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Characterization of Unexpected ICP27 Recombinants and Revertants of Herpes Simplex Virus

by

Scott M. Bunnell 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

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What a piece of work is a man! How noble in reason! How infinite in faculty! In form and moving how express and admirable! In action how like an angel! In apprehension how like a god! The beauty of the world! The paragon of animals!

Hamlet (act 2, scene 2) from
"Hamlet" by William Shakespeare

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Characterization of Unexpected ICP27 Recombinants and Revertants of Herpes Simplex Virus* submitted by Scott M. Bunnell in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Stephen Rice

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Dr. David Stuart

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Abstract

In cells infected with herpes simplex virus type 1 (HSV-1), one of the first proteins to be produced is infected cell polypeptide (ICP) 27. This 512-amino acid protein, which localizes predominantly to the infected cell nucleus, is absolutely essential for viral replication in cell culture. ICP27 plays an important role in the *trans*-modulation of the expression of many HSV-1 and cellular genes, and appears to effect its gene-regulatory activity in a post-transcriptional manner by affecting the processes of polyadenylation and splicing. The protein binds to RNA, and is believed to mediate the transport of viral RNAs from the nucleus to the cytoplasm of the infected cell.

The process of propagating ICP27-mutant viruses in the helper-cell line V27, which provides ICP27 in *trans*, allows for recombination between cellular and viral genomes, and the possibility of the generation of replication-competent (RC) virus within the high-titre stock. Analysis of existing high-titre stocks of growth-defective ICP27 mutants revealed that all contain RC virus, but that it is present at low levels, at a frequency approximating 10^{-7}. RC virus present within the high-titre stock of the ICP27-deletion mutant *d*27-1 was found to possess both its original deletion allele, as well as the wild-type allele of ICP27. The generation of RC virus appears to depend upon the presence of DNA-sequence homology between genomes.

The existing literature on ICP27 suggests that the carboxy (C)-terminal half of ICP27 is functionally important. A revertant of the C-terminal ICP27 mutant M16 was isolated. The revertant, termed M16R, contains an

intragenic mutation as well as one or more extragenic mutations. The intragenic mutation, a base addition at codons 215–217, frameshifts the sequence such that a new stop codon is read at position 290. The extragenic mutation(s) are unknown.

The truncated ICP27 of 289-amino acids produced by M16R is able to complement the growth of *d*27-1, but is unable, in the absence of the extragenic mutation(s), to support viral replication in a non-helper–cell line. The novel stretch of 72-amino acids, which comprises the C-terminus of the truncated protein and is arginine and glycine rich, contributes significantly to its function.

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List of Abbreviations

BSA	Bovine serum albumin
C	Carboxy
CNS	Central nervous system
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocyte
DE	Delayed-early
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
dpi	Days post-infection
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EHV-1	Equine herpesvirus type 1
EHV-2	Equine herpesvirus type 2
ER	Endoplasmic reticulum
FBS	Fetal-bovine serum
$\gamma 1$	Leaky-late
$\gamma 2$	True-late
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
HANR	Homology-associated nonhomologous recombination
HCF	Host cell factor
HCMV	Cytomegalovirus
HHV-6	Herpesvirus 6
HHV-7	Herpesvirus 7
HHV-8	Herpesvirus 8
HIV	Human immunodeficiency virus
hnRNP K	Heterogeneous nuclear ribonucleoprotein K
hpi	Hours post-infection
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
Hve	Herpesvirus entry mediator
HVS	Herpesvirus saimiri
ICP	Infected cell polypeptide
IE	Immediate-early
IFN-α	Alpha interferon
IFN-β	Beta interferon
IRES	Internal ribosome entry site
kb	Kilobases

kbp	Kilobase pairs
kDa	Kilodalton
KH	K homology
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late
LAT	Latency associated transcript
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
N	Amino
NES	Nuclear export signal
NGF	Nerve growth factor
NLS	Nuclear localization signal
NuLS	Nucleolar localization signal
Oct-1	Octamer transcription factor 1
PEL	Primary effusion lymphoma
PFU	Plaque forming units
PKR	Double-stranded RNA-dependent protein kinase
PRV	Pseudorabies virus
RC	Replication-competent
RNA pol II	RNA polymerase II
SDS	Sodium dodecyl sulfate
snRNP	Small nuclear ribonucleoprotein particle
TAP	Transporter associated with antigen processing
Taq	*Thermus aquaticus*
tk	Thymidine kinase
TNF	Tumour necrosis factor
ts	Temperature-sensitive
UTR	Untranslated region
UV	Ultra-violet
vhs	virion-induced host shutoff
VP16	Virion protein 16
VZV	Varicella-zoster virus

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Introduction

The *Herpesviridae* Family

Herpesviruses are eukaryotic viruses (the word herpes is derived from the Greek verb *herpein*, to creep). Over 100 herpesviruses have been isolated to date, from a wide variety of vertebrate species. Species-association is usually highly specific, although inter-species transmission of herpesviruses can occur and can cause severe disease (e.g. infection of humans with the non-human primate virus cercopithecine herpesvirus 1 (289), also known as B-virus). Following primary infection, herpesviruses can establish a latent infection within the natural host; that is, they are able to persist in the host for life in the absence of detectable infectious virus.

A herpesvirus virion is characterized by the presence of a single, double-stranded, linear DNA genome of 120–240 kilobase pairs (kbp), contained within an icosahedral nucleocapsid. The nucleocapsid is embedded in a protein layer—known as the tegument—which is surrounded by a protein-containing lipid membrane (Figure 1-1A). DNA replication, transcription, and capsid assembly occur within the nucleus of the host cell.

The Herpesvirus Virion

The virion is the infectious particle produced during the lytic cycle. Herpesvirions range in diameter from 102–200 nm, and are unstable in

detergents and other lipid solvents (330, 333). This instability results from the presence of, and requirement for, a lipid envelope. The lipid envelope is acquired from the cell during virion maturation. Viral glycoproteins, embedded in the envelope, appear as spikes on the virion surface under electron micrography (338); the glycoproteins mediate attachment of the herpesvirion to the target cell. Penetration into the target cell is effected through viral glycoprotein-induced fusion of the envelope with the plasma membrane of the target cell (226).

Sandwiched between the lipid envelope and the nucleocapsid is a structural layer unique to herpesvirions: the tegument. This often asymmetric layer is poorly characterized with respect to function, but contains important viral regulatory proteins.

Nucleocapsids of herpesviruses are icosahedral in shape, and are composed of 162 capsomers; 150 of the 162 capsomers are hexameric, while the remaining 12 are pentameric. Nucleocapsids are typically 100 nm in diameter.

Contained within the nucleocapsid of the herpesvirion is the genomic core. The viral DNA is associated with protein, and appears as a toroid under electron micrography (96, 236). Little is known of the precise arrangement of the viral DNA in the toroid; the genomic termini are not linked covalently to protein, as they are in adenoviruses (274, 284), nor do the ends of the genome form terminal hairpin loops, as is the case in poxviruses (8). The G+C base composition of herpesvirus DNAs ranges from 32–75%.

Herpesvirus Taxonomy

The current classification scheme applied to the *Herpesviridae* family has evolved from one based solely on biological properties (e.g. host-cell range, efficiency of multiplication), to one focusing more on molecular criteria, such as sequence data and genome structure. Three subfamilies exist: *Alpha-*, *Beta-*, and *Gammaherpesvirinae*.

Alphaherpesvirinae

Members of this subfamily (also known as α-herpesviruses) exhibit a wide and variable experimental host range, a relatively short reproductive cycle (allowing for relatively rapid growth in cell culture), a high degree of efficiency with respect to host cell destruction, and the ability to establish latent infections in sensory ganglia. Examples of α-herpesviruses are herpes simplex virus type 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), equine herpesvirus type 1 (EHV-1), pseudorabies virus (PRV), and B-virus.

Betaherpesvirinae

Members of this subfamily (also known as β-herpesviruses) are classified on the basis of a narrow experimental host range, relatively long reproductive cycle (infection progresses slowly in cell culture), production of an enlarged infected cell morphology (cytomegalia), and the ability to establish latent infections in secretory glands, kidneys, and lymphoreticular cells. Examples of β-herpesviruses are human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV), and human herpesviruses 6 and 7 (HHV-6 and HHV-7).

Gammaherpesvirinae

Members of this subfamily (also known as γ-herpesviruses) are lymphotropic, with specificity for either T- or B-lymphocytes. *In vitro*, lytic infections can be effected in some types of fibroblastic and epithelioid cells, in addition to lymphoblastoid cells. Latency is often established in lymphoid tissue; lytic-stage infections rarely produce infectious progeny. Examples of γ-herpesviruses are Epstein-Barr virus (EBV), human herpesvirus 8 (HHV-8; commonly referred to as Kaposi's sarcoma-associated herpesvirus, KSHV), equine herpesvirus type 2 (EHV-2), and herpesvirus saimiri (HVS).

The Human Herpesviruses

To date, eight herpesviruses have been associated with humans as their natural host: the α-herpesviruses HSV-1, HSV-2, and VZV; the β-herpesviruses HCMV, HHV-6, and HHV-7; and the γ-herpesviruses EBV and HHV-8.

HSV-1 and HSV-2

HSV-1 and HSV-2, while antigenically distinct, display a high degree of similarity at the coding-sequence level (approximately 70–80% identity) (203). Primary HSV infection normally occurs at a mucocutaneous surface—usually oral-facial for HSV-1 and genital for HSV-2—where the virus undergoes a period of productive replication (49). Viral replication within infected cells, accompanied by the host immune response, often leads to tissue damage that is manifest as painful vesicular lesions. Not all virus is contained at the epithelial tissue, however, as adjacent sensory nerve endings exposed to progeny are sensitive to infection; nucleocapsids can travel, through retrograde axonal transport, to neuronal cell bodies in

sensory ganglia. Acute ganglionic infection can be destructive to the infected cells, but usually the viral genome enters a state of latency, hallmarked by the absence of detectable genome replication and infectious progeny production. The establishment of latency allows for the sequestration and persistence of viral DNA within the infected neurons for the life-span of the host.

Following primary HSV infection, an infected individual may never again experience recurrent HSV disease. Most infected individuals, though, do experience recurrent infections, although these are usually less severe than the primary infection in the immuno-competent individual. The factors contributing to the reactivation of virus replication are poorly understood, but reactivation has been associated with a variety of stimuli, such as physical damage to mucocutaneous tissue, exposure to ultra-violet (UV) light (e.g. a sunburn), exposure to an unrelated disease-agent, emotional stress, and hormonal changes. During the reactivation process, viral genomes are replicated in the sensory ganglia and progeny are transported back through nerve cell axons to epithelial sites at or near the site of the primary infection. Recurrent viral infections of epithelial tissue are manifest as vesicular lesions (commonly referred to as cold sores for oral-facial infections). Unfortunately, infectious virus may be transmitted from the lesions during, or before, the vesicular phase of disease via direct contact with a mucocutaneous surface of another individual; transmission of virus is very efficient, and often occurs before the infectious individual even realizes that a recurrent infection is in progress.

Rarely are HSV infections debilitating in the immuno-competent host. Primary HSV infection of the eye can lead to blindness (50), and on very rare occasions, a primary HSV infection can result in encephalitis (50, 159). HSV infections can have serious consequences in an immuno-compromised individual, as well as in newborns (337, 377).

VZV

VZV is the etiological agent responsible for two distinct diseases: varicella (chickenpox) and zoster (shingles) (4). Primary VZV infection usually occurs during childhood, as the virus is transmissible in the aerosolized respiratory secretions of infected individuals displaying the varicella-disease state. Infection with VZV initially results in varicella, a disease characterized by widespread mucosal and cutaneous lesions. As is the case for HSV, VZV can establish a latent infection in neural ganglia; recurrent infections are manifest as zoster. The zoster-disease state is different from varicella, and is presented as a painful skin rash. The severity of disease experienced by

individuals exposed to primary VZV infection increases with the age of exposure. In immuno-compromised individuals, VZV infections can be fatal.

HCMV

HCMV is a ubiquitous human pathogen. Most primary infections with the virus are inapparent, and infected individuals usually remain asymptomatic (256, 275). When symptomatic, primary infections in children and adults can result in a wide variety of clinical manifestations, such as myelitis, carditis, and hepatitis (160). HCMV is the most common cause of viral birth defects in congenitally infected babies; both deafness and mental retardation can result from congenital HCMV infections (335). In immuno-compromised individuals, HCMV can cause severe disseminated disease.

Viral transmission is effected through milk, semen, urine, saliva, and cervical secretions, and the virus can be acquired sexually, transplacentally, perinatally, as well as via blood transfusion and organ or bone marrow transplantation (46, 176, 272, 336). A number of cell types can be infected by the virus, including leukocytes, endothelial cells, epithelial cells, and connective tissue cells (135, 233, 272).

HHV-6

HHV-6 is a T-cell–tropic virus that was originally isolated from AIDS patients with lymphoproliferative disorders (154, 293). HHV-6 isolates are differentiated into two groups—variants A and B—on the basis of antigenic specificity, genetic polymorphism, and *in vitro* growth (113). The virus is believed to be widespread in the population, but, like HCMV, is not thought to cause disease in the healthy individual. HHV-6 infection of infants can cause exanthem subitum (a generally non-serious skin rash) (386). In adults, HHV-6 infection has been linked with meningoencephalitis (145), infectious mononucleosis (13), autoimmune disorders (166), chronic fatigue syndrome (26), and multiple sclerosis (38). HHV-6 has been proposed to be a cofactor in AIDS progression (190), and may play a role in the reactivation of latent human immunodeficiency virus (HIV) (77).

HHV-7

Another T-cell–tropic virus is HHV-7, a close viral relative of HHV-6 (238). Primary infection with HHV-7 normally occurs in infancy (seroprevalence reaches at least 80%) (383), and is associated with exanthem subitum (349). In rare instances, HHV-7 infection can cause encephalitis and seizures due to invasion of the central nervous system (CNS), but usually the virus is not pathogenic beyond the self-limiting childhood disease.

EBV

EBV is a near-ubiquitous human herpesvirus (114), first discovered because of its association with Burkitt's lymphoma (157), a B-cell lymphoma common in sub-Saharan Africa. EBV strains are classified as either type A or type B based upon DNA sequence and *in vitro* growth (114, 298). Primary EBV infection initially occurs in oropharyngeal epithelial cells (virus is acquired from infected saliva), and after an initial round of productive replication, proceeds to B-lymphocytes (116). Infection of B-lymphocytes transforms them, and the resulting proliferation can result in a state of infectious mononucleosis (usually only observed in individuals infected later than early childhood). Although EBV infections are generally asymptomatic, the virus has been shown to contribute to the genesis of several malignant tumours of hematopoietic or epithelial origin, including Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease (157, 193). EBV is sufficient for the formation of B- and T-cell lymphomas in immuno-compromised individuals.

HHV-8

HHV-8 is the most recent human herpesvirus identified; it is commonly referred to as KSHV as it was discovered during an investigation into the causative agent of Kaposi's sarcoma (KS) (40, 361). In addition to its association with KS, HHV-8 has been linked to two types of B-cell tumours: primary effusion lymphoma (PEL) and multicentric Castelman's disease (67). The virus appears to be sexually transmitted.

HSV-1

Because HSV-1 can be propagated to high titres in tissue-culture systems, it serves as a prototype for understanding how the herpesviruses replicate and effect disease within their hosts.

The genome

The HSV-1 genome is 152 kbp in size, and consists of two covalently linked components, L and S (Figure 1-1B) (287). Both components are similar in arrangement, in that they are composed of a unique sequence (U_L and U_S) flanked by a pair of oppositely oriented repeat elements (R_L and R_S). The U_L section is much larger than the U_S section (108 kbp versus 13 kbp,

respectively). Present at the termini of the genome are 3' single-base extensions (225).

A short sequence, known as *a*, is present at the junction of the L and S components, and is also present at the termini of the genome; one to several copies of the *a* sequence are present at the L–S junction and at the end of the L component, while only a single copy is present at the end of the S component (55, 185, 365). The L–S junction *a* sequence is inversely oriented with respect to the orientations of the *a* sequences of the termini (224). The *a* sequence is an essential *cis*-acting element that is involved in a number of important events, including the circularization of viral DNA post-infection, the cleavage and packaging of replicated viral DNA, and the isomerization of the viral genome (57, 69, 235, 303, 320, 323, 360).

Preparations of HSV-1 DNA contain equimolar amounts of four sequence-orientation isomers, illustrated in Figure 1-1C (129). The presence of inverted repeat elements (i.e. the *a*, R_L, and R_S sequences) allows for recombination during replication, leading to L–S inversion (isomerization). An infectious virion contains a single genome in one of the four possible orientations, but post-replication, the infected cell will contain equimolar amounts of the four isomers as a result of the isomerization process. All isomeric forms are functionally equivalent (149).

The lytic cycle

Entry into the target cell is effected through envelope glycoproteins present on the surface of the virion (329). First, membrane glycoproteins C (gC) and B (gB) bind to the heparan-sulfate moieties of cell-surface proteoglycans (123). The presence of gC and heparan-sulfate is important for virion adsorption, as loss of either results in a significant reduction in viral attachment (117, 131, 317, 382). Next, another membrane glycoprotein, glycoprotein D (gD), binds tightly to a herpesvirus entry mediator (Hve) present on the surface of the target cell; three Hves have been identified (A, B, and C) (167, 304, 369, 376, 380). HveA is a member of the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily, whereas HveB (also known as poliovirus receptor-related protein 2) and HveC (also known as poliovirus receptor-related protein 1) are members of the immunoglobulin superfamily. The binding of gD to a Hve leads to viral and cellular membrane fusion, effected through additional envelope glycoproteins (329), and delivery of the tegument and nucleocapsid into the cytoplasm of the cell.

Once in the cytoplasm, the tegument disassembles and the nucleocapsid binds to microtubules via the microtubule-motor molecule dynein; it is believed that the nucleocapsid is transported along the microtubule network

to the cell's nucleus (226, 324). Disassembly of the tegument releases several important viral proteins, including virion protein 16 (VP16) and the virion-induced host shutoff (vhs) protein. VP16 is a powerful *trans*-activator of the immediate-early (IE or α) genes of HSV-1 (10, 35), while vhs effects shutoff of host macromolecular synthesis through the degradation of host mRNA (162, 169, 242, 318).

Viral DNA leaves the nucleocapsid and enters the cell's nucleus at a nuclear pore (9). Inside the nucleus, the termini of the viral genome fuse, yielding a circular DNA molecule (152). The genome is now primed for procession through the viral regulatory cascade.

The viral regulatory cascade is defined by the sequential activation of three distinct temporal classes of viral genes: IE, delayed-early (DE or β), and late (L or γ) (136-138). The IE genes are the first to be expressed, and for the most part function as *trans*-regulators of DE and L genes. The rates of synthesis of IE polypeptides are highest 3–4 hours post-infection (hpi). Transcription of the IE genes is stimulated by VP16. Each IE gene contains within its promoter one or more copies of the *cis*-acting element **TAATGARAT** (where **R** is a purine residue) (103, 191). VP16 has been shown to bind directly to the cellular proteins Octamer transcription factor 1 (Oct-1) (340) and Host cell factor (HCF) (105), and it is the complex of these three proteins that binds to the **TAATGARAT** motifs and activates transcription. Transcription of all viral genes is carried out by the host RNA polymerase II (RNA pol II) (11).

Production of the IE proteins results in the expression of the DE genes. The rates of synthesis of DE polypeptides are highest 5–7 hpi. The protein products of the DE genes are generally responsible, both directly and indirectly, for effecting replication of the viral genome. Unlike the dependence on the host-cell RNA pol II for transcription, the virus supplies its own DNA polymerase (374).

Induction of viral DNA replication occurs when sufficient quantities of DE proteins have been synthesized. Three origins of replication exist within the genome; the U_L section contains one, while the R_S elements each contain another (342, 375). Interaction of the replication machinery with any of the origins of replication results in the initiation of bi-directional DNA replication. It is believed that this theta type-like replication is the mechanism initially responsible for viral DNA replication (37, 312, 395). At later stages, however, viral DNA replication is thought to proceed via a rolling-circle mechanism, as large concatemeric DNA molecules are generated (147, 152).

Viral DNA synthesis stimulates expression of the L genes. L polypeptides are made at increasing rates until at least 12 hpi. Most of the L genes encode structural proteins, as well as proteins involved in the assembly and egress

of progeny virions. The L genes are separated into two sub-classes based upon the requirement of DNA replication for expression. Leaky-late (γ1) gene expression occurs in the absence of viral DNA replication, but is enhanced by DNA replication. True-late (γ2) gene expression is strictly dependent on the onset of viral DNA replication. The VP16 and vhs proteins are γ1 proteins.

Accumulation of L proteins leads to the self-assembly of viral capsids in the nucleus of the host cell (237). The concatemeric DNAs generated by rolling-circle replication are then cleaved prior to, or during, packaging into the capsids to yield mature linear genomes (360); cleavage of the viral DNA and its encapsidation are believed to be tightly coupled events effected by the viral cleavage-packaging system. The signals that direct site-specific cleavage into unit-length genomes are *cis*-acting sequences—*pac1* and *pac2* sequences—present within the *a* sequence (57, 358). It has been proposed that viral DNA is spooled into a capsid until a second *a* sequence matching the orientation of the first is encountered, triggering cleavage (57). Site-specific cleavage generates the 3′ single-base extensions found at the termini of the genome.

Assembly of a HSV-1 virion is not complete until the tegument and lipid envelope have been acquired. Nucleocapsids bud through the inner nuclear membrane, and in the process are enveloped (54, 309). Two models have been proposed to account for the fate of the enveloped nucleocapsids; the models are not mutually exclusive, and it is possible that both are being effected in the host cell. In one model, the tegument and lipid envelope are acquired as a result of passage through the inner nuclear membrane (34, 150, 352, 368). The virions then pass through the endoplasmic reticulum (ER)/Golgi apparatus secretory pathway, and are transported to the cellular membrane in membrane-bound vesicles. In the alternate model, the enveloped nucleocapsids in the perinuclear space de-envelope via fusion with the outer nuclear membrane, and are thus delivered into the cytoplasm as naked nucleocapsids (24, 43, 74, 75, 334, 357). The tegument and lipid envelope are not acquired until the nucleocapsids bud into the ER and/or Golgi apparatus. As with the first model, the assembled virions are transported to the cellular membrane.

Latency

Infection of sensory neurons with HSV-1 establishes a state of latency in which none of the viral genes of the lytic cycle are expressed (92, 341). The VP16 protein may play a key role in the establishment of latency. VP16 is able to effect the *trans*-activation of IE gene expression in non-neuronal cells because of its interaction with HCF; VP16 relies on HCF for import into the nucleus of the non-neuronal cell (170). In sensory neurons, HCF is non-

nuclear in its localization (165). VP16 may therefore be unable to enter the neuronal cell nucleus because of this HCF localization profile—the lytic cycle is abrogated and latency results.

In latently infected neurons, the viral genome is maintained as an episome (212, 285). As the lytic-cycle viral genes are not expressed, the viral genome is not actively replicated, and exists essentially in a quiescent state. However, three latency associated transcripts (LATs) can be detected in latently infected cells. One is a minor LAT, or mLAT, of approximately 8.3 kilobases (kb), transcribed from a region of the R_L (221). The other two LATs, approximately 2.0 and 1.5 kb in size, map to the 5′ region of the mLAT and are thought to be the stable splice-products of the mLAT (89, 332, 363, 364, 372). These RNAs are unusual in that they are not capped or polyadenylated, remain in the nucleus, and do not produce any known protein products (63, 364). The role of the LATs in latency remains largely undetermined, but evidence exists suggesting a contribution to reactivation (16, 18, 66, 134, 249, 250).

The IE Genes

The IE genes of HSV-1 are the first to be expressed during the lytic cycle, and are operationally defined as those transcribed in the absence of prior viral protein synthesis (370). Five IE genes exist: infected cell polypeptide (ICP) 47, ICP22, ICP0, ICP4, and ICP27. The locations and orientations of the IE mRNAs for one of the four possible HSV-1 isomers are presented in Figure 1-1B. Alternative IE gene monikers are presented in Table 1-1.

ICP47

ICP47 is a 12-kilodalton (kDa) protein that localizes to the cytoplasm of the infected cell (195, 261). It is not essential for HSV-1 replication in cell culture (188), but almost certainly contributes to the pathogenicity of the virus during infection of a human host. In cells that express major histocompatibility complex (MHC) class I molecules (e.g. epithelial cells), ICP47 has been shown to bind to the transporter associated with antigen processing (TAP), preventing viral peptide translocation into the ER (95, 133). Because of this, loading of viral peptides onto MHC class I molecules does not occur, and the empty MHC molecules remain in the ER (393). Antigen presentation by MHC class I molecules on the infected cell surface is required for recognition by $CD8^+$ T cells; by inhibiting antigen presentation, ICP47 effects a shielding of HSV-1–infected cells from HSV-specific $CD8^+$ cytotoxic T lymphocytes (CTLs).

ICP22

ICP22 is a 68-kDa protein that localizes predominantly to the nucleus of the infected cell (181, 260). It has been shown to exist in several phosphorylated states, and can be nucleotidylylated by casein kinase II (219, 220, 265, 266). Viral dependence on the protein for viability varies amongst infected cell types—ICP22 is essential for productive HSV-1 replication in confluent resting HEL cells, as well as RAT-1 and BHK cell lines, but is dispensable for virus growth in Vero and HEp-2 cells (257, 310). In infected cells, ICP22 plays a role in the post-translational modification of the large subunit of RNA pol II. The presence of ICP22 induces the formation of a novel phosphorylated form of the subunit, which is hypothesized to aid in the transcription of the viral genome (187, 280, 281, 331).

ICP0

ICP0 is a 110-kDa phosphoprotein that localizes to the nucleus of the infected cell (201). While the protein is not absolutely essential for viral growth in cell culture, ICP0 mutants are impaired for IE, DE, and L gene expression at low multiplicity of infection (MOI) infections; the absence of ICP0, however, can be overcome by carrying out infections at high MOI (79, 291, 343). ICP0 most likely effects viral gene *trans*-activation at the transcriptional level (31, 153, 184, 297). In transient co-transfection assays, ICP0 can stimulate gene expression from a variety of promoters (33, 81, 82, 102, 104, 184, 240, 241, 270).

The actions of ICP0 are not confined to the viral genome. ICP0 has been shown to localize to discrete nuclear domains, known as ND10, causing disruption and dispersion of their constituent proteins (78, 85, 200, 201). In addition, ICP0 has been shown to directly interact with a number of cellular proteins and to induce the degradation of key cellular factors (42, 83, 84, 87, 155, 156, 177, 213, 214, 232, 244). It is possible that this alteration of the cellular environment facilitates entry of HSV-1 into the lytic cycle (28, 84, 86). Recently, it was demonstrated that ICP0 is required to overcome the antiviral effects of the immune-system molecule alpha interferon (IFN-α) (228).

A number of lines of evidence suggest that ICP0 and VP16 are functionally related with respect to their roles in the lytic cycle (1, 30, 33, 53, 184, 229, 291, 319, 343, 390). ICP0 also appears to play an important role in the establishment and maintenance of latency; ICP0 mutants establish latency much less efficiently than wild-type virus and exhibit reduced reactivation frequencies (29, 47, 121, 128, 178, 288, 378, 399). It is

possible that ICP0 substitutes functionally for VP16 in the *trans*-activation of IE genes during the reactivation process.

ICP4

ICP4 is a 175-kDa protein that localizes primarily to the nucleus of the infected cell (51, 248). Several cellular kinase-consensus sites, as well as an autophosphorylation site, have been identified in the protein (384, 385)—these most likely contribute to the observed existence of the multiple electrophoretic species of the protein (2, 248). ICP4 can homodimerize (215, 314). It is absolutely required for productive infection, as virus mutants lacking functional ICP4 are not viable (56, 58, 59, 64, 262, 264, 344).

ICP4 functions as the major transcriptional activator of DE and L genes (60, 82, 104, 110, 240, 270), but can also act as a repressor of its own expression (61, 241, 245). The protein can be dissected into discrete functional domains that are responsible for nuclear localization, dimerization, DNA binding, and transcriptional activation (61, 88, 245, 316). Although the DNA-binding activity of ICP4 appears to be required for both *trans*-activation and *trans*-repression (88, 119, 283, 316), *trans*-activation is not associated with specific binding sites (48, 73, 80, 122, 322). *Trans*-activation, however, is dependent upon the presence of the carboxy (C)-terminal region of ICP4, which has been shown to support the interaction of the protein with the general transcription factor TFIID (36, 59, 60, 118, 314).

ICP27

ICP27 is a 63-kDa protein (137, 290), composed of 512-amino acids (204). The theoretical molecular weight of the protein is 56-kDa, and it is believed that this difference between observed and theoretical molecular weights is due to a highly acidic region located near the amino (N)-terminus of the protein (279), as well as one or more post-translational modifications. Modification by phosphorylation has been demonstrated (379) involving various serine residues present within ICP27 (397). Nucleotidylation (14, 15) and methylation (211) have also been observed. Although ICP27 can act as a substrate for phosphorylation by a number of different kinases (397), and can be nucleotidylated and methylated, it is unclear what contribution these modifications make to protein function.

Like most of the IE proteins, ICP27 localizes predominantly to infected cell nuclei (2). The staining pattern within the nucleus can be described as speckled, or punctate, in appearance (282), and appears to be the result of colocalization of ICP27 with small nuclear ribonucleoprotein particle (snRNP) clusters (253, 301); snRNPs constitute the major subunits of the spliceosome (313).

ICP27 is absolutely essential for viral replication in cell culture. This conclusion was first drawn from studies of temperature-sensitive (*ts*) mutants of HSV-1 whose lesions were found to map to the ICP27 gene (290). The construction and characterization of ICP27-deletion mutants confirmed the fundamental importance of the protein in the production of infectious progeny (202, 277). Viral mutants defective in ICP27 production are unable to: (i) efficiently express some DE genes, and most L genes (202, 205, 277, 290, 356); (ii) down-regulate IE and DE gene expression at late times post-infection (202, 277, 290); (iii) efficiently replicate the viral genome (202, 277, 356). Therefore, ICP27's roles in the lytic process include the *trans*-modulation of gene expression from all three temporal gene classes, as well as the enhancement of DNA replication.

Studies investigating the region(s) of ICP27 responsible for its gene regulatory and replication-enhancing activities have revealed that its ability to stimulate DNA replication can be genetically separated from its ability to effect expression of the L genes. Analysis of ICP27-*ts* mutants—which are able to replicate their DNA to substantial levels, but are unable to synthesize appreciable levels of L proteins—first provided evidence that DNA-replication stimulation and L gene *trans*-activation are genetically separable functions (290). Analysis of ICP27 mutants that contain C-terminal truncations revealed that the ability of the protein to activate L gene expression is sensitive to even a very small C-terminal deletion, but that this same deletion has no effect on the ability of the protein to enhance DNA replication (277). In addition, a number of C-terminal ICP27-codon-substitution mutants display phenotypes defined by wild-type levels of DNA replication, but defects in L gene expression (278). In contrast, ICP27 mutants bearing a N-terminal deletion (codons 12–63) are deficient in their ability to replicate their viral genomes, but exhibit only modest reductions in L protein production (279). Therefore, the data suggest that the C-terminus of ICP27 encodes an activity crucial for the activation of L gene expression, whereas the N-terminus of the protein contributes to the stimulation of viral DNA replication. Not surprisingly, it has been demonstrated that ICP27 is required for the efficient expression of a subset of the DE genes responsible for effecting DNA replication (205, 356). As such, it appears that ICP27's ability to stimulate DNA replication is indirect in nature, and is a function of its ability to *trans*-activate the DE genes responsible for DNA replication.

The repression of host-cell protein synthesis observed in cells lytically infected with HSV-1 is a bi-phasic process, initially effected by the vhs protein, and later requiring one or more viral proteins produced as a result of viral gene expression (90, 169, 239, 273, 345). ICP27 contributes to the shutoff of host-cell gene expression, as cells infected with ICP27-mutant

viruses—in contrast to cells infected with wild-type HSV-1—exhibit elevated levels of both cellular mRNA (124, 325) and protein (290, 325).

Recently, ICP27 has been associated with another function during lytic infection. Infection of a number of human cell lines with an ICP27-deletion virus resulted in cellular death hallmarked by the signs of apoptosis (6), and not the normally observed cytopathic effect (CPE) (286). Apoptotic death was not observed in human and non-human cells infected with wild-type HSV-1, nor in non-human cells infected with the ICP27-deletion virus (6). In infected human cells, therefore, ICP27 appears to be required, either directly or indirectly, for the prevention of apoptosis.

Insights into ICP27's *modus operandi* were initially formulated from plasmid-based transfection studies, which demonstrated that the protein could effect the activation and repression of reporter genes under the control of viral promoters (125, 208, 276, 278, 279, 282, 311, 347). Attempts at mapping the physical domains of ICP27 responsible for the observed *trans*-activation and *trans*-repression have yielded complex and often contradictory data. Two sets of experiments led to the conclusion that the regions important for repression and activation map to the C-terminal half of ICP27 (125, 208). However, this postulation is challenged by three additional sets of experiments. The first showed that plasmids encoding ICP27 proteins C-terminally truncated retain the ability to *trans*-activate and *trans*-repress (282). The second revealed that an ICP27-encoding plasmid containing a N-terminal deletion is defective in *trans*-repression (but not *trans*-activation) (279). The third demonstrated that plasmids encoding ICP27 proteins with codon substitutions in the C-terminus, while able to *trans*-repress, are unable to *trans*-activate (278). Thus, with respect to the localization of the region(s) of ICP27 responsible for effecting *trans*-activation, most of the data indicate that the C-terminal section of the protein is important; the presence of multiple *trans*-activation domains within the protein cannot be rigidly ruled out, however. With respect to the localization of the region(s) of ICP27 responsible for effecting *trans*-repression, the data support a multiple *trans*-repression–domain model, with both the N- and C-terminal sections of ICP27 containing a domain.

The *trans*-activity displayed by ICP27 was first believed to be transcriptionally based, much like the *trans*-activity displayed by ICP0 and ICP4. However, while some existing evidence suggests a transcriptional role for ICP27 (202, 243, 331), the bulk of the recently accumulated data strongly support a scenario in which ICP27 controls gene expression in the infected cell post-transcriptionally by affecting the processes of polyadenylation (effecting *trans*-activation) and splicing (effecting *trans*-repression).

ICP27's ability to *trans*-activate gene expression appears to be inversely proportional to the basal-level efficiency at which its target pre-mRNAs are 3' processed. Constructs bearing weak (non-consensus) polyadenylation sites can be activated in their expression by ICP27 in a promoter-independent fashion (41, 205, 207, 302). The polyadenylation sites of some L genes have been shown to be inefficiently 3' processed at the basal level, and constructs containing them are responsive to ICP27, resulting in an increase in 3' processing (205). In contrast, the polyadenylation sites of some IE and DE genes have been shown to be (more) efficiently 3' processed at the basal level, and constructs containing them are unresponsive to ICP27. It is possible that ICP27 modulates L gene expression through its ability to stimulate 3' processing. Stimulation of 3' processing coincides with an increase in the binding to RNA of proteins responsible for effecting 3' processing, and ICP27 is required for this observed increase in binding (205).

A splicing-inhibition function was initially ascribed to ICP27 based upon analysis of transfection assays. Target genes containing an intron were repressed in their expression in the presence of ICP27, irrespective of the nature of their polyadenylation region (302). Further investigation into the relationship between ICP27 and the splicing process revealed that ICP27 could: (i) facilitate the redistribution of snRNPs in infected cells (253); (ii) contribute (post-transcriptionally) to a decrease in the accumulated levels of three spliced cellular mRNAs (124); (iii) contribute to the nuclear accumulation of viral pre-mRNA transcribed from the intron-containing genes ICP0 and UL15 (a L gene) (126); (iv) effect the nuclear reassortment of the non-snRNP splicing factor SC35, an essential member of the SR-splicing factor family (115, 301); (v) coimmunoprecipitate with anti-Sm antisera-reactive splicing factors and alter the phosphorylation state of at least two of these splicing factors (anti-Sm antisera precipitates nucleoplasmic snRNPs) (252, 300); (vi) promote the nuclear retention of intron-containing transcripts from the ICP0 and UL15 genes at nuclear locales that colocalize with snRNP clusters (255). ICP27, therefore, appears to directly interact with the splicing machinery of the infected cell, causing an indiscriminate inhibition of pre-mRNA splicing. This indiscriminate inhibition of splicing may be analogous to vhs's indiscriminate degradation of both cellular and viral mRNAs (169, 242). Since only five of approximately 80 genes expressed by HSV-1 contain introns (these are the genes responsible for the production of the ICP0-, UL15-, ICP22-, ICP47-, and gC-protein products), a viral protein with a splicing-inhibition function would have little effect on the expression of the majority of viral genes, but would have an enormous effect on the expression of most cellular genes and certainly would play a role in the observed shutoff of cellular gene expression.

Recently, investigations focusing on ICP27's role in the post-transcriptional processing of the α-globin transcript (which contains two introns) induced by HSV-1 infection have provided evidence arguing against ICP27 acting as an indiscriminate inhibitor of pre-mRNA splicing. Rather than reducing the accumulation of the spliced RNA, expression of ICP27 did not detectably alter the levels or rate of accumulation of the fully spliced α-globin RNA in infected cells (44). The observed ability of ICP27 to inhibit splicing, therefore, does not appear to be global in nature.

ICP27 most likely binds to RNA in the infected cell. This conclusion is based upon the finding that purified ICP27 from *Escherichia coli* (*E. coli*) expression systems can bind *in vitro* to various RNA substrates (23, 144), as well as the findings that *in vitro*-translated ICP27 can bind to poly(G) and poly(U) RNA homopolymers (211) and that ICP27 can be UV crosslinked to RNA in the infected cell (299). The region of the protein that appears to mediate RNA binding locates to codons 138–152, and is composed of 15 consecutive arginine and glycine residues (211); runs of arginine and glycine residues are characteristic of RGG boxes, RNA-binding motifs found in a number of cellular nuclear proteins (27, 158, 198). It has also been proposed that the C-terminus of ICP27 contains three K homology (KH)-like RNA-binding motifs (326), but the evidence supporting this claim is not substantial.

Emerging from an ever-expanding collection of work is a model in which ICP27 mediates the transport of viral RNAs from the nucleus to the cytoplasm of the infected cell. The protein contains a strong nuclear localization signal (NLS), which maps to codons 110–137, as well as a nucleolar localization signal (NuLS), which maps to codons 138–152 (the RGG box); one or more weaker NLSs are believed to exist in the C-terminal portion of the protein (209). A leucine-rich nuclear export signal (NES), which maps to codons 5–17 and resembles the leucine-rich NES of the HIV protein Rev (216), has also been identified (299). Disruption of the strong NLS, NuLS, or NES results in viral mutants that are growth defective (209, 279).

The presence of the NLS and the NES allow ICP27 to shuttle between the nucleus and cytoplasm of infected cells (210, 254, 299, 325). The demonstration that ICP27 can bind to RNA, coupled with the observations that shuttling of ICP27 requires the presence of a viral RNA cofactor (325), that it is able to bind and affect the nuclear export of a number of intronless HSV-1 transcripts (but not intron-containing transcripts) (299), and that an export-block of ICP27 results in a reduction of L RNAs in the infected cell cytoplasm (326), has resulted in the proposal that ICP27 promotes the specific transport of viral intronless RNAs. However, this model of intron-

based transport has recently been challenged by data collected from studies involving the processing of α-globin and ICP0 RNAs in infected cells. Central to these studies is the unexpected finding that ICP27 can promote, to different degrees, the cytoplasmic accumulation of both spliced and intron-bearing α-globin and ICP0 RNAs in infected cells (44, 76). This suggests that the ICP27-dependent nuclear export of RNA may not be a function of the presence or absence of an intron, but may instead be based upon specific *cis*-acting transcript sequences; such *cis*-acting sequences have been alluded to as being present in the ICP0 (255), beta interferon (IFN-β), and c-*myc* transcripts (23). In this scenario, ICP27 would effect transport of RNA in a splice-independent manner, similar to the Rev protein of HIV (259, 346).

Few proteins are completely autonomous, and ICP27 is no exception. The protein can be coimmunoprecipitated with anti-Sm antisera, suggesting an interaction with one or more snRNPs (300). Interactions have also been demonstrated with the cellular proteins heterogeneous nuclear ribonucleoprotein K (hnRNP K), casein kinase II (362), and p32 (25). The hnRNP K protein is thought to serve as a pre-mRNA–processing protein in the cell (199), casein kinase II is an ubiquitous eukaryotic kinase (146), and p32 functions as a RNA-splice regulator (251). The only HSV-1 protein for which evidence exists describing a direct physical interaction is ICP4 (243). Recently, the ability of ICP27 to self-associate *in vivo* has been demonstrated (398). A known C-terminal zinc-finger-like region may be key to this interaction (359).

Recombination and Reversion

Recombination and reversion are two important biological events that enlarge the genetic repertoire of an organism, thereby increasing its chances of propagating under the continued pressure of natural selection.

Recombination of HSV-1

HSV-1, like any other organism, relies on recombination both for the generation of genetic diversity, as well as for the maintenance of genetic integrity. Four types of recombination exist: homologous, site-specific, transposition, and illegitimate (132). Homologous recombination, also known as "general" or "legitimate" recombination, occurs between DNA molecules that have an extended stretch of nucleotide homology. Site-specific recombination occurs between DNA molecules that share little or no sequence homology; this type of recombination differs from homologous recombination in that the basis for recognition is protein-DNA or protein-

protein in origin, and not the DNA sequence itself. An example of site-specific recombination is the insertion of HIV-1 cDNA into cellular chromatin (111). Transposition occurs for defined DNA sequences, known as transposable elements, which are recognized by transposon-encoding proteins and moved to random locations within the genome. Illegitimate recombination defines recombination events that do not fit into the first three types of recombination. Because this type of recombination occurs in an environment where little or no sequence homology exists between the recombining molecules, it is often referred to as non-homologous recombination. Of the four types of recombination, only homologous and illegitimate are believed to apply to HSV-1.

The HSV-1 genome is composed of two unique sections, each of which is bracketed by repeat regions (Figure 1-1B). Homologous recombination between repeat regions—both inter- and intra-molecularly—can occur, and has been observed to result in the deletion (321) and inversion (258) of unique sequences, as well as the transfer of a genetic marker from one repeat region to another (306). Homologous recombination is thought to contribute to the generation of the four isomers of HSV-1 (68, 303, 320, 371). The process of homologous recombination is central to the generation of defined-mutation viruses (33, 112, 260). Because homologous recombination involving HSV-1 is associated with viral DNA replication (68, 223), it is likely that many of the DE proteins produced by the virus are involved in the homologous recombination process (19, 20, 70).

Unlike homologous recombination, illegitimate recombination occurs between regions that share very little, if any, sequence homology. Key to the illegitimate recombination process is the ligation of non-homologous DNA termini, generated either through enzymatic error (e.g. DNases gone awry), or by micro-homology associated strand invasion (72). The presence of inverted repeats within the HSV-1 genome can be explained by illegitimate recombination between two oppositely oriented genomes. In addition, like homologous recombination, illegitimate recombination is thought to contribute to the generation of the HSV-1 isomers. Unusual HSV-1 variants containing either enlarged inverted repeat regions (353), shifted inverted repeat regions (127), or a short inverted repeat duplication (355), are believed to be the products of illegitimate recombination events. Class I defective HSV-1 DNAs, small replication-competent units of viral DNA, are defined by the presence of DNA sequences from the S component of the genome—including an origin of replication and an *a* sequence (308). It is assumed that illegitimate recombination between an *a* sequence at the L–S junction and the U_S is responsible for the generation of class I DNAs (222).

Reversion of HSV-1

The genetic integrity of an organism is continuously being compromised by mutations. Reversing the effects of mutations is known as reversion, and organisms that have undergone reversion are known as revertants. Three types of reversion exist: primary, second-site intragenic, and second-site extragenic. A mutant phenotype that has been suppressed by a mutation at the site of the original mutation is said to have undergone a primary reversion (the organism is therefore referred to as a primary revertant). A mutant phenotype that has been suppressed by a mutation elsewhere in the same gene as the original mutation is said to have undergone a second-site intragenic reversion (second-site intragenic revertant). Suppression by a mutation in a completely different gene refers to a second-site extragenic event (second-site extragenic revertant).

Revertant viruses can be useful in the investigation of the functional properties of a protein. Characterization of the ICP4 (315) and VP5 (62) proteins of HSV-1 has been aided by the study of revertants. In the case of the characterization of ICP4, examination of a second-site intragenic revertant of a growth-defective ICP4 mutant revealed that the regulatory activity associated with the protein could be restored without a concomitant restoration of DNA-binding activity; this suggests that that ICP4 may interact with a molecule other than DNA to promote transcriptional activation. In the case of the characterization of VP5, examination of second-site extragenic revertants of a growth-defective UL26/UL26.5 mutant revealed a partial map of VP5 residues that are important for its interaction with the protein products of the UL26/UL26.5 genes.

Often, in order to provide evidence that a particular mutation is responsible for a particular phenotype, "revertant" HSV-1 viruses are created by homologous recombination of a wild-type–allele fragment in place of a mutant fragment (189, 350, 373). This is an incorrect use of the revertant label—said viruses should be properly referred to as rescuants, and not revertants.

HSV-1 and Modern Medicine

HSV-1 is slowly establishing itself as a valuable tool in the era of modern molecular medicine. Two approaches for HSV-1 use have been developed thus far: one based upon the use of mutant viruses, and the other based upon the use of amplicons.

Mutant virus approach

Replication of HSV-1 during the lytic cycle kills cells, and it is this ability that is being harnessed as a novel cancer therapy. Of concern is the establishment of replication selectivity, such that only tumour cells, and not the surrounding normal cells, are affected. A number of strategies have been formulated to address this concern; one of the most successful involves the inactivation of viral genes associated with neurovirulence. Viral mutants containing inactive thymidine kinase (tk) or ICP6 (the large subunit of ribonucleotide reductase) genes cannot replicate in post-mitotic cells (e.g. neurons), but can replicate in actively dividing tumour cells, as the tumour cells provide cellular tk and ICP6 equivalents (21, 197, 217, 268). Additionally, inactivation of the γ34.5 gene produces viral mutants that grow well in tumour cells, but which are extremely retarded for growth in neurons (3, 39, 271). This growth disparity can be partially attributed to the inability of most tumour cells to induce an antiviral response based upon the double-stranded RNA-dependent protein kinase (PKR). A multi-mutated HSV-1 (containing both ICP6 and γ34.5 inactivations) has shown much promise for the treatment of recurrent gliomas (142, 218, 348).

HSV-1 mutants are being developed as vectors for gene therapy (173); the virus is particularly well suited for the treatment of neurological disorders, due to its capacity for latency. HSV-1–derived vectors must be defective for IE gene expression, however, as expression of the IE proteins (with the exception of ICP47) is toxic to the infected cell (151, 263, 295, 381). To effect propagation of the vectors, cell lines providing the IE proteins in *trans* are employed. Unfortunately, initial attempts to achieve long-term expression of transgenes yielded mostly disappointing results, as transgene promoters were unable to maintain transgene expression, and became silenced during latency (17, 71, 171, 186). Problems associated with transgene silencing during latency have recently been successfully addressed through the use of the LAT promoter to drive expression of an internal ribosome entry site (IRES)-linked transgene (174, 196).

Amplicon approach

Amplicons are plasmid-derived vectors that contain the minimum *cis*-acting elements of HSV-1 required for replication (an origin of replication) and packaging (the *pac* sequences) as infectious particles (99, 328). Carrying the desired transgene, amplicons are dependent upon a helper virus to provide, in *trans*, the viral proteins necessary for generation of the infectious particle. Early systems for amplicon generation were plagued by helper virus contamination—resulting in toxicity to the cells targeted for gene therapy—and low amplicon yield (100, 182, 247, 396). Improvement was

introduced through the use of overlapping HSV-1 cosmid clones as helper virus (52); the cosmids lack the *pac* sequences needed for DNA cleavage and packaging, and thus when transfected along with the amplicon vector, allow for the selective packaging of the amplicon plasmid and not the helper virus (91). Most recently, the system has been refined to increase the yield of packaged vector (339). Because amplicons are free of virus-encoded genes, their use as vectors for gene therapy is not complicated by the cytotoxicity and promoter silencing associated with HSV-1 mutants.

Preface to Thesis Research

The work presented in this thesis details investigations into recombination and reversion events involving the ICP27 gene of HSV-1. Chapter 3 examines the presence of replication-competent viruses within growth-defective stocks of HSV-1, and reveals that replication-competent virus can exist at very low levels in high-titre stocks and can be generated in unusual and unexpected circumstances. Chapters 4 and 5 focus on the characterization of a second-site revertant of an ICP27-mutant virus. The results of this characterization lead to a surprising conclusion regarding the C-terminal half of ICP27.

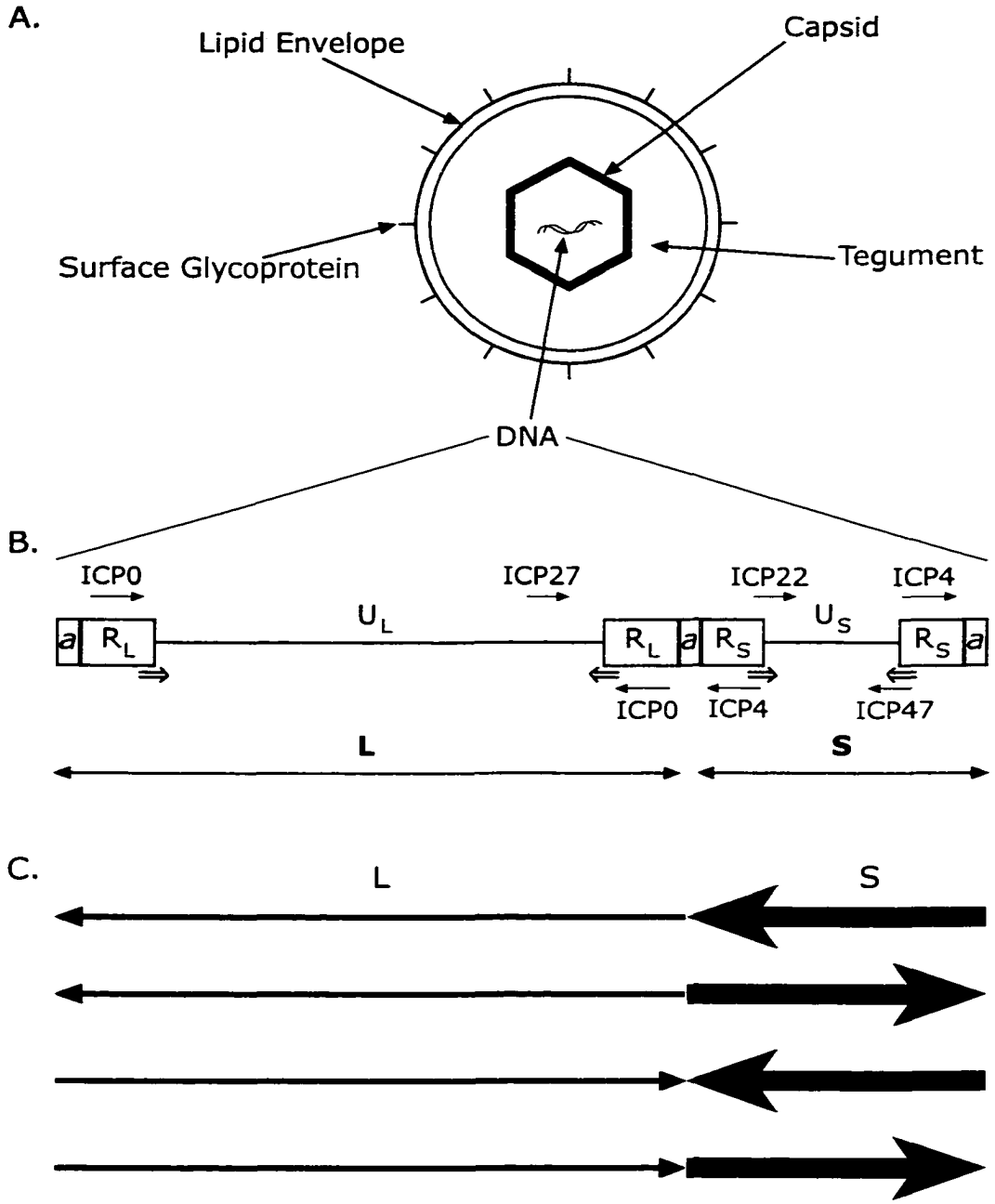

Figure 1-1. HSV-1 virion and genome schematics (A) Herpesvirion architecture; (B) Structure of HSV-1 DNA. The genome consists of two components, L and S, each of which is composed of a unique sequence (U_L and U_S) bracketed by inverted repeat sequences (R_L and R_S). Double-line arrows indicate the orientations of the repeat sequences. One or more *a* sequences are present at the ends of the L and S components. Also shown are the locations and 5′→3′ orientations of the IE mRNAs; (C) The four possible isomers of HSV-1 DNA.

ICP number	Molecular weight of polypeptide	Location of ORF in genome
ICP0	IE110 or Vmw110	RL2
ICP27	IE63 or Vmw63	UL54
ICP4	IE175 or Vmw175	RS1
ICP22	IE68 or Vmw68	US1
ICP47	IE12 or Vmw12	US12

Table 1-1. IE gene nomenclature Several systems of IE gene nomenclature exist. The IE genes can be referred to by their infected cell polypeptide (ICP) number, by the molecular weight of their polypeptide, or by the location of their coding region in the genome.

2

Materials and Methods

Cells

Vero cells, obtained from the American Type Culture Collection, and V27 cells—a Vero-cell derivative (277) containing one or more stably transfected copies of the ICP27 allele from KOS1.1—were maintained in Dulbecco's minimal essential medium (DMEM) containing 200U penicillin per mL and 200 μg streptomycin per mL (Gibco-BRL), supplemented with 5% fetal-bovine serum (FBS) and 5% FBS plus 100 μg per mL Geneticin (G418; Gibco-BRL), respectively.

Viruses

HSV-1 KOS1.1 (141) was propagated on Vero cells. HSV-1 *d*27-1 (277), *d*4–5 (209), M11, M15, and M16 (278) were propagated on V27 cells. High-titre stocks of the revertant viruses M16R, exCd305R1, and exCd305R2, and the recombinant viruses *d*27-2, M16exC, exCd305, and *n*217d, were prepared using V27 cells. Viral infections were maintained in medium 199 (Gibco-BRL) containing 2% heat-inactivated newborn-calf serum (Gibco-BRL) with 200U penicillin per mL and 200 μg streptomycin per mL. For viral plaque assays, 1% pooled normal human serum (ICN) was present in the medium 199 in addition to the newborn-calf serum, penicillin, and streptomycin.

Plasmids

Plasmid pPs27pd1, containing the ICP27 allele from KOS on a *Pst*I fragment, has been described previously (282). This plasmid was used to construct pPsd27-2, the vector employed in the creation of the recombinant virus *d*27-2. Briefly, pPs27pd1 was digested with *Aat*II and *Stu*I; this generates two *Aat*II fragments, and two *Aat*II–*Stu*I fragments (encoding wild-type ICP27). The two *Aat*II fragments were ligated together, resulting in the plasmid pPsd27-2. pPsd27-2 lacks the entire ICP27 allele, as well as a majority of the UL55 coding region.

Plasmid pM16R27 was created by cloning the *Bam*HI–*Sac*I ICP27-allele fragment from the revertant virus M16R into pUC19. The plasmids p27 and pM1627 were constructed by cloning the *Bam*HI–*Sac*I ICP27 allele fragments from pM27, encoding wild-type ICP27 (278), and pM16, encoding substitution-mutation-inactive ICP27 (278), respectively, into pUC19.

The two extra cytosine-containing plasmids, p27exC and pM1627exC, are both derivatives of the engineered plasmid pM27exC. Oligonucleotide-directed mutagenesis (Altered Sites system; Promega) was used to introduce an extra cytosine into pM27 at codon position 215–217 of the ICP27 allele, thus creating pM27exC. The plasmids p27exC and pM1627exC were then created by ligating the *Sac*I–*Bam*HI fragment from pUC19 and the *Bam*HI–*Sal*I ICP27-allele fragment from pM27exC to either the *Sal*I–*Sac*I ICP27-allele fragment from p27, or the *Sal*I–*Sac*I ICP27-allele fragment from pM1627.

Plasmids pexCd305 and pn217d possess the same C-terminal–codon deletion in their ICP27 alleles. To create this deletion, the plasmid pPsd10–11 (180)—which contains the *Bam*HI–*Sac*I ICP27-allele fragment from KOS1.1 with an engineered *Xho*I site at codons 305–306—was digested with *Xho*I and *Eco*NI. The 3′-recessed ends were filled in with Klenow (the large fragment of *E. coli* DNA polymerase I) and then ligated together resulting in a plasmid, pPsd305, with an ICP27 allele missing codons 306–512. Plasmid pexCd305 is the product of the ligation of the *Sac*I–*Bam*HI fragment from pUC19, the *Bam*HI–*Sal*I ICP27-allele fragment from pM27exC, and the *Sal*I–*Sac*I ICP27-allele fragment from pPsd305. To create pn217d, oligonucleotide-directed mutagenesis was used to introduce an ochre-termination signal into pM27 at codon position 218 of the ICP27 allele. The *Bam*HI–*Sal*I ICP27-allele fragment containing this stop codon was then ligated to the *Sal*I–*Sac*I ICP27-allele fragment from pPsd305 and the *Sac*I–*Bam*HI fragment from pUC19, resulting in pn217d.

The plasmids employed for recombinant HSV-1 production are based upon pPs27pd1 (282). pPsM1627exC, pPsexCd305, and pPsn217d were constructed by cloning the *Bam*HI–*Sac*I ICP27-allele fragments from pM1627exC, pexCd305, and pn217d, respectively, in place of the *Bam*HI–*Sac*I ICP27-allele fragment from pPs27pd1.

Plasmid pd305R1 was created by cloning the *Bam*HI–*Sac*I ICP27-allele fragment from the revertant virus exCd305R1 into pUC19; plasmid pd305R2 was created identically, save for the fact that the revertant virus used was exCd305R2.

PCR analysis and PCR primers

Vero or V27 cells growing in wells of a twelve-well plate were infected with virus, and at one to three days post-infection (dpi) were harvested into 400 μL of 1X lysis buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 2% sodium dodecyl sulfate [SDS], 100 μg of proteinase K per mL). Following incubation at 37°C for approximately 24 hours, 48 μL of 3 M sodium acetate (pH 5.2) were added to the lysates, which were then extracted once with phenol-chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 95% ethanol, resuspended in TE (10 mM Tris [pH 7.6], 1 mM EDTA), and subjected to RNase A digestion (50 ng per μL) at 37°C for approximately one hour. Five to fifty percent of total DNA isolated was used as template in the amplification reactions, which were composed of 10 μL of "magic" PCR buffer (300 mM Tricine [pH 8.4], 20 mM $MgCl_2$, 50 mM β-mercaptoethanol, 1% Thesit), 10 μL of dNTP mix (2 mM each), 10 μL of Primer1 (2.5 μM in TE), 10 μL of Primer2 (2.5 μM in TE), 10 μL of dimethyl sulfoxide (DMSO), 10–50 μL of DNA, and 5U *Thermus aquaticus* (*Taq*) DNA polymerase (Gibco-BRL), in a final volume of 101 μL. Amplifications, following an initial denaturation-25 cycle (denaturation, annealing, elongation)-final elongation profile, were carried out using a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). Products were visualized using agarose-gel electrophoresis.

The HSV-1 primers R51 (gtgctcgtggcgcttcacta), R62 (tagcccctgggccccggtgc), R63 (ggttggtgaggggggtcgct), R64 (aaacagggagttgcaataaa), R65 (gtttggcgtcgtgagtcccg), R66 (tggcagtggtacacgatccc), R72 (gagcggcagggccccaacga), R78 (tgccgtgcacgtacgggggg), R96 (gacgggtctcctgggaaacc), R97 (gcgctcggagatggagcgca), and R99 (ggtgtcatagtgcccttagg) were synthesized at the Biochemistry Core DNA facility of the University of Alberta using an Applied Biosystems 394 DNA/RNA Synthesizer.

Sequence analysis

Plasmid constructs were sequenced at the Biochemistry Core DNA facility of the University of Alberta using an Applied Biosystems 373A DNA Sequencer. DNA primers for sequencing were synthesized at the same locale using an Applied Biosystems 394 DNA/RNA Synthesizer.

Construction of recombinant viruses

Viruses were constructed using a homologous-recombination procedure, described previously (277-279). Co-transfections were carried out using a modified lipofectAMINE (2 mg per mL; Gibco-BRL) protocol. Briefly, 1 μg of *Pst*I fragment and 1 μg of infectious *d*27-*lacZ*1 DNA, in a final volume of 200 μL of serum-free DMEM, were mixed with 10 μL of lipofectAMINE per μg of DNA, in a final volume of 200 μL of serum-free DMEM, and allowed to sit for 15–45 minutes at room temperature. Each DNA-lipofectAMINE mixture was made up then to a final volume of 2 mL with serum-free DMEM and added to Vero cells that had been rinsed with serum-free DMEM. After a five hour incubation at 37°C, the transfection mixtures were removed and replaced with DMEM (5% FBS). One day later the DMEM was removed and replaced with medium reserved for viral infections (described above). For each mutant, two independent viral isolates, designated a and b, were obtained. The genomic structures of all viral isolates were confirmed by PCR.

Isolation of cell-line derived recombinant viruses

Aliquots of M16—which had been UV irradiated for 0, 18, 30, 60, 120, 240, or 480 seconds in a UV Stratalinker 2400 (Stratagene) at 120 mJ per cm^2—were used to infect Vero cells growing in 150-cm^2 flasks at a MOI of one. After allowing the infections to proceed for 48 hours, the flasks were subjected to three rounds of freeze-thawing, and used to infect, at a 1:5 dilution, 75-cm^2 flasks of Vero cells. Plaques indicative of a growth-competent virus appeared in all flasks two dpi. Virus present within the cells of the flasks was released through three rounds of freeze-thawing, and used to infect Vero cells for the purpose of individual plaque selection. Three dpi, a single plaque from each of the infections—all seven of the UV time points—was isolated, subjected to three rounds of freeze-thawing, and used

to infect, at a 1:5 dilution, Vero cells growing in wells of a twelve-well plate. Vero cells were infected also with M16 at a MOI of one. Two dpi, total DNA was harvested for PCR and restriction-enzyme-digest analysis.

Vero cells growing in 75-cm^2 flasks were infected with M11, M15, M16, *d*4–5, *d*27-1, and the zero-second-UV-irradiated M16 aliquot, at a MOI of one. Two dpi, the infections were harvested, subjected to three rounds of freeze-thawing, prepared as stocks, and used to infect, at a 1:5 dilution, Vero cells growing in 75-cm^2 flasks. Plaques indicative of a growth-competent virus appeared in all flasks two dpi. For the purpose of individual plaque isolation, the stocks were used then to infect Vero cells. Three dpi, two plaques from each of the infections were isolated, subjected to three rounds of freeze-thawing, and used to infect, at a 1:5 dilution, Vero cells growing in wells of a twelve-well plate. Vero cells were infected also at a MOI of one with KOS and *d*27-1. Two dpi, total DNA was harvested for PCR, restriction-enzyme digest, and Southern blot analysis. The two plaques isolated from the *d*27-1 stock were plaque purified two additional times in Vero cells, yielding the isolates *d*27rc-1a and -1b.

Eight *d*27-1 plaques growing on V27 cells were isolated, subjected to three rounds of freeze-thawing, and used to infect, at a 1:5 dilution, V27 cells growing in 25-cm^2 flasks. Infections were maintained until five dpi, at which time small working stocks were prepared; these stocks were used then to prepare high-titre stocks in V27 cells. To address the question of the presence of cell-line recombinants within these high-titre stocks, 75-cm^2 flasks of Vero cells were infected at a MOI of one. After allowing the infections to proceed for 48 hours, the flasks were subjected to three rounds of freeze-thawing, prepared as stocks, and used to infect, at a 1:5 dilution, Vero cells growing in 75-cm^2 flasks. By three dpi, two of the eight flasks had viral plaques forming on their cell monolayers. The stocks corresponding to the two plaque-positive flasks were used then to infect Vero cells growing in wells of a six-well plate for the purpose of individual plaque isolation. Two plaques from each infection were isolated; all four were plaque purified two additional times in Vero cells, yielding the isolates *d*27rc-2a, -2b, -3a, and -3b.

The genomic structures of *d*27rc-1a, -2a, and -3a were examined by infecting Vero cells at a 1:5 dilution, along with KOS (at a MOI of one) and *d*27-1 (at a MOI of ten). Two dpi, total DNA was harvested for PCR analysis.

Isolation of revertant viruses

The genesis of M16R was as follows: six M16 plaques growing on V27 cells were isolated, subjected to three rounds of freeze-thawing, and used to infect, at a 1:5 dilution, Vero cells growing in 25-cm^2 flasks. After allowing the infections to proceed for 48 hours, the flasks were subjected to three rounds of freeze-thawing, and used to infect, at a 1:5 dilution, 25-cm^2 flasks of Vero cells. Plaques indicative of a growth-competent virus appeared within one of the flasks three dpi. Following initial plaque purification in Vero cells, PCR and restriction-digest analysis were used to confirm the presence of the *Xho*I site within the ICP27 allele. The virus was plaque purified in Vero cells one additional time and a small working stock of M16R was prepared in Vero cells.

The genesis of exCd305R1 and exCd305R2 was as follows: four exCd305 plaques growing on V27 cells were isolated, subjected to three rounds of freeze-thawing, and used to infect, at a 1:5 dilution, V27 cells growing in wells of a twelve-well plate. Six dpi, the plate was subjected to three rounds of freeze-thawing and used to infect, at a 1:5 dilution, Vero cells growing in 75-cm^2 flasks. After allowing the infections to proceed for 48 hours, the flasks were subjected to three rounds of freeze-thawing and used to infect, at a 1:50 dilution, 25-cm^2 flasks of Vero cells. Four dpi, one of the flasks was deleted from the experiment as it was observed to contain wild-type virus (which was confirmed using PCR), while the remaining three flasks were subjected to three rounds of freeze-thawing and used to infect, at a 1:5 dilution, 25-cm^2 flasks of Vero cells. At five dpi, the flasks were subjected to three rounds of freeze-thawing and used to infect, at a 1:10 dilution, 25-cm^2 flasks of Vero cells. Four dpi, two of the three flasks contained plaques indicative of a growth-competent virus. The two new viruses were then plaque purified three times in V27 cells and small working stocks of exCd305R1 and exCd305R2 were prepared in V27 cells. PCR analysis confirmed the presence of their engineered ICP27 alleles and the absence of the wild-type–ICP27 allele.

Complementation assay

The *d*27-1 virus-complementation assays were carried out as described previously (279, 282), except that Vero cells were transfected following a modified-lipofectAMINE (2 mg per mL; Gibco-BRL) protocol. Briefly, 1 μg of pUC19, wild-type–, or mutant-ICP27 plasmid, in a final volume of 200 μL of serum-free DMEM, was mixed with 10 μL of lipofectAMINE per μg of DNA, in a

final volume of 200 μL of serum-free DMEM, and allowed to sit for 15–45 minutes at room temperature. Each plasmid-lipofectAMINE mixture was made up then to a final volume of 2 mL with serum-free DMEM and added to Vero cells that had been rinsed with serum-free DMEM. After a five hour incubation at 37°C, the transfection mixtures were removed and replaced with DMEM (5% FBS). All other incubations were carried out at 37°C for approximately 24 hours.

Virus yield assay

Revertant and recombinant viruses were examined for their ability to produce infectious progeny through the course of a single replication cycle, as described previously (278, 279). Briefly, parallel cultures of Vero and V27 cells were infected at a MOI of ten with virus and at two hpi were treated with a glycine-saline solution (pH 3.0) (32). Following incubation for approximately 24 hours, virus yield was determined by plaque assay on V27 cells.

Immunofluorescence

Vero cells were infected at a MOI of ten and at four hpi processed for indirect immunofluorescence as described previously (269). H1113 (Goodwin Institute for Cancer Research, Plantation, Fla.) (2), at a 1:600 dilution, was the primary antibody used. The secondary antibody used was tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories Inc., Mississauga, Ontario, Canada) at a 1:200 dilution. The cells were visualized with a Zeiss Axioskop 20 fluorescence microscope equipped with a Plan-Neofluar 63X objective lens.

Southern blot analysis

V27 or Vero cells growing in wells of a twelve-well plate were infected with virus at a MOI of ten, and harvested at either 48 hpi or at the times indicated into 400 μL of 1X lysis buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 2% SDS, 100 μg of proteinase K per mL). Following incubation at 37°C for approximately 24 hours, 48 μL of 3 M sodium acetate (pH 5.2) were added to the lysates, which were then extracted once with phenol-chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). DNA was

precipitated with 95% ethanol, resuspended in TE, and subjected to RNase A digestion (50 ng per μL) at 37°C for approximately one hour. Based upon quantitation by UV absorption at 260 nm, 3 μg of DNA were cleaved with *Pst*I and *Sal*I and separated on a 1% agarose gel. Following acid cleavage and alkali denaturation, DNA was transferred to a nylon membrane and hybridized to a ^{32}P-labeled probe—generated by random priming—in Church buffer (250 mM sodium phosphate buffer [pH 7.2], 7% SDS, 1% bovine serum albumin [BSA], 1 mM EDTA) at 65°C for approximately 12 hours. The probe examining the structures of the *d*27rc viruses was *Eco*RI-linearized pPs27pd1. The probes examining revertant-genome structure were a 510-base pair PCR fragment spanning N-terminal codons 90–259 of wild-type ICP27, and a 588-base pair PCR fragment spanning C-terminal codons 308–503 of wild-type ICP27. The probe examining levels of viral DNA replication was a 480-base pair PCR fragment spanning codons 1–142 of UL55, plus an additional 55 nucleotides upstream within the non-coding region. Membranes were washed twice for 15 minutes at 68°C in 2X SSC-0.1% SDS and twice for 15 minutes at 68°C in 0.1X SSC-0.1% SDS prior to film exposure. Signal quantitation was measured by phosphorimage analysis using a Fujix BAS100 bioimaging analyzer with MacBAS imaging software.

Western blot analysis

Vero cells growing in 25-cm^2 flasks were infected with virus at a MOI of ten, and at either four or six hpi were harvested as described previously (177, 281). Total proteins were separated on 15% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blocked with 10% skim milk in TBS-T (1X Tris-buffered saline, 0.2% Tween 20). The membranes were washed twice with TBS-T and probed with H1113, at a 1:1000 dilution in TBS-T, for 45 minutes. Membranes were washed again twice with TBS-T, and protein was visualized using a horseradish peroxidase-conjugated secondary antibody, at a 1:3000 dilution in TBS-T for 30 minutes, and an enhanced chemiluminescence system (Amersham Pharmacia).

Plaque morphologies

A single plaque/focus growing on Vero cells was photographed using an Olympus SC 35 Type 12 camera mounted on an Olympus CK40 microscope at four dpi.

3

Investigation of Unexpected Recombinants Present in ICP27-Mutant Stocks

Vectors based upon HSV-1 hold great promise for the treatment of certain genetic defects and human diseases. Neurological conditions, such as Alzheimer's, Parkinson's, and Huntington's diseases, are well suited to therapeutic manipulation through the use of HSV-1 as a delivery vehicle for required genetic material (22, 93, 98, 101, 106-109, 172, 173, 179). Conversely, HSV-1 has the potential to effect the destruction of unwanted neurological tumours; glioblastomas—the most common form of brain tumour—are extremely difficult to treat successfully, with current treatments (surgery, radiation, and chemotherapy) changing little the high mortality associated with the affliction (246, 267, 294). HSV-1 directly introduced at the tumour site would effectively kill the tumour cells via its naturally cytotoxic process of viral replication (161, 206, 351). Alternatively, the HSV-1 genome could be engineered to express specific exogenous gene products necessary for the activation of a specific anti-tumour pro-drug (161, 218).

One caveat associated with the use of HSV-1 and HSV-1 vectors is the potential for uncontrolled, rampant viral replication and spread. HSV-1 is extremely effective at killing cells that it infects. A rare but crippling form of encephalitis is the result of viral spread in the brain (5, 120, 307); any HSV-1 introduced at this site would have to be unable to replicate and spread beyond the confines and conditions of its therapeutic purpose. At present, two strategies have evolved that address this concern: the use of growth-attenuated HSV-1, and the use of replication-defective HSV-1. The

inactivation of the tk, ICP6, or γ34.5 genes of HSV-1 severely hampers viral replication and spread in normal, non-tumour cells; inactivation of these genes seems to have little effect on viral replication and spread in actively dividing tumour cells (7, 65, 161, 175, 206, 218, 227, 351, 366, 367, 392). However, the use of growth-attenuated HSV-1 that preferentially replicates in tumour cells still carries with it the risk of infection of healthy cells, and the subsequent destruction of those cells. The more favoured strategy involves the use of replication-defective HSV-1. Not only is the risk of spread to healthy tissue obviated, in situations where long-term expression of a gene product is desired, target cells can be spared from the normally cytotoxic process of viral replication. HSV-1 strains that are replication-defective usually contain one or more IE gene—ICP4, ICP22, and ICP27—deletions (97, 140, 163, 164, 194, 196), although other replication-defective strains exist as well (196, 389). Common to all replication-defective HSV-1 is the fact that the virus must be propagated in helper cell lines, which express stably integrated copies of the missing viral gene(s).

Whenever a replication-defective virus is propagated in a helper-cell line, there exists the possibility that the final stock will contain a sub-population of virus that has obtained replication-competency through the process of homologous recombination between the viral genome and the integrated viral gene(s) present in the cellular genome (130, 394). Any replication-competent (RC) virus, which would behave phenotypically as wild-type virus, would pose a serious problem to the therapeutic use of replication-defective stocks.

The high-titre stock of M16 contains replication-competent virus

The investigation into recombination events in our laboratory stocks of HSV-1 stemmed from the initial purpose of isolating second-site revertants of ICP27 mutants—the results of that investigation are presented in Chapters 4 and 5. As second-site revertants had never spontaneously arisen in our ICP27-mutant stocks, it was believed that changes to the viral genome had to be introduced artificially using a mutagenic process. While use of a chemical mutagen was considered, for procedural reasons it was dismissed in favour of the energy mutagen, UV light. UV light is capable of inducing the formation of thymine dimers and DNA breaks (391); this damage must be repaired in order for the integrity of the genetic material to be preserved. Repair of DNA damage is an inherently error-prone process, leading to the possibility of permanent genetic changes (183). The right mutations can effect a suppression of the defective parental phenotype, resulting in progeny that exhibit a growth-competent phenotype.

The virus initially chosen for second-site–revertant isolation was M16, a substitution-mutation inactive ICP27 mutant (278). M16 cannot grow in Vero cells—an African green monkey kidney-cell line commonly used to propagate HSV-1 stocks—but can grow in V27 cells, which contain one or two copies per-haploid-Vero-genome of a cloned HSV-1–genomic fragment encoding ICP27 (277). Aliquots of high-titre M16 ($>10^8$ plaque forming units [PFU] per mL), which had been prepared using V27 cells, were exposed to UV light for defined periods of time. The ability of the UV light to introduce genetic changes was reflected in a plaque assay of the UV-exposed viral aliquots on V27 cells. Exposure for 240 seconds resulted in a two-log decrease in the viral titre.

All experiments screening viral stocks for RC variants followed a two-round-infection procedure whereby Vero cells were infected initially with the virus(es) of interest (amplification round), allowed to incubate for two days, and then harvested for a subsequent Vero-cell infection (detection round). In the initial experiment, Vero cells were infected at a MOI of one with the UV-exposed M16-viral aliquots, and at two dpi were harvested for subsequent Vero-cell infection. Two dpi, RC variants were observed in all flasks. Virus from the flasks was harvested and used to infect, in a serial-dilution series, Vero cells. One viral plaque from each infection was isolated. To determine whether these plaques represented second-site revertants, a small number of Vero cells were infected either with the plaque lysates, or with the original M16 high-titre stock. Total DNA was harvested two dpi, and PCR analysis was performed using HSV-1 primers R62 and R63; these primers amplify a 920-base pair region demarcated from codon 289 of UL54 (ICP27) to codon 9 of UL55 (Figure 3-1). Approximately one-fifth of the post-PCR DNA was cut with *Xho*I and subjected to agarose-gel electrophoresis, as shown in Figure 3-2. Only the PCR product amplified from the high-titre M16-stock DNA digested successfully with *Xho*I. Digestion of the M16-PCR DNA was expected, as M16 is defined genotypically by a *Xho*I site at codons 488–489 of its ICP27 allele. The fact that none of the other PCR products could be digested with *Xho*I indicated that their ICP27 alleles had lost the *Xho*I site, and suggested that replication-competency had been obtained either as a result of primary reversion, or as a result of homologous recombination between viral and cellular ICP27 alleles. The probability that all six of the RC variants had been generated independently by primary reversion seemed highly unlikely; one or more of the RC variants were most likely wild-type recombinants that had acquired the wild-type ICP27 allele via recombination with the V27-cell genome. Lacking any allelic markers, however, it was impossible to distinguish between a RC variant generated by primary reversion, and one generated by homologous recombination. If one or more of the RC variants

was indeed recombination-derived, this would mean that RC virus is present in the high-titre stock of M16, but that it is present at low enough levels to escape detection by a standard plaque assay on Vero cells (i.e. less than approximately 0.001% of the high-titre stock is RC virus).

Replication-competent virus is present in other high-titre stocks

If RC virus exists at low levels within the M16 stock, is this also the case for the other high-titre stocks of ICP27 mutants? To address this question, flasks of Vero cells containing approximately $1x10^7$ cells were infected at a MOI of one with the following V27-cell propagated viruses: the substitution-mutation inactive ICP27 mutants M11, M15 (278), and M16; the non-UV irradiated M16 aliquot; the RGG-box deletion mutant *d*4–5 (209); and the ICP27-deletion mutant *d*27-1 (277). Two dpi, virus from the flasks was harvested and prepared as working stocks for each mutant. Vero cells were infected with the working stocks for the purpose of RC virus detection, and allowed to incubate for two days. Surprisingly, when the flasks were checked for evidence of RC-virus plaquing, all flasks were found to contain viral plaques (a mock-infected flask was plaque-free). Again, these RC variants could have arisen either through reversion, or through recombination with the V27-cell genome.

It is relatively easy to envision how homologous recombination between viral and cellular genomes could account for RC virus present within the high-titre stocks of M11, M15, M16, and *d*4–5 (Figure 3-3A). It is more difficult to envision how homologous recombination could account for RC virus present within the high-titre stock of *d*27-1 (Figure 3-3B); homology between the *d*27-1 genome and the cloned cellular insert exists only at one end of the insert. Introduction of the ICP27 allele from the cellular insert into the viral locus would have to be effected through a combination of homologous and illegitimate recombination processes.

To characterize the RC viruses, two plaques from working-stock infected cells were isolated for each virus and used to infect a small number of Vero cells along with high-titre KOS and high-titre *d*27-1. Total DNA was isolated from each infection, and a portion of the DNA from one infection for each virus—with the exception of the *d*27-1 RC DNA isolates, where both were examined—was subjected to PCR amplification using HSV-1 primers R62 and R63 (Figure 3-1). Agarose-gel electrophoresis of the post-PCR DNA revealed the presence of the 920-base pair product in all samples except for the high-titre *d*27-1–DNA preparation and the mock-infection DNA preparation (Figure 3-4). The presence of the 920-base pair product in the KOS lane was expected, as this is the appropriate amplification-product size for the wild-type genome. Since primer R62 is homologous to sequences deleted in the

*d*27-1 virus, it was also expected that the high-titre *d*27-1–DNA preparation would not be able to produce the 920-base pair product. The presence of the 920-base pair product in the *d*4–5, M11, M15, and M16 lanes was expected; native, revertant, and recombinant genomes would all produce the same-sized product. Presence of the 920-base pair product in the two *d*27-1 RC DNA lanes indicates that the RC variants present in the high-titre *d*27-1 stock contain at least the C-terminal–encoding section of the ICP27 allele, as well as the beginning section of the UL55 allele. It is almost certain that the entire ICP27 allele is present within the genome of these RC variants given that the N-terminal half of ICP27 is essential for protein function, and therefore viral viability (209, 279).

To determine whether the RC variants from the M11, M15, and M16 infections had retained their respective *Xho*I sites—M11 contains an engineered site at codons 340–341, and M15 contains an engineered site at codons 465–466 (278)—aliquots of post-PCR DNA were digested with *Xho*I and subjected to agarose-gel electrophoresis. As Figure 3-5 clearly shows, only the 920-base pair product amplified from high-titre M16-stock DNA could be digested with *Xho*I; products in the M11, M15, and M16 lanes were unaffected, as was the product in the *d*27-1 RC-DNA lane (negative control). As before, loss of *Xho*I sites could have occurred either through primary reversion, or as a result of recombination with the V27-cell genome. It is unlikely that replication-competency was achieved in all cases through primary reversion knowing that recombination-derived RC virus can be generated and exists in the high-titre stock of *d*27-1.

The data presented here reveal that RC virus, most likely derived through recombination with the viral fragment present within the genome of the V27 cells, commonly exists in V27-cell prepared high-titre stocks of ICP27 mutants. Based upon the fact that RC virus was found when $1x10^7$ cells were infected at a MOI of one, RC virus must constitute greater than or equal to 0.00001% of an examined high-titre stock.

Replication-competent *d*27-1: one virus, two ICP27 alleles

The RC virus present within the high-titre stock of *d*27-1 has acquired the wild-type–ICP27 allele, presumably via a combination of homologous and illegitimate recombination with the V27-cell genome. To confirm that the wild-type allele had recombined in at the ICP27 locus of the viral genome, a portion of *d*27-1–RC DNA from each isolate (hereafter known as *d*27rc-1a and -1b), along with high-titre *d*27-1 DNA, was amplified using PCR and HSV-1 primers R51 and R63 (Figure 3-1). The resulting PCR products were subjected to agarose-gel electrophoresis (Figure 3-6). Primer R51 was chosen for the reaction because it anneals to a portion of the UL53-coding

region, which is not found within the cloned viral fragment present in the V27 cells, and therefore can be employed in an examination of the nature of the viral ICP27 locus. PCR amplification using primers R51 and R63 should yield a 757-base pair product for high-titre *d*27-1 DNA. A product of this size is present in the *d*27-1 lane. Unexpectedly, a product of this size is also found in the two d27rc lanes. Since both *d*27rc-1 isolates are RC, a product of 2382-base pairs was expected for PCR amplification across a supposed wild-type–ICP27 locus (see Figure 3-8). The 757-base pair product can only be generated for amplification across the deletion (*d*27-1) allele. Its presence in the *d*27rc lanes means that these isolates have retained the ICP27-deletion allele at the viral ICP27 locus; the wild-type–ICP27 allele conveying replication-competency has recombined in elsewhere in the viral genome.

As a validation of the PCR results, and verification that the *d*27rc isolates possess unique genomic structures, Southern blot analysis of DNA representing KOS, *d*27-1, and the *d*27rc isolates was carried out. Duplicate DNA samples were cut either with *Pst*I or *Sal*I, and fragments were separated, transferred, and probed with the plasmid pPs27pd1 which had been linearized with *Eco*RI and labeled with radioactive phosphate (Figure 3-7). Plasmid pPs27pd1 contains the large ICP27-encoding *Pst*I fragment from KOS (282); because a section of this is R_L sequence, it is able to hybridize to fragments containing R_L sequence from both R_L–U_L junctions (see Figure 1-1). The hybridization profiles presented in Figure 3-7 are thought to demonstrate exactly this phenomenon—the common bands present at approximately 5.7 kb for the *Pst*I digestions and approximately 9.4 kb for the *Sal*I digestions are most likely the result of hybridization with the first R_L–U_L junction (as oriented in Figure 1-1). In addition to the common bands, expected hybridization patterns were obtained for both wild-type and *d*27-1 genomes: the pPs27pd1 probe hybridizes to a 6.0 kb fragment for *Pst*I-digested KOS DNA and a 4.3 kb fragment for *Pst*I-digested *d*27-1 DNA, and to 7.1 and 6.4 kb fragments for *Sal*I-digested KOS DNA and a 11.8 kb fragment for *Sal*I-digested *d*27-1 DNA (the 11.8 kb fragment is generated because the *d*27-1 deletion includes a *Sal*I site). While the hybridization patterns for the wild-type and *d*27-1 genomes were as expected, a novel hybridization pattern presented itself for the *d*27rc isolates. Therefore, the Southern blot analysis confirmed that the *d*27rc isolates are genotypically distinct from both KOS and *d*27-1.

The issue of recombination and DNA-sequence homology

The *d*27rc isolates contain both the wild-type- and deletion-ICP27 alleles. Simple homologous recombination between viral and cellular genomes cannot explain the presence of the wild-type allele in the *d*27rc isolates; a

less obvious, and perhaps more unusual, recombination mechanism seems to have been responsible for insertion of the wild-type allele in the viral genome. An interesting question is whether apparent DNA-sequence homology between the cellular ICP27 allele and the viral ICP27 allele is essential for the generation of RC virus. To address this question, a new *d*27 virus, completely devoid of apparent homology between itself and the cloned viral fragment present in the V27-cell genome, was created. The recombinant virus *d*27-2 contains an entire deletion of the ICP27 gene, as well as a deletion of most of the coding region—codons 1–119 of 186—of the UL55 gene (Figure 3-3C). Deletion of most of the UL55-coding region was unavoidable due to the UL55 sequence present in the cellular HSV-1 fragment; fortunately, the UL55 allele is not essential for HSV-1 growth in cell culture (192, 234). A marker-transfer protocol based upon homologous recombination between plasmid DNA containing the deletion allele and genomic viral DNA containing the *lacZ* allele in the ICP27 locus was employed in the creation of *d*27-2 (277). Verification of successful marker-transfer was effected through PCR analysis of independently generated viral isolates using HSV-1 primers R65 and R66 (Figure 3-1). Primer R65 anneals to a region of sequence in the UL53 allele which lies outside of the homologous recombination area, and thus when paired with primer R66—which is homologous to the sequence representing codons 136–142 of UL55—allowed for positional confirmation of the deletion allele within the viral genome (data not shown).

Recall that RC virus is present in high-titre *d*27-1 stock at a frequency of greater than or equal to one per $1x10^7$ PFU (or 0.00001% of the viral stock). The ability of *d*27-2 to recombine with the V27-cell genome was examined in parallel with *d*27-1 over the course of two experiments. Eight *d*27-1 and eight *d*27-2 plaques growing on V27 cells were isolated and used to infect $3x10^6$ V27 cells each for the purpose of working stock generation. Titering of the working stocks suggests that *d*27-2 cannot be fully complemented by the ICP27 expressed by the V27 cells. Plaques of *d*27-2–infected cells took markedly longer to develop, and titres of the *d*27-2–working stocks were reduced in comparison to those of *d*27-1 by an average of ten-fold. The relatively poor growth of *d*27-2 in V27 cells may be the result of the predicted lack of expression of the UL55-gene product. However, it is possible that the *d*27-2 phenotype is the result of the lack of expression of another viral protein as well. The *d*27-2 deletion removes a polyadenylation site thought to be associated with the UL53 transcript (204, 230), and as such may alter the production level of the UL53 protein.

As an initial measure of frequency of RC-virus generation, the working stocks of *d*27-1 and *d*27-2 were used to each infect $1x10^7$ Vero cells at a MOI

of 0.2. Two dpi, the infections were harvested and used to infect another set of $1x10^7$ Vero cells. Infections were monitored for the appearance of RC-viral plaques until four dpi. During this period of time, none of the 16 infections showed any signs of containing RC virus. Because $2x10^6$ PFU were initially introduced to the Vero cells, the frequency of RC-virus generation appears to be less than one event per $2x10^6$ PFU (constituting less than 0.00005% of the stocks) for *d*27-1 and *d*27-2.

High-titre stocks were prepared for the eight *d*27-1- and the eight *d*27-2–working stocks. As was the case with the working stocks, when the high-titre stocks were titred, *d*27-2 plaques took longer to develop on the V27 cells and their titres were on average five-fold lower than those of *d*27-1. Whether this growth deficiency is the result of an extension of the lytic cycle of the virus—leading to fewer progeny being produced for a given time—or the result of fewer progeny being produced per lytic cycle is not known.

To further investigate the frequency of RC-virus generation in *d*27-1 and *d*27-2 stocks, $1x10^7$ Vero cells were infected with the high-titre stocks of both *d*27-1 and *d*27-2 at a MOI of one. Two dpi, the infections were harvested and used to infect $1x10^7$ Vero cells. The appearance of RC-viral plaques in these infections was monitored until four dpi, at which time two of the *d*27-1 infections were RC-virus positive. None of the remaining infections had any indication of RC virus being present and were terminated. The fact that only two of eight *d*27-1 high-titre stocks tested were found to contain RC virus suggests that the threshold frequency for RC virus in a *d*27-1 stock approaches one per $1x10^7$ PFU (constituting approximately 0.00001% of the stock). For *d*27-2, RC virus appears to be present at a frequency less than one per $1x10^7$ PFU. While it is tempting to conclude that the absence of RC virus in *d*27-2 stocks is the definite result of an inability of the viral genome to recombine with the cloned viral fragment present in the cellular genome due to a lack of apparent homology between the two, it would be premature to do so. RC virus may exist in *d*27-2 stocks at very low or almost undetectable levels. A more sensitive *d*27-2 stock screening would have to be performed to truly address the question of whether the generation of RC virus via recombination requires apparent DNA-sequence homology. For example, $1x10^7$ Vero cells could be infected at a MOI of ten with the high-titre stocks of *d*27-1 and *d*27-2; RC-virus presence could be detected down to a frequency of approximately one per $1x10^8$ PFU. In the case of *d*27-1, such an assay would be expected to reveal the presence of RC virus in all eight high-titre stocks. The screening of *d*27-1 and *d*27-2 stocks at even more sensitive levels, however, would require (especially in the case of *d*27-2, which grows relatively poorly in V27 cells) the creation of much larger volumes of more concentrated stocks.

All three replication-competent *d*27-1 variants appear to be identical

Two RC *d*27-1 variants were observed to be present in the *d*27-1 high-titre stocks tested. The initial RC *d*27-1 variant isolated, *d*27rc-1, distinguished itself with an unusual two-ICP27-allele genotype. Are the two other RC *d*27-1 variants, hereafter referred to as *d*27rc-2 and *d*27rc-3, products of a similar genesis? To address this query, two isolates from each *d*27rc variant (-1, -2, and -3) were plaque purified in Vero cells three times. A small number of Vero cells were infected then with the -a isolate from each variant, along with high-titre KOS (at a MOI of one) and high-titre *d*27-1 (at a MOI of ten), for the purpose of PCR analysis. Infections were allowed to progress until two dpi, at which time they were harvested for total DNA. PCR amplification using HSV-1 primers R51 and R63 (Figure 3-1) was performed on an aliquot of DNA representing each virus; the PCR products that were obtained are shown in Figure 3-8. The expected KOS product of 2382 base pairs and *d*27-1 product of 757 base pairs are both present in their respective lanes. The 757-base pair product was also amplified for *d*27rc-1a, -2a, and -3a, indicating that all three RC variants have retained the parental ICP27-deletion allele at its original locus.

Having established that the three RC variants contain the ICP27-deletion allele, the next logical step was to confirm that replication-competency had been achieved through the integration of the wild-type–ICP27 allele present in the V27 cells. PCR analysis using HSV-1 primers R62 (homologous to ICP27 sequence representing codons 289–296) and R78 (homologous to ICP27 sequence representing codons 497–504) was performed on all five viral genomes. Use of this primer pair allows for the amplification of a DNA region present only in the wild-type allele (Figure 3-1). Figure 3-9 presents the PCR-analysis data. The 643-base pair product amplified in the KOS and *d*27rc lanes indicates that the three RC variants contain at least codons 289–504 of the wild-type allele, and most likely the entire ICP27 allele from the V27 cells as this small region of coding sequence itself cannot effect replication-competency. Inclusion of high-titre *d*27-1 DNA as a negative control in the PCR analysis supports the specificity of the amplification reaction; the deletion allele is unable to provide a template for amplification.

The viral fragment stably integrated into the V27-cell genome is a *Bam*HI–*Hpa*I section encompassing the entire ICP27 allele as well as approximately 50% of the UL55-coding region (Figure 3-3). When *d*27rc-1 was initially characterized using PCR analysis, it was found that its recombined fragment is inclusive of a stretch of sequence from codon 289 of ICP27 to codon 9 of UL55. Of interest was whether the other two RC variants, *d*27rc-2 and -3, also possess recombined fragments containing this

sequence stretch. To investigate this, the aforementioned five viral genomes were subjected to PCR amplification using HSV-1 primers R62 and R63 (Figure 3-1), and the resulting products were visualized using agarose-gel electrophoresis (Figure 3-10). A 920-base pair product was amplified for both *d*27rc-2 and -3 DNAs, indicating that—like KOS and *d*27rc-1—these RC variants also contain the sequence stretch of interest. As was the case in Figure 3-9, the lack of product in the *d*27-1 lane supports the specificity of the amplification reaction.

Together, the data presented in Figures 3-8–3-10 reveal that the *d*27rc variants contain both the original ICP27-deletion allele as well as *at least* the C-terminally–encoding portion of the wild-type allele. While use of PCR analysis in the determination of the structural composition of a genome provides at best a limited picture, all three *d*27rc variants appear to be the result of a similar recombinatory process. The deletion allele is present in all three at its original locus, a fact that vitiates the possibility that replication-competency had been achieved through straightforward homologous recombination between viral and cellular alleles. All three appear to contain the wild-type allele of ICP27, but its location within the *d*27rc genomes cannot be determined from the data presented; a single allelic location could be common for all three, or all three could have unique insertion-positions for the wild-type allele.

A model to explain the *d*27rc variants

The genomic structures of the three *d*27rc variants are unusual in that they (most likely) possess a wild-type–ICP27 allele obtained from the V27-cell genome, as well as the deletion allele indicative of native *d*27-1. The deletion-ICP27 allele has been retained at its original locus. The obvious questions are: i) where are the wild-type alleles located in the respective *d*27rc variants, and ii) by what recombinatory process(es) were they incorporated? The data presented in this chapter reveal that a straightforward homologous recombination event was not responsible for the *d*27rc genotypes. Figure 3-11 illustrates a possible genesis for the *d*27rc variants. This model of tandem recombination, which is an example of nonconservative recombination (two parental DNAs producing one progeny DNA), can be realized either by employing homologous recombination, or by employing a process known as homology-associated nonhomologous recombination (HANR) (292). HANR is a type of illegitimate recombination thought to contribute to the generation of the four equimolar isomers of HSV-1 DNA (354). HANR occurs when a cross-stranded structure (Holliday-type intermediate), moving along a region of homology, encounters a region of nonhomology. A break forms, causing illegitimate recombination between

DNAs. The boundaries between regions of homology and nonhomology, therefore, can be recombinogenic. HANR has been observed to occur between donor and target plasmids in mouse cells (292).

V27 cells contain one or more stably integrated copies of the wild-type–ICP27 allele. As Figure 3-11 illustrates, the allele is located on a *Bam*HI–*Hpa*I viral fragment, flanked in the cell by unknown amounts of pUC19 DNA (the *Hpa*I site was destroyed in the cloning process, and is only preserved in the schematic as a positional marker). Integration into the viral genome begins with the excision and circularization of the viral fragment and its flanking plasmid/cellular DNA through either a homologous or illegitimate recombination event; circularization is necessary for insertion of the wild-type allele without the concurrent displacement of the deletion allele. The homologous region from the *Stu*I site in the ICP27 allele to the *Hpa*I site in UL55—present in both the circularized DNA and the *d*27-1 genome—allows then for the formation of a cross-stranded structure. Under the homologous recombination scenario, resolution of the cross-stranded structure before it migrates to the end of the region of shared homology results in the homologous insertion of the plasmid/cellular DNA and wild-type allele into the *d*27-1 genome. However, if the cross-stranded structure is not resolved before it encounters the nonhomologous region, a recombinogenic point is created (delineated by the arrow), resulting in the illegitimate insertion (via HANR) of the plasmid/cellular DNA and wild-type allele into the *d*27-1 genome. The end-result of either scenario is the same: two alleles essentially existing in tandem in the genome.

The PCR analysis performed on the *d*27rc variants presents evidence for the existence of two ICP27 alleles within the same viral genome, but does not directly validate the proposed model of tandem recombination. The Southern blot data of Figure 3-7 present a *d*27rc genome that is apparently quite different structurally from the genomes of both KOS and *d*27-1. Because the exact size and nature of the proposed plasmid/cellular DNA insert linking the two ICP27-allele sections is not known (see Figure 3-11), it is difficult to reconcile the hybridization patterns of *d*27rc with the model of tandem recombination. However, the presence of the small sub-2.0 kb fragment in the *Pst*I-digest lanes for *d*27rc-1 (Figure 3-7) is predicted by the model (see Figure 3-11), based upon the continued existence of a known *Pst*I-restriction site within the plasmid/cellular DNA insert.

The question marks identifying the genomic area immediately downstream of the inserted sequence (see Figure 3-11) represent the possibility of deleted sequence. Both homologous recombination and HANR allow for insertion and end-ligation to the original recombinogenic point present in the viral genome (thus re-creating the UL55 allele), but HANR also

allows for displacement and loss of genomic DNA downstream of the recombinogenic point. It is possible that the *d*27rc variants contain deletions in the UL55 and UL56 alleles. Their loss would likely not affect replication-competency, as they are both dispensable for HSV-1 growth in cell culture (192, 234). This displacement and loss of genomic sequence may eliminate HANR-insertion of the wild-type allele in the opposite direction (at the recombinogenic point delineated by the star), as the UL53 allele *is* essential for viral viability (143, 148).

Synopsis

Because of its penchant for infecting non-dividing cells, especially neurons, and its abilities to tolerate large exogenous DNA insertions and to persist in the infected cell in a state of latency, use of HSV-1 is attractive from a gene-therapy perspective. One concern, however, deals with the abrogation of viral replication; not only is the process of viral replication toxic to the cell, viral progeny are produced which are able to infect adjacent cells. This concern has lead to the development of replication-defective strains of HSV-1. Replication-defective HSV-1 must be propagated in a complementing-cell line (e.g. V27 cells). Use of such a cell line raises the spectre of wild-type–recombinant virus generation as a direct result of recombination between the viral genome and the cloned viral fragment present in the cellular genome. To ensure the safety of therapeutic HSV-1 stocks, therefore, the potentiality of recombination occurring must be addressed, with the goal of minimization and/or elimination.

The detection of RC virus in supposedly replication-defective HSV-1 stocks is well documented (182). When our existing laboratory stocks of replication-defective HSV-1 were examined for the presence of RC virus, all were found to be positive for RC virus at a frequency of approximately 10^{-7}. Interestingly, in the case of RC *d*27-1 (i.e. *d*27rc), both the original ICP27-deletion allele and the wild-type allele were detected in the viral genome. While the location of the deletion allele is known, the location of the wild-type allele is not. A proposed model for the genesis of *d*27rc places the wild-type allele in a tandem orientation with the deletion allele. This genomic structure could be confirmed through the following methodology: *d*27rc DNA is digested with various restriction enzymes and subjected to Southern blot analysis for the purpose of identifying restriction fragments containing ICP27 N-terminal–coding sequence. Pertinent restriction fragments are then cloned into pUC19 and sequenced. Based upon the sequence analysis, the recombinogenic location(s) are identified.

The issue of whether apparent DNA-sequence homology—between viral genome and cellular insert—is required for the generation of RC virus was

examined by screening high-titre stocks of *d*27-1 and *d*27-2 for the presence of RC virus. *d*27-2, which shares no apparent sequence homology with the cellular insert, was found to be RC-virus negative at a frequency of less than approximately 10^{-7}. While not entirely unexpected, given the lack of apparent homology, a more sensitive screening of *d*27-2 will have to be performed in order for the stock to be truly designated RC-virus negative. It is hoped that deletion of apparent homology obviates the ability of RC virus to be generated. If this is found to be true, it will help to ensure the safety of therapeutic HSV-1 stocks.

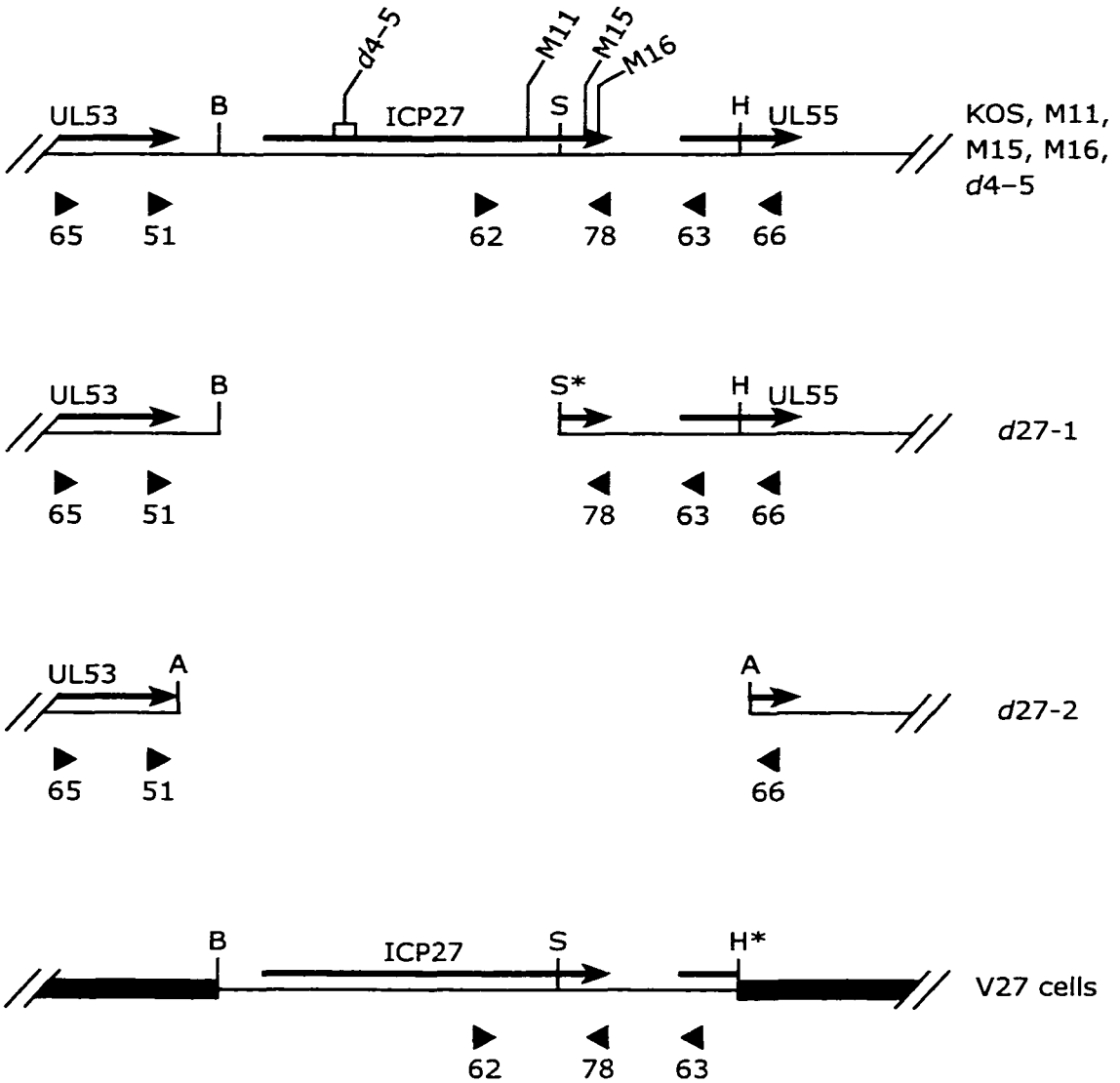

Figure 3-1. Annealing locations of HSV-1–PCR primers Illustrated are the annealing locations of PCR primers (represented as numbered triangles) for HSV-1 and V27-cell genomes. HSV-1 sequence and plasmid/cellular DNA are represented by thin and thick horizontal lines, respectively. (*) denotes a lost restriction-enzyme site, present for positional purposes. B—*Bam*HI; S—*Stu*I; H—*Hpa*I; A—*Aat*II.

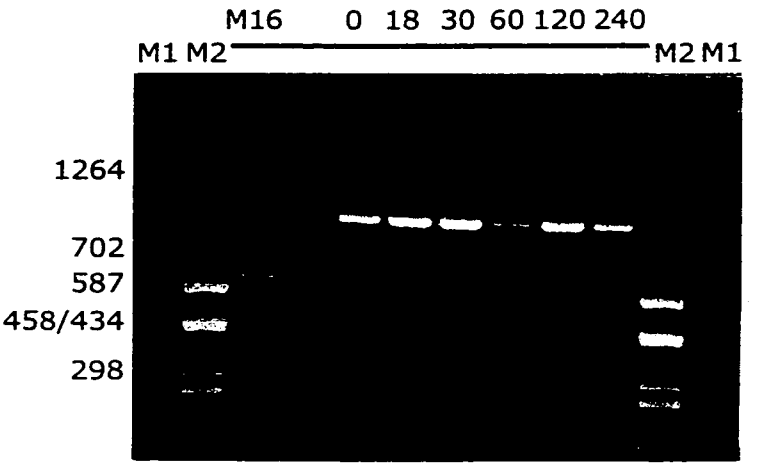

Figure 3-2. The RC variants of M16 have lost their engineered *Xho*I sites Viral DNAs were subjected to PCR amplification using HSV-1 primers R62 and R63. Post-PCR DNA was digested with *Xho*I and visualized using agarose-gel (1.4%) electrophoresis. Lanes are represented as follows: M1—*Bst*EII-digested λ DNA; M2—*Hae*III-digested pUC19 DNA; M16—High-titre–M16 post-PCR DNA; 0, 18, 30, 60, 120, 240—UV-exposed M16-RC variant post-PCR DNAs. All marker sizes are in base pairs. PCR amplification yields a 920-base pair product; digestion with *Xho*I results in a 595- and a 325-base pair product.

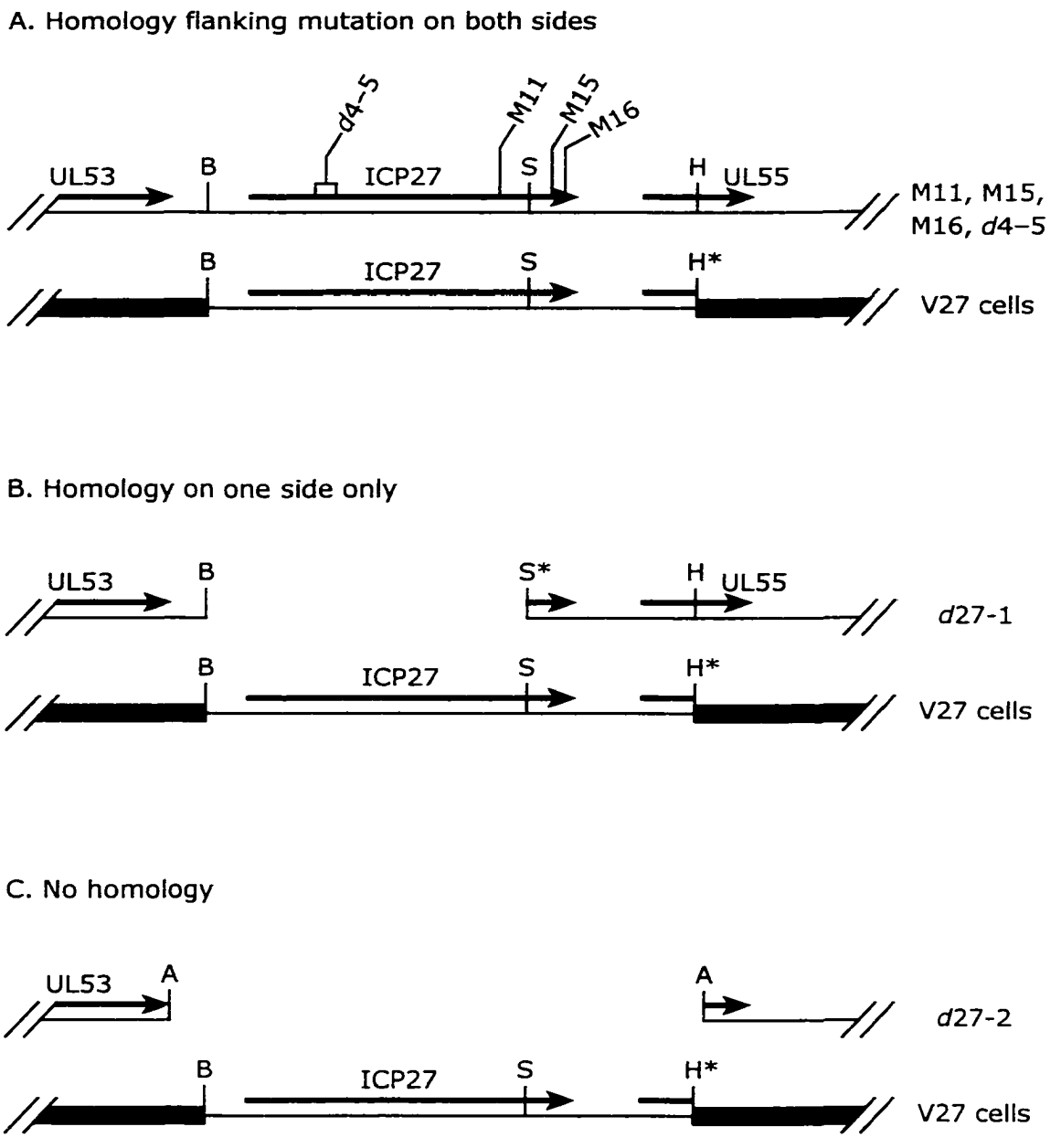

Figure 3-3. Regions of homology between viral constructs and V27 cells Illustrated are genomic schematics representing HSV-1 mutants and V27 cells. HSV-1 sequence and plasmid/cellular DNA are represented by thin and thick horizontal lines, respectively. (*) denotes a lost restriction-enzyme site, present for positional purposes. B—*Bam*HI; S—*Stu*I; H—*Hpa*I; A—*Aat*II.

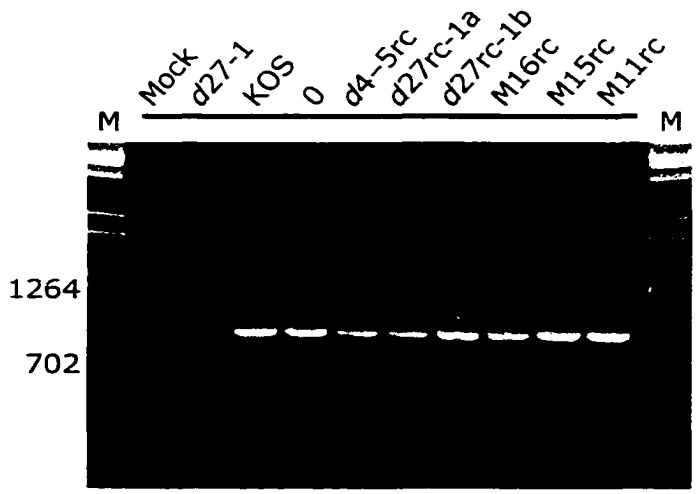

Figure 3-4. The RC variants present in HSV-1 mutant stocks contain the C-terminal-encoding section of the ICP27 allele Viral DNAs were subjected to PCR amplification using HSV-1 primers R62 and R63. Post-PCR DNA was visualized using agarose-gel (1.2%) electrophoresis. Lanes are represented as follows: M—*Bst*EII-digested λ DNA; Mock—cellular DNA only; *d*27-1—High-titre *d*27-1; KOS—High-titre KOS1.1; 0—UV-exposed M16-RC variant; *d*4-5rc, *d*27rc, M16rc, M15rc, M11rc—RC variants of *d*4-5, *d*27-1, M16, M15, and M11, respectively. All marker sizes are in base pairs. PCR amplification yields a 920-base pair product.

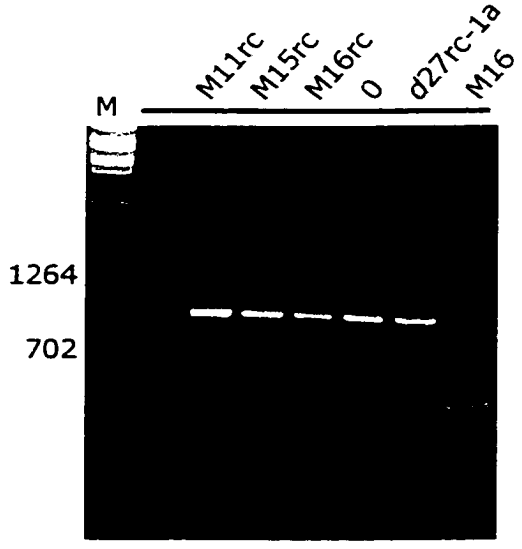

Figure 3-5. The RC variants of M11, M15, and M16 have lost their engineered *Xho*I sites Post-PCR DNA was digested with *Xho*I and visualized using agarose-gel (1.5%) electrophoresis. Lanes are represented as follows: M—*Bst*EII-digested λ DNA; M11rc, M15rc, M16rc, *d*27rc-1a—RC variants of M11, M15, M16, and *d*27-1, respectively; 0—UV-exposed M16-RC variant; M16—High-titre M16. All marker sizes are in base pairs. PCR amplification yields a 920-base pair product; digestion with *Xho*I results in a 595- and a 325-base pair product.

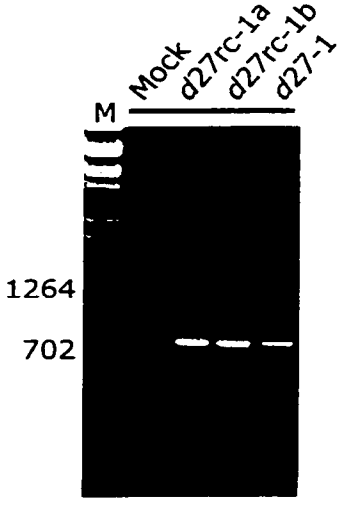

Figure 3-6. The *d*27rc-1 isolates have retained the ICP27-deletion allele Viral DNAs were subjected to PCR amplification using HSV-1 primers R51 and R63. Post-PCR DNA was visualized using agarose-gel (1.2%) electrophoresis. Lanes are represented as follows: M—*Bst*EII-digested λ DNA; Mock—cellular DNA only; *d*27rc-1a, -1b—RC isolates of *d*27-1; *d*27-1—High-titre *d*27-1. All marker sizes are in base pairs. PCR amplification across the ICP27-deletion locus yields a 757-base pair product.

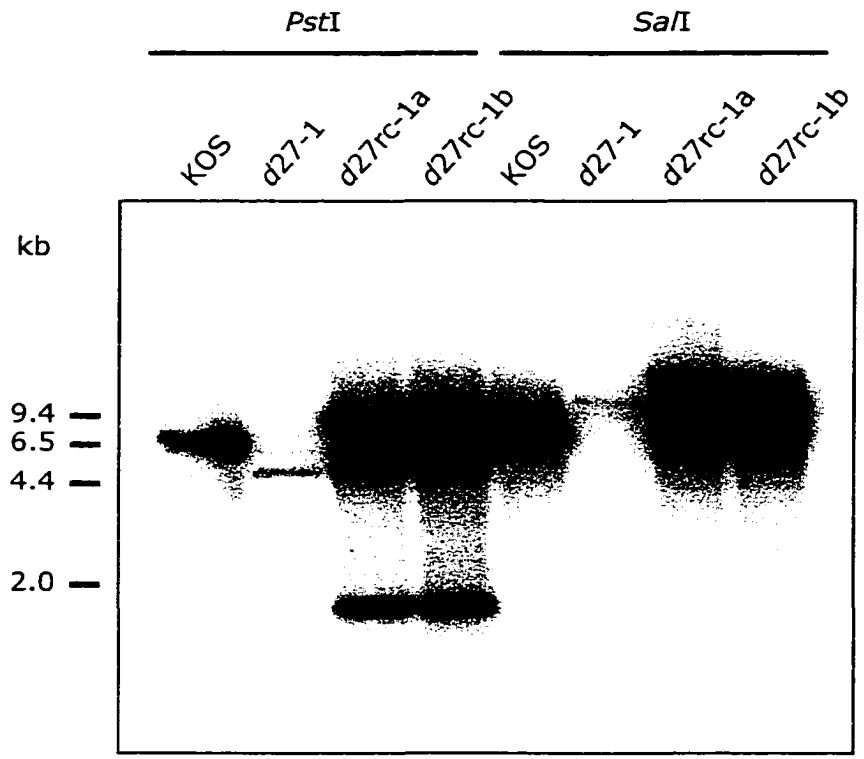

Figure 3-7. The *d*27rc isolates are genotypically distinct from both KOS and *d*27-1 Viral DNAs were digested with either *Pst*I or *Sal*I and separated on a 1% agarose gel. Following acid and alkali treatment, fragments were transferred to a nylon membrane and hybridized to ^{32}P-labeled pPs27pd1 (linearized with *Eco*RI). Shown is the resulting autoradiograph. Size markers are in kb. Lanes are represented as follows: KOS—High-titre KOS1.1; *d*27-1—High-titre *d*27-1; *d*27rc—RC isolate(s) of *d*27-1.

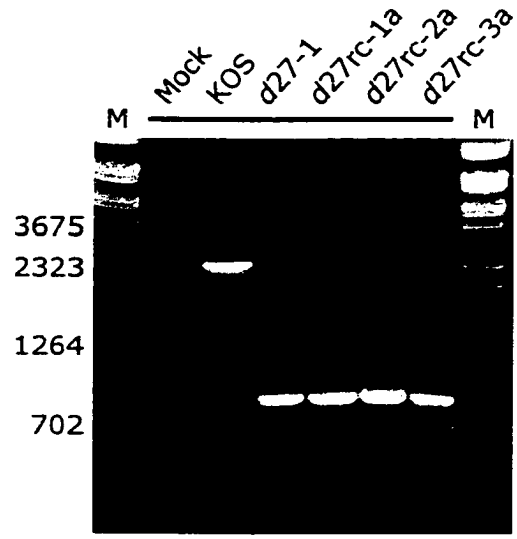

Figure 3-8. The three RC variants have retained the ICP27-deletion allele at its original locus Viral DNAs were subjected to PCR amplification using HSV-1 primers R51 and R63. Post-PCR DNA was visualized using agarose-gel (1%) electrophoresis. Lanes are represented as follows: M—*Bst*EII-digested λ DNA; Mock—cellular DNA only; KOS—High-titre KOS1.1; *d*27-1—High-titre *d*27-1; *d*27rc-1a, -2a, -3a—RC variants of *d*27-1. All marker sizes are in base pairs. PCR amplification across the ICP27 locus yields a 2382-base pair product for the wild-type allele, and a 757-base pair product for the deletion allele.

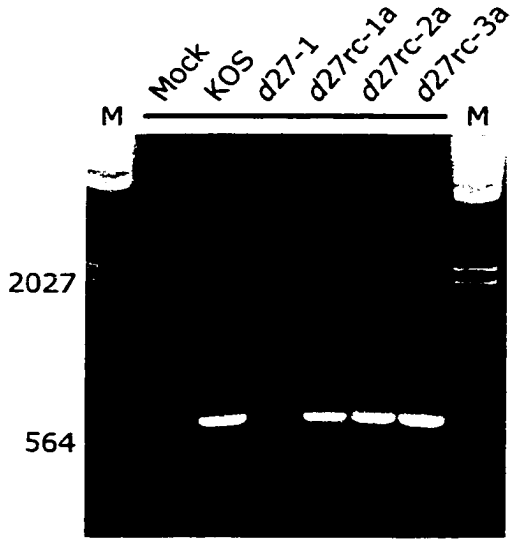

Figure 3-9. The three RC variants contain the C-terminal–encoding section of the ICP27 allele Viral DNAs were subjected to PCR amplification using HSV-1 primers R62 and R78. Post-PCR DNA was visualized using agarose-gel (1.2%) electrophoresis. Lanes are represented as follows: M—*Hin*dIII-digested λ DNA; Mock—cellular DNA only; KOS—High-titre KOS1.1; *d*27-1—High-titre *d*27-1; *d*27rc-1a, -2a, -3a—RC variants of *d*27-1. All marker sizes are in base pairs. PCR amplification yields a 643-base pair product.

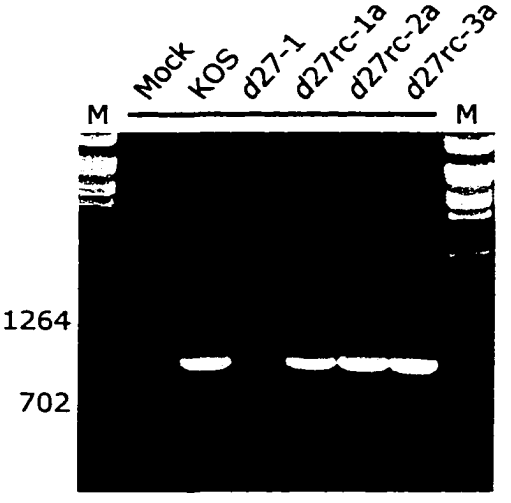

Figure 3-10. The three RC variants contain a recombined stretch of sequence from codon 289 of ICP27 to codon 9 of UL55 Viral DNAs were subjected to PCR amplification using HSV-1 primers R62 and R63. Post-PCR DNA was visualized using agarose-gel (1.2%) electrophoresis. Lanes are represented as follows: M—*Bst*EII-digested λ DNA; Mock—cellular DNA only; KOS—High-titre KOS1.1; *d*27-1—High-titre *d*27-1; *d*27rc-1a, -2a, -3a—RC variants of *d*27-1. All marker sizes are in base pairs. PCR amplification yields a 920-base pair product.

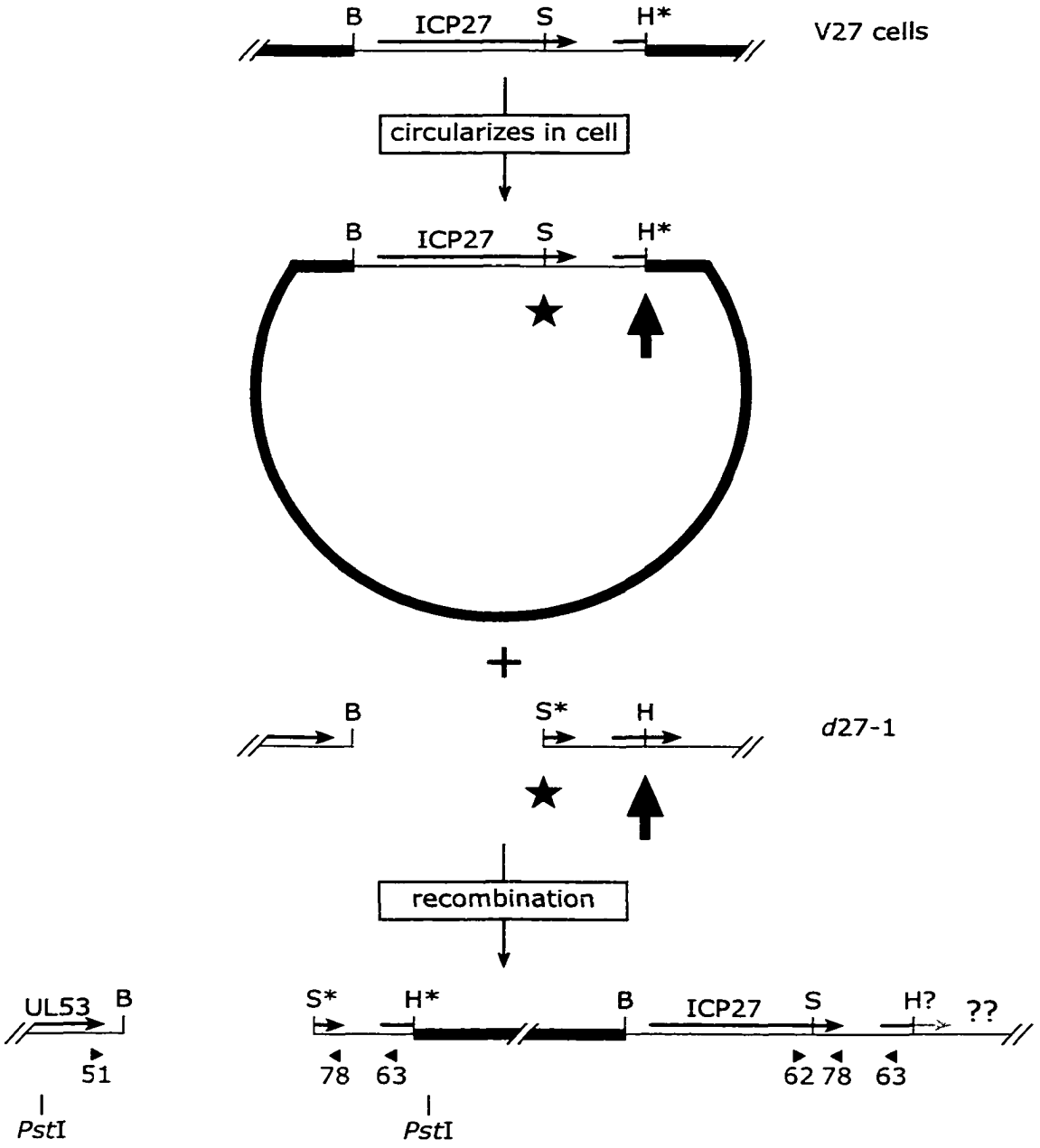

Figure 3-11. A model of recombination in tandem for *d*27rc
Proposed is a diagram illustrating the genomic structure of *d*27rc as a result of recombination between V27 cells and *d*27-1 (see text for details). HSV-1 sequence and plasmid/cellular DNA are represented by thin and thick horizontal lines, respectively. (*) denotes a lost restriction-enzyme site, present for positional purposes. B—*Bam*HI; S—*Stu*I; H—*Hpa*I. Present also are the annealing sites for various HSV-1–PCR primers (solid triangles).

4

Characterization of the HSV-1 –ICP27 Revertant M16R: Part I*

Sequence homologues of ICP27 are found not only in viruses from the three human herpesvirus classes, but also in non-human herpesviruses as well (12, 23). In fact, ICP27 has the distinction of being the only IE protein for which sequence homologues exist in all known herpesviruses (238). The portion of the protein most conserved between the various homologues is the C-terminal section of approximately 200 residues. The N-terminal half of ICP27 is not well conserved amongst herpesviruses. This close degree of conservation of the C-terminal portion of ICP27 suggests that this portion is functionally important—a supposition supported by the experimental data. Genetic manipulation (insertions, alterations, and deletions) of the C-terminal portion of ICP27 abrogates its ability to effect the activation and repression of transfected reporter genes (125, 208, 282), as well as its ability to complement the growth of ICP27 deletion viruses (208, 282). In the viral context, construction of recombinant viruses bearing ICP27 alleles containing C-terminal–codon deletions results in non-viable virus (208, 277); a number of C-terminal substitution mutations are also lethal (278).

The isolation of second-site revertants of growth-defective viruses is useful for two reasons. Firstly, in the case of point mutants, second-site

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* A version of this chapter has been published: **Bunnell, S. M., and S. A. Rice.** 2000. Journal of Virology. **74:**7362–7374.

intragenic revertants can potentially provide insights into the 3° structure of the mutated protein in question. Secondly, second-site extragenic revertants can potentially identify protein-protein interactions. Our laboratory was interested in isolating second-site revertants of the growth-defective viruses M11, M15, and M16 (278). These viruses are characterized by codon substitutions in the C-terminal half of ICP27, which inactivate protein function. By selecting for growth in non-complementing cells, a second-site revertant of M16—designated M16R—was isolated; characterization revealed that M16R contains both an intragenic reversion and one or more extragenic mutations. This chapter addresses the nature of the intragenic reversion. Contribution(s) by the extragenic mutation(s) to M16R's phenotype are presented in the following chapter (Chapter 5).

M16R contains an intragenic reversion

As mentioned in Chapter 3, second-site revertants had never been observed to have spontaneously arisen in our high-titre stocks. Because of the presence of RC virus in our high-titre stocks, generated as a result of recombination with the V27-cell genome, it was not practical to subject any high-titre stocks to a revertant-screening procedure. Instead, six plaques of M16 were individually isolated from a V27-cell infection. The probability that any one of these six plaques contained RC virus was extremely small. Virus contained within the plaques was liberated through three rounds of freeze-thawing, and a small number of Vero cells were infected with the plaque lysates. After a 48-hour period, the infections were terminated by freeze-thawing, and a portion of the resulting lysates was used to infect another set of flasks containing Vero cells. Growth-competent virus (M16R) appeared within one of the Vero flasks three dpi. Selection for growth in non-complementing cells produced a revertant surprisingly easily considering that the amount of virus screened was small (six plaques of M16, each plaque containing on the order of 10^3–10^4 PFU).

M16 cannot grow in Vero cells because of a codon-substitution mutation at position 488 of ICP27 (278). The introduction of a *Xho*I site altered the native-cysteine codon at this position to a leucine codon (Figure 4-1C). As cysteine residues can be important in the maintenance of the 3° structure of a protein, it is relatively simple to envision how such a substitution could affect protein functionality. It was possible that the growth-competency observed was the result of a primary reversion event. If true, then M16R would have lost the *Xho*I site. To examine this possibility, the M16R virus was plaque purified in Vero cells and its genome was subjected to PCR amplification along with viral DNA from KOS and M16 infections. The region amplified was a C-terminal–codon stretch of ICP27, inclusive of the *Xho*I site.

Digestion of the PCR products with *Xho*I revealed that M16R had retained the *Xho*I site (data not shown). It was concluded that the virus is therefore the result of second-site reversion(s).

To address whether or not M16R contains an intragenic reversion, the ICP27 allele from M16R was cloned into pUC19—yielding the plasmid pM16R27—and tested for its ability to complement the growth of *d*27-1 (Figure 4-1B) (277, 279). Duplicate samples of Vero cells were transfected with either pUC19 (None), p27 (WT), pM1627 (M16), or pM16R27. One day later, the cells were infected with *d*27-1 and allowed to incubate for approximately 24 hours. Infections were terminated by subjecting the cells to freeze-thaw cycles, and *d*27-1 titres were determined using complementing V27 cells. Figure 4-2 shows the result of the complementation experiment. Wild-type ICP27 (WT), expressed in *trans*, is able to complement the growth of *d*27-1 approximately five logs greater than no ICP27 (None). As was expected, M16 ICP27 was unable to complement (278). However, transfection of the M16R-ICP27 allele resulted in a significant level of complementation—approximately three logs greater than the complementation observed by the M16-ICP27 protein—indicating that M16R does indeed contain an intragenic reversion.

The intragenic reversion of M16R is a base addition

In order to identify the intragenic reversion, the entire M16R-ICP27 allele was sequenced. A point reversion was expected, given the point-mutation nature of the parent. Unexpectedly, the only deviation that was found from the M16 sequence is an extra cytosine base within an existing run of cytosines at codons 215–217. A stretch of eight cytosines (WT and M16) has become a stretch of nine cytosines for the M16R-ICP27 allele (Figures 4-1A, 4-1D, and 4-3A). As a result, starting at codon 218, 72 new amino acids are coded for, ending with a stop codon at position 290 (Figure 4-3B).

M16R produces a truncated ICP27 protein

To confirm that M16R expresses a truncated ICP27 protein during the lytic cycle, western blot analysis was performed. ICP27 production was examined at four hpi for KOS, M16, and M16R infections in Vero cells. Total protein, which had been separated on a 15% SDS-polyacrylamide gel, was transferred to a nitrocellulose membrane and probed with the monoclonal antibody H1113; H1113 recognizes residues 109–137 of ICP27 (209). H1113-bound ICP27 was visualized using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system (Figure 4-4). As expected, KOS and M16 produce ICP27 proteins approximately 63 kDa in mass (2). M16R produces an ICP27 protein

approximately 38 kDa in mass, consistent with the highly truncated ICP27 predicted by sequence analysis to be produced by M16R.

The truncated protein is strongly nucleolar in its localization

The intracellular localization of the ICP27 proteins produced by KOS, M16, and M16R during infection was examined. Vero cells were infected with the three viruses, and at four hpi were processed for indirect immunofluorescence using H1113 and a rhodamine-conjugated secondary antibody (Figure 4-5). During a KOS infection, ICP27 is found predominantly in the nucleus of the infected cell (277); the pattern of staining is speckled in appearance, and is often referred to as punctate (253). A low level of diffuse cytoplasmic staining is also observed. In the case of infection with M16, the localization pattern of ICP27 is altered from the wild-type–localization pattern in two distinct ways: i) there is a negligible amount of cytoplasmic staining; and, ii) ICP27 is excluded from the nucleolus of the infected cell. Nucleolar-exclusion is assessed based upon a corresponding phase-contrast field, not presented in the figure. M16 is growth-defective in Vero cells. Other ICP27 mutants exhibiting a similar localization pattern of nucleolar-exclusion are also growth-defective (209, 277). Although the functional significance of nucleolar-exclusion is unknown, it is possible that ICP27 plays an essential role in the post-transcriptional modification of viral transcripts in the nucleolus, or that it facilitates its shuttling function through a nucleolar partnership.

Infection with M16R produces an ICP27-localization profile dramatically different from the localization profiles observed for KOS and M16 infections. The 289-amino acid protein produced by M16R, in stark contrast to the M16-localization profile, is strongly nucleolar in its localization during infection. One other ICP27 mutant, the C-terminal–truncation virus *n*263R, exhibits a similar intracellular localization profile (277). Therefore, the frameshifted amino acids, which comprise the new C-terminus of the ICP27 protein produced by M16R, do not appear to effect the localization of the protein to the nucleolus of the infected cell. It is possible that the truncation of ICP27 to a protein approximately half-the-size of the wild-type protein enhances the ability of ICP27 to interact with rRNA and ribonucleoprotein particles present in the nucleolus. The two viruses, however, are the antithesis of one another with respect to growth-competency; M16R can grow in Vero cells, whereas *n*263R cannot (277).

Figure 4-3C presents a comparison of the percent content of arginine and glycine residues present from codon 217 to end for both wild-type and M16R ICP27 proteins. The novel stretch of 72-amino acids coded for by M16R is extremely arginine- and glycine-rich and resembles the RGG-box region

located in the N-terminal section of the protein (codons 139–153). It has been shown that the RGG box of ICP27 mediates a RNA-binding activity (209, 211, 299). If indeed ICP27 performs an essential RNA-based function within the infected cell, then perhaps the ICP27 protein produced by M16R effects that function in a heightened capacity. This heightened function may partially substitute or replace the regulatory activity associated with the normal C-terminal half of ICP27.

Only an extra cytosine is required for complementation

Sequence analysis of the cloned M16R-ICP27 allele revealed only a single change from the parental M16-ICP27 allele: a run of eight cytosine bases at codons 215–217 for M16 has become a run of nine cytosines for M16R. In order to verify that an extra cytosine alone is responsible for M16R's complementation phenotype, an extra cytosine was introduced into both M16- (yielding the plasmid pM1627exC) and wild-type– (yielding the plasmid p27exC) ICP27 alleles. These new alleles (Figures 4-1E and 4-1F) were tested then for their ability to complement the growth of *d*27-1. Duplicate samples of Vero cells were transfected with either pUC19 (None), p27 (WT), pM1627 (M16), pM16R27 (M16R), pM1627exC, or p27exC. One day later, the cells were infected with *d*27-1, and after allowing for a single replication cycle, were subjected to three freeze-thaw cycles. Resulting *d*27-1 progeny were titred using V27 cells (Figure 4-6). The data show that the new alleles can complement a *d*27-1 infection to the same levels as the original M16R allele, and therefore indicate that an extra cytosine is the only intragenic reversion required for M16R's complementation phenotype.

The truncated ICP27 protein is functional

M16R produces a truncated ICP27 protein. It is possible that the truncated ICP27 is functional and is itself responsible for the observed *d*27-1 growth complementation. However, it is also possible that a modicum of C-terminal–bearing ICP27 is being produced via an alternate mechanism, such as translational frameshifting (139), and that this re-coded protein accounts for the growth complementation. To address the question of whether the truncated ICP27 protein of 289 amino acids is itself unequivocally complementation-competent, codons 306–512 were deleted from an extra-cytosine-containing allele (Figure 4-1G). This new allele (contained within the plasmid pexCd305) was tested then for its ability to complement the growth of *d*27-1. Duplicate samples of Vero cells were transfected with either pUC19 (None), p27 (WT), p27exC (WTexC), or pexCd305. One day later, the cells were infected with *d*27-1, and after allowing for a single replication cycle, were subjected to three freeze-thaw cycles. Resulting *d*27-1 progeny were

titred using V27 cells (Figure 4-7). The data show that the exCd305-deletion allele can complement a *d*27-1 infection to the same levels as the non-deletion allele. Therefore, the truncated ICP27 protein produced by M16R is functional and accounts for the observed *d*27-1 growth complementation.

The novel 72-amino acids are required for protein function

The ICP27 protein produced by M16R is composed of 217 wild-type–amino acids, followed by a novel 72-amino acid C-terminal section. To determine if this novel stretch of amino acids contributes to protein function, a stop codon was engineered at position 218 of the exCd305-deletion allele (yielding the plasmid pn217d); this new allele (Figure 4-1H) was tested for its ability to complement the growth of *d*27-1. Duplicate samples of Vero cells were transfected with either pUC19 (None), p27 (WT), pexCd305 (exCd305), or pn217d. One day later, the cells were infected with *d*27-1 and incubated for a single replication cycle. Resulting *d*27-1 progeny, released through freeze-thawing, were titred using V27 cells (Figure 4-8). The data reveal that the *n*217d allele is only marginally better then pUC19 in effecting the production of *d*27-1 virus—it is approximately two logs reduced in its ability to complement in comparison to the exCd305 allele, which produces the 289-amino acid truncated ICP27 protein. Therefore, the data support the conclusion that the novel stretch of 72 amino acids is required for protein function.

Each recombinant virus produces its appropriate ICP27 protein

The truncated ICP27 protein of 289 amino acids, produced by ICP27 alleles containing an extra cytosine at position 215–217, can complement the growth of *d*27-1; the novel stretch of amino acids is required for this complementation. To assess the functionality of both the 289-amino acid and the 217-amino acid (produced by the *n*217d allele) ICP27 protein in the viral context, three HSV-1 recombinant viruses were created. ICP27 alleles M16exC, exCd305, and *n*217d, contained on *Bam*HI–*Sac*I viral DNA fragments, were cloned from their respective transfection plasmids into the plasmid pPs27pd1. Plasmid pPs27pd1 is useful for the creation of recombinant ICP27 viruses because of the fact that cloned ICP27 alleles are flanked by relatively large regions of viral DNA. The resulting plasmids, pPsM1627exC, pPsexCd305, and pPsn217d, were employed then in a homologous recombination procedure with *d*27-*lacZ*1 DNA. *d*27-*lacZ*1 is a HSV-1 mutant that contains an ICP27-*lacZ* fusion gene in the place of the wild-type–ICP27 gene (27). Two independent isolates for each new recombinant were obtained and plaque purified in V27 cells, yielding the viruses M16exC, exCd305, and *n*217d. Extensive PCR characterization was

performed on viral DNA isolated from the plaque purified viruses (data not shown). This characterization determined the following for the exCd305 and *n*217d viruses: i) the allele of interest is present in the viral genome; ii) the allele is located at the ICP27 locus; iii) no wild-type–ICP27 allele is present. PCR characterization of M16exC determined that a wild-type–length allele is present at the ICP27 locus, and sequence analysis of the allele determined that the allele is the M16exC variant.

The M16exC and exCd305 viruses possess the same extra-cytosine addition in their ICP27 alleles as M16R does, and therefore were predicted to produce ICP27 proteins identical to the one produced by M16R. The recombinant *n*217d was predicted to produce a less massive ICP27 protein because of the stop codon present at position 218. To verify that the new recombinant viruses produce ICP27 proteins of the correct apparent molecular weight, Vero cells were infected with either KOS, *d*27-1, M16R, M16exC, exCd305, or *n*217d. Six hpi, total protein was isolated, separated on a 15% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Detection of ICP27 was effected through incubation with the monoclonal antibody H1113, followed by incubations with a horseradish peroxidase-conjugated secondary antibody and reagents from an enhanced chemiluminescence system (Figure 4-9). All three HSV-1 recombinants produce ICP27 proteins of expected apparent molecular weight. The pattern of faster mobility bands present in addition to the primary protein product for KOS, M16R, M16exC, exCd305, and *n*217d, is likely the result of degradation of the full-length ICP27 produced by each respective virus. Faster mobility bands have been observed previously (282), but are often undetected on ICP27 immunoblots (Figure 4-4) (277, 279).

The HSV-1–recombinant viruses are growth-defective in Vero cells

High-titre stocks of M16exC, exCd305, and *n*217d were prepared using V27 cells. V27 cells are used to propagate ICP27-mutant viruses as expression of the cellular ICP27 protein alleviates any potential replication-defective phenotype. This allows for high viral titres of the desired mutant to be obtained. Virions produced using V27 cells maintain their mutant identity, as the ICP27 protein supplied in *trans* during cellular infection does not become encapsulated in the viral tegument (387), unlike some of the other IE proteins (387, 388).

The ability of the HSV-1 recombinant viruses to grow in Vero and V27 cells was examined and compared to M16R, KOS, and *d*27-1. Cells, which had been infected with serial dilutions of the viruses, were screened for the presence of viral plaques four dpi. The resulting titres for each virus are shown in Table 4-1. KOS, as expected, is able to replicate equally well in

both Vero and V27 cells (277). The ICP27-deletion mutant, *d*27-1, is unable to grow in Vero cells (titres below 10^3 cannot accurately be presented as low serial dilution infections destroy the cell monolayer). M16R, like KOS, is able to replicate equally well in both Vero and V27 cells, but produces slightly smaller plaques on Vero cells than KOS. The three recombinant viruses are unable to form plaques on Vero cells; all are able to form small foci of infected cells, but the number of foci produced for each virus is low and difficult to count. The data from Table 4-1 clearly show that the production of the truncated ICP27 protein is insufficient for growth-competency.

The inability of M16exC and exCd305 to grow in Vero cells was surprising, given the fact that both of these viruses contain the same intragenic reversion as M16R does. Both alleles are quite functional in transient assays complementing the growth of *d*27-1, yet are not able to effect growth-competency in the wild-type background. One possible explanation for these seemingly incongruent observations stems from the likelihood that protein expression from a plasmid in the complementation assays is much greater than protein expression in a genomic background—plasmid expression is not regulated by the same *cis*-acting environment that governs genomic expression. In addition, there are more than likely multiple plasmid copies present within the transfected cell, allowing for abnormally high expression levels. Higher expression levels could compensate, in a growth-complementing fashion, for a protein that is normally only moderately functional.

Viral plaque morphologies on Vero cells at four dpi are presented in Figure 4-10. Plaques (or foci) selected to be photographed were chosen from a large population at random, and are representative of the general plaquing phenotype of that virus. The boundary of the KOS plaque is located just beyond the field of view; a KOS plaque on Vero cells is approximately twice the size of a M16R plaque. Infection with M16exC (not shown) produces foci identical in morphology to those produced by exCd305.

M16R contains one or more extragenic mutations

The inability of M16exC and exCd305 to grow in Vero cells, in contrast to M16R's growth-competency in Vero cells, suggests that M16R—in addition to its intragenic reversion—possesses one or more extragenic mutations. To investigate this possibility further, M16R and the recombinant viruses were subjected to a virus-yield assay. This assay measures the production of viral progeny through the course of a single replication cycle. Duplicate samples of Vero and V27 cells were infected with either KOS, *d*27-1, M16R, M16exC, exCd305, or *n*217d. V27 cells are infected, in addition to Vero cells, to demonstrate that equal multiplicities of infection have been applied to the

cells and that, in the case of engineered ICP27 mutants, extragenic mutations are not contributing to the phenotype. After allowing for a single replication cycle, viral progeny were released by multiple freeze-thaws and titred using V27 cells (Figure 4-11). Data are presented as virus yield (in PFU per infected cell) for each virus tested. In Vero cells, KOS produces a virus yield of approximately 100 PFU per cell, M16R produces a virus yield of approximately 1 PFU per cell, M16exC and exCd305 produce virus yields of approximately 0.01 PFU per cell, and *d*27-1 and *n*217d produce virus yields of approximately 0.0001 PFU per cell. There are a number of conclusions that can be drawn from the data. Firstly, M16R, as expected, is not able to grow as well as KOS in the infected cell; it is not known whether this is a function of the inability to successfully replicate its genome, or whether assembly of functional progeny has been affected. Secondly, the two-log difference in virus yield between M16R and M16exC (and exCd305) must be due to the presence of one or more extragenic mutations within the M16R genome. Thirdly, even though the M16exC and exCd305 viruses only produce 0.01 PFU per cell (note that it is impossible to produce one one-hundredth of an infectious particle, and so this number possibly reflects the production of many progeny by only a few of the infected cells), this is a great deal more than the virus yield achieved with the *d*27-1 infection. Therefore, within the viral context, the truncated ICP27 protein of 289-amino acids has some functionality. Fourthly, the aforementioned functionality is entirely dependent upon expression of the frameshifted amino acids—truncation of ICP27 to a protein only 217-amino acids in size (the *n*217d infection) is as detrimental to the production of infectious progeny as not producing any ICP27 protein at all.

The novel 72-amino acids stimulate viral DNA replication

One possible explanation for the inability of the recombinant viruses to grow in Vero cells is that they are unable to replicate their DNA within the infected cell. Some C-terminal–truncation mutants that are growth-defective are unable to efficiently replicate their viral DNA (277, 356). Is this the case for the recombinant viruses? To address this question, Vero cells were infected with KOS, *d*27-1, exCd305, or *n*217d. At various time points, viral DNA was isolated. An equal mass of total DNA for each time point was digested for each virus with *Pst*I and *Sal*I, and viral fragments were separated on a 1% agarose gel. Following acid and alkali treatment, fragments were transferred to a nylon membrane and hybridized to a radiolabeled DNA probe. DNA for the probe was generated using PCR amplification and HSV-1 primers R66 and R99; these primers amplify a majority of the UL55 gene. Shown in Figure 4-12a is the resulting

autoradiograph. Hybridization signal intensities for each virus were quantified by phosphorimage analysis (Figure 4-12b). The ability of each virus to replicate its DNA through the course of 24 hours is presented as a fold-DNA-replication value for each post-one hour harvest-time point. In comparison to KOS, *d*27-1 replicates its viral DNA poorly (approximate 25-fold reduction in ability). This result is consistent with published viral DNA-replication data (277-279, 356). Surprisingly, exCd305 is able to replicate its DNA to levels comparable to those of KOS (although it is somewhat delayed in the onset of DNA replication—compare eight-hpi values). This suggests that the growth-defective phenotype displayed by exCd305 (and most likely by M16exC too) is not a function of an inability to replicate viral DNA. It is interesting that the novel stretch of 72-amino acids coded for by exCd305 is required to replicate DNA efficiently—*n*217d displays a marked reduction (approximately eight fold) in its ability to replicate viral DNA. The ICP27-truncation mutant *n*263R, which replicates its viral DNA at similar levels to *n*217d (277, 356), is DNA-replication deficient due to an inability to stimulate a subset of the DE genes responsible for DNA replication (356). It is possible that exCd305 is able to replicate its DNA efficiently because the frameshifted amino acids are able to effect, either directly or indirectly through the modulation of the activity of another *trans*-activating protein (such as ICP4), *trans*-activation of the DE genes responsible for DNA replication.

Synopsis

Little is known regarding both the intra- and intermolecular protein interactions that define ICP27. In order to gain insight into these interactions, the growth-defective ICP27-mutant virus M16 was subjected to a revertant-screening procedure. A revertant of M16 was identified based upon its ability to grow in Vero cells, and was successfully isolated and propagated. Cloning and sequence analysis of the ICP27 allele of the new revertant, designated M16R, revealed that its reversions are second-site in nature. Knowing this, the ICP27 allele of M16R was tested for its ability to complement the growth of the ICP27-deletion virus, *d*27-1. The allele was able to complement the growth of the deletion virus to a significant degree, indicating that it contains a second-site intragenic reversion. Sequence analysis identified the intragenic reversion as a cytosine-base addition at codon position 215–217 of the ICP27-coding region—this addition increased the existing stretch of cytosines from eight to nine.

The +1 frameshift defining the ICP27 allele of M16R predicted the production of a highly truncated protein of 289-amino acids (the wild-type protein consists of 512-amino acids). The amino-acid stretch coded for by the frameshift (72-amino acids) is arginine- and glycine-rich and resembles

ICP27's RGG box (209). Immunoblot analysis of the ICP27 protein produced by M16R confirmed that the virus produces a highly truncated ICP27 protein. Examination of the intracellular localization of the truncated protein during infection revealed a strongly nucleolar localization profile, similar to another C-terminal–truncation virus, *n*263R (277).

A series of additional plasmid-based complementation assays were performed to further characterize the intragenic reversion. These assays evinced that the extra cytosine is the only intragenic reversion required for the M16R phenotype, that the truncated protein is itself functional, and that the 72-amino acids comprising the protein's C-terminus are required for *d*27-1–growth complementation.

When alleles coding for the truncated protein were introduced into the ICP27 locus (in the wild-type background), the resulting viruses (M16exC and exCd305) were found to be growth-defective in Vero cells, despite producing ICP27 proteins of the correct apparent molecular weight. M16exC and exCd305, when compared to M16R and KOS, were unable to form plaques on Vero cells, and instead produced small foci of infected cells. Suspected presence of one or more extragenic mutations within the M16R genome was validated when M16R displayed a significantly more robust single-cycle–viral yield than both M16exC and exCd305. These two viruses, however, were able to produce much more progeny than *n*217d, a virus able to express an ICP27 protein only 217-amino acids in size. This indicated that the frameshifted amino acids confer a limited, but measurable, degree of functionality to the ICP27 protein in the viral context. This conclusion is in agreement with that derived from the complementation data.

Insight into the *modus operandi* of the truncated protein was provided by an analysis of its ability to effect viral DNA replication. The virus exCd305 was found to be able to replicate its DNA at levels comparable to those of KOS, indicating that the block in its achievement of growth-competency lies outside of the province of DNA replication. *n*217d demonstrated replication levels approaching those of *d*27-1.

The truncated ICP27 protein has function, but it is not enough to effect the level of growth-competency displayed by M16R. It is believed that the truncated protein acts in concert with one or more extragenic mutations within M16R to produce a growth-competent virus, but what function(s) can be ascribed to the truncated protein itself? It is possible that the novel amino acids have a definable function themselves, perhaps substituting (poorly) for regulatory tasks that had been the domain of the now absent C-terminal half of the protein (23, 124, 125, 208, 210, 277, 278, 282, 301, 302). It is also possible that the novel amino acids have no inherent function, but simply act to stabilize a functional N-terminal section (279). Interestingly, the novel 72-

amino acid stretch is rich in arginine and glycine residues, and resembles ICP27's known RNA-binding domain (the RGG box), located in the N-terminal half of the protein. Therefore, the novel amino acids may effect functionality through an enhancement of the protein's purported post-transcriptional activities (23, 126, 205, 207, 253, 255, 299, 301, 302, 325).

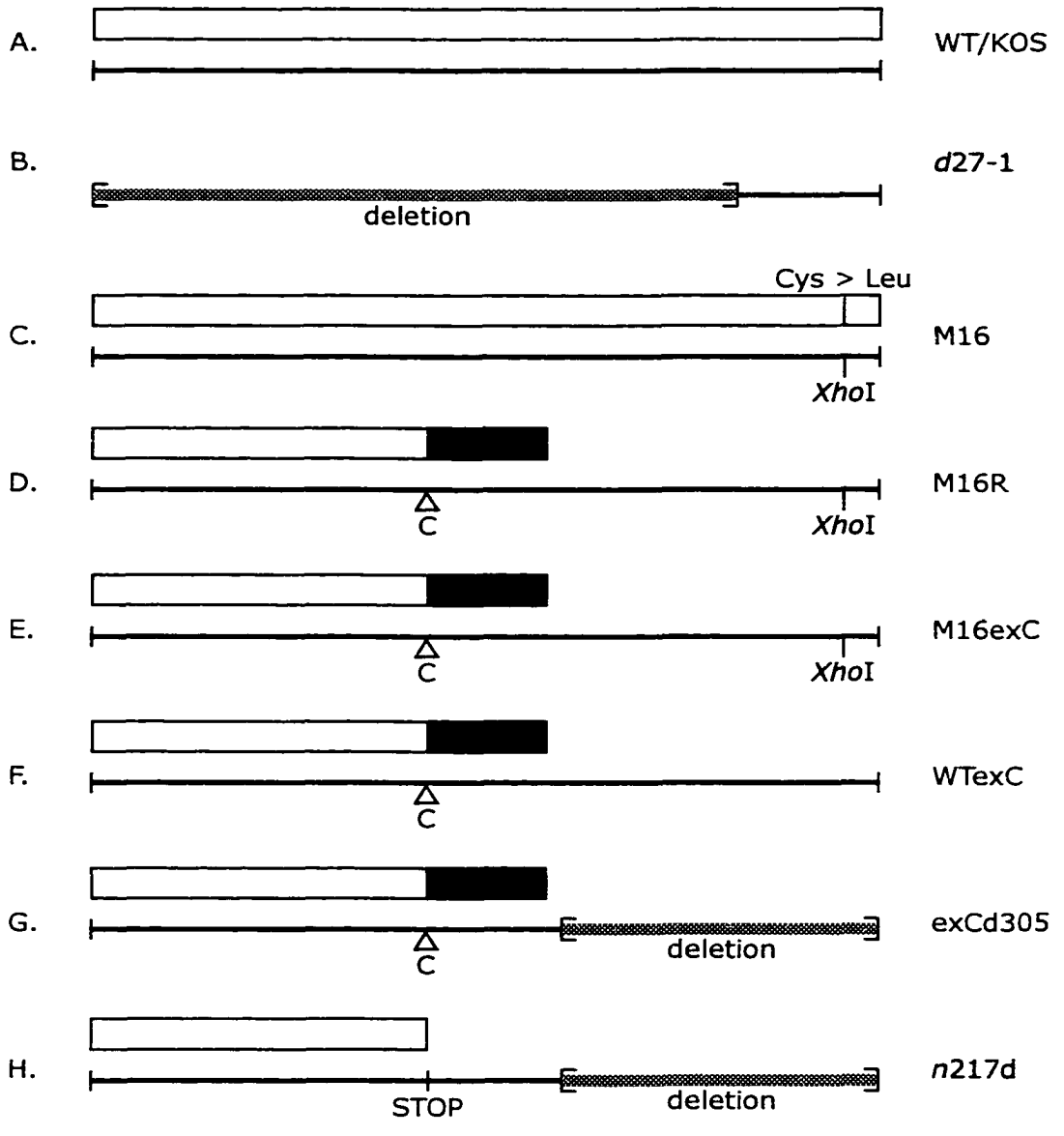

Figure 4-1. ICP27 alleles Presented is an overview of the various ICP27 alleles (lines) and their respective protein products (bars). White bars denote wild-type–protein sequence; black bars denote frameshift sequence translated from the +1 reading frame. C—cytosine-base insertion.

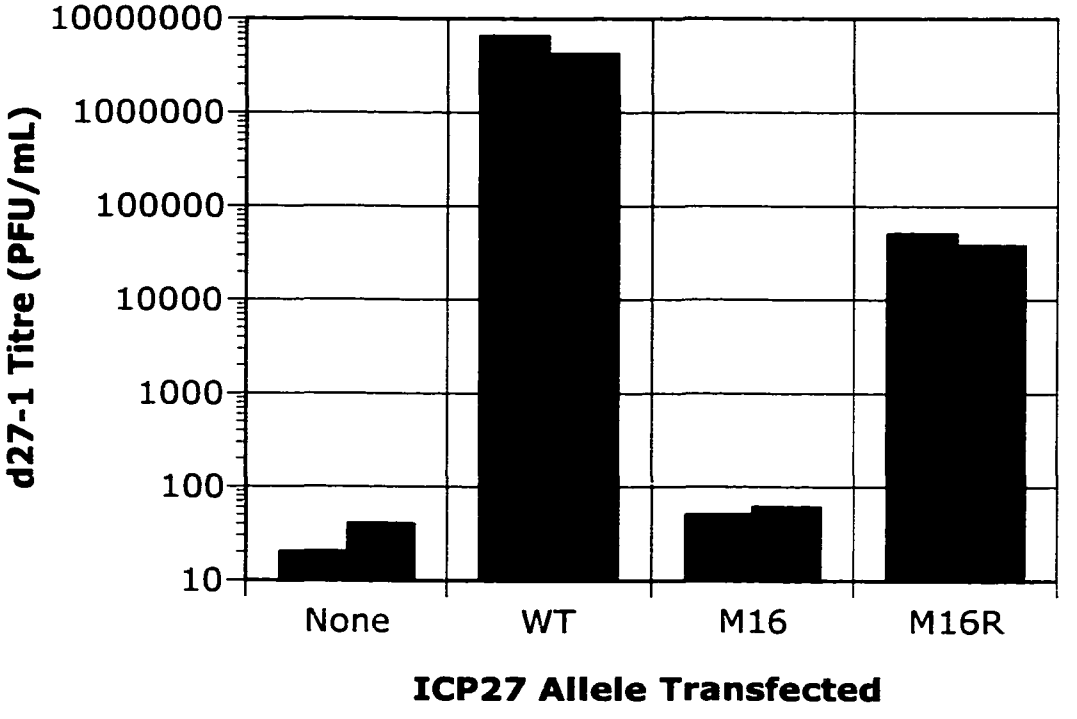

Figure 4-2. The M16R allele can complement the growth of *d*27-1
Vero cells were transfected in duplicate either with pUC19 (None), or with pUC19 containing an ICP27 allele. One day later, the cells were infected with *d*27-1. After allowing for a single replication cycle, the cells were subjected to three freeze-thaw cycles and *d*27-1 progeny were titred using V27 cells.

A. Frameshift mutation of M16R

	214	215	216	217	218
-GCA	CCC	CCC	CCG	CTA-	WT
Ala	Pro	Pro	Pro	Leu	
-GCA	CCC	CCC	CCC	GCT-	M16R
Ala	Pro	Pro	Pro	Ala	

B. Amino-acid sequence of frameshifted region

218

- ANDAGDCPPARGPPR
PGPGAKGARRRHHRR
HHAVGPALHLRARGG
RPHQRELRPQRTGHA
RPLWGAAVSRRE

289

C. Analysis of the percent content of arginine and glycine residues

		Allele	
		WT	M16R
# of residues from position 217 to stop	:	295	72
% content of arginine and glycine residues	:	15	**36**

Figure 4-3. The M16R-frameshift mutation predicts the expression of a highly truncated, arginine- and glycine-rich ICP27 protein (A) The insertion of an extra cytosine into the M16R-ICP27 allele at codons 215–217 results in the alteration of the encoded protein at residue 218; (B) a novel stretch of 72 amino acids (shown in single letter code), and a pre-mature truncation of the protein at residue 289, result from the frameshift; (C) the novel stretch of 72 amino acids is extremely arginine- and glycine-rich.

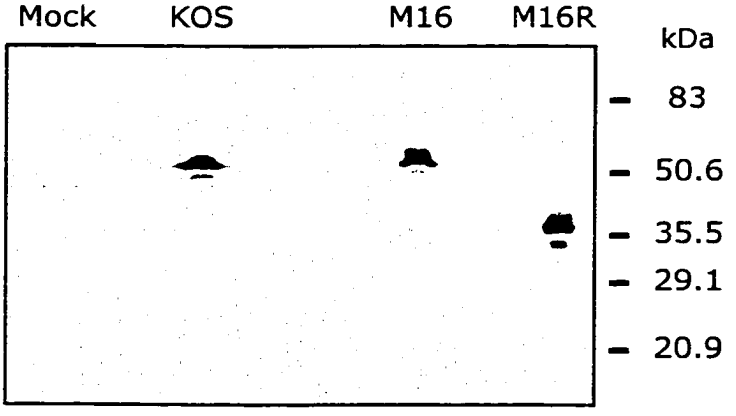

Figure 4-4. M16R produces an ICP27 protein with a mobility consistent with that expected for a truncated protein of 289 residues
Vero cells were either mock infected, or infected with KOS, M16, or M16R. At four hpi, total protein was isolated and subjected to immunoblotting using H1113. Size markers are listed in kDa. The apparent molecular weight for the ICP27 protein produced by KOS and M16 is 63 kDa; a protein of approximately 38 kDa is produced by M16R.

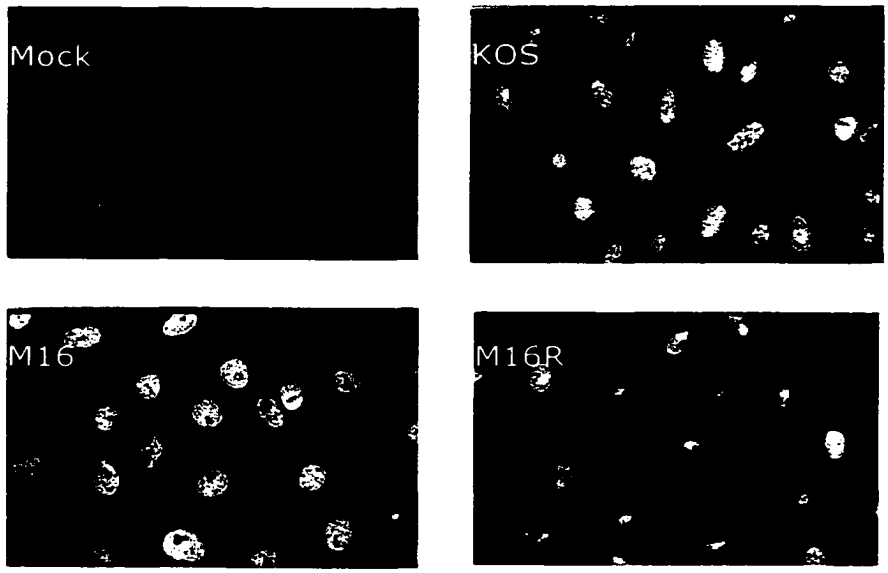

Figure 4-5. The truncation of ICP27 leads to a dramatic shift in its cellular localization during infection Vero cells were either mock infected, or infected with KOS, M16, or M16R. At four hpi, the cells were processed for immunofluorescence using H1113. The highly truncated ICP27 protein produced by M16R localizes preferentially to the nucleolus of the infected cell. This is strikingly different from the localization profile of the wild-type protein (punctate nuclear) and the M16 variant (nucleolar-exclusion).

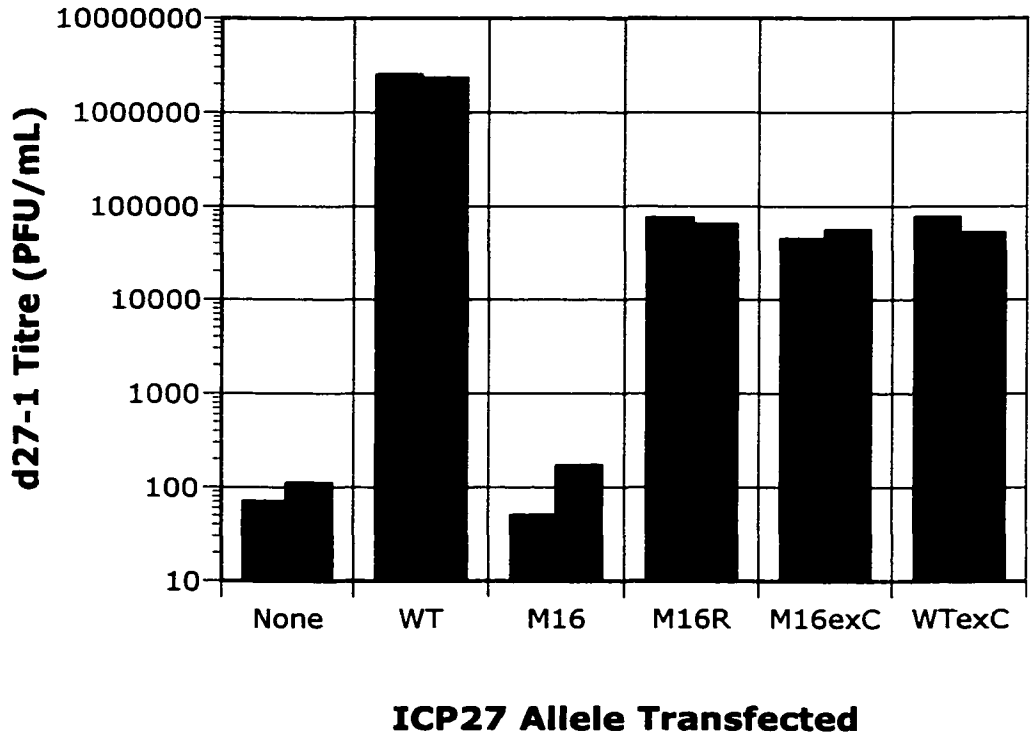

Figure 4-6. Engineered ICP27 alleles containing an extra cytosine can complement the growth of *d*27-1 to the same levels as the M16R allele Vero cells were transfected in duplicate either with pUC19 (None), or with pUC19 containing an ICP27 allele. One day later, the cells were infected with *d*27-1. After allowing for a single replication cycle, the cells were subjected to three freeze-thaw cycles and *d*27-1 progeny were titred using V27 cells.

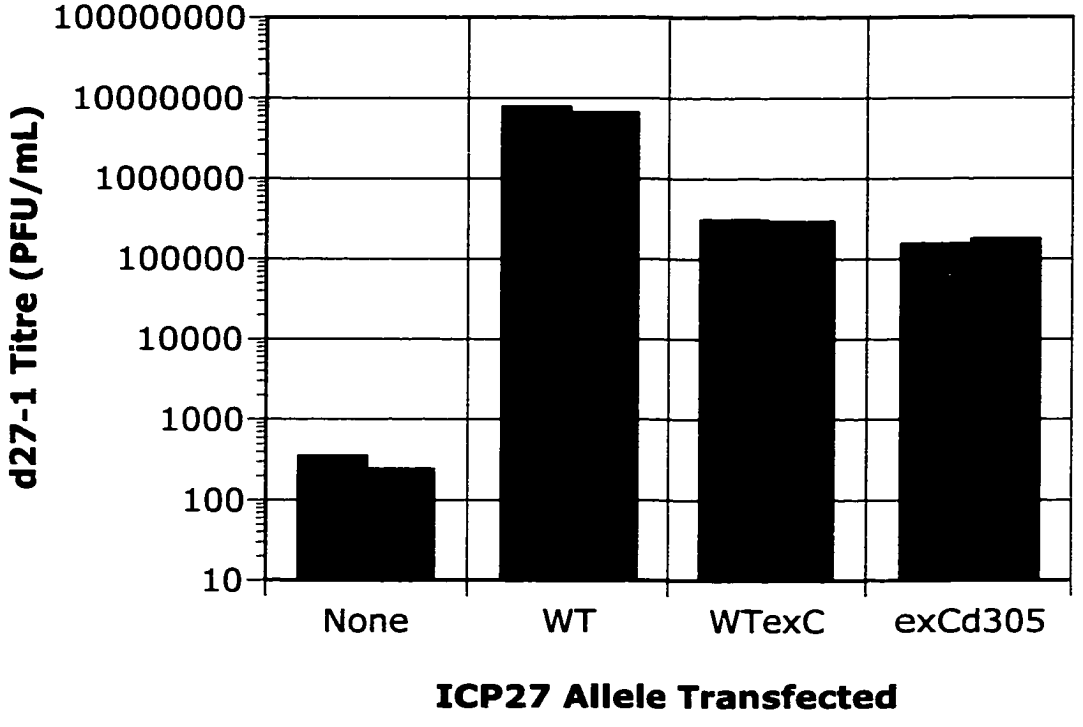

Figure 4-7. Deletion of codons 306–512 of ICP27 has no effect on the ability of an engineered ICP27 allele containing an extra cytosine to complement the growth of *d*27-1 Vero cells were transfected in duplicate either with pUC19 (None), or with pUC19 containing an ICP27 allele. One day later, the cells were infected with *d*27-1. After allowing for a single replication cycle, the cells were subjected to three freeze-thaw cycles and *d*27-1 progeny were titred using V27 cells.

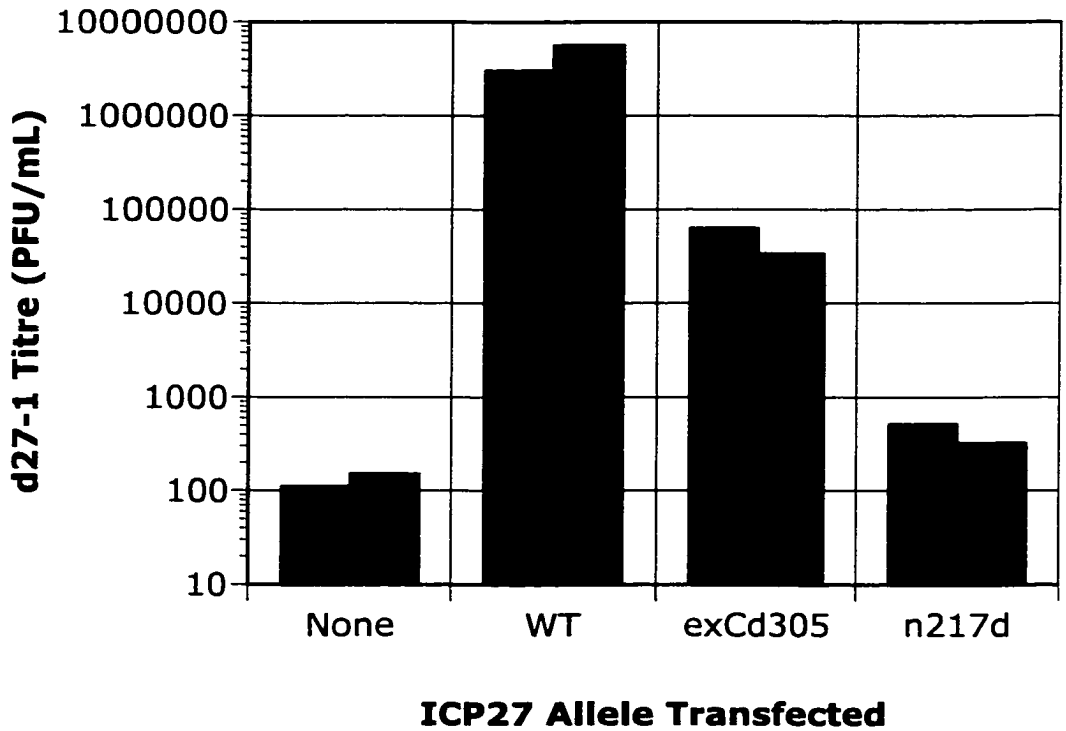

Figure 4-8. The novel stretch of 72-amino acids is required to complement the growth of *d*27-1 Vero cells were transfected in duplicate either with pUC19 (None), or with pUC19 containing an ICP27 allele. One day later, the cells were infected with *d*27-1. After allowing for a single replication cycle, the cells were subjected to three freeze-thaw cycles and *d*27-1 progeny were titred using V27 cells.

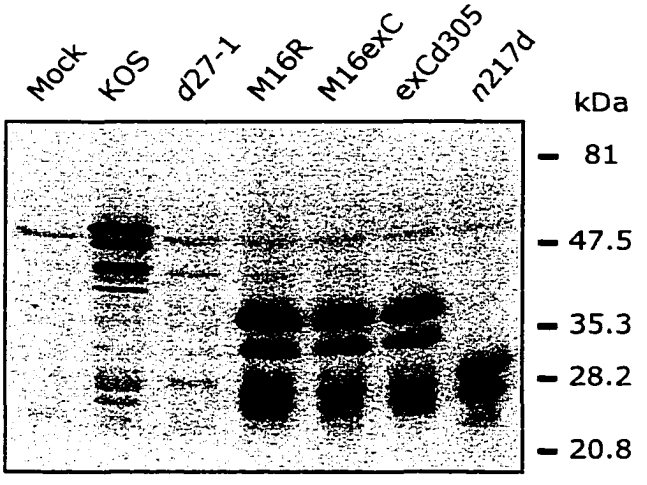

Figure 4-9. The HSV-1 recombinants produce ICP27 proteins of expected apparent molecular weight Vero cells were either mock infected, or infected with KOS, *d*27-1, M16R, M16exC, exCd305, or *n*217d. At six hpi, total protein was isolated and subjected to immunoblotting using H1113. Size markers are listed in kDa. The apparent molecular weight of the ICP27 protein produced by KOS is 63 kDa; a protein of approximately 38 kDa is produced by M16R, M16exC, and exCd305. *n*217d produces a 30-kDa protein.

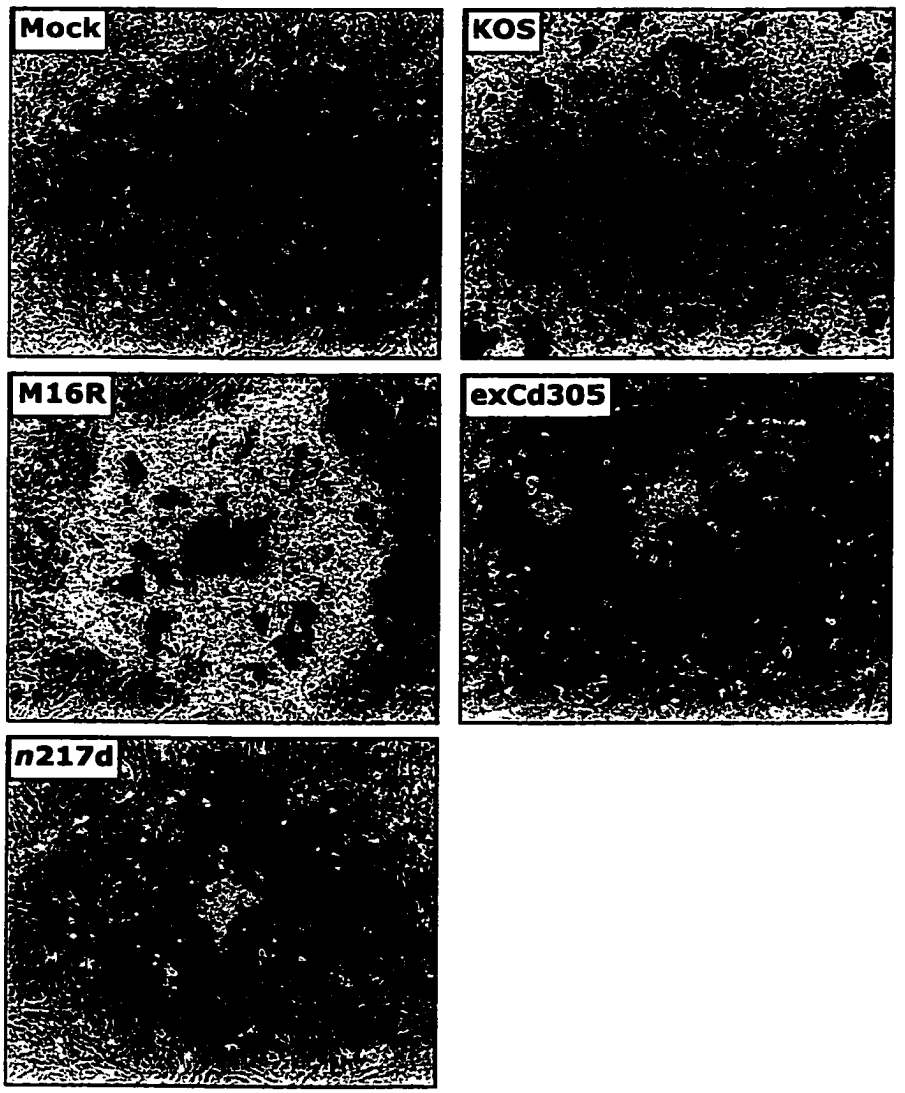

Figure 4-10. M16R can produce a plaque, but the HSV-1 recombinants can only produce small foci of infected cells Vero cells were infected with mock, KOS, M16R, exCd305, and *n*217d. Four dpi, a single plaque or focus from each infection was photographed. All photographs were taken under an identical field of magnification. The boundary of the KOS plaque is located just beyond the field of view.

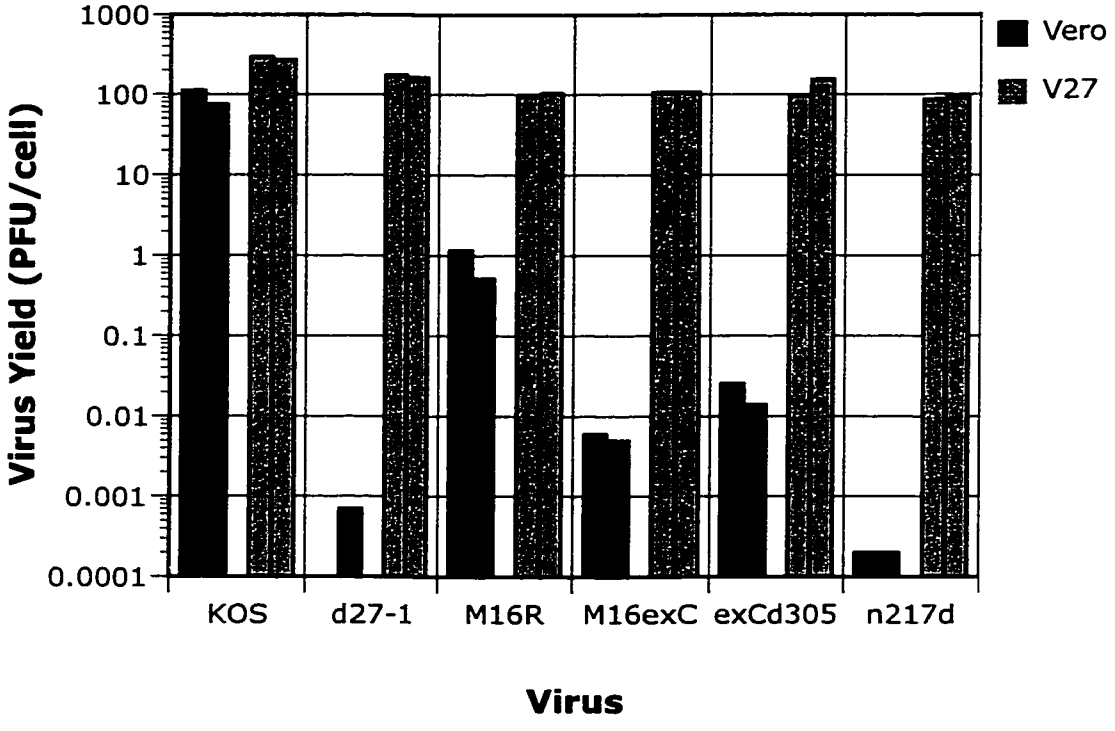

Figure 4-11. The HSV-1 recombinants generate fewer infectious progeny then does M16R Vero and V27 cells were infected in duplicate with either KOS, *d*27-1, M16R, M16exC, exCd305, or *n*217d. After allowing for a single replication cycle (approximately 24 hours), viral progeny were released by freeze-thawing, and titred using V27 cells. The data are expressed as virus yield (in PFU) per infected cell.

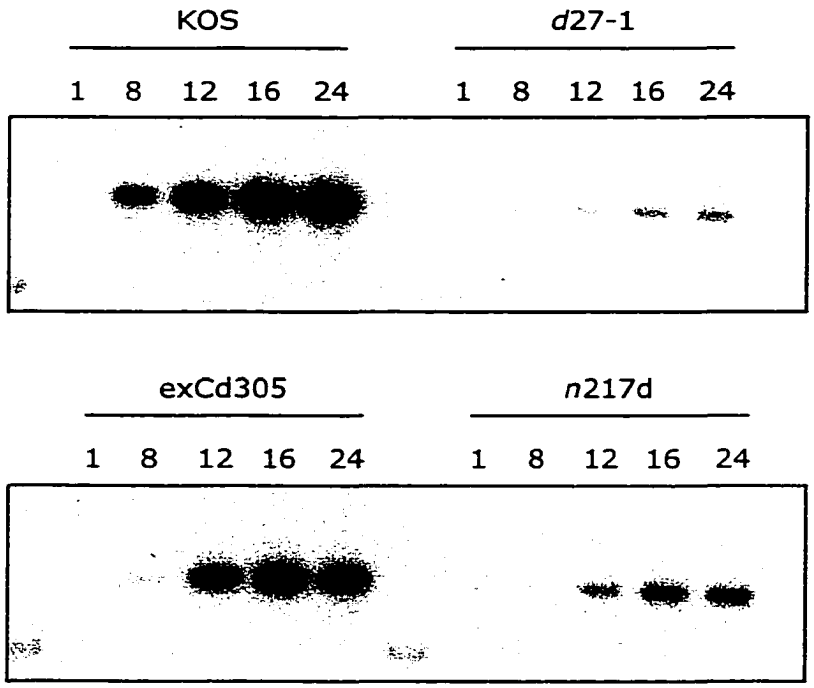

Figure 4-12a. The novel stretch of 72-amino acids stimulates viral DNA replication Vero cells were infected with KOS, *d*27-1, exCd305, or *n*217d. At one, eight, twelve, sixteen, and twenty-four hpi, viral DNA was isolated and digested with *Pst*I and *Sal*I. Following separation on a 1% agarose gel, and acid and alkali treatment, fragments were transferred to a nylon membrane and hybridized to a ^{32}P-labeled 480-base pair PCR-generated probe. Shown is the resulting autoradiograph.

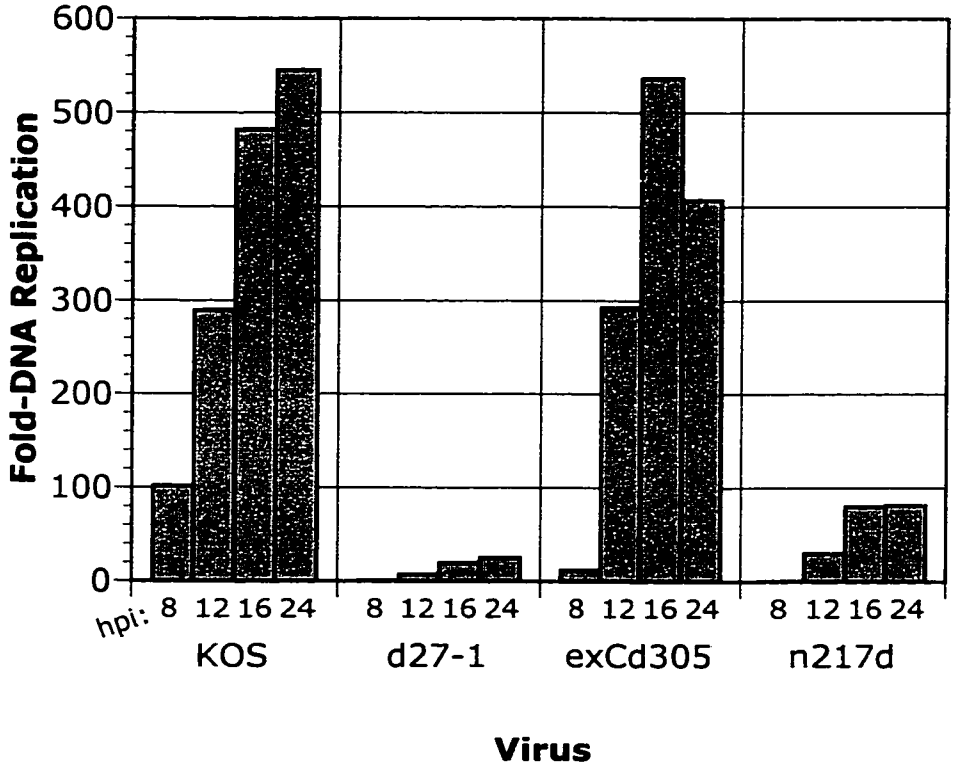

Figure 4-12b. The novel stretch of 72-amino acids stimulates viral DNA replication Hybridization-signal intensities were quantified by phosphorimage analysis of the data shown in Figure 4-12a, and are presented for each virus as fold-DNA replication (i.e. ratio of signal at *X* hpi to signal at one hpi).

Virus Stock	Titre on Vero cells (PFU/mL)	Titre on V27 cells (PFU/mL)	Plaquing Efficiency
KOS	4.1×10^8	2.7×10^8	1.5
*d*27-1	$<2.0 \times 10^3$	2.9×10^8	<0.0000069
M16R	$3.6 \times 10^{8\dagger}$	5.0×10^8	0.72
M16exC	$<2.0 \times 10^{4\S}$	2.1×10^8	<0.000095
exCd305	$<2.0 \times 10^{4\S}$	2.1×10^8	<0.000095
*n*217d	$<2.0 \times 10^{3\S}$	1.2×10^8	<0.000017

† plaques are small compared to KOS plaques.

§ discrete plaques are not visible; small foci of infected cells can be seen.

Table 4-1. The HSV-1 recombinants cannot grow on Vero cells The ability of the HSV-1 recombinant viruses M16exC, exCd305, and *n*217d to form plaques on Vero and V27 cells was examined and compared to KOS and M16R (both growth-competent in Vero cells), as well as to *d*27-1 (growth-defective in Vero cells). Shown are titres on Vero and V27 cells for viral high-titre stocks. Viral plaquing efficiencies, as a measure of Vero-to-V27 titre, are also presented.

5

Characterization of the HSV-1 –ICP27 Revertant M16R: Part II*

M16R, in addition to its intragenic reversion, contains one or more extragenic mutations. This/these extragenic mutation(s) are necessary for growth-competency. This chapter focuses on the phenotypic changes associated with the acquisition of (an) extragenic mutation(s) for viruses expressing the truncated ICP27 protein of 289-amino acids.

Intra- and extragenic mutations convey growth-competency

The virus M16R was isolated based on its ability to form plaques on Vero cells. A high-titre stock of M16R, prepared using V27 cells, was used in conjunction with high-titre stocks of KOS and M16 in an examination of viral growth characteristics. Table 5-1A provides a summary of the ability of the three viruses to form plaques on both Vero and V27 cells. KOS can grow equally well on either cell line, with high-titre stocks of the virus commonly yielding titres on the order of 10^9 PFU. The ICP27-mutant virus M16 cannot form plaques on Vero cells, but is able to on V27 cells due to the cellular ICP27 provided in *trans*. M16R can form plaques on both Vero and V27 cells at nearly identical titres, but produces plaques on Vero cells that are roughly half the diameter of those produced by KOS on Vero cells (see Figure 4-10); this may be a reflection of the reduced virus yield observed for M16R (Figure

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* A version of this chapter has been published: **Bunnell, S. M., and S. A. Rice.** 2000. Journal of Virology. **74:**7362–7374.

5-1). Although M16 and M16R titre to levels approaching those of KOS, they cannot be propagated in V27 cells to the same extent as KOS in Vero cells. The most likely explanation for this is that the ICP27 provided in *trans* is not able to fully restore viral viability to wild-type levels. ICP27 may not be as efficiently expressed in a cellular context as it is in a viral one.

In addition to the plaque assay, viral viability can be measured as a function of the number of infectious progeny produced by a given virus over the period of a single replication cycle (i.e. virus yield). To further the growth characterization of M16R, the three aforementioned viruses (KOS, M16, and M16R) were subjected to a virus-yield assay. Briefly, duplicate samples of Vero and V27 cells were infected with either KOS, M16, or M16R, and allowed to incubate long enough for a single wild-type–replication cycle to be completed (approximately 24 hours). Viral progeny were released by freeze-thawing and titred using V27 cells (Figure 5-1). Data are presented as virus yield (in PFU) per infected cell. As expected, in Vero cells, growth-competent KOS produces approximately 100 PFU per infected cell, whereas growth-defective M16 produces approximately four logs fewer progeny. Interestingly, growth-competent M16R exhibits a virus-yield profile in between that of KOS and M16, generating two logs more infectious progeny than M16 (or conversely two logs fewer infectious progeny than KOS). As the assay reflects virus yield for the cellular population as a whole, it is not known if each infected cell is producing approximately one PFU, or if there is a progeny-production disparity between infected cells. It is also not known if the reduced virus yield displayed by M16R is due to a true reduction in the number of infectious progeny that are able to be produced by the virus, or if the viral lytic cycle has been lengthened or delayed such that only a limited number of infectious progeny are available for titring at the fixed assay-time point.

Because of intra- and extragenic mutations present within its viral genome, M16R is growth-competent. It can grow to a high titre in Vero cells, can form discernable viral plaques, and can generate many more infectious progeny within an infected cell population than can growth-defective M16. Interpretation of the yield-assay data for M16R (see both Figures 4-11 and 5-1) leads to one other extremely interesting conclusion: the ICP27 provided in *trans* by V27 cells can enhance the growth phenotype of M16R. In Figure 5-1, V27 cells infected with M16R are able to produce approximately ten fold more infectious progeny than Vero cells infected with M16R; in Figure 4-11, the difference is a factor of 100. This suggests that wild-type ICP27 is able to functionally vitiate extragenic mutation contributions to the growth phenotype.

The exCd305 virus can revert to growth-competency

The observation that the engineered HSV-1–recombinant viruses that produce the truncated ICP27 protein of 289-amino acids are growth-defective in Vero cells provides a compelling argument for the presence of one or more extragenic mutations within the M16R genome. Attempts to map the extragenic mutation(s) of M16R using a marker rescue approach with M16R genomic fragments and the M16exC- and exCd305-recipient viruses failed to yield conclusive results, as the recipient viruses demonstrated a propensity for spontaneous reversion to a state of growth-competency. Therefore, in order to provide additional evidence in support of the conclusion that M16R contains one or more extragenic mutations that convey growth-competency, small V27-cell prepared stocks of exCd305 were screened for the presence of growth-competent virus through sequential passages in Vero cells. Two revertants of exCd305 were isolated and plaque purified. Preliminary genetic analysis performed on their respective viral genomes using PCR suggested that the viruses had retained the original deletion allele and had not acquired the wild-type–ICP27 allele (data not shown). Table 5-1B presents the titering profiles of the two exCd305-derived revertant viruses, exCd305R1 and exCd305R2, in relation to the titering profiles of the parental virus, exCd305, and the original revertant virus, M16R. Both exCd305R1 and exCd305R2 are able to form plaques on Vero cells, in contrast to exCd305, but they differ significantly in their plaquing morphologies (see below). In addition, the high-titre stock of exCd305R2 appears to be composed of a heterogeneous population of virus, as approximately ten percent of the viral plaques display a syncytial morphology on both Vero and V27 cells. The differing plaquing phenotypes exhibited by exCd305R1 and exCd305R2 suggest that they are the products of different mutation events.

The exCd305R1 and exCd305R2 reversions are extragenic

To identify the nature of the reversions present within the exCd305R1 and exCd305R2 viruses, the ICP27 alleles from exCd305R1 and exCd305R2 were cloned as *Bam*HI–*Sac*I fragments into pUC19. The resulting plasmids, pd305R1 and pd305R2, were then subjected to sequencing analysis to determine if any intragenic changes had occurred during their genesis. Sequencing of the ICP27 allele from exCd305R1 revealed no changes from the expected sequence (i.e. from that of exCd305). Sequencing of the ICP27 allele from exCd305R2 revealed a single base change in the promoter region of ICP27, upstream of the 5'-untranslated region (UTR)—a cytosine base at map position 113378 (204) has become an adenine. Whether this base change effects the phenotype of exCd305R2 is not known; it does not,

however, appear to affect the expression level of the truncated ICP27 protein (see Figure 5-2).

The lack of sequence change in the ICP27 allele of exCd305R1 indicates that its growth-competency is a result of extragenic reversion(s). And while a single base change was discovered in the ICP27 allele of exCd305R2, more than likely this alteration is not responsible for the growth-competency observed for the virus. If both viruses are growth-competent because of extragenic reversions, their production of a truncated ICP27 protein should be unchanged. To verify that the exCd305-revertant viruses indeed produce the expected ICP27 protein, Vero cells were infected with either KOS, *d*27-1, M16R, exCd305, exCd305R1, or exCd305R2. Six hpi, total protein was isolated, separated on a 15% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Incubations with the monoclonal antibody H1113, a horseradish peroxidase-conjugated secondary antibody, and reagents from an enhanced chemiluminescence system, allowed for the detection of ICP27 (Figure 5-2). As predicted by the sequencing results, the exCd305-revertant viruses produce ICP27 proteins of identical apparent molecular weight to those of the exCd305 and M16R viruses. The patterns of faster mobility bands present in addition to the full-length products are again likely the result of protein degradation (also seen in Figure 4-9).

exCd305R2 plaques are much smaller than exCd305R1 plaques

The fact that the exCd305 virus was able to obtain, in two separate occasions, a state of growth-competency without the aid of intragenic reversions lends strong credence to the conclusion that the growth-competency of M16R is owed to both intra- and extragenic mutations. But while both exCd305R1 and exCd305R2 have achieved a state of growth-competency because of one or more extragenic reversions, Figure 5-3 presents data that suggest that the viruses are products of differing extragenic mutations (or sets of). Photographs of a single plaque for mock, KOS, M16R, exCd305R1, and exCd305R2 infections in Vero cells were taken four dpi—plaques were selected at random for photo-documentation and represent the general plaquing phenotype of the particular virus. The M16R plaque is approximately one-half the diameter of the KOS plaque (the boundary of which extends just beyond the presented field of view). Infection of Vero cells with exCd305R1 produces viral plaques that are essentially identical to those produced on Vero cells by a M16R infection. Interestingly, infection of Vero cells with exCd305R2 produces viral plaques that deviate markedly in their plaque morphology from those produced by exCd305R1; as is the case for M16R and KOS, exCd305R2 produces plaques approximately one-half the diameter of exCd305R1 plaques. Although it is possible that the

single base change found within the ICP27 allele of exCd305R2 is somehow contributing to the plaquing morphology of the virus, it is highly unlikely that it is the *sole* contributor. It is therefore hypothesized that the different plaquing morphologies exhibited by the two exCd305-revertant viruses are the result of differing extragenic mutations present within the two viruses. One possibility is that one or more completely different extragenic reversions are effecting growth-competency for the two viruses; it is also possible that exCd305R1 and exCd305R2 possess the same extragenic reversion(s), but that exCd305R2 contains one or more additional mutations as well. The identities of the extragenic reversions are not known.

M16R and exCd305R1: the same extragenic mutations?

As both exCd305R1 and exCd305R2 are growth-competent viruses, they should produce greater numbers of infectious progeny than their parent, exCd305. To validate this supposition, the two exCd305-revertant viruses were subjected to a virus-yield assay along with KOS, *d*27-1, M16R, and exCd305. Duplicate samples of Vero and V27 cells were infected with each virus. After allowing for a single replication cycle, viral progeny were released by multiple freeze-thaws and titred using V27 cells (Figure 5-4). Data are presented as virus yield (in PFU) per infected cell for each virus tested. Consistent with previous results, KOS is able to produce the highest virus-yield values, with approximately five logs difference between it and the virus-yield values produced by *d*27-1 (see Figure 4-11). Also consistent with previous results are the virus-yield deficiencies of approximately two logs exhibited by M16R in comparison to KOS, and by exCd305 in comparison to M16R (see Figures 4-11 and 5-1). exCd305R1 produces virus-yield values approximately identical to M16R's; exCd305R2 produces virus-yield values in-between those of exCd305R1 and exCd305 (i.e. approximately one log lower than exCd305R1's and one log higher than exCd305's). Both exCd305R1 and exCd305R2 produce significantly more infectious progeny during V27-cell infections than during Vero-cell infections.

The virus-yield data allow for the exploration of a number of interesting hypotheses: (i) exCd305R1 and exCd305R2 produce greater virus-yield values than exCd305, but the fact that exCd305R1 is more proficient than exCd305R2 in the production of infectious progeny adds support to the proposed theory that the two viruses are products of differing extragenic mutations (note again the possible contribution to exCd305R2's virus-yield numbers by the single-base change in the ICP27 allele); (ii) M16R and exCd305R1 are essentially indistinguishable with respect to their virus-yield values. Although their extragenic mutations have not been mapped, it is tempting to speculate that they possess the same extragenic mutation(s);

this speculation is based upon the observation that their viral phenotypes are almost identical (see Table 5-1B and Figures 5-2–5-4); (iii) comparison of the viral yield data in Figure 5-4 with the plaque morphologies presented in Figure 5-3 reveals a definite correlation between viral yield and plaque diameter—the greater the viral yield, the larger the viral plaque. This is a reasonable association, as an infected cell producing a relatively high number of infectious progeny (e.g. a cell infected with KOS) would have a higher probability of infecting the surrounding cells in direct contact with it in comparison to an infected cell producing a relatively low number of infectious progeny (e.g. a cell infected with M16R); (iv) both exCd305R1 and exCd305R2 are able to produce at least 100 fold more infectious progeny in V27-cell infections, therefore indicating that—as is the case for M16R—the ICP27 provided in *trans* by V27 cells can suppress their respective phenotypes.

The exCd305 revertants possess only ICP27-deletion alleles

The parental virus of the two exCd305 revertants, exCd305, contains an ICP27 gene in which the sequence corresponding to codons 306–512 has been deleted. Since an ICP27 protein of 289-amino acids is produced by exCd305, this deletion of C-terminal–encoding sequence is of no consequence to the expression of the truncated protein. exCd305 is not growth-competent in Vero cells, but exCd305R1 and exCd305R2 are, and although PCR, DNA-sequence, and immunoblot data show no indication of the presence of the wild-type allele within the revertant genomes, it is possible that the revertant viruses contain within their genomes a portion of the wild-type–ICP27 allele capable of expressing a C-terminal–protein fragment. Such a C-terminal–protein fragment could be contributing to the growth-competency of exCd305R1 and exCd305R2. To investigate whether the C-terminal–encoding section of the wild-type–ICP27 allele is absolutely absent from the viral genomes of exCd305R1 and exCd305R2, Southern blot analysis was performed. Briefly, V27 cells were infected with either KOS, *d*27-1, M16R, exCd305, exCd305R1, or exCd305R2. Following a 48-hour incubation period, viral DNA was isolated, digested with *Pst*I and *Sal*I, and separated on a 1% agarose gel. The DNA fragments embedded within the gel were then subjected to acid-cleavage and alkali-denaturation treatments and transferred to a nylon membrane.

Two hybridizations were carried out—one involving ORF sequences coding for the N-terminal half of ICP27, and one involving ORF sequences coding for the C-terminal half of ICP27. Figure 5-5A details a schematic of the various ICP27 alleles present within the test viruses, indicating the hybridization locations of the N- and C-terminal probes, as well as the expected fragment

sizes for a *Pst*I and *Sal*I digest. The C-terminal probe spans codons 308–503 of ICP27, whereas the N-terminal probe spans codons 90–259. Both DNA probes were generated as PCR products from the template plasmid p27; PCR primers R96 and R78 (which hybridize to codons 308–314 of ICP27 and codons 497–503 of ICP27, respectively) were employed in the creation of the C-terminal probe, and PCR primers R72 and R97 (which hybridize to codons 90–96 of ICP27 and codons 253–259 of ICP27, respectively) were employed in the creation of the N-terminal probe. Prior to hybridization, each probe was ^{32}P-labeled through random priming.

Initial hybridization was carried out using the C-terminal probe. Figure 5-5C presents the autoradiograph of the C-terminal–probe hybridization. Hybridization signals of the approximate correct size are present in the KOS, *d*27-1, and M16R lanes, with no detectable hybridization signals present in the remaining lanes. This result initially suggested that the growth-competent viruses, exCd305R1 and exCd305R2, lack any C-terminal–encoding sequence. However, it is possible that the negative hybridization results are the consequence of missing viral DNA samples. To confirm that exCd305, exCd305R1, and exCd305R2 viral DNA fragments had been successfully transferred to the nylon membrane, the membrane was stripped of the C-terminal probe, and DNA fragments were subjected to hybridization with the N-terminal probe. Figure 5-5B shows that viral DNA from the exCd305, exCd305R1, and exCd305R2 viruses is indeed present on the surface of the nylon membrane; as expected, hybridization signals corresponding to the predicted fragment size are present in all lanes (except for *d*27-1, which cannot yield a N-terminal–probe signal). Therefore, the Southern blot analysis confirms that, not only have the exCd305-revertant viruses retained the original ICP27 allele, they are also devoid of any C-terminal–encoding sequence.

Synopsis

M16R is a multi-mutation virus, with both an ICP27-intragenic reversion and one or more extragenic mutations. The intra- and extragenic mutations are almost certainly both required for growth-competency. It is formally possible that the extragenic mutation(s) alone can effect growth-competency in the absence of ICP27, but given what is known of the contribution of the protein to viral viability, the scenario of extragenic mutations circumventing the requirement of an ICP27 protein is extremely unlikely. Contrary to its parental virus, M16, M16R can spread and form plaques on non-complementing Vero cells, and is able to produce approximately 100 fold more infectious progeny per cell, on average, than M16. However, M16R does not grow as well as the wild-type virus, KOS; Vero-cell infections with

KOS not only produce much greater virus yields, but also result in viral plaques of a larger diameter (approximately twice the size of M16R plaques).

Because the extragenic mutation(s) present within the M16R genome has/have not been identified, its/their existence relies on a collection of circumstantial evidence. To strengthen the conclusion that M16R contains one or more extragenic mutations that effect growth-competency, stocks of the recombinant virus exCd305 (which produces the truncated ICP27 protein of 289-amino acids) were examined for the presence of growth-competent virus. Two independently generated revertants of exCd305 were isolated: exCd305R1 and exCd305R2. Analysis of their genotypes revealed that neither virus had obtained a single sequence deviation within their respective ICP27-coding regions; exCd305R2, however, does contain one base alteration within the promoter region of its ICP27 gene. Both revertants also lack any ICP27 sequence capable of producing the C-terminal half of the wild-type protein. These discoveries indicate that extragenic reversions are responsible for the growth-competent phenotypes of the exCd305 revertants. If extragenic mutations can co-operate with an (engineered) intragenic mutation to effect growth-competency for exCd305R1 and exCd305R2, it is highly likely that the same situation is occurring in the case of M16R.

The exCd305 revertants are phenotypically similar to M16R—all three viruses are able to form plaques on Vero cells, and exhibit nearly identical ICP27 steady-state protein-production profiles. Interestingly, though, while exCd305R1 is essentially indistinguishable phenotypically from M16R (which suggests that the two viruses possess the same extragenic mutation(s)), exCd305R2 displays two important characteristics that distinguish it from exCd305R1 and M16R. Firstly, exCd305R2 differs markedly in its plaquing morphology; plaques produced by the virus on Vero cells are approximately one-half the size of those produced by exCd305R1 and M16R. Secondly, exCd305R2 is not as robust as exCd305R1 and M16R with respect to the production of infectious progeny over a fixed time period. The phenotype of exCd305R2, therefore, appears to have been achieved through the acquisition of one or more extragenic mutations that differ from those acquired by exCd305R1 and M16R.

Assuming that M16R contains one or more extragenic mutations, the growth-competency observed for each of the three revertant viruses may be explained by one of two scenarios. The first involves the truncated ICP27 protein directly interacting with the mutated protein(s) produced by the extragenic-mutation allele(s). The second involves the truncated ICP27 protein and the mutated protein(s) produced by the extragenic-mutation allele(s) acting in concert to effect growth-competency, but not through a direct physical interaction. One attractive protein candidate for the scenarios

is ICP4 (315). ICP4 is an essential protein, required for the expression of DE and L genes (58, 64). There is some evidence that ICP27 and ICP4 effect HSV-1 gene *trans*-regulation through a physical interaction (231, 243, 296). Under the first scenario proposed, truncation of ICP27 would abrogate its interaction with ICP4, and would result in a growth-defective phenotype (e.g. exCd305). Restoration of growth-competency would come about as a result of a mutation in the ICP4 gene—the mutated ICP4 protein would now be able to physically interact with the truncated ICP27 protein well enough to allow for a moderate growth phenotype (e.g. exCd305R1 and M16R). This scenario could also be played out substituting ICP0 for ICP4. However, the fact that ICP0 is not essential for viral replication in cell culture (291, 343), and that the evidence for a physical interaction between ICP27 and ICP0 is not very compelling (231, 243, 400), weakens the plausibility of this situation.

Under the second scenario proposed, growth-competency could be achieved because one or more mutation-derived proteins (e.g. a mutant form of ICP4) are now able to carry out essential functions that were once the province of the wild-type–ICP27 protein. Alternatively, growth-competency could be associated with extragenic mutations that have affected protein expression, rather than protein activity. For example, certain essential L genes could acquire mutations that uncouple their expression from a dependence upon a full-length ICP27.

Unlike the exCd305 revertants, which have acquired only extragenic mutations, M16R has acquired both extragenic and intragenic (ICP27) mutations—presumably during its genesis from growth-deficiency to growth-competency. The possibility exists, however, that the original M16 virus (278) contains the extragenic mutation(s), and that M16R acquired only the intragenic mutation during its genesis. While it is not thought that the original M16 virus contains the extragenic mutation(s), this possibility can be addressed through a replacement of the M16-ICP27 allele in the M16 virus with the M16R-ICP27 allele. If the resulting recombinant virus is growth-competent in Vero cells, then the extragenic mutation(s) is/are present within the M16 genome. This allele-swapping experiment could also be performed with the M16R-ICP27 allele and the *d*27-1 virus (277) as an investigation into the functional disparity displayed by the extra-cytosine alleles in the transient plasmid and virus-plaquing assays (see Figure 4-6 and Table 4-1); at issue is whether or not the *d*27-1 virus contains the extragenic mutation(s), which allow(s) for complementation with the M16exC and exCd305 alleles.

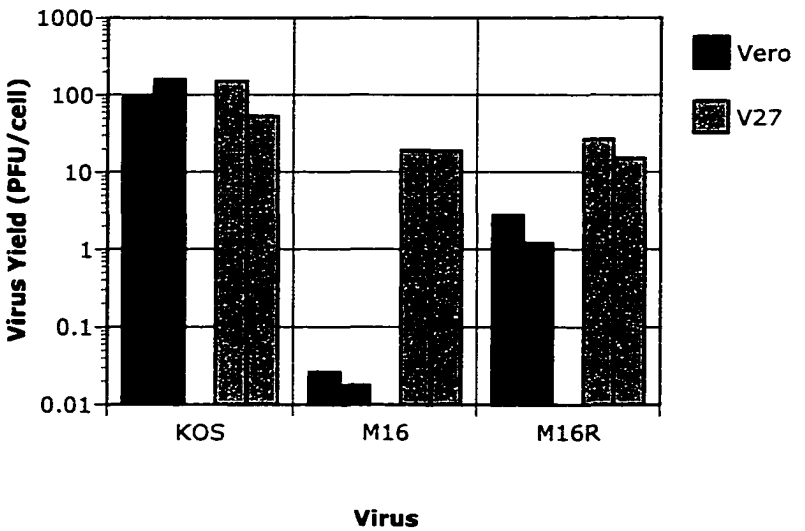

Figure 5-1. M16R generates 100 fold more infectious progeny than does the progenitor virus M16 Vero and V27 cells were infected in duplicate with either KOS, M16, or M16R. After allowing for a single replication cycle (approximately 24 hours), viral progeny were released by freeze-thawing, and titred using V27 cells. The data are expressed as virus yield (in PFU) per infected cell.

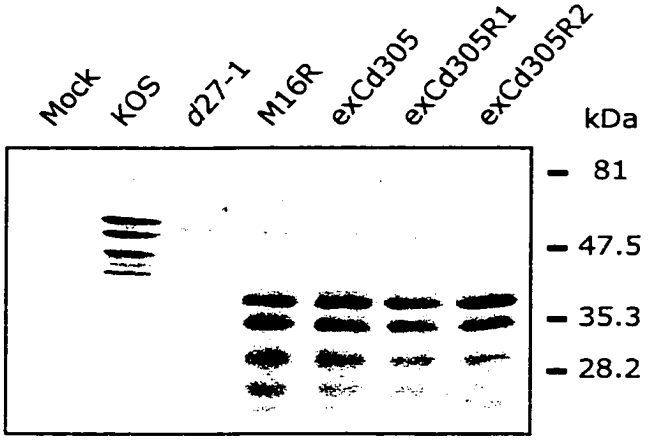

Figure 5-2. The exCd305 revertants produce ICP27 proteins of expected apparent molecular weight Vero cells were either mock infected, or infected with KOS, *d*27-1, M16R, exCd305, exCd305R1, or exCd305R2. At six hpi, total protein was isolated and subjected to immunoblotting using H1113. Size markers are listed in kDa. The apparent molecular weight of the ICP27 protein produced by KOS is 63 kDa. M16R, exCd305, and the two exCd305 revertants produce a 38-kDa protein.

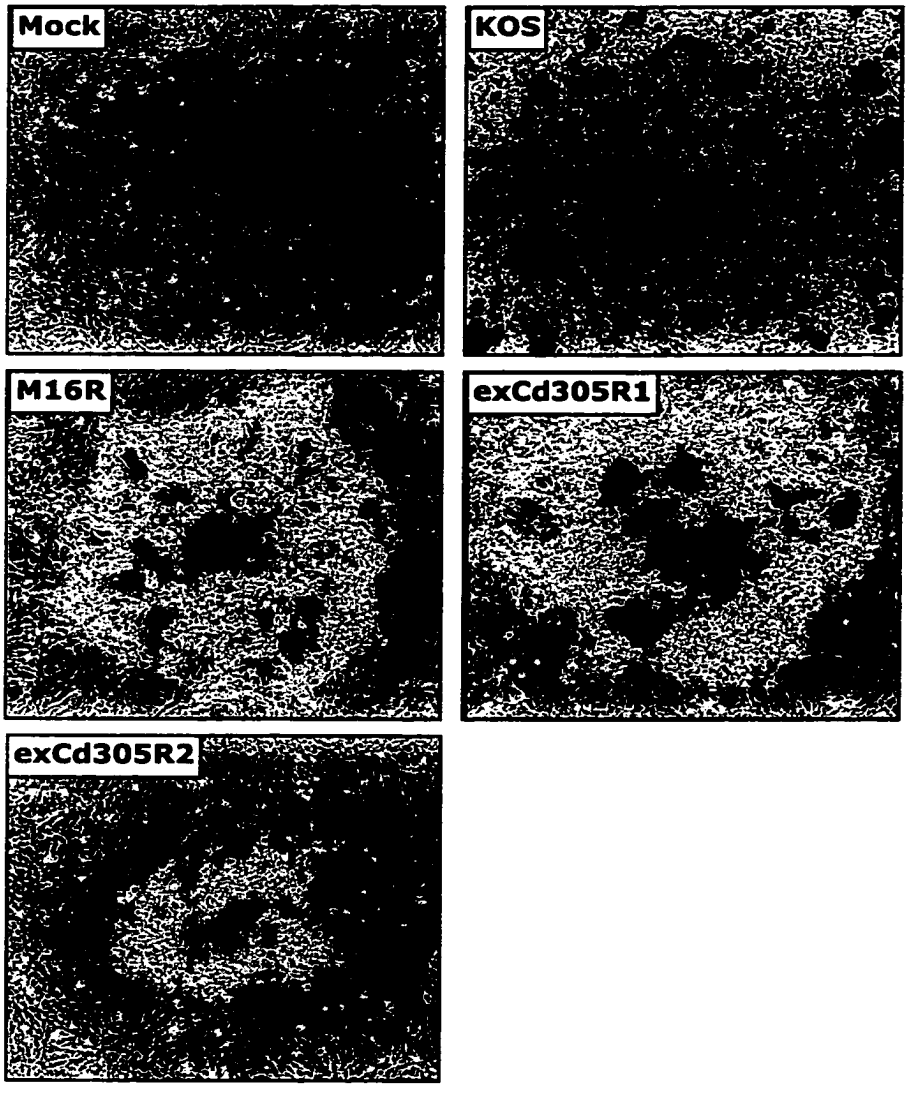

Figure 5-3. The exCd305 revertants exhibit very different plaquing morphologies Vero cells were infected with mock, KOS, M16R, exCd305R1, and exCd305R2. Four dpi, a single plaque from each infection was photographed. All photographs were taken under an identical field of magnification. The boundary of the KOS plaque is located just beyond the field of view.

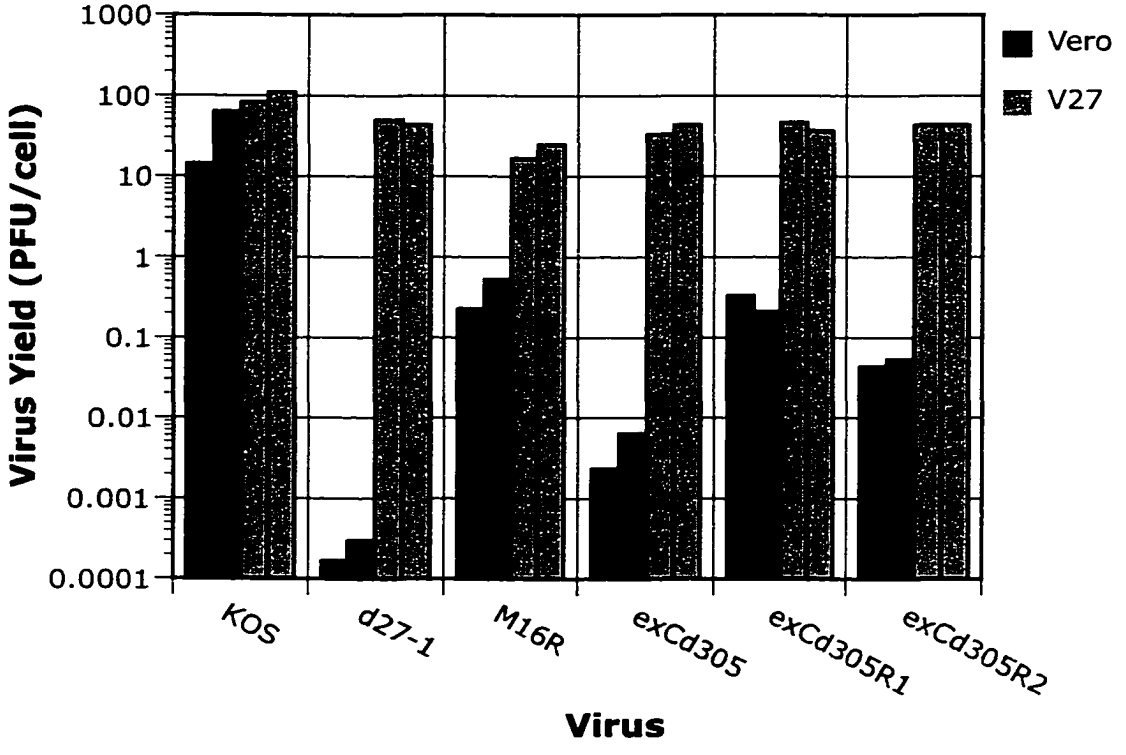

Figure 5-4. exCd305R1 is similar to M16R in the production of infectious progeny Vero and V27 cells were infected in duplicate with either KOS, *d*27-1, M16R, exCd305, exCd305R1, or exCd305R2. After allowing for a single replication cycle (approximately 24 hours), viral progeny were released by freeze-thawing, and titred using V27 cells. The data are expressed as virus yield (in PFU) per infected cell.

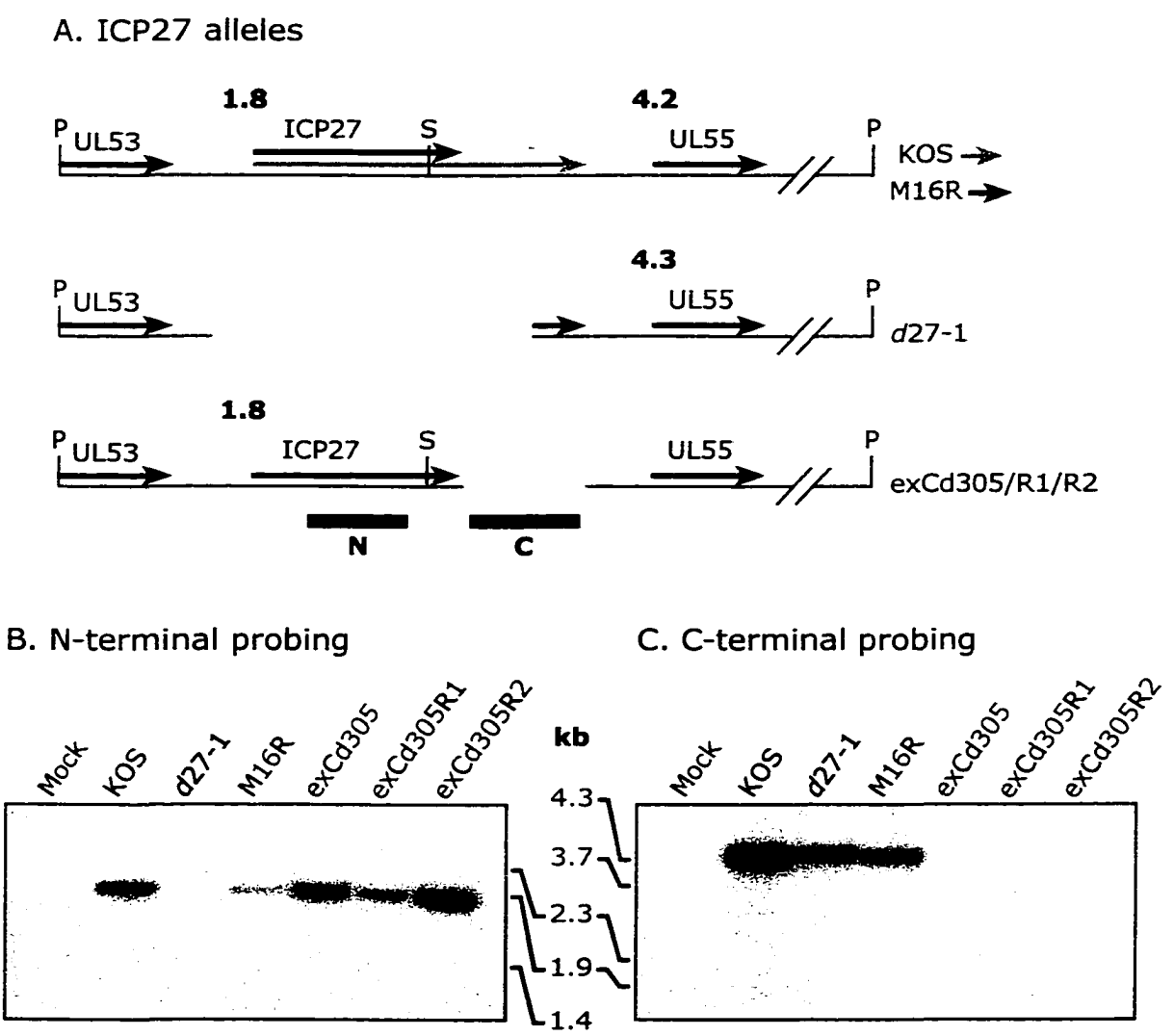

Figure 5-5. The exCd305 revertants have retained the exCd305-ICP27 allele (A) Schematics of the ICP27 alleles present within their respective viruses, indicating hybridization-fragment size (in kb) and the annealing locations of the DNA probes; (B) and (C) V27 cells were either mock infected, or infected with KOS, *d*27-1, M16R, exCd305, exCd305R1, or exCd305R2. At 48 hpi, viral DNA was isolated and digested with *Pst*I and *Sal*I. Following separation on a 1% agarose gel, and acid and alkali treatment, fragments were transferred to a nylon membrane and hybridized to ^{32}P-labeled PCR-generated probes specific for (B) N-terminal codons 90–259 of ICP27, or (C) C-terminal codons 308–503 of ICP27. Shown are the resulting autoradiographs. Size markers are in kb. P—*Pst*I; S—*Sal*I.

	Virus Stock	Titre on Vero cells (PFU/mL)	Titre on V27 cells (PFU/mL)	Plaquing Efficiency
A.	KOS	4.5×10^{9}	4.2×10^{9}	1.1
	M16	$<2.0 \times 10^{4}$	4.9×10^{8}	<0.000041
	M16R	$6.3 \times 10^{8\dagger}$	9.5×10^{8}	0.66
B.	M16R	$8.6 \times 10^{8\dagger}$	8.9×10^{8}	0.97
	exCd305	$<2.0 \times 10^{4\S}$	8.5×10^{8}	<0.000024
	exCd305R1	$9.9 \times 10^{8\dagger}$	8.2×10^{8}	1.2
	exCd305R2	$2.8 \times 10^{8*,\infty}$	$6.3 \times 10^{8\infty}$	0.44

† plaques are small compared to KOS plaques.

§ discrete plaques are not visible; small foci of infected cells can be seen.

* plaques are minute compared to KOS plaques.

∞ a fraction of the plaques display a syncytial morphology.

Table 5-1. Growth properties of wild-type and mutant viruses (A) The ability of M16R to form plaques on Vero and V27 cells was examined and compared to KOS (growth-competent in Vero cells), as well as to M16 (growth-defective in Vero cells); (B) The ability of exCd305R1 and exCd305R2 to form plaques on Vero and V27 cells was examined and compared to M16R (growth-competent in Vero cells), as well as to exCd305 (growth-defective in Vero cells). Shown are titres on Vero and V27 cells for viral high-titre stocks. Viral plaquing efficiencies, as a measure of Vero-to-V27 titre, are also presented.

6

Final Thoughts

ICP27 is a complex, multi-functional protein, whose contributions to the lytic cycle of HSV-1 have yet to be fully elucidated. The original research presented in this thesis details: i) the examination of RC virus present within growth-defective stocks of ICP27 mutants; ii) the characterization of an unexpected second-site revertant of an ICP27 growth-defective mutant. A summary of the major findings and conclusions surmised from the data collected on the two projects is detailed below.

Investigation of Unexpected Recombinants

Preparation of high-titre stocks of growth-defective ICP27 mutants, unable to replicate because of engineered changes to the ICP27 gene, requires the use of a cell line (V27 cells) able to express a virally inducible wild-type–ICP27 protein (277). This allows for the possibility of recombination between the cellular ICP27 gene and the viral ICP27 gene, leading to the creation of a wild-type–rescuant virus that is now RC. When five ICP27-mutant stocks, which had been prepared using V27 cells, were examined for the presence of RC virus, all were found to contain RC virus. Therefore, recombination between cellular and viral genomes, leading to RC virus creation, is not an uncommon event.

The RC virus present in most growth-defective stocks could be the result of two-crossover event homologous recombination between the V27 cell and

viral genomes. However, in the case of the ICP27-null mutant, *d*27-1, RC virus isolated from this high-titre stock contains both the original deletion allele at the ICP27 locus, as well as the wild-type–ICP27 allele at an unknown position. It is proposed that the two alleles exist in tandem within this RC virus, the result of either homologous or illegitimate recombination (via HANR) between the viral ICP27-deletion allele and a circular section of cellular DNA containing the wild-type–ICP27 allele.

Although the existence of RC virus in the growth-defective stocks can be explained by recombination processes that are associated with the presence of apparent DNA-sequence homology between viral and cellular genomes, it is possible that apparent homology between genomes is not absolutely essential for the genesis of RC virus. To address this issue, a new ICP27-deletion virus was created: *d*27-2. This virus is characterized by a deletion of the *entire* ICP27 gene and flanking sequences (as opposed to *d*27-1), and therefore shares no apparent sequence homology with the cellular ICP27 gene. When examined in parallel with *d*27-1 for the presence of RC virus, high-titre stocks of *d*27-2 were found to be devoid of RC virus, suggesting that the generation of RC virus is dependent on apparent DNA-sequence homology.

Summary

1) Replication-competent virus exists in growth-defective stocks.
2) Replication-competent virus from *d*27-1 contains two ICP27 alleles.
3) The generation of RC virus appears to require sequence homology.

Future Directions

A number of unanswered questions remain. (i) Does the propagation of high-titre stocks of growth-defective virus using V27 cells result in the inevitable creation of RC virus? If so, then RC virus should be present in all ICP27-mutant stocks. Subjecting Vero cells to sequential infections with stocks of virus would provide answers to this question. (ii) What is the genomic structure of the RC virus present in the *d*27-1 stock(s)? To address this question, RC-viral DNA would be digested with restriction enzymes and analyzed by Southern blotting using a radio-labeled probe specific for ICP27 sequence absent in *d*27-1—this would identify genomic restriction fragments that contain the wild-type–ICP27 allele. Restriction fragments containing the wild-type allele would then be cloned and sequenced to delineate the genomic locale of the wild-type allele. Of interest is whether the proposed tandem arrangement of alleles exists, or whether the insertion of the wild-type allele occurs at one or more different locations throughout the genome. (iii) Can RC virus be generated during the creation of high-titre stocks of

*d*27-2? Preliminary results suggest that RC virus is not being generated. However, to address the question satisfactorily, a much more sensitive screening of high-titre stocks of *d*27-2 would have to be performed. If RC virus were found to be present, even at very low levels, this would suggest that HSV-1 can acquire genetic material from the cellular genome without the benefit of apparent sequence homology (i.e. via illegitimate recombination). It is important to determine the prevalence of RC virus in growth-defective stocks, especially if such stocks are being developed as vectors for gene therapy.

Characterization of M16R

The isolation and characterization of revertant viruses arising in growth-defective stocks can yield insight into the structural and functional relationships of the originally mutated protein. A revertant of the ICP27-mutant virus M16 (278) was isolated based upon its ability to form plaques on a non-complementing cell line. This revertant, known as M16R, contains an intragenic reversion within its ICP27 gene; sequence analysis of the gene revealed a single cytosine-base addition in an existing stretch of cytosines at codons 215–217. Addition of the cytosine base creates a +1 sequence frameshift such that a new stop codon is read at codon 290. In *d*27-1 growth-complementation assays, the truncated ICP27 protein is functional, although not to the same degree as the wild-type protein.

M16R produces an ICP27 protein in the infected cell consistent in mass with that expected from sequence analysis of the mutated allele. During infection, the ICP27 protein of M16R localizes preferentially to the cell's nucleolus—this localization profile is in stark contrast to those observed for infections with either KOS (where ICP27 is predominantly nuclear, but is dispersed throughout the nucleus in a speckled pattern) or M16 (where ICP27 is *excluded* from the nucleolus). The significance of the novel ICP27-localization profile produced by a M16R infection is unknown.

The ICP27 protein produced by M16R lacks what would normally be considered to be the C-terminal section of the wild-type protein. Instead, a novel C-terminal section of 72-amino acids has been created as a result of the +1 frameshift. The new 72-amino acids contribute significantly to the functionality of the truncated ICP27 protein, as their removal results in: (i) a protein unable to complement the growth of *d*27-1 in a transient transfection assay, and (ii) a virus (*n*217d) unable to efficiently replicate its viral DNA.

Three recombinant HSV-1 viruses were created to assess the contribution(s) of the truncated ICP27 protein to the viral phenotype. Two of

the recombinants, M16exC and exCd305, contain the extra cytosine addition and produce ICP27 proteins identical to the one produced by M16R, while the third, *n*217d, possesses a stop codon at position 218 and produces an ICP27 protein lacking the novel 72-amino acid stretch. All three are unable to form plaques on a non-complementing cell line, and generate much lower levels of infectious progeny than M16R. The most likely explanation for these observations is that M16R, in addition to the intragenic reversion, contains one or more extragenic mutations that are required for growth-competency. The truncated ICP27 protein of 289-amino acids is not completely devoid of function in the absence of the extragenic mutation(s), however, as it is able to effect the replication of viral DNA at near wild-type levels.

Two revertants of exCd305, named exCd305R1 and exCd305R2, were isolated and characterized. The two are phenotypically distinct from one another. exCd305R1 is almost indistinguishable from M16R with respect to phenotype; comparisons of plaquing morphology, truncated ICP27-protein production, and virus-yield values between the two growth-competent viruses reveals very little difference. Genotypically, the ICP27 allele of exCd305R1 was found to be identical in sequence to that of its parent, exCd305. The growth-competency of exCd305R1, therefore, must be the result of one or more extragenic reversions. It is possible that M16R and exCd305R1 contain the same extragenic mutation(s).

exCd305R2 displays a phenotype different from those of M16R and exCd305R1. On a non-complementing cell line, exCd305R2 produces plaques that are approximately half the diameter of the plaques produced by either M16R or exCd305R1. In addition, the virus-yield values generated by exCd305R2 are markedly lower than the values generated by both M16R and exCd305R1. Examination of the sequence of the ICP27 allele of exCd305R2 revealed a single base substitution in the non-coding promoter region of the allele. It is not known whether this single sequence deviation contributes to the phenotype, however the level of expression of the truncated ICP27 protein appears unaffected by the mutation. The fact that exCd305R2 displays a unique phenotype almost certainly means that its extragenic mutation(s) is/are different from the mutation(s) of exCd305R1.

Summary

1) M16R produces a functional ICP27 protein of 289-amino acids.
2) The new C-terminal 72-amino acids are functionally important.
3) One or more extragenic mutations exist within M16R's genome.
4) The exCd305 virus can revert to growth-competency.

Future Directions

The characterization of M16R is far from complete, as the data accumulated from the preliminary analysis of the revertant presents numerous unanswered questions and areas of investigation. An obvious question is: what is/are the extragenic mutation(s) of M16R? This could be addressed either by the cloning and sequencing of selected genes thought to be good reversion candidates (e.g. the ICP4 gene), or by a co-infection system involving the exCd305 virus and a library of amplicon constructs (168, 327, 328) bearing genomic sections of M16R. Resulting virus-yield levels from the co-infections would indicate which genomic section(s) contain the extragenic mutation(s). The use of a direct protein-protein interaction assay, such as the two-hybrid system (45), would also be useful in the identification of extragenic-mutation proteins. This approach assumes that the truncated ICP27 protein directly interacts with the extragenic-mutation protein(s), however, which may not be the case.

It is possible that the extragenic mutations present within the M16R, exCd305R1, and exCd305R2 genomes are effecting L gene expression. This could be investigated with a combination of [^{35}S] protein labeling experiments and analysis of mRNA levels of specific γ1 or γ2 genes (such as glycoprotein C (94)) using viruses that contain only the intragenic mutation (e.g. M16exC and exCd305) and those that contain both mutation types (e.g. M16R and exCd305R1).

Of interest is whether the truncated ICP27 protein produced by M16R shuttles between the nucleus and the cytoplasm of the infected cell. Evidence exists to support the requirement of shuttling for the expression of L gene products (325). Preliminary shuttling experiments with M16R suggest that the truncated ICP27 protein does not shuttle.

ICP27 is known to be able to bind to RNA *in vitro* through a RGG box (211). The truncated ICP27 protein contains this RGG box, so it is presumed to be able to bind to RNA. Using a well-described homopolymer system for *in vitro* RNA-binding analysis (158, 211), the ability of the truncated ICP27 protein to bind RNA could be investigated. It would also be interesting to determine whether the RGG box is essential for any RNA-binding activity, as the novel C-terminal region encoded by the extra-cytosine containing ICP27 allele resembles the RGG box.

Is the truncated ICP27 protein able to effect the *trans*-activation and *trans*-repression of co-transfected reporter genes in transient assays to the same degree as the wild-type protein? ICP27 has been shown to be able to enhance the expression of constructs bearing weak polyadenylation signals, while inhibiting the expression of reporter genes bearing certain introns (302). What role does the novel C-terminal region play, if any?

A number of experiments could address the role of the novel C-terminal region itself. For example, what effect on activity does the introduction of a stop codon in various places throughout the region have? The region, as mentioned previously, resembles a RGG box; as such it would be interesting to investigate whether other known RNA-binding domains (198, 209) can be substituted functionally. The possibility also exists that the function of the novel C-terminal region is dependent on the fact that it is a basic sequence. Can the 72-amino acid tail be attached to the truncated ICP27 proteins of other growth-defective ICP27 mutants (such as *n*263R and *n*406R) and effect an exCd305-like phenotype?

The frameshift mutation expanded an existing homopolymeric run of eight cytosine residues to nine in the ICP27-coding strand at codons 215–217. Such homopolymeric sequence expansions and contractions may be common mutational events (305). The homopolymer at codons 215–217 is the largest of the ICP27 ORF, and so may represent a mutational "hot spot". If so, revertants bearing the identical intragenic reversion should be able to be selected for using, not only M16, but also other growth-defective viruses defined by mutations in regions downstream of the homopolymer at codons 215–217 (e.g. M11, M15, and *n*406R). It would also be interesting to see whether revertants can be obtained that possess frameshifts at other homopolymeric runs, as the ICP27-coding region contains a number of homopolymeric runs only one or two residues smaller than the run at codons 215–217.

7

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