells. The intermediate phase of gene expression is concomitant with DNA replication and downregulation of early gene synthesis. Poxviruses are unique in that all DNA replication occurs inside the cytoplasm independent of the host cell nucleus (41, 162). A cellular protein, Vitf2, which translocates from the nucleus, is essential for transcription of the intermediate genes which suggests tissue type specificity (54). Once DNA replication has ensued the structural components of the virus are expressed by the intermediate genes and the immature virion becomes enveloped through the *trans*-Golgi network. Subsequent transport to the cell surface and actin polymerization either directly transports the virus to surrounding cells or it is released as an extracellular enveloped virion. The kinetics of viral replication and egress are quite fast compared to other DNA virus with poxviruses producing up to 10000 infectious virions per cell in approximately 7 hours (54, 120).

Poxviruses as gene therapy vectors have been mainly applied to oncolytic approaches to tumor models as well as vaccination strategies. The most widely used poxvirus is vaccinia virus since its inception as a successful vaccine for smallpox ((81). This vector has many advantages over other viral systems such as the large genome and subsequently large transgene capacity. There is also the possibility of a vector with multiple gene inserts due to the large encoding capacity. One limitation is the need for a safe permissive cell line for large scale generation of recombinant viruses. This cell line must be able to generate progeny poxvirus with the correct post translational modifications for correct antigen presentation in the host as well as support

high titer growth. Another drawback of Poxvirus gene therapy is limited lifecycle within the host leading to short term transgene expression. Poxvirus vector transduction may therefore be more amenable to transient expression of an antigen for vaccine application. Herpes Simplex Virus

1.2 HERPES SIMPLEX TYPE 1 VECTORS

Herpes Simplex Type 1 is an enveloped 152 kb double strand DNA virus that can infect a wide variety of tissue types. This virus is amenable to gene therapy applications for a number of reasons. The large genomic size can house multiple gene or large transgene cassettes with up to 40 kb inserts. The virus has two distinct phases of infection that can be taken advantage of for a number or different maladies. In the latent state of infection the genome remains in a dormant state within primary neurons and transcribes a short 2 kb transcript referred to as the latency associated transcript (LAT). This LAT promoter can therefore be used to drive expression in the neuron for the life of the host. Such applications include treatment of chronic pain and ion channel modulation (10, 25). The lytic phase of replication can be taken advantage of for oncolytic therapy in particular in the brain. Since the virus remains dormant in post mitotic cells, vectors can be made to selectively replicate a wide variety of neoplasia (89, 124, 164, 180). The growth of these vectors is also straightforward since typically they are only missing one or two essential immediate early gene products. However, these vectors can be quite toxic and therefore impaired for gene transfer capacity. The current state of the art of HSV-1 gene therapy is described in the following sections highlighting the importance of this vector and the rationale for investigations into bioprocessing this type of gene therapy vector.

1.2.1 Basic Biology of HSV-1

The HSV-1 virion is on the order of 200 nm in diameter and is enveloped by a glycoprotein coat. The viral envelope contains 11 glycoproteins of which glycoprotein D, glycoprotein B, the complex of glycoprotein L/H are essential for entry into the host cell (8, 20, 95). An amorphous collection of proteins referred to as the tegument lies between the nucleocapsid and the glycoprotein envelope. The tegument contains transactivators necessary to initiate productive infection as well as alter the cell state for selective replication of the HSV-1 genome. Upon attachment and entry, the virus sheds the glycoprotein envelope and is transported to the nucleus through association with microtubules (45, 91). Tegument proteins are also liberated upon capsid release. These proteins serve to alter host function during viral replication. Once the nucleocapsid reaches the nucleus, the genomic content is released and transcription occurs through host RNA polymerase II. HSV-1 gene expression is coordinately regulated in an ordered temporal cascade (53). Three kinetic classes of gene expression illustrate the pattern of HSV-1 protein production. Immediate early (IE), early (E), and late (L) are the temporal categories assigned to each class. IE genes are characterized by expression in the absence of host or *de novo* protein expression. These gene products' functions comprise transcriptional regulation, mRNA processing, and host immune evasion. Dynamics of IE gene expression are regulated at the promoter level through viral and host protein DNA interactions. Tegument protein VP16 directs the assembly of viral and host proteins at the site of IE promoters. Host cellular factor (HCF), a proteolytically cleaved protein, associates with VP16 and accumulates host transcription factors to *cis* acting TAATGARAT (R represents any purine) sequences on IE promoters (83, 195, 196). Early genes represent the replication machinery encoded by the virus which includes DNA polymerase and ribonucleotide reductase. Late genes denote the structural components of the virion such as capsid and glycoproteins as well as tegument proteins packaged

in the virion. In the absence of IE gene expression, the virus cannot initiate a productive infection and the cascade is stalled. The next three sections highlight the roles of three critical immediate early gene products.

1.2.1.1 ICP4

Of all immediate early genes, ICP (infected cell protein) 4 has been implicated as a requirement for productive infection (6, 11, 37, 39). This gene is present in two copies within the genome on each side of the unique short region adjacent to an origin of replication (121). ICP4 contains different functional domains which permit direct DNA binding, transactivation, dimerization, and nuclear localization (40). ICP4 has been shown to interact directly with basal transcription factors such as TATA binding proteins (TBP), Transcription Factor IID (TFIID), and TFIIA. The indirect binding of ICP4 to early (thymidine kinase) and late (glycoprotein C) promoters infers its ability to transactivate essential downstream genes (98, 165). Additionally, this protein is immunogenic in mouse models and imparts cytotoxicity through sequestration of transcription factors by inhibiting host transcription (62, 108).

1.2.1.2 ICP0

ICP0 has been shown to transactivate both cellular and viral promoters in a non-specific fashion (50, 136). This viral protein is also a mediator of cytotoxicity most likely due to the E3 ubiquitin ligase activity that has been implicated in setting the cell cycle and altering the host response to infection (65, 113). The open reading frame of ICP0 contains multiple domains necessary for biological activity. The amino terminus contains a zinc binding RING finger motif with a ubiquitin specific protease, nuclear localization signal, and a self multimerization site at the carboxy terminus (48). These functional domains have been removed to create various insertional mutants whose phenotype has allowed clarification of ICP0 activity. In the absence

of the RING finger domain, inhibition of host cellular interferon stimulated genes is ablated (100). Growth phenotypes of ICP0 null mutants are MOI and cell type dependent. Infections at MOIs at or above 1 tend to grow normally in comparison with wild type in Vero cells while infections below this threshold display 3 to 4 log differences in productivity (19). However, in osteosarcoma cells, such as U2OS, the ICP0 null mutant grows similar to wild type which implicates these cells as having some function that can either rescue the growth phenotype or is missing some function that ICP0 specifically targets for selective expression of the viral genome(202). Another feature of this viral protein is the ability to combat host immune response to infection. Several studies have shown that ICP0 can degrade promyelocytic leukemia (PML) or nuclear domain (ND10) structures that form at the nuclear membrane in punctuate organelles in response to physiological stress such as heat shock or viral infection (47, 61). These PML bodies have been shown to repress transcription and gene expression from incoming pathogens which has implications on viral spread and productive infection. The toxicity imparted by ICP0 can be attributed to it's ubiquitin ligase activity. ICP0 has been shown to target specific host response molecules to allow selective replication of the virus It is therefore, critical to disseminate the role of ICP0 and its contribution to the spread of the virus for safe clinical mutants that lack this function.

1.2.1.3 ICP27

The majority of HSV-1 post-transcriptional processing has been shown to be directed by one viral gene, ICP27. Analogous to the other IE gene products, ICP27 is multifaceted with different domains that direct viral functions. This 63 kDa protein has been shown to direct viral transcript nuclear transport during infection and is conserved among all herpesviruses. The N-terminus contains an RNA binding domain as well as nuclear import and export signals (112, 156). Transactivation and early to late gene transition functionalities have been mapped to the

C-terminus (169, 170). Novel studies using fluorescence imaging reveal that this protein has a self-interacting domain connecting the N and C termini that is essential for full ICP27 functionality (207). These investigations highlight the nature of typical HSV-1 IE genes in that they are multifunctional to efficiently associate with host cell machinery to regulate the genetic cascade of expression.

Timely expression of ICP27 is necessary, in conjunction with ICP4, for propagation of HSV-1 in culture. The transcriptional transition from the early to late gene class requires the presence of ICP27 in that it can downregulate early transcripts and promote late gene transcription (80, 144, 191). Furthermore, detailed work on prereplicative ND10 bodies reveal that ICP27 colocalizes with ICP0 and ICP4 in these nuclear structures upon infection indicating that this protein may serve a regulatory role in the viral life cycle (123). Along these lines of evidence, ICP27 interaction with splicing regulatory factors and global inhibition of transcript splicing has been implicated by numerous studies (18, 23, 109, 159). Likewise, ICP27 has been shown to shuttle between the nucleus and cytoplasm during infection. This transport of ICP27 is associated with cellular factors linked to RNA binding and RNA export from the nucleus indicating that HSV-1 can efficiently exploit the natural RNA transport pathways to aid in gene expression. Relocalization and host shut off function of ICP27 most likely serves to alter the host cell in such a way as to selectively allow viral transcription and repress cellular functions that could inhibit the progression of infection.

1.2.2 Construction of HSV-1 Vectors

HSV-1 vectors can be utilized in three different ways depending on the application and method of construction. Outlined below are the current methodologies for construction of HSV-1 based gene therapy vectors.

1.2.2.1 Amplicon Vectors

The safest way to deliver genetic material is by eliminating pathogenic effects of the virus while maintaining stable expression of the transgene. Amplicon vectors are essentially the viral DNA backbone in a plasmid form with an origin of replication, a packaging sequences, and cis acting elements to drive expression of the exogenous gene. Since this DNA structure is minimal, the payload capacity is up to 100 kb which makes this strategy quite attractive. Likewise, since there are no viral genes associated with amplicons, the toxicity profile is superior due to the lack of reactivation, complementation, or recombination with latent genomes. To propogate these vectors helper virus must be supplied to provide *in trans* gene functions that amplify and package the amplicon DNA into virions. This helper virus, however, can contaminate viral stocks leading to poor toxicity profiles and difficulties in scale up (84). Currently, bacterial articificial chromosomes (BAC) are used to provide the missing HSV-1 gene functions. These BACs lack a packaging signal and one essential gene, usually ICP27. This approach leads to fewer contaminating helper virus particles since (i) the BAC genome cannot be packaged into virions and (ii) the virus is defective without the essential gene, and (iii) the helper genome containing the BAC is considerably larger so packaging is not as efficient. Co-transfection of complementing cell lines with BAC helper virus and amplicons can yield up to 10^7 transducing units/ml, but large scale production is limited due to the need for expensive transfection reagents.

1.2.2.2 Conditional Replication Vectors

HSV-1 vectors that replicate in certain cellular microenvironments are normally devoid of viral replication machinery. The selective replication of HSV-1 vectors has direct application

in oncolytic therapy. These viruses are replication defective in normal post mitotic tissue such as primary neurons. However, in actively dividing neoplasms these viruses are supplied the missing functions *in trans* which include viral gene necessary for DNA metabolism such as thymidine kinase, ribonucleotide reductase, and the viral DNA polymerase. Mutations in these genes along with the neurovirulence factor (ICP34.5) which is diploid in the viral genome, allow selective replication in the microenvironment of a brain tumor in contrast to normal non-dividing glial cells. HSV-1 mutants lacking neurovirulance factor ICP34.5 have been shown to efficiently infect and reduce tumor mass in both mouse, primate, and human models (110, 117). This vector, G207, has been deleted for ICP6, the viral ribonuceotide reductase, and both copies of ICP34.5 allowing it to only replication once in the glioma. Through homologous recombination of wild type virus, KOS, with a cotransfected targeting plasmid that encodes the LacZ gene flanked by upstream regulatory sequences of ICP6 and a stop codon introduced into the downstream elements after the LacZ insertion (58). This strategy allows for marker transfer and relatively simple selection of blue plaques in the presence of beta galactosidase staining. Removal of ICP34.5 was confirmed by restriction digest and southern blot analysis (28). The added advantage of using HSV-1 for this mode of therapy is that latency associated genomes is apathogenic to the surrounding healthy glial cells. Oncolytic therapy is an attractive approach as it allows selective replication specific to the disease state. This method of viral construction and application is now being extended to other DNA viruses such as adenoviruses and vesicular stomatitis viruses (189, 197).

1.2.2.3 Replication Defective Vectors

Replication defective vectors represent a broad area of research for the delivery of exogenous DNA (160, 205). These vectors are typically mutated by one or more essential functions that will preclude viral DNA replication and thus render the virus replication defective.

Normal construction of these viruses is carried out in a similar fashion to selective replication vectors. Marker transfer remains a convenient method to removing a particular locus and replacing the viral gene with a colorimetric marker such as EGFP, lacZ, or RFP flanked by the homologous sequences in a targeting plasmid via co-transfection and infection with the parent virus (92, 93).

These viruses are normally propagated to high titer through the use of a complementing cell line that provides the missing gene products *in trans*. The complementing producer cell lines are constructed to express the viral genes only under the context of infection. Varied complementation/mutation strategies have been employed to generate and propagate replication defective HSV-1 mutants depending on the tissue type and kinetics of expression. Of the immediate early genes, ICP4 and ICP0 are the most toxic in cell culture. In conjunction with deletion of the ICP27 locus, removal of ICP4 and ICP0 dramatically removes the toxicity of the vector while allowing multiple or relatively large transgene insertions into the vector backbone. This strategy has led to the generation of triple and quadruple mutant vectors devoid of any immediate early function (150). Another approach is to preclude the expression of the immediate early class of genes by constructing VP16 mutants. Since the tegument protein, VP16, can efficiently transactivate the immediate early class of genes, its absence should theoretically prohibit immediate early synthesis and subsequently viral DNA replication. Multiple deleted viruses that lack the VP16 and U_L9 (origin binding protein) loci are completely incapable of replication within Vero cells up to an MOI of 30 (203). However, the issue of leaky viral promoters in different tissue types remains an issue. Therefore, the safest vectors for gene transfer will have the most cytotoxic IE genes ablated to remove the opportunity for viral replication.

1.2.3 Applications of HSV-1 Vectors

The direct application of HSV-1 for gene therapy has proven successful in a wide variety of tissue types owing to its natural ability to infect many tissue types. Gene transfer is possible in primary epithelia, peripheral nerves, adipose tissue, hepatic tissue, cancerous tissue, and skeletal muscle (1, 33, 55, 59, 135). This array of potential tissue types for therapy highlights the capacity of this vector type for gene transfer applications.

HSV-1 as a gene transfer vehicle has been exploited for numerous applications of human disease. The use of HSV-1 as an oncolytic therapy has proven successful in both the primate model and in humans (106). The use of vectors attenuated for the virulence factor, ICP34.5, and the viral encoded DNA polymerase UL39 have shown improved safety and sensitivity to prodrug administration such as ganciclovir. This strategy allows an extra level of protection since delivery of ganciclovir will inhibit virus replication and reduce possibility of herpes encephalitis once the virus has been delivered. These vectors also show no effects on motor or cognitive development as assayed by open field maze test task (139).

Neuropathies associated with pain also represent an active area of research in the field of HSV-1 gene therapy. Recent efforts have proven useful in ectopically expressing the transient receptor cation potential vanilloid (TRPV1) receptor and subsequently searching for receptor modulators using a selective viral replication strategy (172). These studies have led to enhanced understanding in pain signaling and receptor physiology. Recent efforts have revealed reduced pain response by the hind footpad lifting tests in response to heat in a mouse model. The use of HSV-1 as a tool is also employed in the study of early embryogenesis for identifying critical components in development. A selective replication system has been recently developed with

replication driven by a developmental promoter controlling ICP4. This system allows the identification of cellular factors that can drive the expression of the essential immediate early gene ICP4 that will drive viral replication. cDNA libraries can be routinely constructed and pools clonal isolates of HSV-1 vectors housing these libraries are now being developed. Since HSV-1 can infect and transduce a wide variety of tissue types, a library screen based on certain disease states has the potential to uncover previously unknown mechanisms in disease models. The use of HSV-1 as a tool for investigating molecular biology dynamics as well as virus host cell interactions represents a promising arena of molecular therapy based on HSV-1. Furthermore, insight gained from cDNA library screens based on HSV-1 vectors will serve to direct gene therapy in a high throughput manner.

1.3 MANUFACTURING OF HSV-1 VECTORS

HSV-1 vectors are routinely produced from cell culture and subsequently purified by centrifugation. There are many limitations in both the upstream and downstream components of manufacture which represents a major bottleneck to bringing these vectors to clinic. The main areas under current investigation are scale up and downstream purification of HSV-1 vectors and transfer of this technology from the bench to cGMP environments.

1.3.1 Production of HSV-1 Vectors from Cell Culture

HSV-1 vectors are routinely propagated using a mammalian host cell line. Vero (African green monkey kidney) cells represent the most common cells used for viral vector production owing to their lesion in the interferon response and ease of culture (46). These cells are aneuploid and will

grow indefinitely in culture so there are no passage limitations. Vero based cell culture represents similar species origin to humans and therefore has been used to study broad areas of disease and molecular virology. For instance, these cells have been approved by the FDA and European governments for biologic production of vaccines against smallpox, rotavirus, and poliovirus (85, 140, 192). Culturing these cells is straightforward, however, viral propagation adds another level of complexity. Critical cell culture considerations are half life of the virus in different culture media and relative stability for long term storage. Studies investigating pH and heat sensitivity have outlined important parameters for culture conditions for HSV-1 production (134). Additionally, multiplicity of infection, serum content, and culture confluency at time of infection can drastically alter the efficiency of production. All of these parameters need to be optimized to realize scaleable manufacturing methods to transfer technology from bench scale to the clinic.

1.3.1.1 Complementing Cell Lines

Since most therapeutic viral vectors are missing essential gene functions, complementing cell lines need to be generated to provide these functions *in trans*. The tissue type used to construct these lines is an important parameter for consideration since the FDA imposes strict requirements on the acceptable cell lines used for production of biologics. The most common cell types are chinese hamster ovary (CHO), human epithelial cells, PERC6, and Vero cells. These cells while, not all continuous, should be free of adventitious agents and non-tumorogenic. This last restriction has severely limited production since most tumor lines are able to support viral growth to higher titers than normal cell lines. As well, the limited passage of primary tissue makes cell line development difficult. The more common Vero cells are routinely used since they grow continuously while their immortalization has not yet been characterized.

The complementing character of the cell line is crucial to propagation of the replication defective vector. Stable complementing cell lines are routinely generated by transfection with a plasmid that contains the gene of interest with a drug selectable marker. The lines then are selected by clonal isolation and tested for viral production capacity. The promoter driving the expression of the viral gene is also crucial since uncontrolled expression of the cytotoxic gene would normally negate selection. This promoter must be responsive to infection and be selective for the incoming virus. Since most DNA viruses express their genes in a cascade regulation this gene or set of genes are normally of the immediate early class that preclude viral DNA replication. The stability of the complementing cell lines are routinely tested by drug resistance as the passage number grows and it has been observed that the complementing activity drops as the cell are cultured to higher passages (133).

1.3.1.2 Cell Culture Configurations

The type of cell culture configuration can drastically alter the economy of viral vector manufacture. Most common cell culture systems are T-flasks with cells grown in monolayers. This configuration however is limited by difficulty in realizing the surface area requirement to grow a large scale culture. Considerable effort is required to culture multiple T-flasks to achieve the surface are required to reach manufacturing scale. Additionally, contamination can easily occur as the number of required flasks grows demonstrating this strategy is not efficient for large scale cell culture. An alternative is the use of Nunc cell factories that are monolayers assembled in sheets of polystyrene contained in a single unit with one air vent. While this configuration eases the burden of subculturing multiple flasks, inefficient CO_2 exchange limits the viability, and therefore productivity, of the cells.

Bioreactor culture remains the choice of most large scale operations due to ease of use and limited opportunity for contamination. For most suspension culture, cell passage is routine

while anchorage dependent cell lines require microcarriers to supply substrate for growth. Cytodex-1 and Cytodex-3 beads have been shown to support growth of Vero cells in suspension as well as production of vaccines and viral vectors (147, 182, 194). These beads are crosslinked dextran with diethylamino ethyl groups that can adhere cells through positive charge interactions with the outer cell membrane. Fibracell disks are an alternative system that relies on the use of polyester and polystyrene non-woven fibers that can adhere cells through hydrophobic interactions with the outer cellular membrane. While both microcarrier culture systems support growth of anchorage dependent cells in suspension, Fibracell disks rely on the use of perfusion culture to deliver nutrients. The main disadvantage of this system is entrainment that occurs when cells adhere to the fibers which has been validated through crystal violet staining. More importantly, virus produced from the cells is also detained within the disks. For large scale manufacture of HSV-1 based viral vectors Cytodex beads remain the state of the art for culture configuration. In this system air is either sparged in through a downcomer or through the headspace. The bubbles that arise from sparged air can lead to reduced cell viability due to bursting within the cell monolayer on the microcarriers. Distributor design has led to decreased bubble size that overcome this limitation, however, accurate monitoring of dissolved oxygen within large scale systems remains a limitation to development of this cell culture configuration due to low specific oxygen uptake rates of the cells, sensitivity of the cells to dissolved oxygen, and the kinetics of the response to controlled dissolved oxygen (57, 132, 148). With advances in the areas of membrane detectors and tighter control these issues will become neglible in the future for production cell based biologics.

1.3.2 Purification of HSV-1 Vectors

HSV-1 vectors have been routinely purified through a variety of methods which include high speed centrifugation, sucrose gradient, and column chromatography. These methods are designed to purify the virus from contaminating host cell protein and DNA with advantages for each in regards to efficiency, cost, and scalability. The purification step represents the most active area of research since stringent requirements have been set by the FDA for recombinant biologics approved for use in humans.

The harvest step of virus consists of collecting cellular debris and supernatant once the culture has reached 80-100% cytopathic effect (CPE). This morphology is usually characterized by cellular membrane blebbing, rounding up of the cells, and detachment from the monolayer. At this point, the cells and supernatant are collected by scraping the remaining cells and salt treating the debris with a moderate concentration (0.45 M) of NaCl to compete off any bound virus to the cellular receptors or intracellular organelles. For most HSV-1 viral vectors there will be a significant fraction remaining within the cellular pellet. As the vector becomes more deleted, the ratio retained within the cells is increased due to inefficient complementation during propagation. Triple mutants that are devoid of essential functions ICP4, ICP0, and ICP27 are particularly attenuated for growth and therefore require extensive processing of the pelleted cellular debris while double mutants missing only ICP4 and ICP27 are not as stringent in this requirement. This harvest step includes sequential salt treatment, freeze thawing, and sonication to release cellular bound virions.

Upon harvest, the virions are further purified through step wise filtration through sequentially smaller pore size filters. However, as the filter size approaches the diameter of the virus (0.22

um) significant losses can occur so larger (1.2, 0.8 and 0.45 um) filters are routinely used to prefilter the virus before either centrifugation or column chromatography. Since these methods are not amenable to large scale purification other techniques have been employed to clarify cell debris. Our lab has developed tangential flow flow filtration as a method to take advantage of size separation for separation of virus from cellular contaminants. DNA contamination remains a challenge due to the fact that it can adhere to the viral membrane. Steps taken to remove include detergent and benzonase treatment. However, these protocols are far from optimized since benzonase removal can complicate the harvest by contributing to the contaminating nonviral protein content.

1.3.2.1 Centrifugation

Pre polishing steps are necessary to remove large cellular debris that remains upon filtration. Traditionally, this is carried through gradient centrifugation either through sucrose or cesium chloride (126, 127). While these purification steps yield relatively high purity genome containing virions, their adaptation to large scale is limited. Therefore, centrifugation remains a common intermediate step in viral vector purification. High speed centrifugation can achieve concentrated stocks however aggregation and cosedimented protein complicate this process.

1.3.2.2 Ion Exchange Chromatography

Vigorous purification methods that can separate genome containing vectors which are adaptable to large scale remain critical to advancing gene therapy vectors into clinic. Strict FDA requirements for vector purity and concentration have resulted in numerous investigations into optimal separation technologies. These include immunoaffinity, membrane adsorption, ion exchange, and molecular imprinting. Ion exchange chromatography represents a scalable robust method for purifying proteins as well as viral vectors. This strategy relies on electrostatic

charge-charge interactions to separate molecules of interest. Salt, usually in the form of NaCl, is loaded onto the column and the appropriate ion binds to the resin. By exchanging either a cation such as sodium or an anion such as the chloride ion, the virus can adsorb to the column matrix. These matrices usually consist of crosslinked agarose with functional moieties covalently linked to impart charge specificity. The crosslinked matrix has an added advantage of acting as a molecular sieve separating small molecular mass proteins while allowing the virus to pass through or bind to the functional groups. These functional groups are normally represented as a strong or weak ion exchanger. Strong cation exchangers consist of negatively charged sulfopropyl (SP) functional groups while strong anion exchangers are usually a positively charged quarternary amine (Q). Loading conditions such as pH, salt composition, and flow rate dictate the capture efficiency of the target molecule. In the case of virus purification, in particular enveloped virions such as HSV-1, the glycoprotein layer can be targeted to the matrix by altering the electrostatic interactions through ionic strength and proton concentration of the load material. The salt concentration will also alter the viscosity of the loading material which can affect the hydrodynamic properties of the virus as it binds to the column. Furthermore, mass transfer effects must be considered as the flow rate will affect contact time as well as the shear rate the virus experiences as it traverses the interstitial space of the packed column. All of these parameters are critical to optimizing the purification process and the biophysical properties of the virus must be considered to achieve optimal purity while maintaining scalability.

2.0 MATERIALS AND METHODS

2.1 VIRUSES AND CELLS

All viruses are based on the KOS strain of HSV-1. Nurel P is a double IE mutant missing ICP27 and both copies of ICP4 originating from the d120 vector (38, 111). The promoter of ICP22 has been deleted of the VP16 responsive TAATGARAT motifs rendering its expression profile under the beta regime of HSV-1 temporal cascade (151). This mutation also removes the immediate early toxicity associated with ICP22 in that it can stabilize the transcript processing of the toxic IE ICP0 gene (22). Additionally, the TNF-α gene driven by the HCMV promoter resides in the U_L41 (virion host shutoff gene) locus along with the rat connexin 43 cDNA driven by the ICP0 promoter and the enkephalin gene in the ICP4 locus (128). This vector is propagated on 7B cells which are Vero cells that contain the ICP4 and ICP27 genes on a single transcript driven by their own natural promoters (105). The JDTOZHE vector (provided by David Krisky) is a progeny of TOZHE (93) which is devoid of the IE genes ICP22, ICP27, and both copies of ICP4. The U_L41 locus has been replaced by the LacZ coding sequence driven by the ICP0 promoter. One genomic copy of the joint region spanning U_L54 to U_S2 of the KOS backbone comprising 15 kb is also missing in JDTOZHE constraining the genomic configuration in a single isoform as opposed to the four equimolar inversion isoforms during normal infection. The EGFP transgene is driven by the HCMV promoter and with the SV40 polyA in both of the ICP4 loci. JDTOZHE can be propagated on ICP4 and ICP27 complementing cell lines with different efficiencies relative to the complementation profile of ICP4. Our laboratory uses three

different ICP4 and ICP27 complementing cell lines (7b, 433, and Q5) to propagate triple mutants depending on the backbone. 433 cells are a progeny of N23 cells transfected with the 4BLA plasmid. QOZ22REH1 (provided by David Krisky) is a quadruple mutant vector missing all IE gene functions with the HCMV promoter controlled expression of EGFP in the ICP0 locus flanked by the SV40 polyA. The U_L41 locus contains the LacZ transgene cassette driven by the ICP0 promoter. In addition, the ICP22 promoter controls expression of the dsRED2 gene at the ICP22 locus replacing this gene. JDQOZEH1 (provided by David Krisky) is another quadruple mutant that is a progeny of JDTOZHE that is missing both copies of ICP0 and the HCMV driven EGFP cassette in the ICP0 locus. Both QOZ22REH1 and JDQOZEH1 require ICP4 and ICP27 to replicate in cell culture. In addition, ICP0 is also required since the vector backbone is severely deficient for essential IE gene functions. The Q backbone of the KOS virus is routinely propagated in our laboratory with 01 cells that are progeny of N23 cells that house the ICP4 gene driven by the ICP0 promoter and the ICP0 gene driven by the ICP4 promoter. All cell lines are propagated in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Inc. Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS Atlanta Biologics, Atlanta, GA), 100 U/ml penicillin/streptomycin, and 2mM glutamine (Invitrogen Corporation, Carlsbad, CA) at 37°C in 5% CO_2 humidified air. Q5 cell lines were created by transfecting N23 cells with a plasmid containing the ICP0 promoter driving ICP4 and ring isolated after passage in 50 ug/ml Blasticidin S HCL (Sigma-Aldrich, St. Louis, MO). 01 cells were constructed by transfecting Q5 cells with a plasmid bearing the ICP0 gene driven by the ICP4 promoter and ring isolated with 20 ug/ml of Hygromycin (Sigma-Aldrich, St. Louis, MO).

2.2 CONSTRUCTION OF IE GENE EXPRESSION PLASMIDS

The plasmid (4BLA provided by David Krisky) bearing the ICP4 coding sequence driven by the ICP4 promoter (SphI (131733)to DdeI (126765) of KOS) used to construct the ICP4/ICP27 complementing cell line (433) was used to subclone a plasmid devoid of the ICP4 promoter by a Sal I digestion which gave a 500 bp and a 4.5 kb fragment. This 4.5 kb fragment was gel isolated via the Geneclean kit (QBIOgene, Morgan Irvine, CA), blunted via Klenow enzyme (New England Biolabs, Ipswitch, MA), HindII linker ligation, and transformed into GC5 (GeneChoice, Inc. Gaithersburg, MD) competent E.Coli to create plasmid 4BLA-H. Plasmid pUC19 (New England Biolabs, Ipswitch, MA) was digested with PstI and treated with T4 DNA polymerase to remove 3' overhangs to create pUC19-P. The full ICP0 promoter was subcloned by NcoI digestion of the full ICP0 plasmid 28.4 (provided by Ying Jiang), blunted via Klenow, and gel isolated resulting in a 1.7 kb fragment containing the full ICP0 promoter. The 1.7 kb fragment was ligated into the blunted PstI site of plasmid pUC19-P to create plasmid pUC19-O. Plasmid pUC19-O was digested with XbaI, blunted via Klenow, and HindIII linker ligation to create pUC19-O-H. Plasmid pUC19-O-H was digested with HindIII and gel extracted to isolate the ICP0 promoter flanked by HindIII sites as a 1.7 kb fragment. This 1.7 kb fragment was then ligated into HindIII digested 4BLA-4 via the rapid ligation kit (Roche Diagnostics, Mannheim, Germany) to create plasmid S0-7 which has the ICP0 promoter driving ICP4 with the mammalian selectable marker blasticidin. This plasmid was then amplified in GC5 competent cells under ampicillin 10 ug/ml (Sigma Aldrich St. Louis, MO) selection.

Plasmid HXSc5 (provided by David Krisky) is a sublcone of pREP4 (Invitrogen, Carlsbad, CA) with the following modifications: the SacII-BamHI fragment of the ICP4 promoter from plasmid 4BLA driving the ICP0 coding sequence from the ATG to the HpaI site and a EcoRV to ClaI deletion to remove the EBNA-1 gene. The HpaI site was blunted and changed to BglII via linker ligation. The ICP4 promoter construct driving ICP0 resides as a PmeI to XbaI 3 kb fragment.

2.3 QUANTITATIVE REAL TIME PCR

RNA was harvested from infected monolayers by first washing the cells twice in fresh PBS and subsequently scraping the cells into a lysis buffer provided in the Qiagen RNEasy kit. Cells were homogenized using Qiashredder columns and subject to RNA purification according to the manufacturers instructions. cDNA was generated by first annealing random hexamer primers (Roche) to RNA eluted from the Qiagen columns. Reverse transcription was then carried out in the presence of 10mM dNTPs and 1 Unit of SuperScript III reverse transcriptase (Invitrogen) at 50°C for 50 minutes. Reverse transcription reactions were heat inactivated at 75°C for 15 minutes and kept at -20°C for long term storage. Quantitative PCR reactions were performed through the use of an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The double differential method is used to calculate the amount relative to both an endogenous gene (18s) and a calibrator (plasmid containing the coding sequence) where $\Delta\Delta C_T$ is $(C_{T\ sample} - C_{T\ 18s}) - (C_{T\ background} - C_{Tcalibrator})$. $C_{Tbackground}$ is obtained from an RNA sample without reverse transcription. The calibrator plasmid also serves as a control from assay to assay. Primer and probe sequences for HSV-1 and cellular genes are as follows: ICP4 Forward Primer: GGTGGCTCCAGAACCCG, ICP4 Reverse Primer: AAGGAGCTGCTGTTGCGC,

ICP4 Probe: CAGGCCTGCTTCCGGATCTCGG, ICPO Forward Primer: AACGCCAAGCTGGTGTACCT, ICP0 Reverse Primer: TCACGATCGGGATGGTGC, ICP0 Probe: TGACGCCCAGCGGGTCGTTC, ICP27 Forward Primer: TTGATATGCTAATTGACCTCGGC, ICP27 Reverse Primer: ATTCCAGGTCGTCGCGG, ICP27 Probe: TGGACCTCTCCGACAGCGATCTGG, TK Forward Primer: UL1 Forward Primer: GGGTTTTTTGGAGGACTTGAGTT, UL1 Reverse Primer: CAAGCGCGTTTCTGTTTCC, UL1 Probe: CCCCGCGTTTCCTGCCAACA, gD Forward Primer: CCCCGCTGGAACTACTATGACA, gD Reverse Primer: GCATCAGGAACCCCAGGTT, gD Probe: CTTCAGCGCCGTCAGCGAGGA, 18s Forward Primer: CCCGAAGCGTTTACTTTGAAA, 18s Reverse Primer: CCTCAGTTCCGAAAACCAACA, 18s Probe: CGCCTGGATACCGCAGCTAGGAATAAT.

2.4 PROTEIN EXPRESSION ASSAY

Infected monolayers were rinsed twice with fresh 1X PBS and overlayed with RIPA Lysis Buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1.0% Deoxycholate, 5 mM EDTA, 1X Protease Inhibitors (Sigma Aldrich, St. Louis, MO) at 5.5 uL/cm^2. Collected cell lysate was incubated at 5°C for 15 minutes vortexing every 5 minutes. Cell lysate was collected by centrifuging at 10,000 rpm for 10 minutes and collecting the supernatant. Protein concentrations were determined by BioRad BSA kit and the lysates stored in aliquots at -20°C if not used immediately. Equal amounts of protein were boiled for 15 min in NuPAGE LDS Sample Buffer (106mM Tris HCL, 141 mM Tris Base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red) and subjected to

resolution by NuPage precast Bis Tris polyacrylimide (4-12%) gels at 100 V run in MOPS SDS Running Buffer (50mM MOPS, 50 mM Tris Base, 0.1% SDS, 1mM EDTA). Resolved proteins were transferred to a PVDF membrane using the XCell Blot Module at 22 V for 1.5 hours in NuPAGE Transfer Buffer (25 mM Bicine, 25mM Bis Tris (free base), 1mM EDTA). Membranes were blocked in 1X PBS containing 6% FBS and incubated overnight with primary antibody concentrations (1/1000 ICP27, ICP4, and ICP0 (Fitzgerald, Concord, MA), 1/1000 VP5 (Virusys Corporation, Sykesville, MD), 1/1000 ICP22 (kind gift from P. Schaffer), gB (gift from W. Goins)) at 5°C. Membranes were washed three times for 15 minutes each in 1X PBS and incubated with secondary antibodies (anti-mouse HRP (ICP4), anti-rabbit HRP (ICP0), anti-mouse alkaline phosphatase (VP5) at 1/1000 concentration or anti-rabbit alkaline phosphatase (ICP22 and gB) in 6% FBS in 1X PBS (ICP4 and ICP0) or 1X TBS (VP5, ICP27, ICP22, and gB). Bands were developed by either NBT/BCIP (VP5, ICP27, ICP22, or gB) or horseradish peroxidase (ICP4 and ICP0). Goat anti-SAG (Everest Biotech, Oxfordshire, UK) was used at a primary concentration of 1/100 and developed via anti-goat HRP at 1/750. A prestained molecular marker (Biorad Laboratories, Hercules, CA) was used to match molecular weights.

2.5 VIRAL GROWTH AND CELL GROWTH ANALYSIS

Complementing cell lines were assayed for growth by trypan blue exclusion dye at selected times post seeding in tissue culture flasks or well plates. Viral propagation was carried out by seeing cells at subconfluency in either tissue culture flasks or well plates in accordance with the experiment. For large scale propagation T-150 flasks were seeded with 13E6 cells and infected at an MOI of 1 to 0.01 one day post seeding. For each T-150, 2 ml of virus was incubated at

37°C and agitated by rotating the flask every 15 minutes to distribute the virus along the monolayer. 10 ml of growth medium was then added to the flask and at 24 HPI the flask was moved to 33°C. JDTOZHE was propagated on Q5 (0d4) cells, Nurel P was grown on 433 (4d4) cells, JDQOZEH1 and QOZ22REH1 were grown on 01 and 0440 cells respectively. JDTOZHE, JDQOZEH1, and QOZ22REH1 were grown at an initial MOI of 1 and media was supplemented at 400 nM Trichostatin A (91). Nurel P was grown on complementing cells at an initial MOI of 0.01. As CPE approached 100% cells and virus were harvested and treated with 0.45 M (final concentration) NaCl to release cellular associated virus. Salt treatment was carried out by rotating cells on a LabQuake rotary shaker (Becton Dickinson, San Diego, CA) for 45 minutes at room temperature. Once in 0.45 M NaCl the virus can be stored for up to a month at 5°C before further purification. As needed the virus stock can either undergo high speed centrifugation or ion exchange chromatography. Prior to either treatment, the virus stock is clarified by passing through a 0.8 micron bottle top filter (VWR, West Chester, PA) under house vacuum. High speed centrifugation is carried out in a Sorvall S100 Rotor (Thermo Fisher, Scientific, Pittsburgh, PA) at 20,000 rpm at 5°C for 30 minutes in Oakridge tubes (Nalgene, Rochester, NY). Pelleted virus is gradually reconstituted by incubation in PBS overnight at 5°C before vortexing to resuspend.

2.6 VIRAL TITRATION

Titers of viral stocks were carried out on appropriate complementing cell lines by serial dilution of virus into culture media and subsequent infection on 0.8E6 cells per well in 35 mm plates. Cells were rotated in a rotisserie manner on a LabQuake rotary shaker at 37°C for one hour before plating in 6 well plates. One day post seeding infected cells, media was removed and

replaced with DMEM containing 1.25% methyl cellulose to prevent secondary spread of virus while moving plates for viewing the progression of infection as evidenced by cytopathic effect (CPE). Titration of virus was quantified by counting plaque forming units (PFU) by removing media when CPE was verified by confocal microscopy and staining with Crystal Violet (2 mg/ml) in a 50:50 mixture of methanol and deionized water. One day post staining, the wells were washed out with water and plaques counted.

2.7 ION EXCHANGE PURIFICATION

Viruses used in all experiments were purified via ion exchange. Virus stocks either stored at 5°C in 0.45 M NaCl or resuspended pelleted virus were delivered to either a prepacked HiTrap SP (cation exchange) or Q (anion exchange) column (GE Lifesciences, Piscataway, NJ) via a P1 peristaltic pump (GE Lifesciences, Piscataway, NJ) at 0.3 ml/min. The columns were conditioned before virus loading by washing out the manufacturer's storage material (70% ethanol) with 5 column volumes of 0.22 filter-sterilized deionized water. Columns were sterilized by contacting with 0.5 N NaOH for 1 hour at 0.3 ml/min. Upon sterilization, NaOH was washed out with 5 column volumes of 0.22 filter-sterilized 1X PBS pH = 7.0. 5 column volumes of 1 M NaCl in 1X PBS pH = 7.0 was then passed through the column at 0.3 ml/min to charge the column with the exchange ion (Na^+ for SP and Cl^- for Q). Excess NaCl was washed out of the columns with 5 column volumes of 1X PBS pH = 7.0. Upon virus loading the flow through was collected and labeled "Load". Unbound virus was then washed out with 5 column volumes of 1X PBS pH = 7.0 and this fraction was collected and labeled "Flow Through". Bound virus was eluted with 0.22 filter-sterilized 0.45 M NaCl in water. Fractions were

collected manually in 1.5 ml eppendorf tubes at 0.5 ml per fraction. Upon collection of 13 column volumes, each fraction was diluted 1/10 in 1X PBS pH = 7.0 and assayed for protein and DNA content by UV spectrophotometry (Perkin Elmer, Waltham, MA). Fractions with the highest 260 absorbance were pooled and resuspended in glycerol 10% v/v final concentration and stored at -80°C in cryovials (Corning Life Sciences, Lowell, MA).

3.0 DEVELOPMENT OF EFFICIENT COMPLEMENTING CELL LINES

Herpes Simplex Virus Type 1 is a promising vector for gene therapy applications for a variety of reasons. Large scale propagation of this vector is critical for clinic application. To provide high titer virus requires efficient cell line substrates that can stably provide missing gene functions *in trans* with the appropriate timing. Replication defective HSV-1 vectors require specialized cell lines for propagation. The replication defective genetic backbone is widely varied depending on end application and certain gene products must be supplied in adequate level with appropriate kinetics. These cell lines must also adhere to current FDA guidelines if the vectors are to be clinical grade. Ideally, these cell lines also provide the cytotoxic gene products only in the context of infection so as to avoid cell death under normal cell growth conditions. These genes should also lack any homology with the input virus to escape recombination events that could lead to replication competent virus (RCV). An efficient complementing cell line is described that provides the IE HSV-1 genes ICP4 and ICP27. The ICP0 promoter was chosen to drive the major transactivator ICP4 in an effort to provide higher levels and faster kinetics of expression. Quantitative PCR and western blotting reveals a better complementation profile with respect to ICP4 under control of the ICP0 promoter in the context of Vero cells infected with JDTOZHE as compared to its natural promoter. Furthermore, the viral yields of the JDTOZHE vector were increased by an order of magnitude as assayed by plaque forming units as well as genomic copy number. By optimizing this complementing cell line, we have created a reagent to propagate highly defective HSV-1 vectors. A progeny of this cell line is also described that can be used to

propagate HSV-1 mutants that are devoid of all IE functions. This cell line has the ICP0 gene driven by the ICP4 promoter in addition to ICP4 driven by the ICP0 promoter and ICP27 driven by its natural promoter. This cell line provides a further 10-fold improvement in yields with respect to triple HSV-1 mutants but remains the only substrate that can be used to propagate quadruple mutants without rescue in our lab.

3.1 INTRODUCTION

Herpes Simplex Virus type 1 (HSV-1) is a 152 kb double stranded DNA virus in which humans are the natural host. This virus has been extensively developed for gene therapy applications related to diseases of the central and peripheral nervous system. HSV-1 infects a variety of cell types with high efficiency such that single vector particles can deliver and express transgenes in infected cells. The principal utility of HSV-1 vectors is the treatment of diseases of the nervous system (72, 160). This is based on the ability to engineer viruses that establish long-term persistence in neurons without neuronal damage or the induction of harmful immune responses. HSV-1 can persist in the host in a quiescent state referred to as latency and indeed the majority of the population are infected with latent virus. Neurons comprising peripheral ganglia are the natural sight for HSV-1 latency and virus persistence can be established without replication. Thus the removal of virus genes that prevent reactivation from the latent state circumvents spread to other individuals and provides an ideal platform for local expression of therapeutic genes in absence of viral lytic functions. The vector can be delivered to peripheral sensory neurons by simple inoculation of the skin where nerve terminals take up the vector with subsequent transport to the nerve cell body in a retrograde manner (56, 99). Selection of the dermatome for

inoculation thus serves a means of vector targeting to specific ganglia without spread to other tissues. Transgene expression can be transient lasting for weeks or long-term lasting for many months depending on the promoter system used for transgene expression. Of particular interest is the natural viral latency promoter which can express the viral latency transcript for many years and can be similarly exploited for transgene expression (9). Preclinical studies have shown that HSV-1 vectors are effective in the treatment of animal models of glioblastoma (64, 71), spinal cord injury (101), chronic pain (68), and peripheral neuropathies (24, 114) For patient applications involving the use of highly defective HSV-1 vectors, it is essential to develop scalable manufacturing processes that provide high titer, purified vectors without contamination by replication competent virus.

Natural HSV-1 infection proceeds in a coordinated fashion which is regulated at the gene expression level temporally in three distinct classes (12). The first class of genes, termed Immediate Early (IE), comprise the necessary functions for efficient initiation of the infection cycle and proper timing and level of expression of the remaining viral functions. The IE gene promoters contain one or more copties of an enhancer sequence that is recognized by a viral structural component termed VP16 which together with several cellular transcription factors specifically activates expression of the IE genes in the absence of any viral protein synthesis. Two of the IE genes, ICP4 and ICP27, are essential for expression of the virus lytic gene program while two additional IE genes, ICP0 and ICP22, are not absolutely required for virus growth but increase the efficiency of virus replication through the control of cell cycle genes and innate immune responses. ICP4 is the major transactivator encoded by the virus which has roles in DNA binding as well as repression of IE transcription(7, 8, 15, 23, 27). ICP27 has been implicated in RNA transport and overall inhibition of mRNA splicing (25, 26, 28). These genes sequentially activate the expression of the early (E) class of genes which support viral DNA synthesis and the late (L) class of genes that encode the viral structural proteins. Efforts over the past decade have

shown that removal of the immediate early genes ICP4 and ICP27 render the virus completely replication defective (16). Large scale production of this type of vector requires the use of complementing cells that provide ICP4 and ICP27 *in trans* to permit vector replication. The further elimination of additional IE genes and other nonessential functions provides vectors that are increasingly inert. However, the production of these vectors is more difficult and less efficient (21, 22). Since the ICP4 and ICP0 genes are found in repeat elements surrounding the unique components of the virus genome, these genes are diploid in the natural virus. The repeat elements allow for recombination to take place which results in isomerization of the unique long and short sequences of the virus genome. This can pose difficulties in modifying genes in the repeat elements and thus removal of one copy of each of the repeats will stabilize the genome into a specific form, provide more space for insertion of foreign DNA sequences, and prevent repair events.

In this study we evaluated the role of complementing gene promoters in supporting vector production. Vero cells engineered to express ICP4 and ICP27 differed greatly in their ability to support the growth of a highly compromised vector (JDTOZHE) which was deleted for ICP4, ICP27, ICP22 and the internal repeat elements separating the unique long and short components of the vector genome. The results indicate that the use of the enriched copy number of the VP16 recognition motif found in the ICP0 promoter was useful for driving ICP4 expression in complementing cells following vector entry. These data support the conclusion that the timing and level of ICP4 expression is critical to high titer vector production. Furthermore, experiments with different histone deacetylase inhibitors support the notion that genome de-repression through chromatin remodeling can lead to even more enhanced virus yields. These studies highlight the dynamic nature of the host cell response and how these interactions can be exploited to improve vector growth.

3.2 RESULTS

3.2.1 HSV-1 IE Complementing Cell Lines

The choice of cell line is crucial for the translation of vector from bench to clinic. We compared the productivity of different ICP4 and ICP27 complementing cell lines for the double mutant, Nurel P, which is missing both copies ICP4 and ICP27. These cell lines are derived from the same parental ICP27 complementing cell line, N23. A cell line constructed by stable transfection with a plasmid that contains the ICP0 promoter driving ICP4 was compared to a cell line with the ICP4 promoter driving ICP4 with respect to complementation over a 12 hour time course at an MOI of 3. Cell lysates were collected (See chapter 2 materials and methods for details) and assayed by western blot for ICP4. The ICP0 promoter allowed faster expression kinetics of ICP4 over the 12 hour time course during infection with Nurel P (Figure 1). It is important to note that during infection with this viral backbone the ICP0 gene is expressed from the virus with efficient kinetics. This gene allows efficient expression of all viral gene products and while not essential provides a cellular environment that is highly permissive for infection.

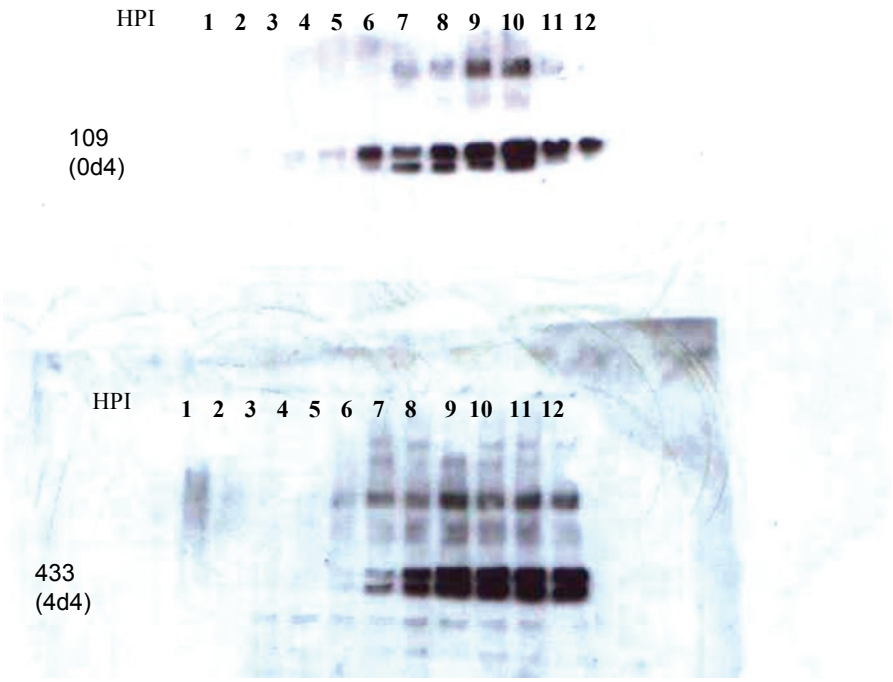


Figure 1. ICP4 Complementation Profile for 0d4 vs 4d4 Cell Lines.

109 and 433 cell lines were infected with Nurel P at an MOI of 3 and lysed at times indicated (HPI). The proteins were resolved on 4-12% SDS-PAGE gels, transferred to PVDF membranes and blotted for ICP4. All samples were normalized to input protein concentration by the BioRad Protein Concentration Assay. The lower mobility bands in both blots represent nonspecific binding of the primary antibody.

Nurel P growth was compared on both cell lines and revealed similar kinetics and productivity as shown in Figure 2. These results are straightforward since the virus contains both copies of ICP0 which can dramatically affect the infectious virus yield but also enhance the cytotoxicity (19, 34).

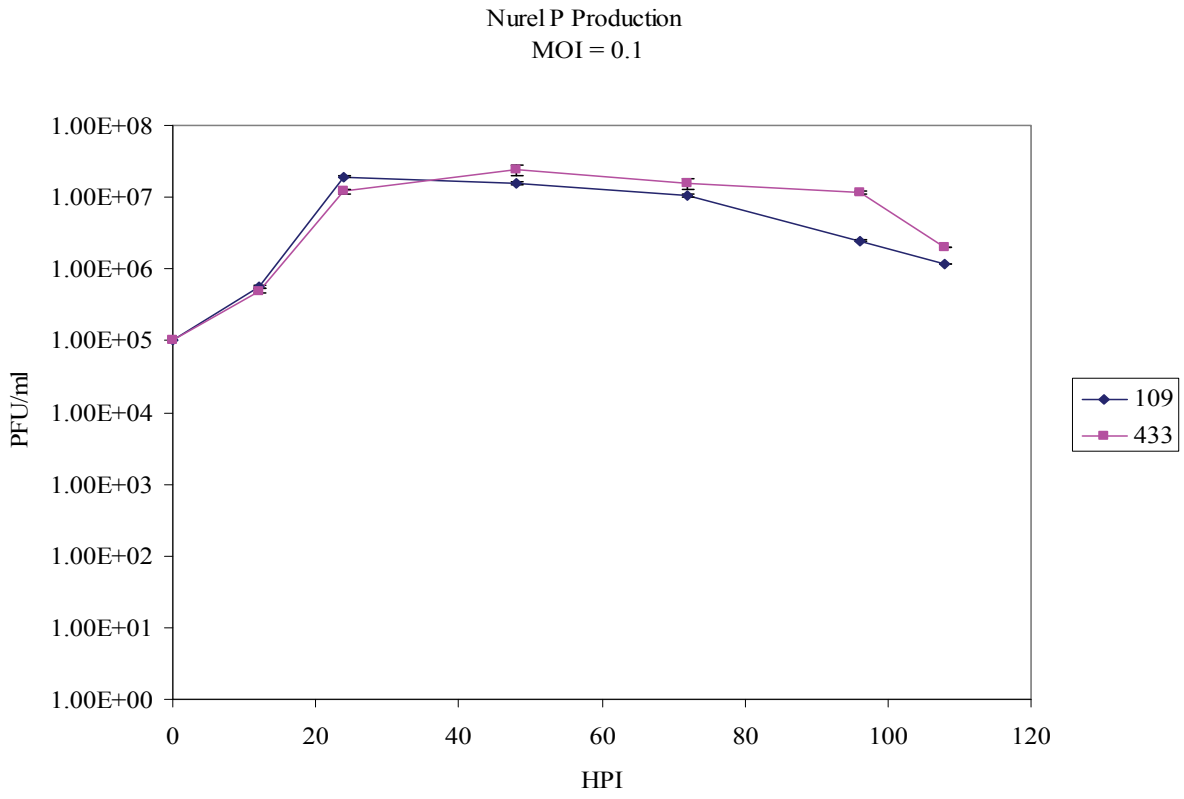


Figure 2. Nurel P Production is equivalent from 433 and 109 Cell Lines.

109 and 433 cells were infected with Nurel P at an MOI of 0.1 and harvested at times post infection indicated.

These data indicate that the earlier ICP4 expression was not as beneficial in the presence of ICP0 delivered by the virus. To test the strength of the ICP0 promoter a new cell line, Q5, was created in a similar manner to the 109 cell line with the full 2 kb ICP0 promoter driving the expression of the major transactivator ICP4. Figure 3 shows the growth properties of another ICP4 and ICP27 mutant, E1G6, on the Q5 and 433 cell lines. E1G6 differs from Nurel P in that the transgene in the ICP4 locus contains the EGFP gene driven by HCMV. There were statistically significant differences between the growth phenotypes of E1G6 on the Q5 cell line suggesting that the full ICP0 promoter allowed even more efficient expression of ICP4 allowing the viral replication cascade to proceed with faster kinetics.

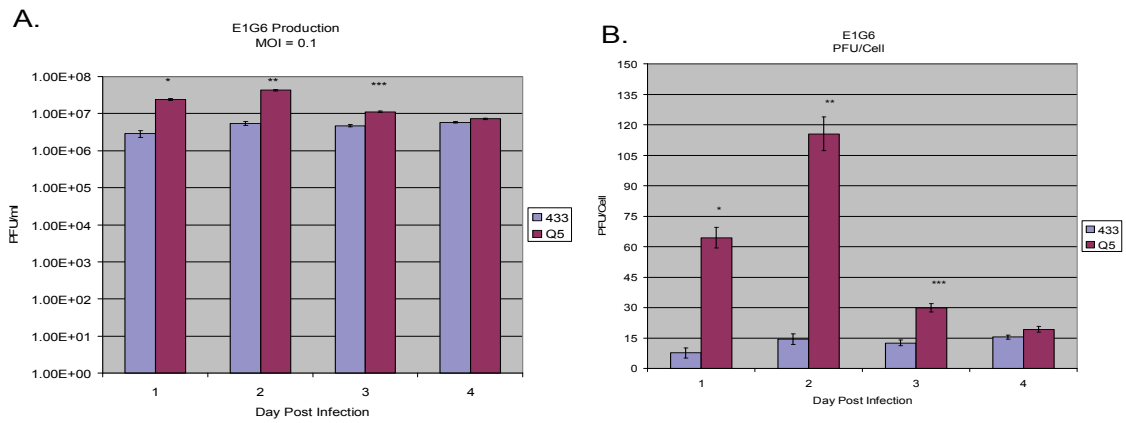


Figure 3. E1G6 Production on 433 and Q5 Cell lines.

433 and Q5 cell lines were infected with E1G5 and harvested at times post infection indicated. * $P < 0.005$, ** $P < 0.01$, *** $P < 0.05$

To verify that the enhanced expression was indeed a result of faster ICP4 complementation kinetics a time course was explored for ICP4 expression at 1-6 hours post infection with respect to transcript copy number. Figure 4 suggests that the ICP0 promoter is more efficient in providing ICP4 transcript. ICP27 and ICP0 RNA copy numbers are similar between the two cell lines indicating that these immediate early gene products may not be responsible for production differences between the two cell lines. The ICP0 transcript data is straightforward since this gene is provided by the input virus and should therefore be similar between both infections. Likewise, for ICP27, the same parental ICP27 cell line was used for construction of both cell lines so its kinetics of expression should be similar.

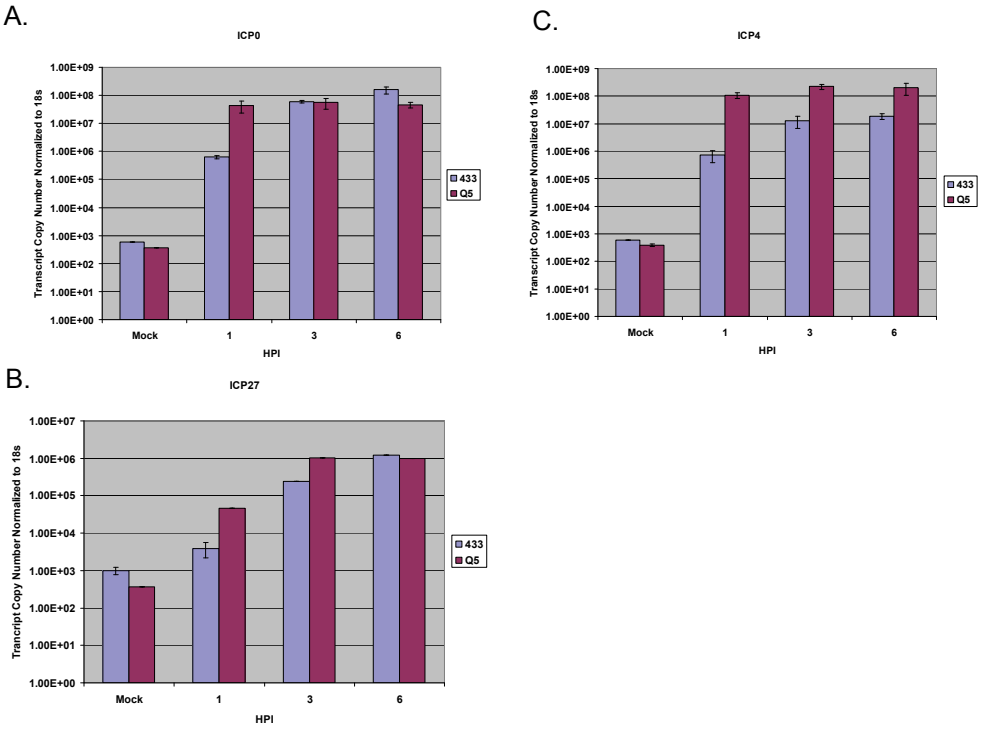


Figure 4. IE Gene Transcription from 433 and Q5 in E1G6 MOI = 0.1.

433 and Q5 monolayers were infected with E1G6 at an MOI of 0.1 and harvested at times post infection indicated. All copy numbers are normalized relative to 18s. Mock refers to uninfected cells under normal cell culture conditions.

To validate that the ICP0 promoter is legitimately responsible for the transcript differences, a characterization of both cell lines was performed to assess the basal copy number. Total genomic DNA harvested by phenol chloroform extraction was subject to quantitative PCR similar to the data presented in Figure 4. Figure 5 indicates that the basal copy number is similar between the two cell lines with respect to both HSV-1 IE genes integrated within the cellular genome. From this point the cell line Q5 will be referred to as 0d4 and 433 cells as 4d4 to indicate the promoter driving ICP4.

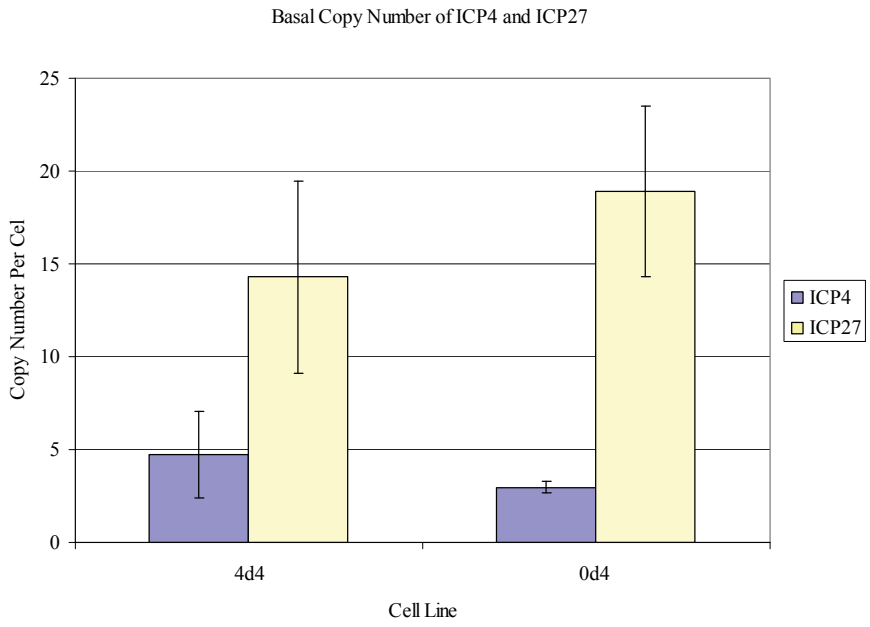


Figure 5. Basal Copy Number of IE Genes within 0d4 and 4d4 Cell Lines.

Phenol chloform extracted genomes were analyzed by quantitative PCR for ICP4 and ICP27 normalized to total cell number. 4d4 represents the ICP4 promoter driving ICP4 cell line and 0d4 represents the full ICP0 promoter driving ICP4.

The higher copy number of ICP27 reflects the relative toxicity of the gene as more copies are able to be tolerated by the host Vero cells. The equivalent copy number of the integrated ICP4 gene per cell suggests that the promoter activity is indeed responsible for the increased transcription and subsequent viral production from the 0d4 cell line.

3.2.2 Growth of Joint Deleted Triple Virus

The strength of these cell lines was tested against a poor growing vector JDTOZHE. Figure 6 shows the genetic structure of both the wild type KOS and the joint deleted triple mutant JDTOZHE.

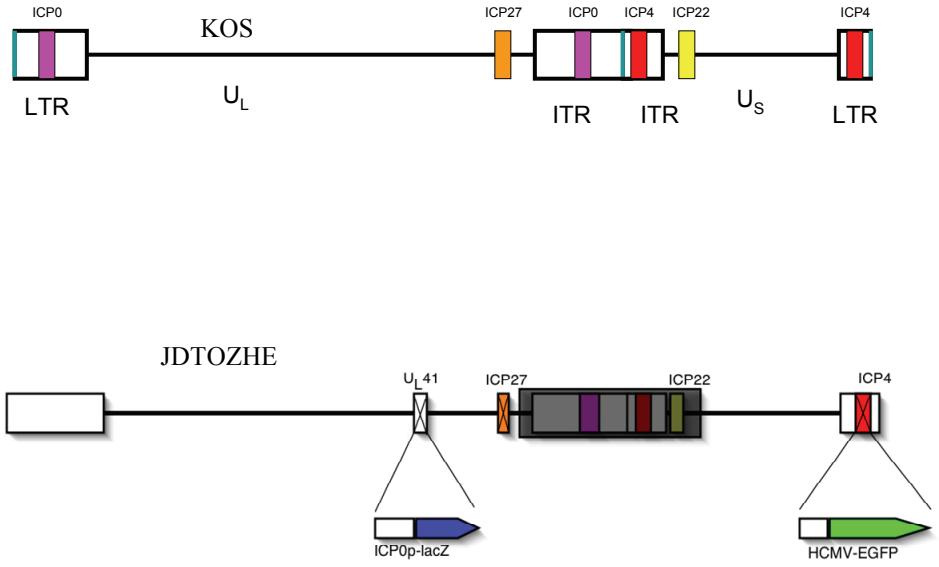


Figure 6. Genetic Backbone of KOS and JDTOZHE.

Map of wild type KOS and recombinant HSV-1 constructs. Schematic of wild type KOS shows segmented genome with unique long (UL) and short (US) segments flanked by long and internal repeats (LTR and ITR respectively). Immediate early genes ICP4, ICP27, ICP0, and ICP22 are highlighted at their natural loci.

Figure 6 illustrates the genomic structures of wild-type HSV-1 KOS virus and the highly defective derivative JDTOZHE. JDTOZHE has a deletion of the ICP27 gene as well as a large deletion spanning the internal repeats between the unique long (U_L) and short (U_S) regions of the genome and extending midway through the ICP22 coding sequence in U_S. The repeat deletion removes one copy each of the ICP0 and ICP4 genes. The second copy of the ICP4 gene, located in the remaining U_S-flanking repeat (TR_S), was replaced with the gene for enhanced green fluorescent protein (eGFP) controlled by the human cytomegalovirus (HCMV) major IE promoter. In addition, an ICP0 promoter-lacZ gene expression cassette is present in place of a portion of the U_L41 locus. Thus, JDTOZHE is deleted for all regulatory IE genes with the exception of a single copy of the ICP0 gene, and contains two reporter genes (eGFP and lacZ)

controlled by different promoters. This vector requires complementation of the essential ICP4 and ICP27 genes for growth.

Complementing cells for JDTOZHE growth were derived from a previously constructed Vero cell line that expresses ICP27 from its own promoter following virus infection (105). This cell line was transfected with plasmids containing a selectable gene (Blastidicin S) along with the ICP4 coding sequence under control of the ICP4 or ICP0 promoter. Drug-resistant colonies were cloned and one of each type, termed 4d4 and 0d4 respectively, were used as the primary lines for the studies described here. Using quantitative PCR, we have found that both lines contain 3-5 copies of the ICP4 gene (Figure 5). Cell lines were characterized for promoter activity by assessing induction of HSV-1 complementing gene transcription in the presence of UV-inactivated virus. Upon crosslinking of viral DNA, the virus is unable to replicate and, therefore, should provide the transactivating tegument protein VP16 to induce the expression of the embedded genes within the cellular chromosome. Figure 7 shows that the UV-inactivation procedure was functional in blocking the expression of ICP4 transcript, determined by randomly primed reverse transcription followed by quantitative PCR, from the wildtype KOS on non-complementing Vero cells at a multiplicity of infection (MOI) of 3 while normal KOS virus was able to amplify ICP4 transcript. Infection with UV-inactivated JDTOZHE on 0d4 and 4d4 cell lines display differential activity up to an order of magnitude at 3 and 6 hpi (Figure 7B) highlighting the strength of this promoter in the context of VP16 induction.

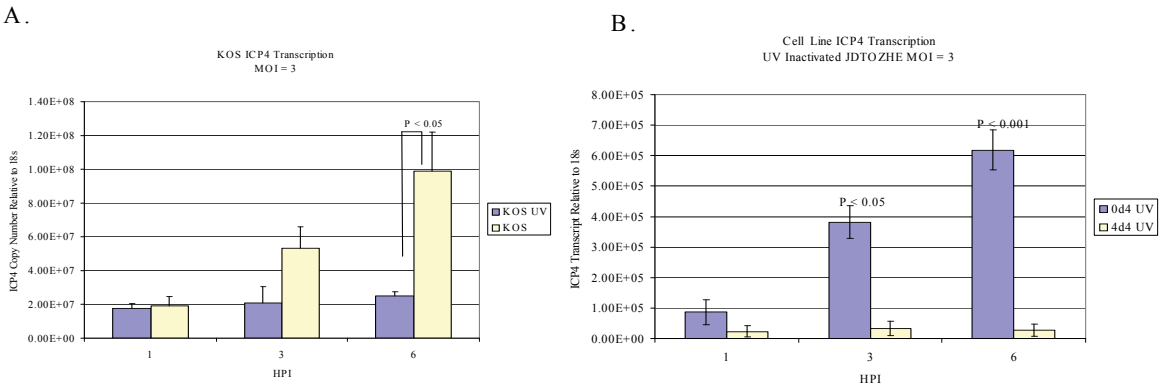


Figure 7. UV-inactivated Virus Induces ICP4 from complementing Cell Lines.

A. Vero cells were infected with UV-crosslinked KOS at an MOI of 3 and harvested for RNA at times post infection indicated. ICP4 copy number was obtained from quantitative PCR as described in the Methods chapter.

The differential ICP4 induction with the UV-inactivated virus and the basal copy number (Figure 5) supports the claim that the full ICP0 promoter is substantially more efficient in driving ICP4 gene expression from the complementing cell line in the context of infection.

3.2.2.1 Complementing activity of 4d4 cells.

To determine the complementing efficiency of 4d4 cells for JDTOZHE virus growth, Vero and 4d4 monolayers were infected with wild-type virus (HSV-1 KOS) and JDTOZHE, respectively, at an MOI of 0.01, the standard MOI used in our lab for virus production. Total virus was collected from replicate wells on each of the first 5 days post-infection and titered on Vero or 4d4 cells. Virus was added to 1E6 cells in triplicate and seeded into 6 well plates. Samples were collected at times indicated by scraping the monolayer with a cell scraper and incubating at room temperature with agitation in 0.45 M NaCl for 25 minutes. After salt treatment, cell debris was spun down at 5,000 rpm for 5 minutes and sonicated for 30 seconds. Cell debris was pelleted by

centrifugation again at 5,000 rpm for 5 minutes and the supernatant was frozen at -80°C until titration was carried out on 0d4 cells (JDTOZHE on 4d4) or Vero cells (Kos on Vero). The results (Figure 8) showed peak yields of KOS on days 2-5 at or above $1x10^8$ plaque forming units (pfu)/ml while the yields of JDTOZHE peaked on day 2 at $1x10^5$ pfu/ml and declined rapidly thereafter. These results showed that complementation of the JDTOZHE growth defect by the integrated ICP4 and ICP27 genes of the 4d4 cell line was short-lived and insufficient for robust virus growth. The limited yields of the mutant virus compared to KOS may not be surprising as the complementing genes in 4d4 cells, unlike their viral counterparts in the KOS genome, are not amplified during virus replication. Hence their expression is not expected to increase as a function of viral genome copy number, resulting in a ceiling that may be far lower than that reached when the genes replicate with the virus. This ceiling, in turn, will limit early and late gene expression from newly synthesized viral genomes and consequently the formation of mature viral particles. This interpretation supported the suggestion that the expression levels of the immediate early complementing genes in 4d4 cells were a key limiting factor in the growth complementation of JDTOZHE.

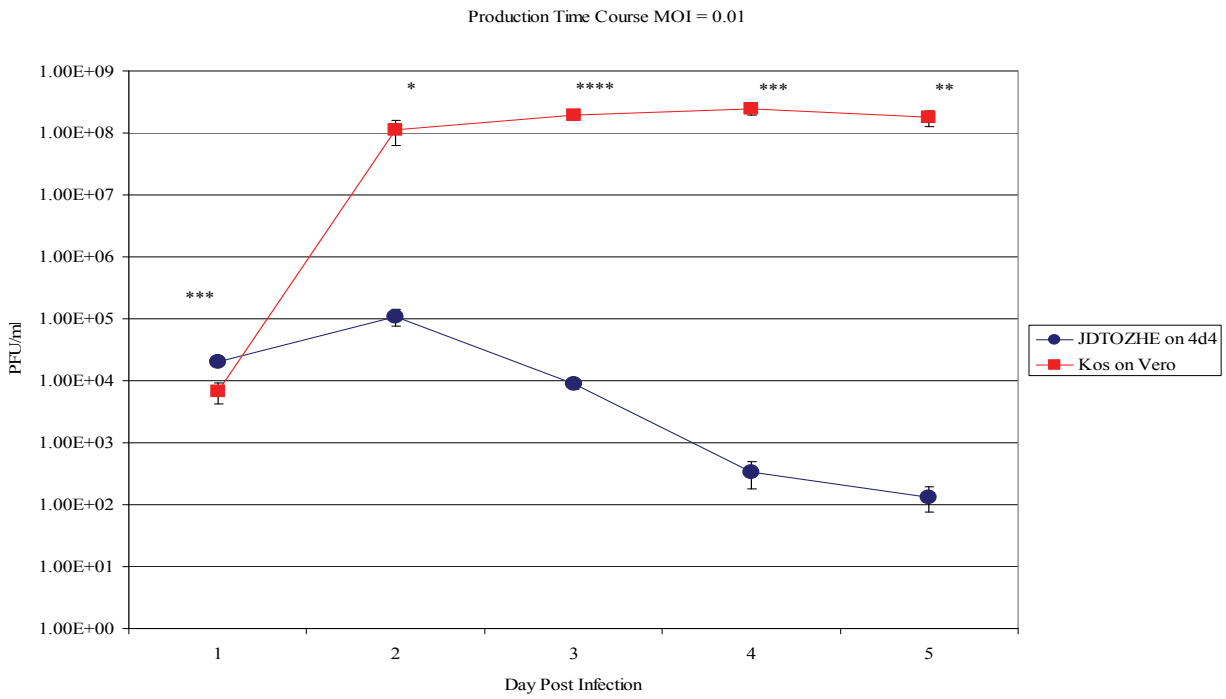


Figure 8. JDTOZHE on 4d4 cells production levels versus KOS on non-complementing Veros

Viral supernatant and cells were harvested and salt treated at times post infection indiated.

P values were calculated using the 1 tailed students t test. * = $P < 0.01$, ** = $P < 0.005$, *** = $P < 0.001$, **** = $P < 1E\text{-}9$.

3.2.2.2 Expression of complementing viral genes in JDTOZHE-infected 4d4 cells.

To explore the causes of the weak complementing activity of 4d4 cells, we examined the kinetics and levels of ICP4 and ICP27 gene expression induced by JDTOZHE infection. 4d4 cell monolayers were infected at an MOI of 3, total RNA harvested at 1, 3, and 6 hpi, and specific RNA levels determined by randomly primed reverse transcription followed by quantitative PCR. As a positive control, RNA from KOS-infected Vero cells was isolated and processed in the same manner. Typical results normalized to 18S ribosomal RNA amplified from the same samples are presented in Figure 9.

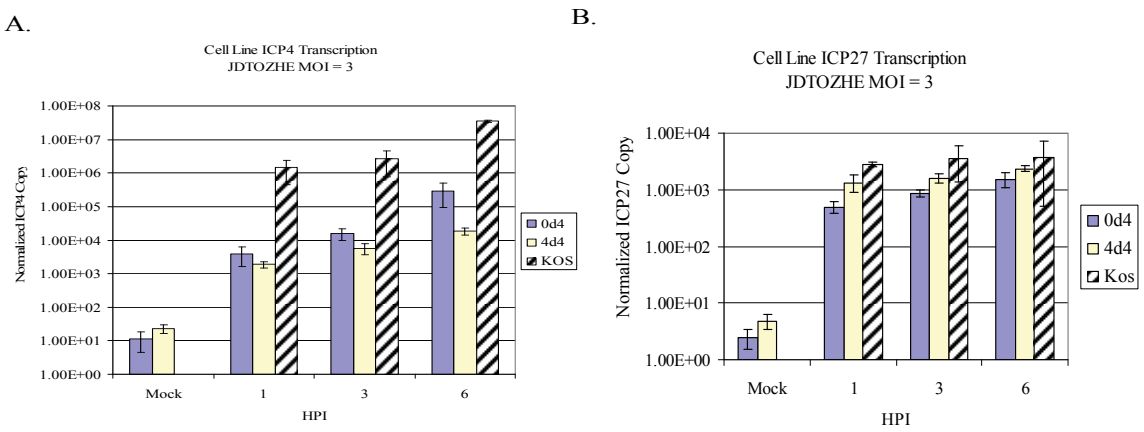


Figure 9. The ICP0 promoter can deliver ICP4 gene under JDTOZHE infection at MOI = 3 with faster kinetics.

A. ICP4 transcription from JDTOZHE at MOI = 3 at immediate early times post infection. B. ICP27 transcription from 0d4 and 4d4 cell lines during JDTOZHE MOI = 3 infection at immediate early times post infection. RNA was harvested and assayed as described in materials and methods. Error bars represent standard error between triplicate samples.

Compared with mock infected cells, ICP4 transcript induction of two orders of magnitude indicate that the increase in copy number is due to infecting JDTOZHE. Figure 9A shows that the ICP4 RNA level increased with slower kinetics in the 4d4 cell lines than in the control wild type samples, resulting in a greater than 25-fold differential at 6 hpi. ICP27 RNA levels as assayed by quantitative PCR (Figure 9B) are reduced as compared to wild type but the differential is a half order of magnitude at a maximum with similar expression kinetics. While the deletion of one copy of the ICP0 gene in JDTOZHE is likely a factor in the lower abundance of ICP4 RNA in the 4d4 samples, we do not know whether the reduced levels of ICP4 and ICP27

RNA play a role as well. Overall, since the ICP4 RNA profile showed the greatest disparity between the experimental and control samples, these results suggested that insufficient ICP4 production from the 4d4 genome could be the principal cause of the limited complementing efficiency of these cells for JDTOZHE growth.

To determine whether the RNA measurements based on RT-PCR correlated with protein levels, Western blot analyses were performed for ICP4, ICP0, and the major capsid protein VP5 encoded by the leaky-late U_L19 gene.

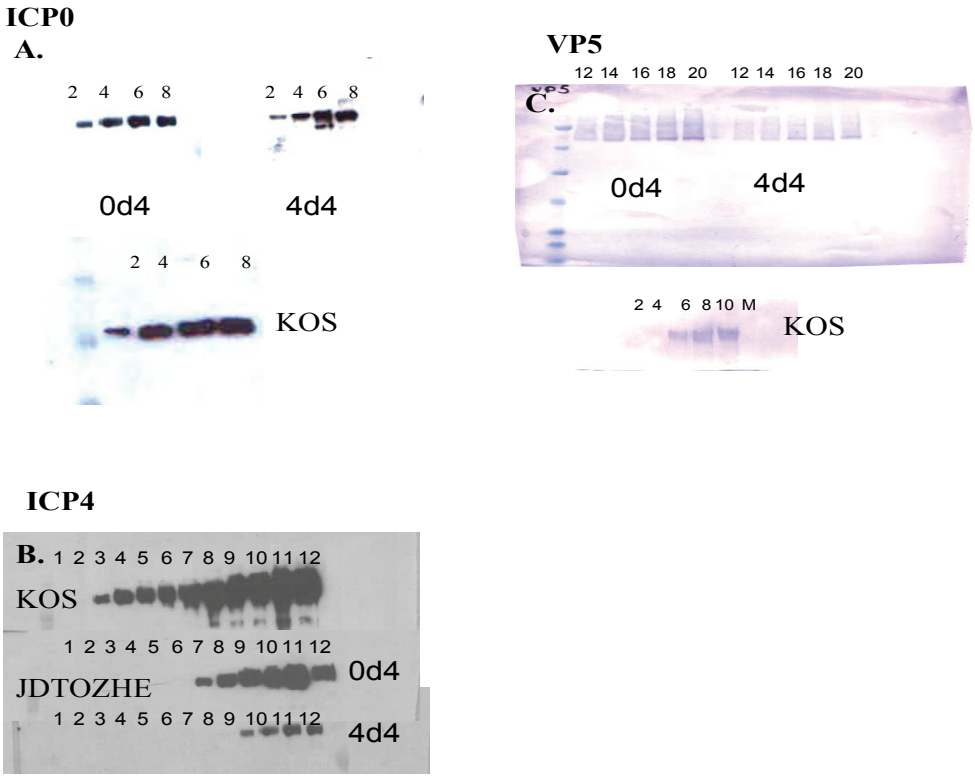


Figure 10. Protein expression kinetics comparing the complementation induction of HSV-1 IE and Late gene expression.

Proteins were collected and resolved on SDS-PAGE gels as described in Materials and Methods. A. ICP0 expression at 2-8 hours post infection . 0d4 and 4d4 cells were infected with JDTOZHE at MOI = 3. Vero cells were infected with KOS at MOI = 3. B. ICP4 expression time course for 1-12 hours post infection. C. VP5 expression from 12-20 hours post infection for JDTOZHE on 0d4 and 4d4 cells. Kos on Vero cells at MOI = 3 during 2-10 hours post infection.

Figure 10A shows that ICP0 accumulated with comparable kinetics in JDTOZHE-infected 4d4 cells and KOS-infected Vero cells indicating that this immediate early protein was not rate limiting in the production discrepancy between JDTOZHE and KOS. In contrast, ICP4 appeared with dramatically delayed kinetics in the mutant-virus-infected cells (Figure 10B). Thus, compared to the single-copy ICP0 gene of the mutant virus, expression of the ICP4 genes integrated in the 4d4 genome was greatly impaired relative to infection with wild-type virus, consistent with the RT-PCR data. VP5 was detected as early as 6 hpi in KOS-infected Vero cells with increased abundance at 8 and 10 hpi (Figure 10C), whereas no signal was detected at these time points in JDTOZHE-infected 4d4 cells (data not shown). VP5 was first observed in infected 4d4 cells at 12 hpi with a minimal increase in level at 16 hpi (Figure 10B). Together, these analyses supported the suggestion that ineffective induction of primarily the integrated ICP4 genes in 4d4 cells precluded robust early and late gene expression from the infecting JDTOZHE genome and thereby vigorous viral progeny production. Given the reduced levels of ICP27 RNA and the documented effects of ICP27 on viral transcription, RNA processing and translation (157, 158), we do not discount the possibility that ICP27 deficiency also contributes to the growth defect of JDTOZHE on 4d4 cells.

3.2.2.3 Complementing activity of 0d4 cells expressing ICP4 from the ICP0 promoter.

In an attempt to gain better infection-dependent inducibility and expression of the ICP4 gene in complementing cells, we tested the 0d4 line described earlier. Briefly, these cells were derived from the same ICP27-complementing line as 4d4 by transfection of an ICP0 promoter-ICP4 gene construct and clonal isolation of drug-resistant colonies. We chose the complete ICP0 promoter to control ICP4 expression because various lines of evidence suggested that this

promoter is significantly stronger than the ICP4 promoter (3, 42, 50), yet like the ICP4 promoter is essentially silent in uninfected cells. Additionally, the ICP0 promoter is downregulated by ICP4 (142), thus preventing run-away overexpression of ICP4 which could result in premature cell death or deregulation of the viral gene expression program. Figure 9 shows a comparison of ICP4 and ICP27 RNA levels detected in JDTOZHE-infected 0d4 cells (MOI=3) with 4d4 cells. The ICP27 (Figure 9B) RNA profiles were similar between the two infected cell lines albeit with slight reduction in comparison to wild type control KOS infections on Vero cells. Unlike the ICP27 transcription profile, ICP4 RNA levels were higher in 0d4 at all times investigated with substantial differentials at 6 hpi (Figure 9A). Protein expression profiles in JDTOZHE-infected 0d4 cells were examined by Western blot analyses, as before, and the results are included in Figure 10. Comparison of the 4d4 and 0d4 profiles of ICP0, ICP4 and VP5 shows the improvement in expression due to higher transcription rates of ICP4. While the kinetics of ICP0 accumulation were similar between the two cell lines, ICP4 was observed 2h earlier in 0d4 cells than in 4d4 cells and reached significantly higher levels in 0d4 cells in the first 12 hpi (Figure 10A). Consistent with this result, VP5 levels were comparatively increased in 0d4 cells at 12-18 hpi (Figure 10C); the protein was not detected in either line at or before 10 hpi (data not shown). ICP27 protein accumulation was similar between the wild type and JDTOZHE infected complementing cell lines (data not shown). Together, these results showed that 0d4 cells provided better complementation of the defects in JDTOZHE gene expression than 4d4 cells and strongly suggested that this was due to better inducibility and greater peak activity of the promoter driving ICP4 expression in these cells.

3.2.2.4 Virus yields on 0d4 cells

To determine whether the increases in complementing viral gene expression observed in 0d4 compared to 4d4 cells translated into better production of the defective JDTOZHE virus,

cells were infected at the standard production MOI of 0.01 and total virus collected daily from replicate wells over a 5-day period for titration on 0d4 cells. Figure 11A shows that the yields on 0d4 cells were some 5-15 fold higher on different days than the yields on 4d4 cells with three fold differences on a per cell basis (Figure 11B).

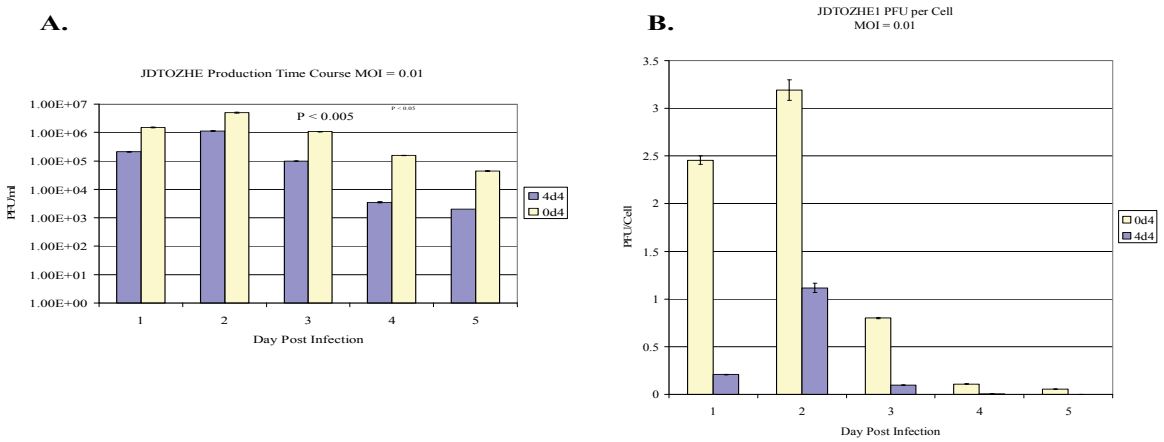


Figure 11. JDTOZHE production is enhanced on ICP4 complementation from the ICP0 promoter versus the ICP4 promoter.

Infections were carried out and harvested as described in Figure 2. A. JDTOZHE production on a per volume basis. B. JDTOZHE Production on a per cell basis.

To validate these results, we used quantitative PCR for the U_L1 gene to determine the number of genome copies in the samples of Figure 11A. Figure 12 shows that the genome yields were up to two orders of magnitude higher on 0d4 cells than on 4d4 cells. The results of these growth experiments are consistent with the interpretation that the earlier kinetics and higher levels of ICP4 expression observed in infected 0d4 cells compared to 4d4 cells provide better complementation of the JDTOZHE growth defect. Further examination of Figure 11 shows that the PFU titers of the virus preparations from both cell lines fell after day 2 (Figure 11A) whereas

the genome yields generally continued to increase, resulting in a decline in PFU:genome ratios and thus the biological quality of the preparations after day 2.

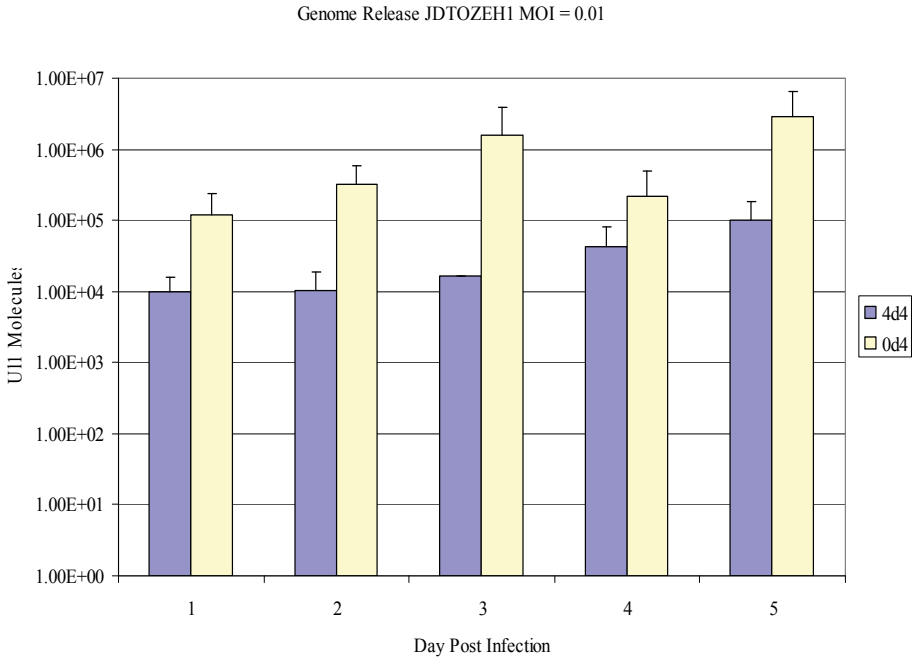


Figure 12. JDTOZHE genome copy number accumulation is enhanced in cells with ICP4 complementation driven by the ICP0 promoter as compared to the ICP4 promoter.

Ul1 was assayed via qPCR from each sample in figure 6 as described in materials and methods.

This may be due in part to virus instability at the production temperature (33^{o}C) or high release of lactic acid from the cultured cells, but additional causes have not been excluded.

Burst experiments were used to determine the virus production capacity per cell for the two cell lines. These experiments were performed at an elevated MOI to accomplish direct infection of all cells. By excluding virus production by cells that are secondarily infected through lateral spread, this design is better suited to detect direct correlations between gene expression and virus synthesis. Infection at an MOI of 3 revealed that peak yields of JDTOZHE on 0d4 cells were higher than on 4d4 cells and were reached earlier (Figure 13A).

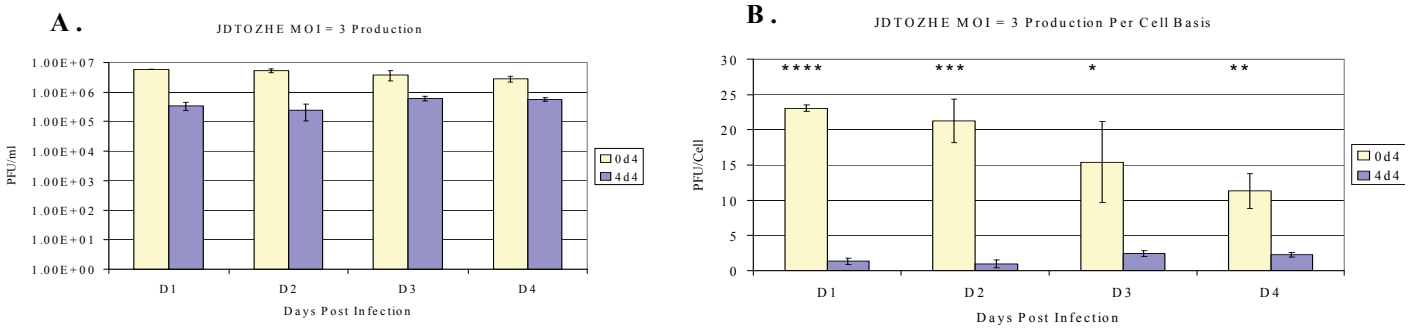


Figure 13. High MOI production reveals enhanced production levels and similar differences between cell lines as with low level infection. A. Four day time course of MOI = 3 production of JDTOZHE on 4d4 vs 0d4 cell lines. B. Data in part A cast as per cell basis. **** = $P < 1.7 E -6$, *** = $P < 0.0005$, ** = $P < 0.005$, * = $P < 0.01$

As illustrated in Fig. 13B, the differences in virus yields per cell ranged from 12-fold at 1 day post infection to just over 4-fold at 4 days post infection. These results are consistent with the earlier production and greater accumulation of ICP4 seen in infected 0d4 cells and thereby support the proposed cause-effect relationship between improved ICP4 expression and better JDTOZHE growth on 0d4 cells compared to 4d4 cells. Furthermore, the normalized infection production data indicates that these cells are not only responsive to input virus in a dose dependent manner but the promoter strength differential is amplified at higher levels of infection.

3.2.3 Heterochromatin Reorganization Effect on Viral Production

The growth phenotype of the triple mutant, JDTOZHE, was investigated in the presence of various concentrations of histone deacetylase (HDAC) inhibitors. These compounds can alter the host chromatin state by binding in the active site of histone deacetylases leading to enhanced global transcriptional activity through lysine acetylation (96, 171). Furthermore, use of HDAC inhibitors have been used to increase transgene expression from adenovirus and AAV (26, 131, 178). HDAC sodium butyrate enhanced viral yield of the highly replication defective vector JDTOZHE in a dose dependent manner (Figure 14).

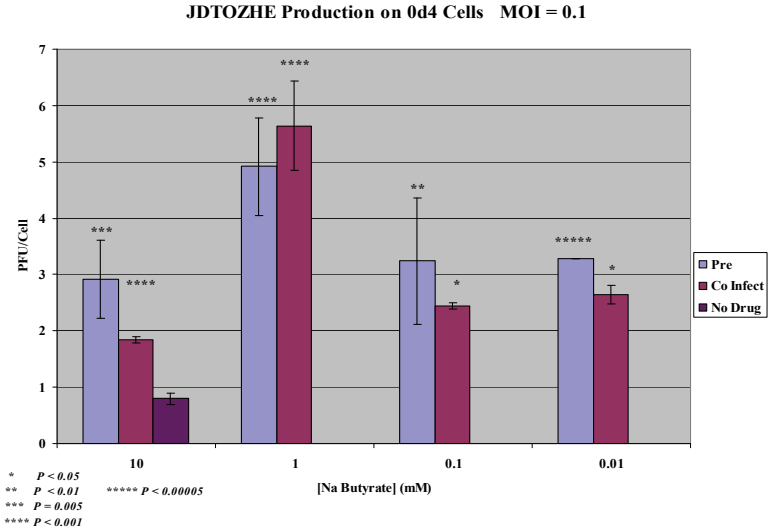


Figure 14. Sodium Butyrate Enhances JDTOZHE Yields.

0d4 cells infected with JDTOZHE exposed to Na Butyrate either 1 hour prior to infection or co administered with virus. Infectious virus recovery at 72 HPI was assayed by titration on 0d4 cells.

JDTOZHE PFU per cell ratios were enhanced up to five fold with either co infection or 1 hour pre treatment with sodium butyrate at micromolar concentrations. Trichostatin A was assessed in a similar manner and Figure 15 shows similar improvements at nanomolar concentrations.

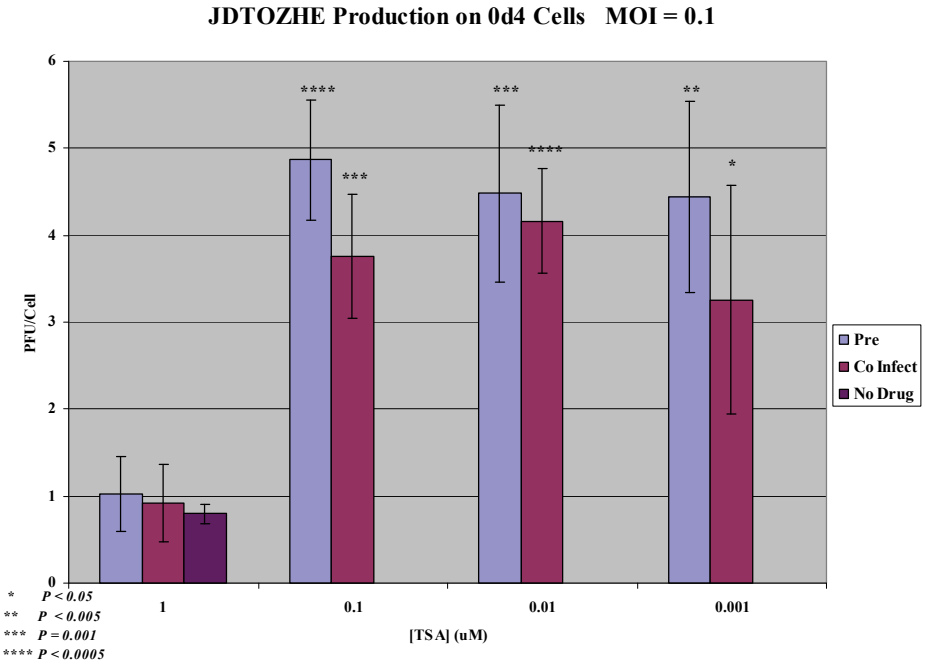


Figure 15. TSA pretreatment leads to enhanced JDTOZHE production at submicromolar concentrations.

0d4 cells infected with JDTOZHE exposed to Na Butyrate either 1 hour prior to infection or co administered with virus. Infectious virus recovery at 72 HPI was assayed by titration on 0d4 cells.

Cells were co infected with each HDAC inhibitor or pretreated to investigate whether the cell state could be induced to a more permissive environment. Statistically significant yields were obtained at all concentrations of both HDAC inhibitors regardless of the pretreatment or co infection regime. However, TSA pretreatment revealed a subtle improvement in pretreatment at much lower concentrations than necessary for sodium butyrate. The reduction in yield at higher concentrations is indicative of drug associated toxicity which is not seen in sodium butyrate.

FDA approval of sodium butyrate for use in humans suggests implementation of this production scheme will not be limited by safety.

3.2.4 Growth and Expression of Quadruple Mutant Virus

Viruses that are highly attenuated are attractive for therapy due to a lower toxicity profile. These vectors are the most challenging to propagate due to the nature and complexity of the gene expression necessary to complement the multiple missing functions. A quadruple mutant virus which is an offspring of JDTOZHE termed JDQOZEH1 is isogenic but devoid of both ICP0 loci replaced with the HCMV promoter driving EGFP cassette. The genetic map of this virus is depicted in Figure 14.

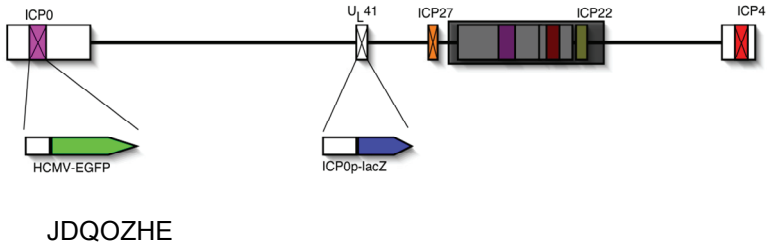


Figure 16. Genetic Map of JDQOZEH1

JDQOZEH1 is an isogenic mutant of JDTOZHE. The joint deleted quadruple version is devoid of ICP0, ICP27, ICP22, and ICP4 with the HCMV-EGFP cassette in the ICP0 locus.

Construction of an ICP0, ICP27, and ICP4 complementing cell line was carried out to provide an efficient substrate for growth for this vector. Normal ICP27 and ICP4 complementing cell lines

are insufficient to grow this vector due the highly crippled backbone. This difficult growth phenotype is hypothesized to be due to lack of sufficient ICP0 expression as this gene can markedly affect growth at low input virus (19). Alternatively, U2OS cells provide a function that can substitute for the ICP0 transactivating function (202). However, since these osteosarcoma are not suitable for clinical applications they are not explored for use as a complementing cell line for gene therapy use. Likewise, these cells are very permissive for gene expression for a wide variety of viruses therefore selecting a silent gene in this environment remains a challenge to cell line construction. 0d4 cells transfected with the ICP4 promoter driving ICP0 expression were selected with hygromycin as described in the methods chapter. These cells also provide ICP27 under its own promoter and ICP4 under the ICP0 promoter. Figure 17 shows the overall productivity from the 4d4, 0d4, and 01 cell lines with respect to transgene expression and cell morphology.

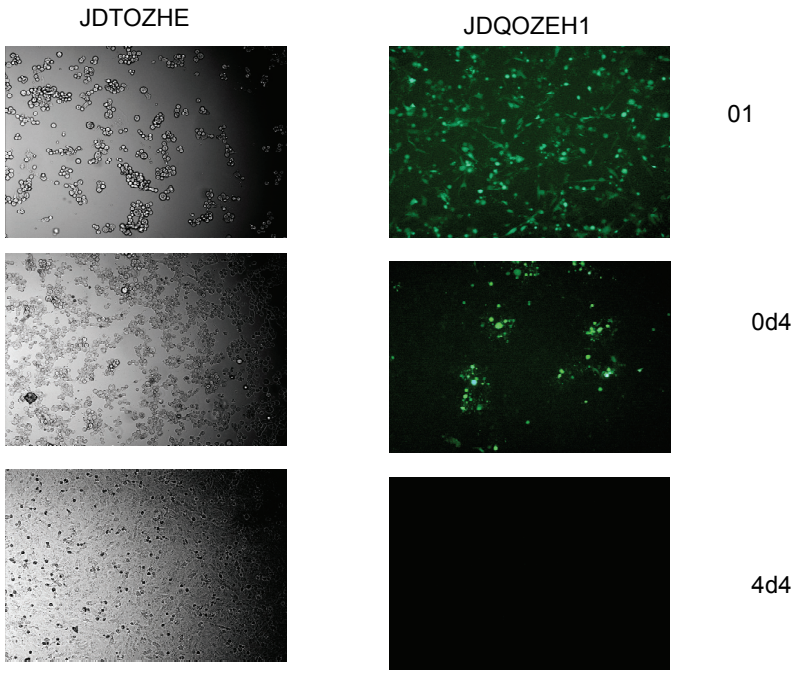


Figure 17. Enhanced Complementation from 01 Cell Lines

01, 0d4, and 4d4 cell lines infected with JDTOZHE and JDQOZEH1 at an MOI of 0.1 3 days post infection.

The presence of ICP0 from the 01 cells allows efficient transduction as evidenced by the overall level of EGFP from the JDQOZEH1 vector. Additionally, this cell line grew the JDTOZHE vector more efficiently as shown by the higher percentage of cells with cytopathic effect at three days post infection. The 0d4 line allowed moderate growth of JDQOZEH1 compared to the 4d4 cells but the lack of ICP0 complementation did not allow sufficient transgene expression as suggested by the low levels of EGFP. Figure 18 shows the productivity differentials for all three cell lines. The 01 cell lines consistently yielded statistically significant yields of this highly defective vector suggesting that this cell line can be used to propagate further non-toxic versions of HSV-1 vectors. Furthermore, this cell line has not yielded replication competent vectors as by testing on non-complementing Vero cells and PCR for the ICP0 region from 01 cells infected with a ICP0 mutant.

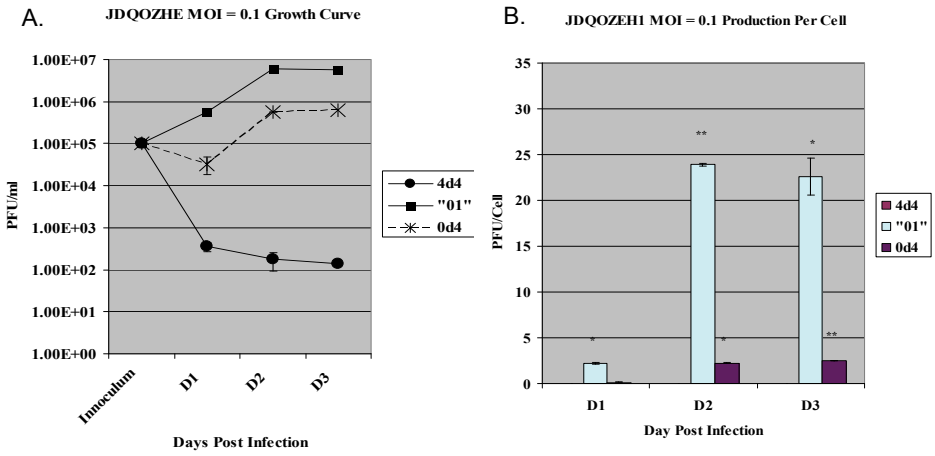


Figure 18. 01 cells can yield relatively high titer of highly defective HSV-1 backbones.

4d4, 0d4, and 01 cells were infected with JDQOZEH1 at an MOI of 0.1 and harvested at times post infection. * $P < 0.05$, ** $P < 0.005$

3.3 DISCUSSION

In this chapter, the production of highly replication-defective HSV-1 vectors was addressed by characterizing the kinetics of RNA and protein expression from cell-based complementing genes for a highly mutated vector, JDTOZHE, compared to the expression of the same genes from the wild type virus genome. Replication-defective vectors are useful for nontoxic delivery of therapeutic transgenes, but these viruses lack essential functions for autonomous propagation which must therefore be supplied *in trans*. Constitutive cellular expression of these essential functions is cytotoxic, thus preventing the healthy growth and use of cell lines modified in this manner. This problem has been overcome in the past by using inducible promoters that are responsive to viral infection, such as the HSV-1 IE promoters (152). On infection with the defective virus, these promoters ideally direct expression of the missing gene products with the same kinetics and to the same levels as in wild-type virus infections. However, since the complementing genes are provided by the cell, their numbers will not increase on infection, unlike their viral counterparts in wild-type virus infections which are amplified by viral genome replication. Thus, it is likely that defective virus grown with the assistance of cell-based complementing genes will have a lower replication ceiling than wild-type virus. This provides a simple explanation for the common observation that complementing genes integrated in the host cell genome do not restore defective virus growth to the levels

achieved by wild-type virus. Since this deficiency directly reflects limiting levels of the complementing gene products, designs to increase expression of the complementing genes will be beneficial. However, the generation of such complementing cell lines remains a challenge due to the lack of control over the number of complementing genes that will integrate or their position in the cellular genome; both of these variables affect expression. While viral vector systems exist that do provide a degree of control over these parameters, these systems introduce additional genetic material into the cell which is undesirable. Compounded with this drawback is the issue of complementation stability for the purposes of cell banking for large scale production. Additionally, as pointed out in this work, the complementation efficiency can drastically alter the production of replication defective vectors which becomes more critical as the vector needs more gene products to be supplied *in trans*.

Vector backbone will also effect vector yields as more essential viral functions are removed growth phenotypes can be dramatically reduced. Certain multiplicities of infection (MOI) will dictate how an infection can proceed (133). As the genotype of the virus is depleted of essential gene functions higher MOIs are necessary to grow high titer virus. This strategy, however, can severely limit large scale production since higher amounts of input virus is needed. Likewise, the backbone of the virus will influence what type of complementing cell line is necessary for growth. ICP0 mutants grow poorly in standard approved cell types (Vero, Hep-2, etc) but an osteosarcoma cell line, U2OS, are highly permissive for reasons yet to be determined (202). Likewise, ICP22 mutants grow poorly in rabbit skin cells but have normal growth phenotype in Vero cells (17). Optimal cell line construction is therefore critical for propagation of different mutants depending on their intended use for *in vivo* gene transfer.

By increasing the level and efficiency of complementation, we have shown that the production of more disabled versions of replication defective mutants of HSV-1 can be improved dramatically. Other studies that have examined the culture conditions and genetic background

for production of double (ICP4 and ICP27 negative) and triple (ICP22, ICP4, and ICP27 negative) mutant HSV-1 strains suggest that scale up be the ultimate solution for production of large scale quantities of replication defective HSV-1 vectors (133). The use of microcarrier based bioreactors has also been shown to increase overall yield for double HSV-1 mutants as well as Vaccinia virus, however, this system has not been explored for the growth of a more deleted vector (12, 181). The authors of this work found that as the virus became more defective the productivity dropped off due to lack of complete complementation. We have shown here that by engineering the architecture of the promoter to incorporate the essential viral functions, a better yield per cell could be achieved. Increased expression of ICP4 presumably not only led to higher levels of early and late classes of genes but may serve as a protection function in the stability of the viral genome as evidenced by enhancement in total HSV-1 genome copy number (173, 174).

There are noticeable differences in RNA translation and protein expression with respect to ICP4 where the RNA levels are quite low in the case of the 4d4 cells but the protein expression occurs only after 9 hours post infection. In the case of 0d4 cells the earlier RNA transcription presumably led to earlier translation into functional ICP4 protein levels detected by western blot analysis. A possibility for this discrepancy in RNA:protein correlation is that the overall copy number of ICP4 has not reached a critical threshold until 7 to 9 hours post infection in these complementing cell lines. In the absence of functional ICP4 there is a significant deficit in transcriptional capacity as which can lead to deficits in translation capacity as well since this gene product represents the major transactivator of the virus. Another possibility is a disjointed cascade of viral gene regulation without sufficient ICP4 expression which could lead to overexpression of viral genes that are normally repressed by ICP4. Additionally, lack of ICP4 can lead to inefficient replication compartments since localization of ICP0, ICP27, and ICP4 has been shown to occur concomitantly with HSV-1 viral DNA replication (50).

Future work on viral genome and ICP4 localization may shed light on the complementation of ICP4 from a cell line versus the natural infection and its ability to enhance stability. This work will serve to bolster future developments in the field of replication defective viruses such as safer vaccine candidates as well as support clinical applications for future gene therapy trials.

4.0 HSV-1 DEREPRESSION

Herpes simplex virus 1 (HSV-1) is a common human pathogen. Following viral infection and release of the viral genome in the nucleus of permissive host cells, lytic infection starts a temporally regulated program of HSV-1 gene expression. The immediate early genes (IE) ICP0, ICP4, ICP22, ICP27, and ICP47 are first expressed and their products are required for expression of early and late HSV-1 genes (7, 21, 75, 76). Among the five IE genes, only ICP0 is known to transcriptionally activate all kinetic classes of genes, i.e. the IE, early and late genes (86).

HSV-1 contains genes classified as essential or non-essential, depending upon their requirement for viral replication in cell culture. However, the so-called non-essential genes play critical roles in completion of the virus life cycle in vivo. Among the non-essential genes, ICP0 is a multifunctional protein which regulates expression of a broad range of viral and cellular genes and proteins. Interestingly, deletion of ICP0 gene alters viral gene expression in a cell-type dependent manner. Earlier studies on infection of Vero and U2OS cells with an ICP0 null mutant, 7134, showed that expression of the IE protein ICP4 and the early protein gD as well as plating efficiency of the mutant virus were significantly higher in U2OS cells than in Vero cells (202).

The mechanism(s) underlying the difference in host cell responses that might contribute to the contrasting effects of ICP0 deletion in U2OS and Vero cells has not been defined. A plausible hypothesis is that untransformed cells contain an innate defense system that represses HSV-1 gene expression and the protein ICP0 counters this innate defense system to allow viral

gene expression and growth. The evidence in support of this hypothesis comes from a recent study (67) in which somatic cell hybrids formed between the permissive U2OS cells and highly restrictive HEL fibroblasts were as non-permissive as HEL cells to infection with KM110 (a mutant that lacks ICP0 and VP16). These observations suggest that the cellular mechanism responsive to ICP0 deletion is important in viral gene expression and, possibly, in tumor growth. One of the reasons for the slow progress in defining this mechanism has been the scarcity of chemical agents that mimic the effects of ICP0 on gene expression. This chapter describes the use of a cell-based, rapid, and simple fluorescence assay for a chemical screen to identify compounds that might be important for defining the repressive cellular mechanism that is negated by ICP0 expression.

4.1 INTRODUCTION

HSV-1 represents an attractive vehicle for gene transfer applications *in vitro* and *in vivo*. A number of facets of HSV-1 biology affirm its utility which include the large genome (84 open reading frames) of which half is non essential, ease of manipulation, and latent state of infection for long term therapy. Naturally occurring infections within the nervous system also make HSV-1 attractive for neuropathies as well as glioma therapy (25, 128). Replication defective HSV-1 vectors have been used for gene transfer in numerous disease states in different tissue models (77, 97, 160). These vectors lack essential IE genes which render them nontoxic and safe for clinical application. Removal of the IE genes will block expression of the virus lytic gene program in non-neuronal cells and therefore transgene expression is severely repressed.

However, viral genomic repression in the quiescent model can be relieved by expression of a single IE gene, ICP0 (44, 116).

HSV-1 mutants that fail to express ICP0 have severe defects in viral gene expression and plaque formation in mammalian cells (16, 52, 63). Despite this critical importance in virus propagation, ICP0 severely reduces cell viability and will induce cell cycle arrest (34, 74). Additionally, the E3 ligase activity attributed to the RING finger domain of ICP0 has been shown to degrade host cell responses to infection (13, 14, 100). These cell responses include accumulation of nuclear organelles which are associated with chromatin reorganization such as PML, Daxx, and Sp100 (2, 49). This effect is clearly marked by punctuate structures associated with nuclear domains (ND10) and ICP0 can effectively degrade these structures in a RING finger dependent manner (51). However, in certain tumor cell types (e.g U2OS) ICP0 is not required for transgene function and in fact ICP0 deletion mutants grow efficiently in these cells and are similar to wild type with respect to virus yields. Thus, U2OS cells appear to be defective in one of more cellular functions that repress the viral genome. This is likely to be a missing function based on fusion of non-permissive HEL cells with U2OS in which the fusogenic progeny were still non-permissive (67). Studies to examine the mechanisms underlying the permissive effect of U2OS cells suggest that they possess an ability to complement ICP0 deficiency (166, 202). However, it is not clear whether this is a direct effect as no molecular mechanisms are currently known. The basis for this interaction has critical importance in cancer therapy as well as HSV-1 biology.

We report the use a high throughput cell based assay for detection of potential agonists that can lead to derepression of HSV-1 genomes in the presence of a non-permissive cell environment. The LOPAC (library of pharmacologically active compounds) library was chosen for the screen due to the well documented activities of each of the compounds. Vero cells preincubated with the LOPAC library and subsequently infected with a quadrurple IE mutant

QOZ22REH1 reveal novel interactions of host cell state that can allow expression of EGFP from the HCMV promoter which is otherwise silent during normal infection. A metal chelator, 1,10 phenanthroline monohydrate, was the only compound that could release Vero induced repression while maintaining a low cytotoxicity profile. The induction of a novel RING finger protein termed sensititive to apoptosis gene (SAG) was observed in the presence of 1 10 phenanthroline both with and without infection suggesting a potential role of this gene in depression of the viral genome. This gene has E3 ligase activity much like the viral gene ICP0 and serves as a potential derepressor of the viral genome in the context of an IE mutant infection. Likewise, proteosome inhibition experiments reveal a common pathway for this gene and implicate the presence of a cellular protein or protein complex in Vero cells that can actively participates HSV-1 gene repression

4.2 RESULTS

4.2.1 Genetic Map of TOZHE and QOZH22REH1

To assess the effect of ICP0 on the derepression of HSV-1 mediated delivery of a transgene in Vero cells two isogenic mutants were used. The control virus, TOZHE, has the ICP0 gene intact but is devoid of ICP27, ICP22, ICP4, and U_L41. This virus is a progeny of TOZ.1 with the EGFP transgene driven by the CMV promoter in the ICP4 locus (193). QOZ22REH1 is a quadruple IE mutant with EGFP driven by the HCMV promoter in the ICP0 locus. Figure 19 shows the genetic map of each vector.

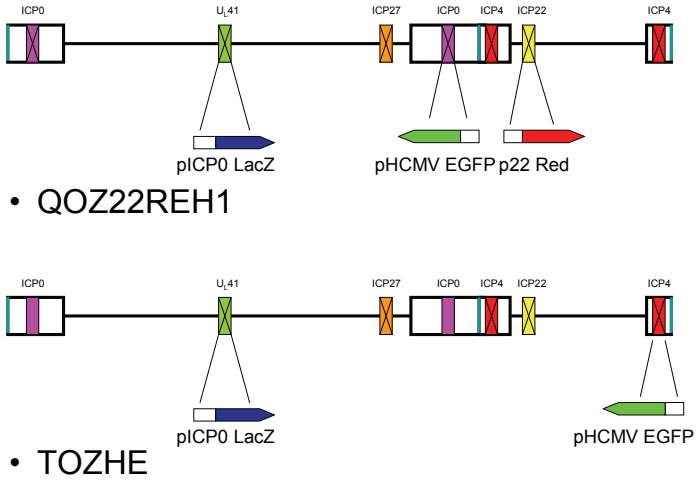


Figure 19. Genetic Map of TOZHE and QOZ22REH1

Schematic map of QOZ22REH1 and TOZHE. The dsRed2 gene is driven by the ICP22 promoter in the ICP22 locus in QOZ22REH1. EGFP driven by HCMV in the ICP4 locus in TOZHE and the ICP0 locus in QOZ22REH1. QOZ22REH1 is propagated on the ICP0, ICP4, and ICP27 complementing cell line 01. TOZHE is propagated on ICP27 and ICP4 the complementing cell line Q5. X at a genetic locus indicates removal of this gene.

4.2.2 Chemical Library Screen

The LOPAC (library of pharmaceutically active compounds) was screened for potential derepressors of HSV-1 gene expression by gain of signal through EGFP expression. Vero cells were plated in clear bottom, black walled 384 well plates in phenol red free media via a Titertek Zoom dispenser. Twenty four hours post seeding cells were administered the LOPAC library via the Vprep mutichannel dispenser at 25 uM. Two hours post-administration with the library, the monolayer was infected with QOZ22REH1 at an MOI of 3 based on transducing units in U2OS cells. Three days post infection, the EGFP signal was assessed by visual confirmation. Pretreatment with 1 10 Phenanthroline (OP) was able to give rise to adequate EGFP expression compared to the control vector (Figure 20). Previous reports indicate that histone deacetylase inhibitors can relieve cellular repression during quiescence from an HSV-1 mutant devoid of any

IE gene function (179). In contrast, pre-incubation with TSA at effective concentrations for growth enhancement of triple mutant viruses yielded no significant increase in EGFP expression from the QOZ22REH1 backbone.

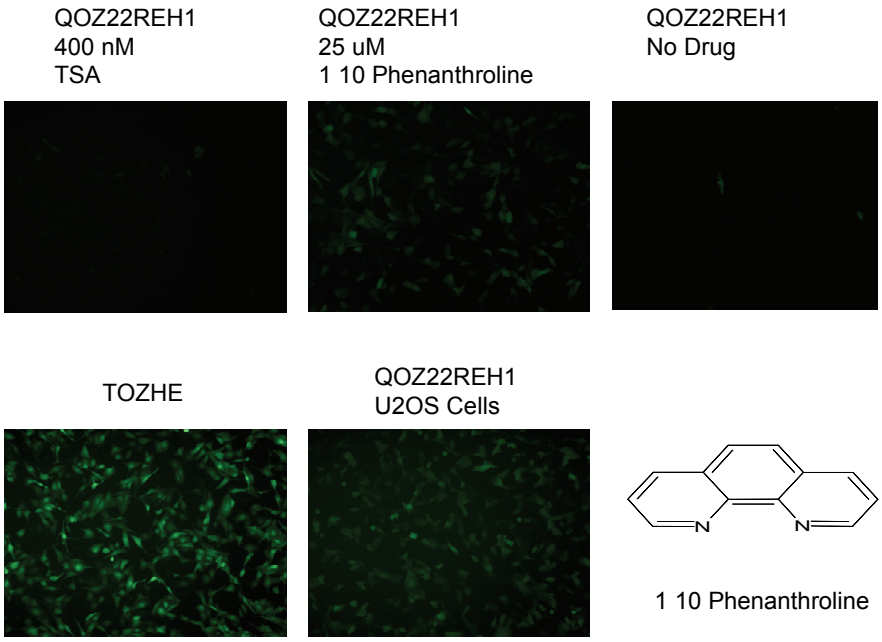


Figure 20. 1 10 Phenanthroline Aids in EGFP Expression from QOZ22REH1.

Confocal fluorescent microscopy of Vero (top row and TOZHE) and U2OS (QOZ2REH1 without drug) monolayers either untreated or pretreated with drug at indicated doses for one hour prior to infection. Chemical structure of 1 10 Phenanthroline.

QOZ22REH1 transgene expression in U2OS cells was similar to TOZHE expression in Vero cells consistent with previous reports suggesting this cell line can substitute for a functionality expressed by ICP0 (202).

4.2.3 Proteosome Pathway Dependency

The proteosomal pathway is a critical component for HSV-1 derepression as evidenced by ICP0 specific degradation of innate cellular response to infection (52, 65). To this end, the effect of

proteosome inhibition was examined for derepression of QOZ22REH1 either alone or in combination with 1 10 phenanthroline (referred to as OP from here on). In the presence of MG132 the expression of EGFP was significantly altered in both U2OS and Vero cells (Figure 21).

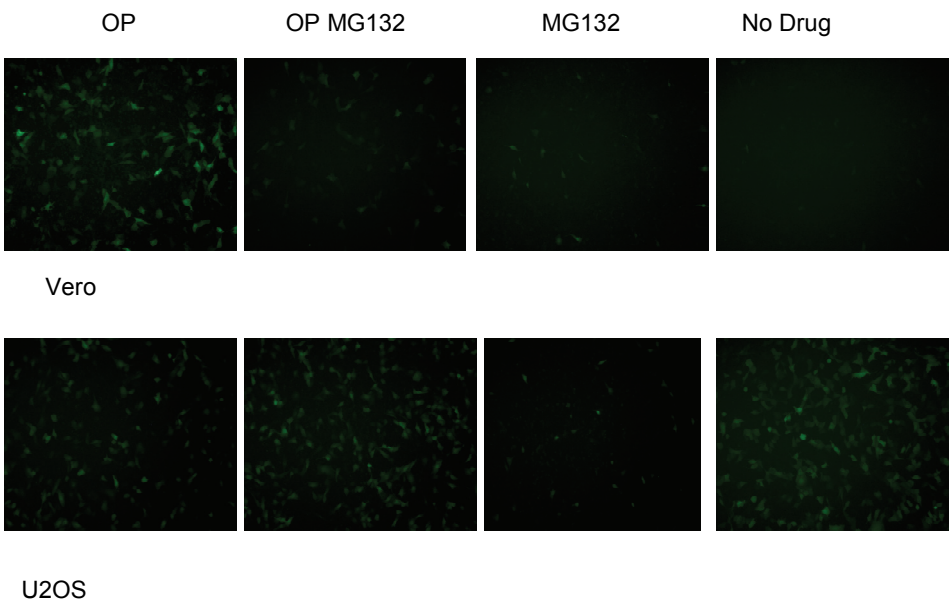


Figure 21. OP Mediated Derepression is Proteosome Dependent

Fluorescence microscopy of EGFP 72 HPI from QOZ22REH1 (MOI = 3) in either OP pretreated (OP), MG132 pretreated (MG132), or OP pretreatment followed by MG132 pretreated (OP MG132) U2OS or Vero cells.

The marked difference in EGFP expression in presence of the proteosome inhibitor MG132 indicates that HSV-1 mediated gene expression from the viral backbone is dependent on this pathway. Furthermore, OP pretreated Vero and U2OS cells were still able to express EGFP from the viral genome in the presence of MG132 suggesting possible degradation of a repressive protein before proteosomal inhibition in both cell types. However, higher viral transgene expression with both OP and MG132 in U2OS cells indicates cell type specificity for this interaction. These data support the hypothesis of the induction of a novel gene product or pathway upon OP administration.

In a similar manner, TOZHE was examined for EGFP expression in the presence of MG132 and OP or a combination thereof.

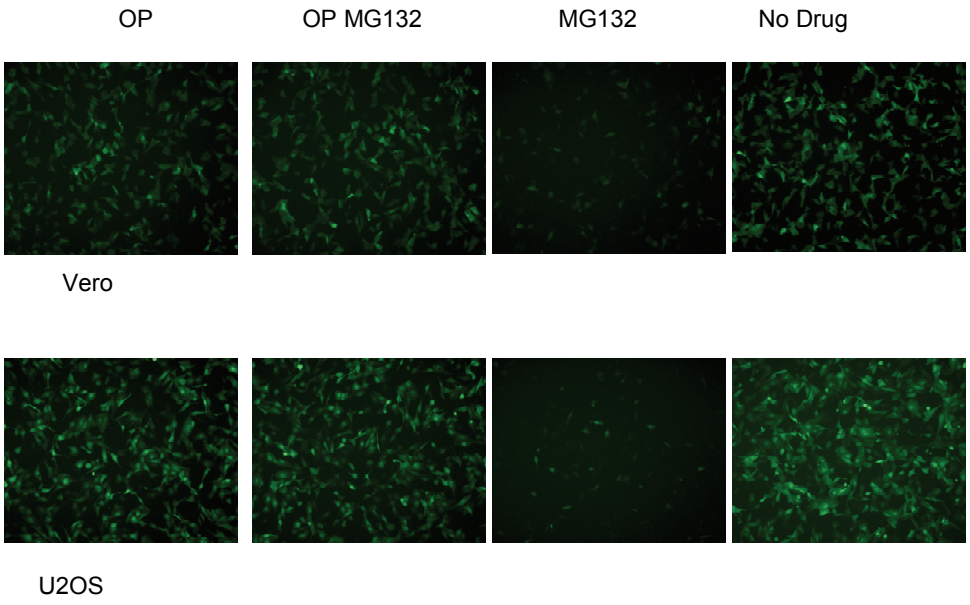


Figure 22. ICP0 Mediated Transgene Expression blocked by MG132 but not by OP

Fluorescence microscopy of EGFP 72 HPI from QOZ22REH1 (MOI = 3) in either OP pretreated (OP), MG132 pretreated (MG132), or OP pretreatment followed by MG132 pretreated (OP MG132) U2OS or Vero cells.

The induction of EGFP from the TOZHE backbone was independent of OP regimen but significant reduction in signal in the presence of MG132 is consistent with reports on the E3 ligase activity of ICP0 through the proteosomal pathway. Interestingly, U2OS cells also displayed a dependency on the proteosome pathway for EGFP expression indicating the degradation of a native moiety involved in HSV-1 genome repression in this cell line.

4.2.4 MOI and OP Dose Response

To further examine the sensitivity of the OP mediated effects of derepression on HSV-1 mediated transgene expression, a dose escalation with input virus and OP was performed on Vero cells.

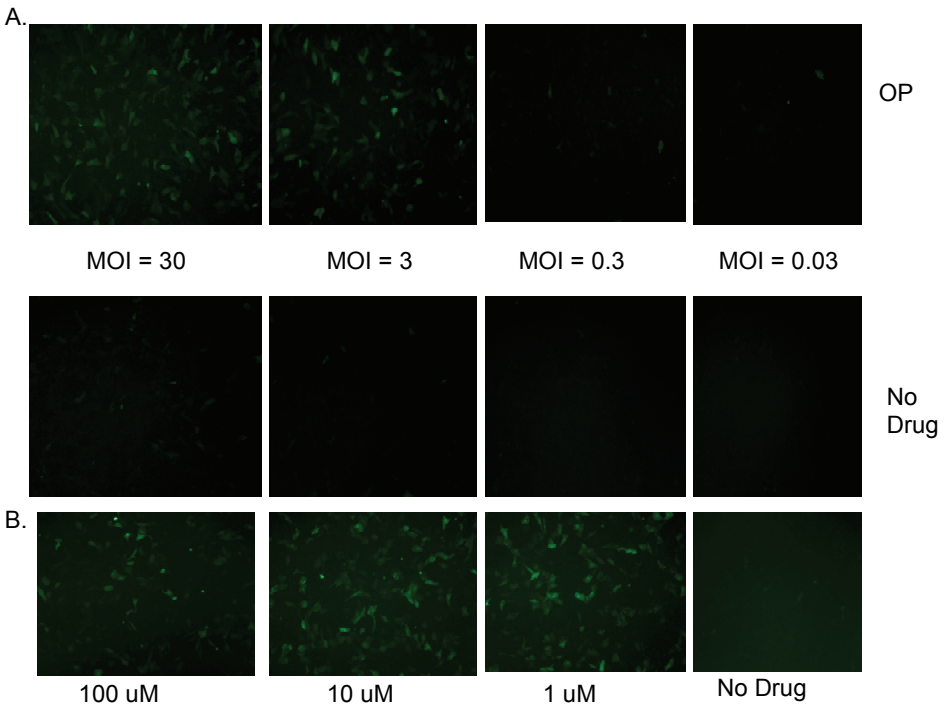


Figure 23. MOI and OP dose response with QOZ22REH1

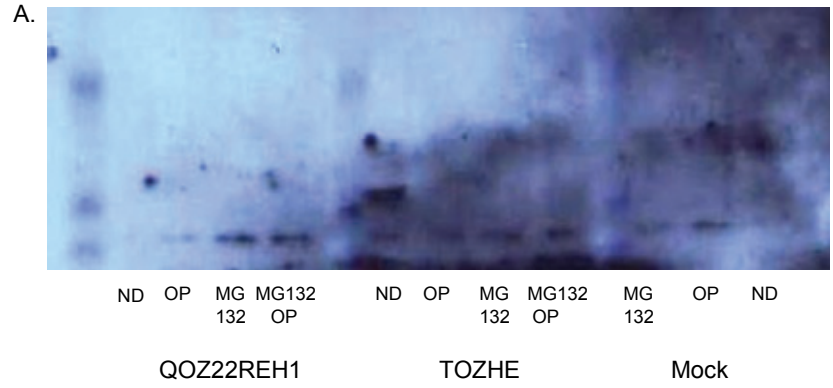
A. Fluorescence microscopy of Vero cells pretreated with 25 uM OP subject to infection with QOZ22REH1 at MOIs indicated. B. Dose escalation of OP. Vero cell were pretreated with concentrations of OP indicated and infected with QOZ22REH1 at MOI = 3.

The enhanced fluorescence at higher MOIs reflects the sensitivity of the pretreated cells to the increasing input viral genomes. Generally, higher input doses of virus can overcome limitations in gene expression for IE mutants, however, this was not the case in absence of OP up to an MOI of 30. This data suggest a strict requirement of cell state to enhanced viral transgene expression

in the absence of all IE genes. Dose response to OP concentration revealed effective phenotype reversal at micromolar concentrations. The lower fluorescence at 100 uM OP is indicative of cytotoxicity of this compound at higher concentrations. These data suggest that OP is effective in manipulation of host cell state to allow HSV-1 mediated transgene expression at moderate MOIs. Furthermore, the dramatic enchancement of EGFP expression at higher MOIs suggest that the OP pretreatment is the limiting factor in derepression since increasing the number of incoming viral particles was not able to overcome this effect in the absence of OP treatment.

4.2.5 Senstitive to Apoptosis Gene (SAG) Induction

Previous reports protection from peroxide and free radical induced DNA damage have suggested that the gene termed sensitive to apoptosis gene (SAG) is expressed with rapid kinetics in both kidney and liver tissue upon stimulation with 10 uM OP (43, 175). Cell lysates collected from the monolayers shown in Figure 21 were assayed for SAG expression by western blot analysis. SAG was enhanced in Vero cells upon normal growth in 25 uM OP (Figure 24 Mock Lanes).



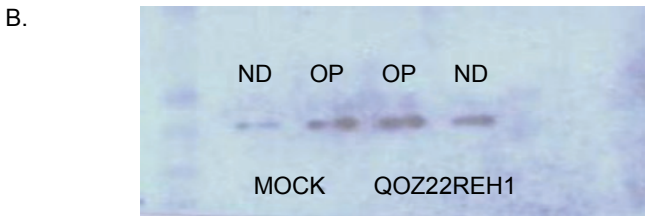


Figure 24. SAG induction upon 25 uM OP culture

A. SAG expression in Vero cell lysates from Figure 22 and 23 were separated on 12% SDS-PAGE blotted for SAG. ND refers to no drug. QOZ22REH1 and TOZHE (MOI = 1) Infections were lysed at 24 HPI. B. SAG expression in U2OS cells infected either Mock or QOZ22REH1 MOI = 3.

SAG bands present in all lanes infected with TOZHE implies that this gene is protected from degradation by the expression of ICP0 and may serve to aid in gene expression from the virus backbone. Increased expression in cultures exposed to MG132 imply that this gene may be degraded by the presence of viral genomes in the absence of ICP0 (QOZ22REH1 lanes in Figure 24). However, a diminished band at 24 HPI in the presence of OP alone (Figure 24 A QOZ22REH1 OP) suggests that at this time point the gene may be active but further degraded at later times post infection. Furthermore, U2OS cells displayed little dependency on OP treatment or infection status for SAG expression indicating this protein is constitutively expressed in this cell line (Figure 24 B). These data suggest that the induction of SAG and its expression during

derepression of the HSV-1 genome may be a novel mechanism for overcoming the innate barrier to viral gene expression.

4.3 DISCUSSION

HSV-1 infects a high percentage of any population groups, commonly causing skin and mucosal lesions only in a fraction of actual infections. Thus, HSV-1 has coevolved with its human host and is rarely life-threatening in immunocompetent individuals. This characteristic makes HSV-1 an attractive platform for therapeutic vector development. Furthermore, HSV-1 efficiently infects a wide variety of cell types and the genome has numerous non-essential genes whose deletion allows insertion of large transgenes. However, deleting genes from a genome that evolved to survive in a hostile niche weakens the vector and tilts the balance in virus-host cell interactions in favor of the repressive mechanisms in the host cell. Transgene expression from ICP0-null mutants in normal cells is inefficient making such vectors of limited applicability. Unraveling the molecular basis of how ICP0 negates repression of gene expression in normal cells and why ICP0 is dispensable in a tumor cell microenvironment is of fundamental importance to our understanding of regulation of eukaryotic gene expression.

Immediately upon release of HSV-1 DNA from capsids, a competition ensues between cellular factors whose mission is to silence viral DNA and viral proteins that aim to block the cell. The innate immune response has been well documented as an active repressive mechanism for viral gene expression (30, 100). ICP0 as a preemptive viral defense mechanism effectively counteracts this response through degradation of key antiviral cellular organelles (52). The E3 ligase functionality is essential for this function of ICP0 indicative of exploitation of the proteosomal pathway.

Efficient infection by ICP0-negative mutants is not limited to cells with a deregulated interferon system. Although ICP0 deletion mutant 7134 (202), displayed reduced plating efficiency in Vero cells at low multiplicities of infection, it grew efficiently in the osteosarcoma cell line U2OS. Furthermore, Western blot analysis showed that the levels of expression of the IE protein ICP4 and the early protein gD in 7134-infected U2OS cells were significantly higher than those in 7134-infected Vero cells. Similar to 7134, a mutant that lacks functional ICP0 and VP16, KM110, is impaired for growth in primary human fibroblasts, but replicates efficiently in U2OS cells (122). The underlying mechanism for the contrasting expression patterns of 7134 and KM110 genes in restrictive and permissive cells is not known. One possibility is that U2OS cells express stimulatory factors that complement ICP0 and VP16 deficiency (202); another is that the permissive U2OS cells lack a repressive mechanism that is targeted by ICP0 which has not been explored. To distinguish between these two possibilities, hybrid cells formed by fusing the permissive U2OS cells with the highly restrictive human embryonic lung (HEL) cells were infected with KM110. The heterokarya were completely non-permissive for KM110 IE gene expression, indicating that restrictive cells contain one or more rapidly acting dominant inhibitory factors that are absent from permissive U2OS cells (67). Additionally, HSV-1 repression has been studied extensively through latency and quiescence in the neuronal as well as other tissue models (29, 116, 179). The current understanding is a mechanism of heterochromatin state within the immediate early viral gene promoters preventing the toxicity of these genes (29). However, deregulation of this repression mechanism through small molecule inhibitors is yields only moderate recoveries implicating multiple repressive phenomena (see Chapter 3 section 3.2.3)

The mutant we have developed, QOZ22REH1, lacks functional ICP0, ICP4, ICP22 and ICP27 and contains EGFP under the control of HCMV. Unlike the mutants described above, 7134 and KM110, QOZ22REH1 is a highly defective non-replicating vector that fails to express

any viral gene products. This vector represents an important tool for investigating regulation of viral gene expression, independent of the process of viral replication. Consistent with the findings described above, we have observed that expression of exogenous enhanced green fluorescent protein (EGFP) reporter gene is repressed in Vero cells but not in U2OS cells. A cell based high throughput chemical library screen allowed the identification of a potent compound (1 10 phenanthroline) which negated gene silencing in Vero cells to enable efficient expression of exogenous genes inserted in the highly defective HSV-1 mutant vector QOZ22REH1. The derepression was specific to the host cell state induced by this compound (Figure 23) and was dose responsive to input virus. Interestingly, at high multiplicities this mutant vector was only available to overcome genomic repression in the presence of drug suggesting a cellular factor that was either degraded or induced upon drug administration. Studies with the proteosome inhibitor MG132 showed that this effect was slightly reduced in the absence of proteosome activity suggesting that this pathway was essential for viral transgene derepression. More importantly, this effect was also observed in U2OS cells indicating the presence of a cellular factor involved in active repression whose gene product was not degraded in the presence of MG132. This gene product has yet to be defined but future experiments outlining the mechanism perhaps through microarray studies will shed light on this previously unknown function.

Previous studies have shown that administration of OP to cell cultures will induce the presence of the gene termed sensitive to apoptosis gene, SAG(43). This gene product much like ICP0 contains a zinc RING (Really Interesting New Gene) finger domain that has been demonstrated to exhibit E3 ligase activity indicating that it could represent a novel target of some repressive function that inhibits viral gene expression (69, 176). The presence of this gene was consistent in all infections that retained the derepressed phenotype of allowing EGFP expression from the viral genome. In addition, induction of SAG was shown to be sensitive to OP treatment

in Vero cells as assayed by western blot. Interestingly, in Vero cells infected with QOZ22REH1 the SAG expression was neglible at the resolution of this assay without drug treatment. This could indicate the necessity of this gene product to the derepressed phenotype of the IE mutant backbone. More importantly, SAG was present in all conditions examined with U2OS cells indicating that this gene may play a role in the permissive nature of this cell line. This is the first report indicating the presence of a novel gene involved in the proteosomal pathway that can lead to enhanced gene expression from an HSV-1 mutant devoid of any IE function. Future experiments investigating the interaction partners and proteosomal targets of this gene will elucidate

5.0 ION EXCHANGE PURIFICATION OF HSV-1 VECTORS

Purification of clinical grade viral based gene therapy products must adhere to strict FDA requirements with respect to contaminating host cellular DNA and protein. In addition, these processes must be scaleable to meet the demand for early phase trials. Common methods of purification for viral vectors include sucrose and cesium gradients, ultracentrifugation, as well as immobilized metal affinity chromatography (IMAC) (90). These strategies are suitable for bench scale production, however, translation to industrial scale is difficult for specific reasons to each application. Gradient centrifugation is costly and not readily amenable to large scale use due to specialized equipment and low yields. Ultracentrifugation, while scalable, can aggregate contaminating cellular debris along with virion particles. IMAC chromatography while improving yields and specificity requires further manipulation of the viral backbone which can add unnecessary complexity to the purification scheme and time to the development process. A common purification strategy for all backbones would greatly benefit the translation of HSV-1 gene therapy into the clinic. HSV-1 is an enveloped DNA virus that requires gentle separation methods so as not to rupture the glycoprotein coat and render the virion non-infectious. Taking these considerations into account, an ion-exchange strategy is proposed that takes advantage of the robust nature of charge-charge based separation along with the freedom to manipulate loading conditions. Described is an investigation into loading conditions using both ion and anion exchange chromatography to purify wild-type HSV-1. By optimizing loading conditions, yields were increased by orders of magnitude. Furthermore, HSV-1 affinity differences between

anion and cation exchange chromatography lead to these differences in recoveries. Additional developments are also suggested.

5.1 INTRODUCTION

Substantial progress in the field of viral mediated gene therapy has resulted in increased pressure to develop methods for large scale production of high titer vectors free of host cell contaminants which can induce immunity and therefore decrease the effectiveness of the gene delivery. In addition to purity, downstream process concerns include total injectable volume, mass transfer effects, and specificity. For genetic manipulation of the central nervous system, there is a lack of space for large deposits. The volumes of purified stocks must therefore be minimized to enable efficient spread of the virus to only the tissue of interest. High speed centrifugation is commonly used to crudely purify viral vectors, but this method lacks specificity for the virus and can co-sediment host cell protein as well as DNA (27). Sucrose gradients are also employed to sediment the virus based on density. This method is attractive in that genome containing virions are separated from empty capsids (126). However, large shear effects are often encountered in high speed centrifugation through sucrose gradients and this strategy is not amenable to large scale operation (149). These shear effects become more critical with respect to enveloped virions since they are generally more labile. Therefore, column based chromatography remains the separation method of choice that allows scalability as well as freedom to dictate the relative concentration of the virus by controlling elution conditions. Loading conditions can be optimized to avoid mass transfer effects by adjusting flow conditions. For instance, the mass transport limitations can be overcome by loading at higher flow rates but this phenomenon must

be balanced by the shear sensitivity of the virus. More importantly, specificity can also be obtained to tailor the separation based on resin density and molecular characteristics (88). The development of ion exchange based chromatography to purify enveloped as well as non-enveloped gene therapy viral vectors has been well established (60, 88, 198). While adenoviral vector purification remains an active area of research due to the prevalence in clinical trials, there is limited work on chromatographic purification of HSV-1 based viral vectors. The work to date consists of either targeting the virus to a ligand based separation such as IMAC (82) or affinity adsorption (129). These techniques are not limited to purification of HSV-1, however, and have been used to produce retroviral (204), adenoassociated (206) and lentiviral vectors (163).

In this chapter, we describe the use of ion exchange chromatography to purify wild type strain of HSV-1, Kos. Infectious virus losses experienced on both anion and cation exchange columns were significant and most likely due to inactivation or irreversible binding to the column matrix. Among all pH conditions tested physiologic pH yielded higher recoveries on both columns. However, the cation exchange column was able to provide higher yields due to lower affinity for the virus. This was verified by investigation of loading virus at elevated NaCl conditions. The higher salt concentrations effectively competed off the virus to both columns albeit with higher efficiency in the anion exchange column. Likewise, yields of both ion exchange strategies were compared with respect to DNA encapsidated virions and genomic copy number. The cation exchange column was able to purify virus with higher concentration of genome containing virions as verified by electron microscopy as well as quantitative PCR for genome copy number. Electron microscopy validated these results showing higher concentration of genome containing virions in the cation exchange elution Loading condition experiments reveal a dose response with flow rate suggesting that the mass transfer limitations are minimal for both cation and anion exchange chromatography. Additionally, the total protein content was substantially lower in the eluted fractions compared to loaded material suggesting that cation

exchange with physiologic pH can yield relatively pure virions that are suitable for clinical applications. .

5.2 RESULTS

5.2.1 Cation and Anion Exchange Chromatography Purification of HSV-1

Ion exchange chromatography was explored as a technique for purification of wild type KOS. This vector was crudely purified by high speed centrifugation outlined in chapter 2. This stock was tested for infectious virus recovery in both anion and cation exchange chromatography to investigate binding properties. Kos was loaded onto HiTrap ion cation (SP) and anion (Q) exchange columns at 0.5 ml/min driven by a P1 pump. All samples were loaded at physiologic pH in 1X PBS at room temperature. Fractions labeled flow through (collected as the virus was loaded), wash (a 5 column volume wash in 1X PBS), and elution (pooled elution of 9 column volumes of 0.45 M NaCl). Viral recoveries as measured by plaque assay on Vero cells are shown in Figure 25 for the anion exchange column.

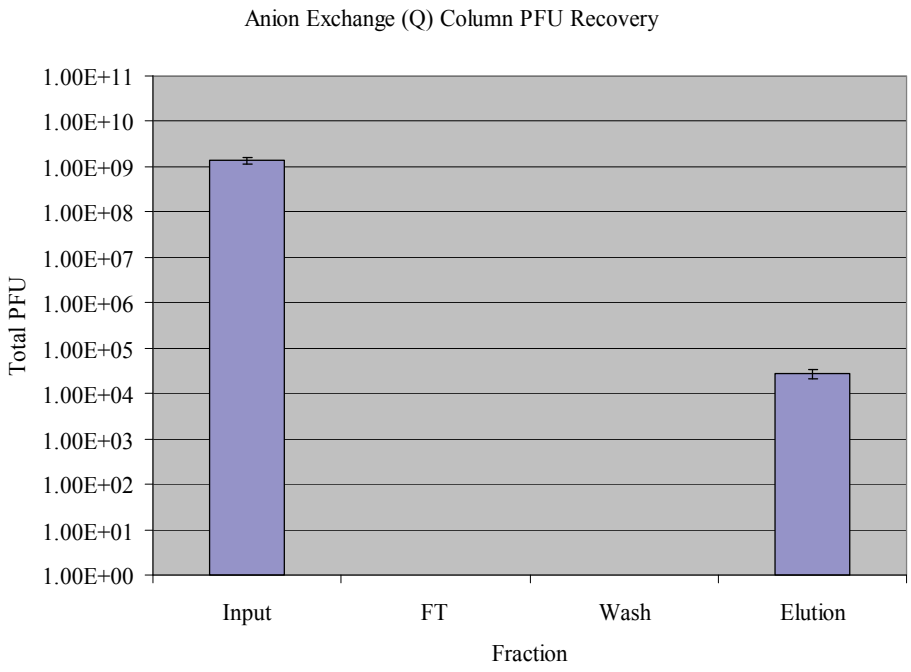


Figure 25. Infectious virus recovery with anion exchange chromatography

1E9 PFU of Kos was loaded at 0.5 ml/min at pH = 7.0 in 1X PBS. Elution was performed at 0.45 M NaCl. Fractions were assayed for infectious virus by titration on Vero cells. Error bars are indicative of three independent experiments.

Viral recoveries are dramatically low with little to no infectious virus appearing in the wash or flow through fractions using this ion exchange strategy. Likewise, recoveries in the elution are substantially low compared with input. Since this column matrix is positively charged, these results suggest that the either virus binds with high affinity and at physiologic pH may be negatively charged or the virus may inactivated within the column matrix. A high salt (1 M NaCl) elution was performed to assess the relative binding strength of HSV-1 to this column matrix. Figure 26 shows the enhanced recovery with this elution scheme, however, a higher contaminating protein level (Table 2) was also experienced compared to the 0.45 M NaCl elution. SP column elution at higher salt concentration was not explored since the higher contaminant level would not be satisfactory for clinical grade virus.

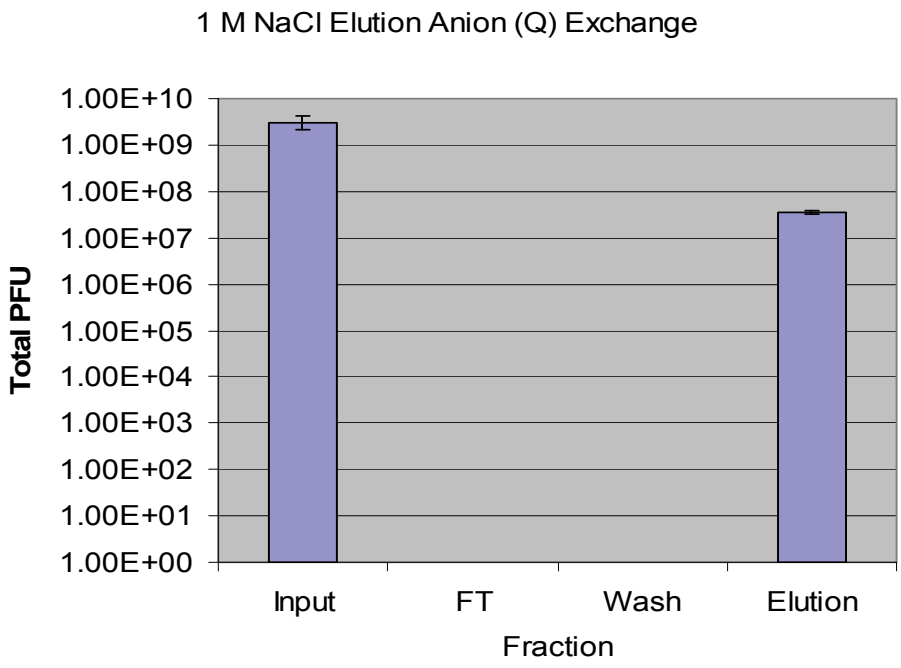


Figure 26. Elevated salt dependency for efficient elution from anion exchange chromatography

1E9 PFU of Kos was loaded at 0.5 ml/min at pH = 7.0 in 1X PBS. Elution was performed at 1.0 M NaCl. Fractions were assayed for infectious virus by titration on Vero cells. Error bars are indicative of three independent experiments.

To investigate these results further, genomic content was assayed by quantitative PCR for the same samples presented in Figure 25. Genomic recoveries in the flow through and wash fractions are enhanced as compared to infectious virus indicating the presence of contaminating DNA from the cellular debris that has cosedimented during the high speed centrifugation. The dramatically high amount of genomes in the elution suggest that viral DNA is bound to the column in addition to infectious virus and could possibly compete for binding sites.

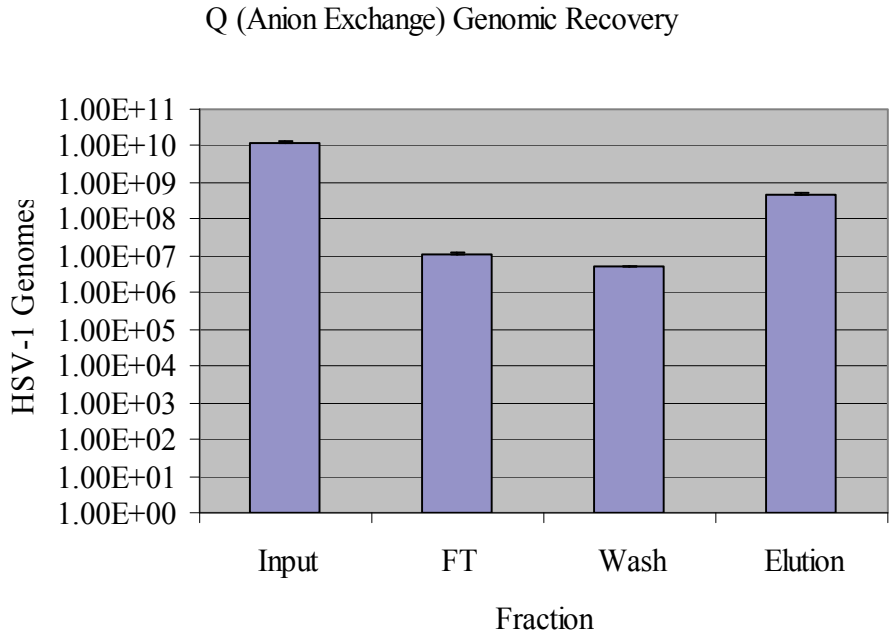


Figure 27. Genomic recovery from anion exchange chromatography

PCR quantitation of viral genomes from Figure 26. Error bars are indicative of three independent experiments.

Cation exchange chromatography was evaluated similarly as for anion exchange chromatography. Infectious virus recovery for cation exchange was dramatically improved with cation exchange chromatography in all fractions tested (Figure 28). The wash and flow through titers indicate weak binding of the virus to this exchange media. Eluted virus recoveries were also enhanced compared to anion exchange further indicating a weaker association within the cation exchange matrix. However, significant losses were experienced in all fractions tested suggesting that the virus could be inactivated within this ion exchange media as well.

Genomic recoveries from cation exchange chromatography were assessed similar to Figure 26. Approximately 20% of the input genomes were recovered in the elution fraction (Figure 29). Likewise, in comparison to anion exchange, there was enhanced recovery in the

wash and flow through fractions further suggesting weak association of the viral genome with the negatively charged resin.

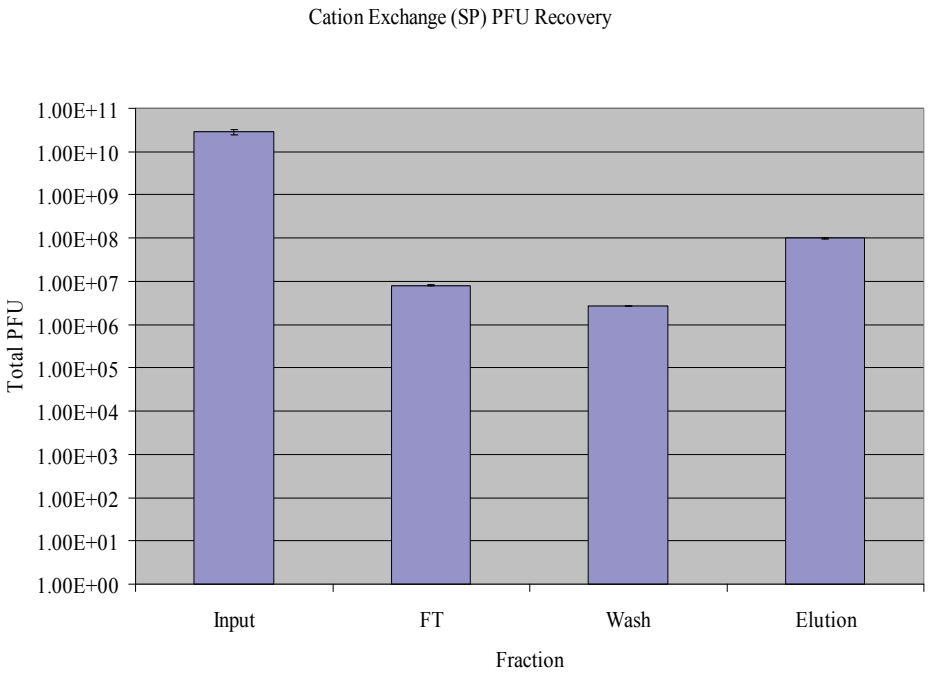


Figure 28. Infectious virus recovery from cation exchange chromatography

1E10 PFU of Kos was loaded onto the cation SP resin at flow rate of 0.5 ml/min and pH = 7.0 in 1X PBS. Elution was performed at 0.45 M NaCl. Error bars are indicative of three independent experiments.

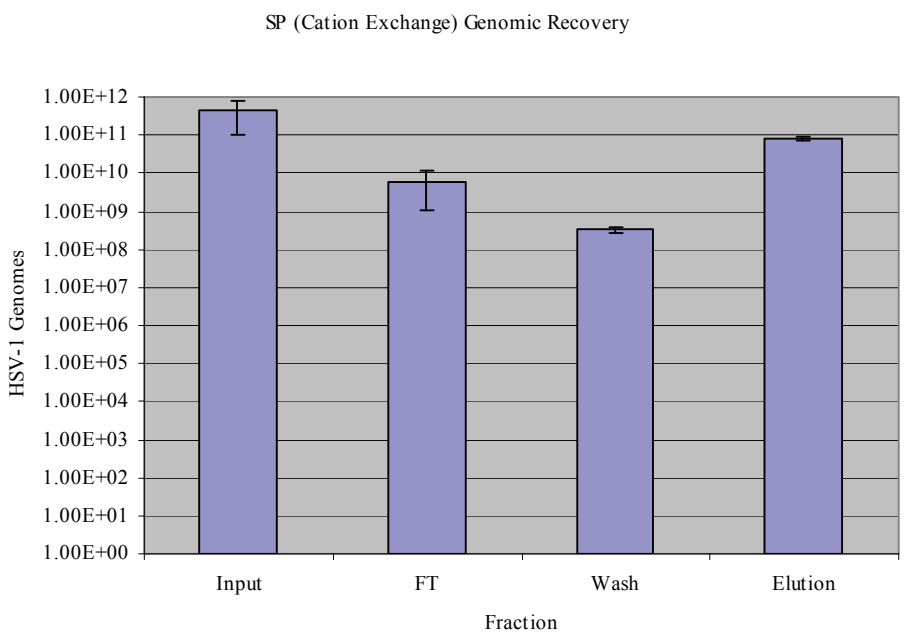


Figure 29. Genomic recovery from cation exchange chromatography

Quantitative PCR analysis from fractions in Figure 28. Error bars are indicative of three independent experiments.

The consistent excess genome copy numbers relative to infectious virus (PFU) indicate there are defective interfering particles that can co-elute from the ion exchange columns. Additionally, this viral DNA can originate from either co-sedimentation (during high speed centrifugation) or viral DNA that is adherent to the virion particles themselves. These sources of viral DNA can contaminate the virus stock prior to loading onto the ion exchange columns.

5.2.2 Proton Concentration Effect on Loading Conditions of Ion Exchange Chromatography

Proton concentration in the load buffer was investigated to assess whether or not there would be a possible pH effect on virus binding to each ion exchange resin. The same flow rate as in

Figures 25-29 was chosen to investigate pH loading conditions. Figure 30 shows the overall recoveries at moderately alkaline loading conditions (pH = 8.0) versus slightly acidic (pH = 5.0)

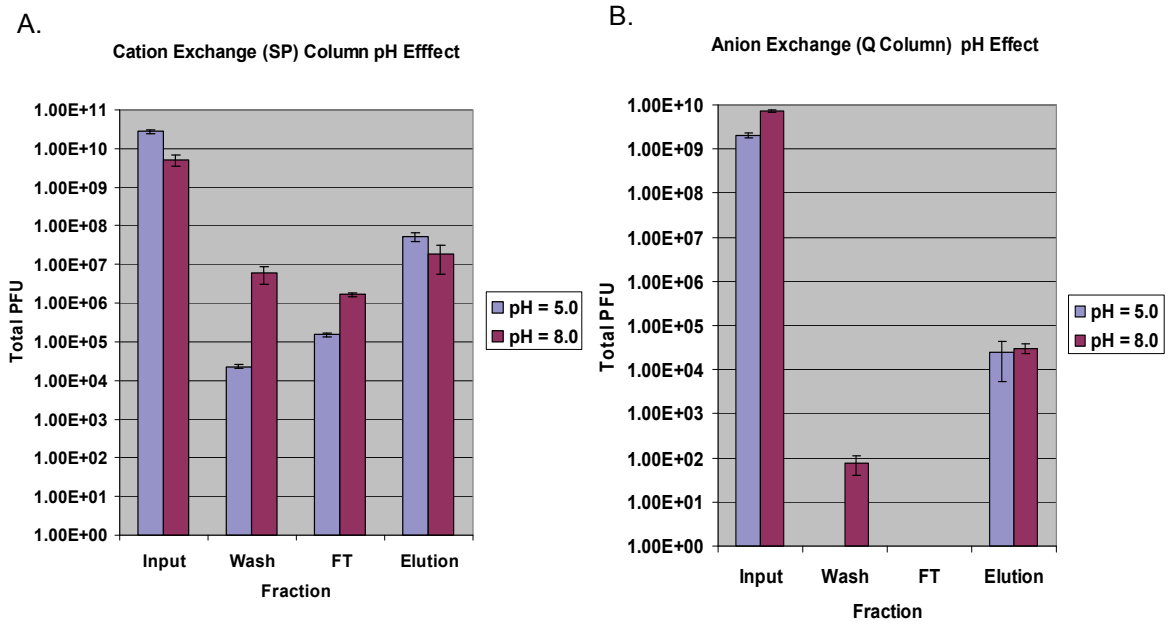


Figure 30. Proton concentration effect on infectious recovery from ion exchange chromatography Kos equilibrated in either pH = 8.0 or 5.0 loaded onto either (A) cation exchange or (B) anion exchange chromatography. Virus was delivered at a flow rate of 0.5 ml/min. PFU was assayed by titration of virus on Vero cells. Error bars are indicative of three independent experiments.

Similar to Figures 25-29, the anion exchange column yields were substantially lower regardless of loading conditions. In contrast, pH values appear to dictate the binding efficiency in the cation exchange column however overall infectious recoveries were still quite low. From the preceding figures, it is clear that regardless of loading pH or resin choice significant infectious

virus losses are experienced. However, cation exchange resins appear to be able to recover virus at higher titers due to lower binding affinity. To further investigate these claims, electron microscopy was undertaken to examine the structural features of the viral recoveries. Figure 31 shows the input virus before dilution and delivery into the column. Figure 32 reflects both a higher concentration of total virions with the cation exchange as well as higher fraction of full capsids as obtained by negative staining with uranyl acetate.

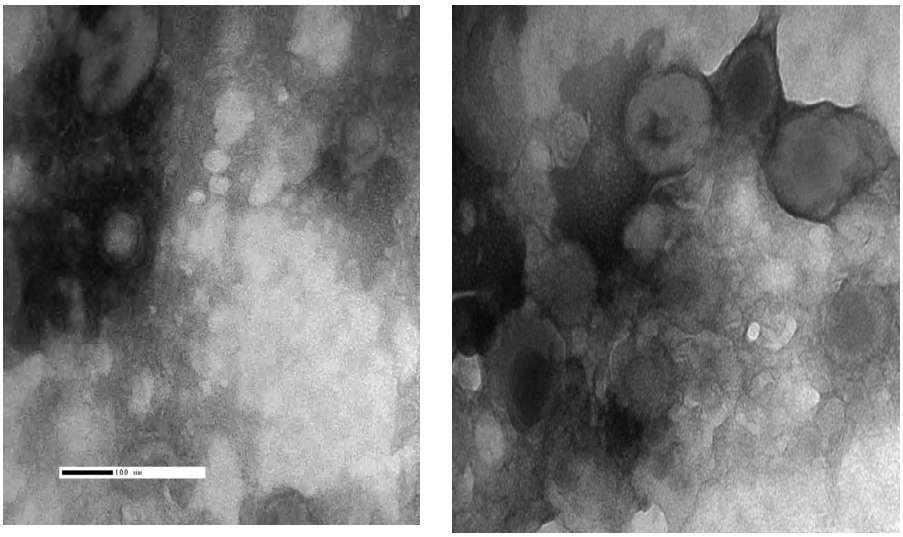


Figure 31. TEM of pelleted virus

1E9 PFU of Kos diluted in 8 ml of 1X PBS stained by uranyl acetate. Scale bar indicates the size of virions. Dark virions are empty capsids. White or light colored virions are genome containing virions.

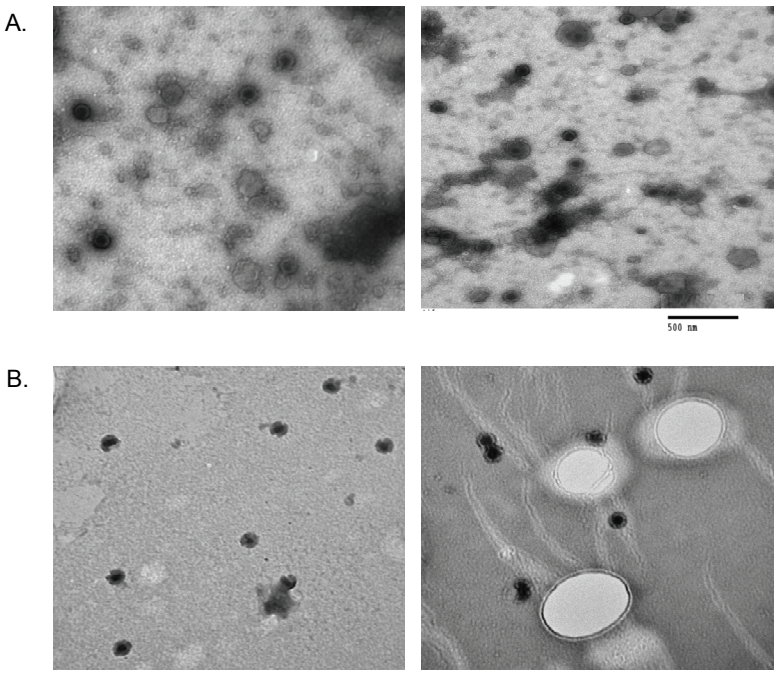


Figure 32. TEM of eluted virus from anion and cation exchange chromatography

Purified virions were diluted 1:1 in water and stained with uranyl acetate. A. Cation (SP) exchange puirifed virions. B. Anion (Q) exchange purified virions

5.2.3 Flow Rate Effect on Loading Conditions of Ion Exchange Chromatography

Mass transfer effects were investigated by varying the feed flow rate into both anion and cation exchange column chromatography. As observed in our laboratory, both columns have previously been shown to bind the virus and are able to purify vectors. A systematic investigation, however, has not been performed with respect to optimal loading conditions. Typical chromatographic flow rates for HSV-1 purification range from 0.5-0.7 ml/min (82, 129) but process economics factors in for larger batches. Thus, faster processing of larger batches with similar recoveries would be dramatically advantageous in the face of time critical applications such as vaccinations for new mutations within pandemic strains.

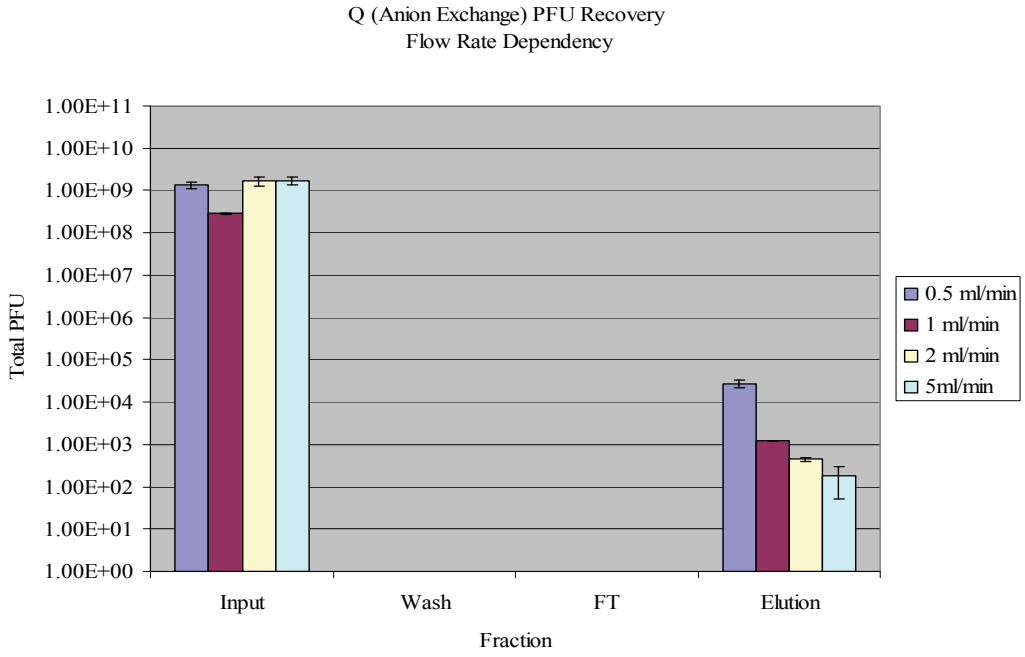


Figure 33. Infectious virus recovery dependency on flow rate in anion exchange chromatography

Infectious virus titered on Vero cells at various flow rates of loading virus at pH = 7.0 1X PBS. Error bars are indicative of three independent experiments.

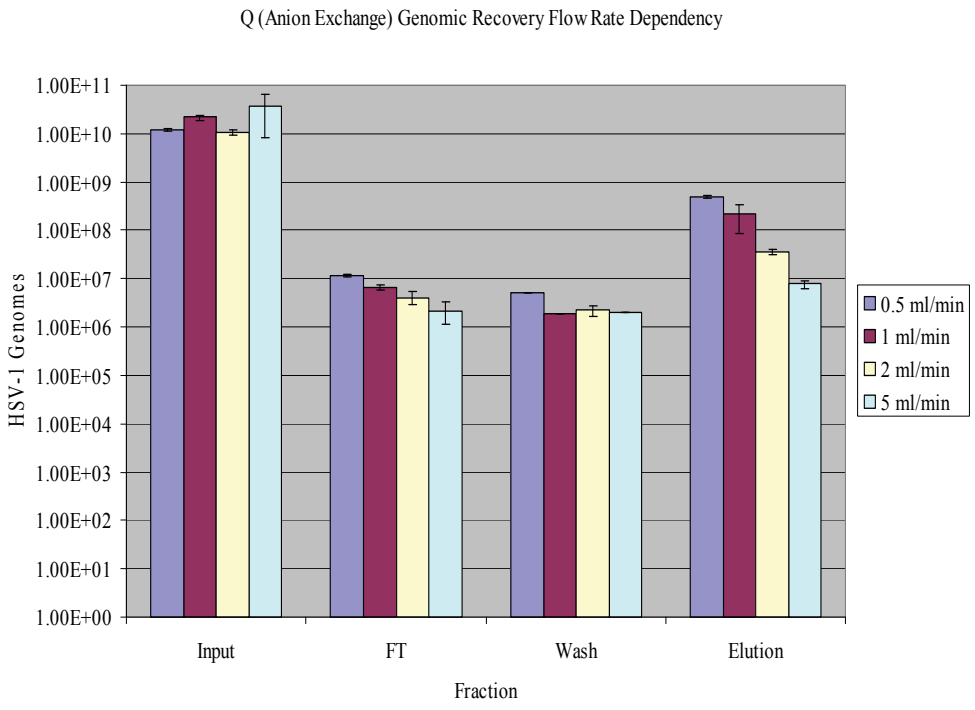


Figure 34. Genomic recovery dependency on flow rate in anion exchange chromatography

PCR quantitation of HSV-1 genomes obtained from samples in Figure 33. Error bars are indicative of three independent experiments.

Slight recoveries were experienced at lower flow rates for anion exchange chromatography but overall this effect is neglible in comparison to total input virus. However, higher flow rates lead to lower genomes recovered (Figure 34). This indicates that there is inactivation within the column matrix as the virus binds and flows through. Likewise, the genomic recovery in the elution was around 2 orders of magnitude in comparison with the input virus at the lowest flow rate while the infectious recovery displayed a 4.5 order of magnitude differential at this flow rate. The lower flow rate correlation with higher recovery suggests that optimal loading conditions for anion exchange chromatography are limited by the binding characteristics of the virus.

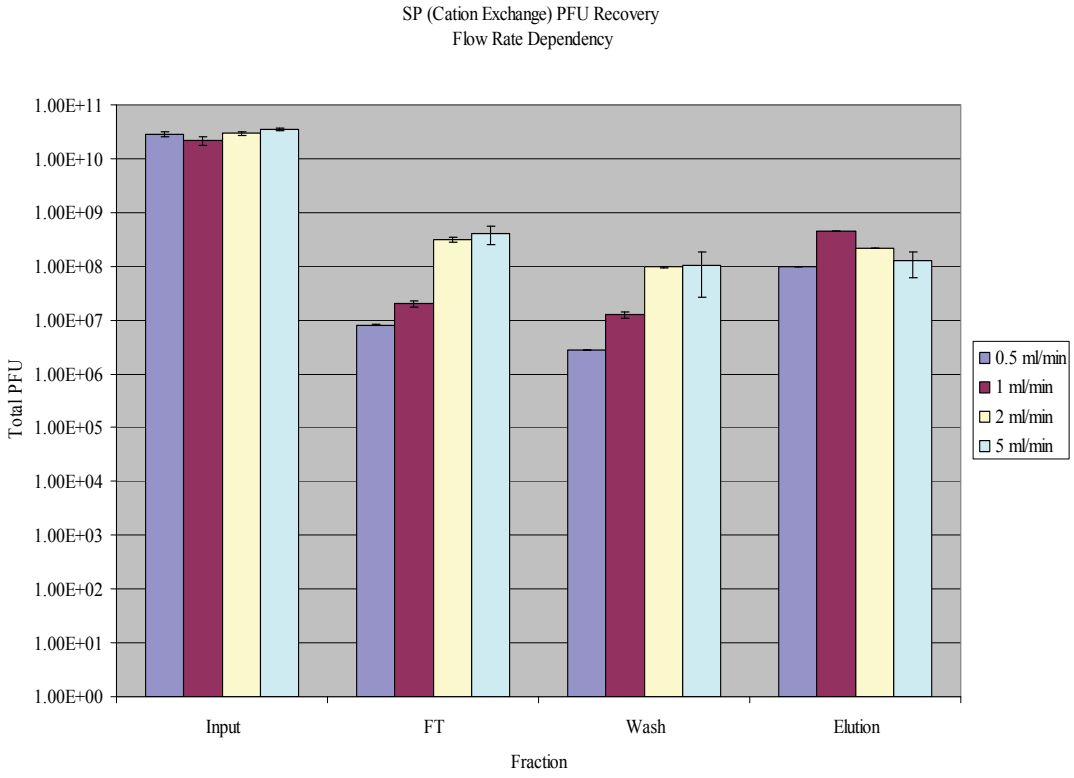


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Figure 35. Infectious virus recovery dependency on flow rate in cation exchange chromatography

Infectious virus recovered at various feed rates assayed by titration on Vero cells. Error bars are indicative of three independent experiments.

Cation exchange chromatography flow rate dependency was evident in the flow through and wash fractions but not the elution fractions suggesting that the kinetics of binding to the column . The elution profile for all flow rates was relatively unaffected even though nearly two orders of magnitude differentials were realized in both the flow through and load indicating an inactivation event within the column since potentially more virus is bound at lower flow rates. Likewise, there is substantial loss experienced within this strategy when comparing input virus and eluted yields which implicates inactivation within the column or irreversible binding. To explore this last point further, genomic data was gathered as described in Figure 35. Figure 36 shows enhanced genomic recovery at higher flow rates in the flow through albeit at a lower level than

the load fraction indicating that the virus is not binding as strongly at higher flow rates to this negatively charged resin.

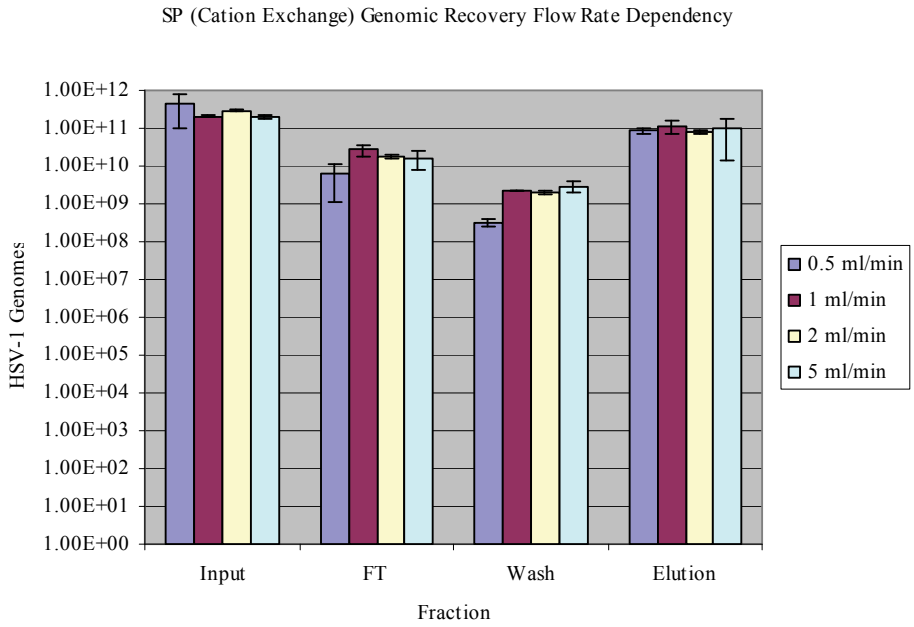

Figure 36. Genomic recovery dependency on flowrate in cation exchange chromatography

PCR quantitation of HSV-1 genomes obtained from samples in Figure 36. Error bars are indicative of three independent experiments

The data point to a strong affinity of the virus for the anion exchange resin with weak binding to the cation exchange resin. Taken together, these date indicate that there is little effect of flow rate on either ion exchange resin with respect to eluted infectious virus recovery. These experiments also suggest that loading conditions are not the limiting factor in virus recovery and elution conditions may be limiting as evidenced by Figure 26.

Table 2 highlights the protein and DNA concentrations for all experiments performed with both ion exchange resins indicating that there was significant improvement in contaminating protein and DNA with all conditions tested adhering to current FDA regulations with regard to cellular contaminants per dose.

Table 2. Protein and DNA Contamination level from Cation and Anion Exchange Chromatography

		Input		Elution		% Reduction	
	Q (anion)	DNA (ug/ml)	Protein (ug/ml)	DNA (ug/ml)	Protein (ug/ml)	DNA	Protein
pH = 7.0 Flow Rate	0.5	11.92 +/- 0.18	183.4 +/- 18.07	0.065 +/- 4.2E-5	2.47 +/- 0.98	99	98
	1	11.99 +/- 0.02	194.8 +/- 1.57	0.000189 +/- 1.55 E-5	4.83 +/- 0.39	99	97
	2	11.53 +/- 1.19	194.1 +/- 4.91	0.000225 +/- 1E-6	6.77 +/- 0.78	99	96
	5	11.90 +/- 0.15	180.94 +/- 17.6	0.000185 +/- 7E-6	8.72 +/- 1.96	99	94
Flow Rate = 0.5 ml/min pH	5	9.37 +/- 0.59	196.77 +/- 1.18	0.0019825 +/- 0.00066	5.94 +/- 1.96	99	96
	8	11.5 +/- 1.00	196.08 +/- 0.196	0.0018 +/- 0.0005	4.13 +/- 1.37	99	97
1M Nacl Elution		12.4 +/- 0.86	193.43 +/- 4.73	0.0123 +/- 0.00454	48.83	99	74
	SP (cation)	SP (cation)					
pH = 7.0 Flow Rate	0.5	11.57 +/- 0.82	196.08 +/- 0.19	0.0017 +/- 0.0002	2.47 +/- 0.98	99	98
	1	12.34 +/- 0.50	194.83 +/- 1.57	0.00195 +/- 7.07E-5	4.83 +/- 0.39	99	97
	2	12.09 +/- 0.16	194.14 +/- 4.9	0.0012 +/- 0.0003	6.77 +/- 0.78	99	96
	5	11.69 +/- 1.01	180.94 +/- 17.6	0.0017 +/- 0.0001	9.41 +/- 0.98	99	95
Flow Rate = 0.5 ml/min pH	5	10.93 +/- 1.49	180.94 +/ 17.67	0.00485 +/- 0.0021	9.41 +/- 0.98	99	94
	8	11.75 +/- 0.48	192.05 +/- 1.96	0.00486 +/- 0.01	5.80 +/- 2.16	99	96

5.3 DISCUSSION

HSV-1 is increasingly becoming the vehicle of choice for gene transfer within the central nervous system (68, 99, 172). Treatments of neuropathic pain, glioma eradication, and sensory nerve damage have been well established in animal models and are soon to enter clinic. To realize the potential of these applications, scalable manufacturing methods must be in place to provide high purity stocks devoid of any host cell contaminants that can potentially induce inflammatory responses to ectopic protein and DNA. This chapter aimed at achieving an improved method for purification of HSV-1 that can provide high titer stocks that conform to current FDA standards for injectable biologics.

Ion exchange chromatography has been used to purify viruses for a wide variety of applications (146, 184, 198). Commercially available HiTrap columns are porous so the strategy of using these columns was investigated for obtaining high titer pure viral preparations to combine the selectivity of ion exchange with the fractionation capability of size exclusion. Anion exchange column liganded with quarternary amines were able to bind the virus with high efficiency at all flow rates examined indicating that the virus is highly negatively charged at physiologic pH. Negligible dependency on flow rate on infectious virus recovery indicated that the binding efficiency was not altered by increasing the contact time within the chromatography media. However, increased infectious virus recoveries in cation exchange chromatography suggest that this resin to be superior in purifying virus at elution in 0.45 M NaCl. Cation exchange chromatography verified these results by yielding higher infectious virus at all flow rates examined in all fractions implying that the slight repulsion by negatively charged regions

within the glycoprotein coat could play a role in binding efficiency to either ion exchange resin. HSV-1 has been shown to exhibit altered electrophoretic mobility in the presence of different ionic buffers indicating that the glycoproteins are flexible with respect to ionization state and the loading conditions can be altered to selectively bind the virus to cation or anion exchange resins (185). Additionally, it is well known that the virus mediates entry into host cells through interaction with heparin sulfate moieties and herpes virus mediators of entry (Hve) that are both positively charged (53, 187) Furthermore, a synthetic polycationic peptide has been shown to bind to the glycoprotein C of the virus, which is necessary for cell surface heparin sulfate binding, suggesting that virus binding to the cation exchange column was most likely mediated through this glycoprotein and there are multiple binding modes of the virus to each ion exchange resin (186). These results implicate a slightly negative charge surrounding the virion glycoprotein coat at physiologic pH with zones of positive charge that allow binding to the cation exchange resin.

Virus binding to both anion and cation exchange resins displayed no dependency on proton concentration while the recoveries in cation exchange showed dramatic differences with respect to the flow through and load fractions. Slightly acidic conditions led to enhanced binding to the cation exchange resin suggesting that the overall virion charge may have been positively shifted under this loading scheme. However, the binding to the anion exchange resin at this pH resulted in no infectious virus recovery indicating that other factors affected binding and elution. These factors include irreversible binding which could explain the low yield of infectious virus and total viral particles as demonstrated with electron microscopy. The genomic data also reveal enhanced recovery from the cation exchange resin compared to anion exchange chromatography further validating the notion that virions could be irreversibly binding to the quaternary amines on the anion exchange resin.

By loading the virus on a strong cation exchange resin, there was no strong flow rate dependency on infectious virus recovery in the eluted fractions indicating mass transfer played little to no role in the capture step to this resin. Furthermore, these elution fractions contained significantly lowered amounts of contaminating protein and DNA with respect to input virus stocks. Weak cation and anion exchangers may be used to further explore relative binding efficiency and recovery of virus preparations at different elution and loading conditions. The details of enhanced binding to anion exhange resins may need to be explored further to elucidate the mechanism for reduced yields of infectious particles. Thus, a more mechanistic description of virus charge-charge interactions would greatly aid the development of optimized purification protocols. These results reflect progress towards optimal purification of the virus for large scale gene therapy applications while maintaining adherence to strict FDA requirements with respect to contaminating host cellular DNA and protein.

6.0 CONCLUSIONS

HSV-1 remains an attractive vehicle for treatment of various neuropathies as well as a oncolytic vector. Replication defective HSV-1 based gene therapy vectors are among the safest since they are missing most of the toxic IE genes necessary for viral replication. Therefore, efficient manufacturing methods to produce these vectors are paramount for realizing their therapeutic potential. Moreover, a basic understanding of host cell interactions with regard to genomic repression in different cellular environments will allow construction of more efficient vectors tailored to specific disease states. Here, we have shown that the engineering of complementing cell lines can be optimized to provide high titer vector and purified to match FDA requirements by ion exchange chromatography. We also demonstrate that the host cell interactions can be exploited to improve transgene expression from highly replication defective vectors in a repressive host cell environment. The outcomes of this study are as follows.

(1) Improving the complementation profile of the essential immediate early gene can yield dramatic results with respect to viral yield. The ICP0 promoter chosen for its inducibility in the context of infection was able to deliver ICP4 gene expression with faster kinetics leading to order of magnitude improvments in production of the highly replication defective vector JDTOZHE. Likewise, this cell line was further optimized through the addition of the ICP0 gene and shown to grow the quadruple mutant JDQOZEH1 up to 1E6 PFU/ml.

(74) A high throughput cell based chemical screen revealed a novel pathway that may lead to enhanced transgene expression in repressive cellular environments. By inducing a more permissive cell state with the molecule 1 10 phenanthroline, enhanced EGFP from the viral backbone was readily observed. The presence of a novel gene, SAG, was observed in the presence of this drug which implicates this function in allowing expression from the viral backbone. Moreover, this gene was expressed consitutively in the permissive U2OS cell environment. This is the first observation of this gene in this cell line which has implications in the field of oncology as well as virology.

(3) Ion exchange purification of HSV-1 was possible by cation exchange. This strategy lead to pure stocks of virus with minimal contaminating protein and DNA. By choosing the correct purification parameters such as pH, flow rate, and elution salt concentration high titers of this virus could be recovered. This purification method is robust and amenable to scaleup which will have dramatic impacts as the pressure on manufacturing methods are increased to meet clinical demand.

In conclusion, the cell lines developed in chapter 3 will serve to aid in the growth an propagation of even more deleted vector backbones that have safer toxicity profiles. These cell lines coupled with the use of ion exchange chromatography represent an efficient manufacturing platform for clinical grade replication defective HSV-1 based vectors. The depression of HSV-1 remains a mystery to the field of virology and the studies described in chapter 4 will aid in the field in that these interactions shed new light on the virus-host cell interactions in Vero cells as well as the enigmatic U2OS cellular environment.

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