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University of Alberta

IDENTIFICATION OF NOVEL PROPERTIES OF THE HERPES SIMPLEX VIRUS REGULATORY PROTEIN ICP27

by

Wendy Elizabeth Mears 

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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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Identification of Novel Properties of the Herpes Simplex Virus Type 1 Regulatory Protein ICP27" submitted by Wendy Elizabeth Mears in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Stephen A. Rice (supervisor)

Dr. Mark Glover

Dr. Charlotte Spencer

Dr. Richard Wozniak

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June 17, 1997

To my father, the late W. Lawrence "Bud" Eamon,
who instilled in me the desired to always pursue new heights,
and my mother Judith Ann (Foan) Hastings,
who constantly demonstrates the courage required to achieve these heights.

ABSTRACT

ICP27 is an essential immediate-early protein of herpes simplex virus type 1 (HSV-1). During infection, ICP27 both positively and negatively regulates the expression of viral genes. Although the exact mechanism(s) by which ICP27 performs its function is not known, much evidence indicates that ICP27 works at the post-transcriptional level. To determine how ICP27 regulates gene expression, we investigated several physical and functional properties of the protein.

First, we investigated ICP27 localization in the cell nucleus. We found that ICP27 contains a strong nuclear localization signal (NLS) sequence which maps to residues 109 - 137. ICP27 also possesses several weaker NLS's within its C-terminal half. We also identified a nucleolar localization signal sequence (NuLS) in ICP27. This NuLS consists of ICP27's strong NLS and a series of arginine and glycine residues which bear a striking similarity to a putative RNA-binding domain, referred to as an RGG box. Second, we examined ICP27's ability to interact with RNA. We found that ICP27 can bind to both poly (G) RNA homopolymers and more complex RNA molecules *in vitro*. Moreover, ICP27's RGG box domain appears both necessary and sufficient for this interaction. Third, we studied the post-translational modifications of ICP27. We found that ICP27 is post-translationally methylated, and that its RGG box is required for this modification. Fourth, we investigated the ability of ICP27 to shuttle between the nucleus and cytoplasm of cells. We used an interspecies heterokaryon assay to demonstrate that ICP27 is a very efficient nuclear shuttling protein. Mutations in ICP27 which result in non-functional protein either decrease or eliminate ICP27's ability to shuttle. Finally, it was found that ICP27 can stimulate gene transcription from an engineered reporter gene in HSV-1-infected cells. This is some of the first evidence that ICP27 can regulate gene expression at the transcriptional level.

In summary, we have used a variety of techniques to identify novel physical and functional properties of ICP27. These findings provide important new clues to the mechanisms by which ICP27 regulates viral gene expression.

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LIST OF ABBREVIATIONS AND NOMENCLATURE

α	Alpha
α-TIF	Alpha transcription intiation factor
β	Beta
C-terminus	Carboxy-terminus
C. tentans	*Chironomus tentans*
CAT	Chloramphenicol-acetyltransferase
DE	Delayed early
DMA	Dimethylarginine
DMEM	Dulbecco modified eagle medium
DNA	Deoxyribonucleic acid
E	Early
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
γ	Gamma
GAG	Glycosaminoglycan
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gH	Glycoprotein H
gL	Glycoprotein L
GST	Glutathione-S-transferase
H + L	Heavy and light
h	Hours
HCMV	Human cytomegalovirus
HHV	Human herpesvirus

HIV	Human immunodeficiency virus
hnRNP	Heterogeneous nuclear ribonucleoprotein
hpi	Hours post infection
HSV	Herpes simplex virus
HTLV	Human T-cell leukemia virus
HVEM	Herpesvirus entry mediator
ICP	Infected cell protein or peptide
IE	Immediate-early
IFN	Interferon
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-thiogalactoside
kDa	Kilodalton
KSHV	Kaposi sarcoma-associated herpesvirus
L	Late
lacZ	β-galactosidase
LPF	Late processing factor
LTR	Long terminal repeat
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
min	Minutes
MOI	Multiplicity of infection
N-terminus	Amino-terminus
NES	Nuclear export signal
NGF	Nerve growth factor
NLS	Nuclear localization signal
NuLS	Nucleolar localization signal
ORF	Open reading frame

PAA	Phosphonoacetic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PFU	Plaque-forming units
PK	Pyruvate kinase
PKI	Protein kinase inhibitor
PMSF	Phenylmethylsulfonyl fluoride
poly(A)	Polyadenylation
RNA	Ribonucleic acid
RRE	Rev-responsive element
rRNA	Ribosomal ribonucleic acid
S. pombe	*Saccharomyces pombe*
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulphate
snRNA	Small nuclear ribonucleic acid
snRNP	Small nuclear ribonucleoprotein
ssDNA	Single-stranded deoxyribonucleic acid
SV40	Simian virus 40
TAD	Transactivation domain
TAP	Transporter associated with antigen processing
TBP	TATA binding protein
TCA	Trichloroacetic acid
TF	Transcription factor
TK, tk	Thymidine kinase
TLCK	Nα-p-tosyl-L-lysine chloromethyl ketone
TNF	Tumour necrosis factor
U_L	Unique long component

U_S	Unique short component
vhs	Virion host shut-off
VP16	Virion protein 16
VZV	Varicella-Zoster virus
WT	Wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X. laevis	*Xenopus laevis*

Herpes viruses used or discussed

KOS1.1	Wildtype HSV-1 strain
Strain G	Wildtype HSV-2 strain
*d*27-*lac*ZI	KOS1.1 with β-galactosidase inserted into ICP27 locus
*d*27-1	KOS1.1 deleted for the ICP27 gene
*d*1-5	KOS1.1 which expresses the ICP27 protein missing amino acid residues 12 - 154
*d*1-2	KOS1.1 which expresses the ICP27 protein missing amino acid residues 12 - 64
*d*3-4	KOS1.1 which expresses the ICP27 protein missing amino acid residues 109 - 139
*d*4-5	KOS1.1 which expresses the ICP27 protein missing amino acid residues 138-154
*d*5-6	KOS1.1 which expresses the ICP27 protein missing amino acid residues 153 - 174
*n*263R	KOS1.1 which expresses the first 263 amino acid residues of the ICP27 protein
*n*406R	KOS1.1 which expresses the first 406 amino acid residues of the ICP27 protein
*n*504R	KOS1.1 which expresses the first 504 amino acid residues of the ICP27 protein plus four extra non-ICP27 amino acid residues

M11	KOS1.1 which expresses the ICP27 protein with point mutations at amino acid residues 340 and 341
M15	KOS1.1which expresses the ICP27 protein with point mutations at amino acid residues 465 and 466
M16	KOS1.1 which expresses the ICP27 protein with a point mutation at amino acid residues 488
KAT	KOS1.1 containing the tkCAT27 reporter gene inserted in ICP27 locus in opposite orientation to the wildtype gene
KATrev	KOS1.1 containing the tkCAT27 reporter gene inserted in ICP27 locus in same orientation as the wildtype gene

CHAPTER 1

GENERAL INTRODUCTION

Chapter One - General Introduction

I. *Herpesviridae*

The *Herpesviridae* family consists of relatively large enveloped DNA viruses which infect a wide range of animal species (reviewed in ref. 73,74). Their linear double-stranded genomes range in size from 120 to 230 kbp and encode anywhere from 60 to 120 proteins. These viruses possess four major biological properties which together distinguish them from other viral families. First, herpesviruses encode a large number of enzymes involved in nucleic acid metabolism (for example, thymidine kinase, alkaline exonuclease) and replication (for example, origin binding protein, ssDNA binding protein, helicase/primase complex). Second, both viral DNA synthesis and viral capsid assembly occur within the nucleus of the infected cell. Third, these viruses possess the ability to become latent in host cells. Finally, productive infection results in the eventual destruction of the invaded host cell.

Herpesvirus particles possess a DNA core contained within an icosadeltahedral protein capsid. This capsid is approximately 100 nm in diameter. Surrounding the capsid is an amorphous layer of variable thickness referred to as the tegument (75). Several viral proteins are present within this layer including VP16, the viral transcriptional activator of immediate-early genes (reviewed in ref. 57,74), and the *vhs* protein which is responsible for initial shut-down of host cell protein synthesis by inducing nonspecific destabilization and degradation of cellular and viral mRNA (36,74). The tegument is surrounded by a host-derived lipid-containing envelope (88) which possesses outwardly protruding spikes. Host cell lipids similar to the cytoplasmic membrane but different from the nuclear membrane are found in the viral envelope (92). The spikes are believed to be glycoproteins encoded by the virus, 11 of which have been identified to date. The size of the intact virion varies between 120-300 nm.

The Herpesviridae are divided into three sub-families: the *Alphaherpesvirinae* (α), *Betaherpesvirinae* (β), and *Gammaherpesvirinae* (γ). These divisions were initially determined according to biological properties and have subsequently been based on DNA sequence homology, similarities in genome arrangement, and relatedness of important viral proteins (reviewed in ref. 73). The validity of some of these classifications is generally under review as more information is obtained for each virus and more viruses are discovered. At present the three classes are distinguished by the following biological properties (reviewed in ref. 35,73,74).

Alphaherpesviruses are neurotropic viruses with a variable host range and a relatively short reproductive cycle. They are often highly cytopathic in cultured cells, and establish latency in sensory ganglia. Of the eight human herpesviruses identified, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and Varicella-Zoster virus (VZV) are classified as alphaherpesviruses.

Betaherpesviruses were initially characterized as salivary gland viruses and later as cytomegaloviruses. With the inclusion of herpesvirus 6 and herpesvirus 7, these descriptions no longer encompass all betaherpeviruses. These viruses possess a more restricted host range, have a relatively long reproductive cycle, are less cytopathic than alphaherpesviruses, and have relatively GC-rich coding regions. Infection frequently results in the enlargement of the invaded cell (cytomegalia). Latency can be established in secretory glands and other tissues such as lymphoreticular cells and the kidneys. Betaherpesviruses include the human cytomegalovirus (HCMV) and the human herpesviruses 6 and 7 (HHV-6 and HHV-7).

The final sub-family, the gammaherpesviruses, consist of lymphotropic viruses with restricted host range and genomes of roughly 150 kbp. These viruses are specific for B- or T-lymphocytes and can establish latency within these cells or can transform them (i.e., they can be tumourigenic). The human Epstein-Barr virus (EBV) and the more

recently described Kaposi sarcoma-associated herpesvirus (KSHV or HHV-8) are examples of this sub-class of herpesvirus.

II. Herpes Simplex Virus

Herpes simplex virus type 1 (HSV-1) is a member of the *Alphaherpesvirinae* (reviewed in ref. 74). This virus is of great benefit to study due to its ease of propagation and its short replication cycle in tissue culture. Analysis of this virus provides insight into both general cellular and viral functions along with specific activities of HSV-1. HSV-1 consists of a GC-rich (68%) genome, approximately 152 kbp in size and encoding over 70 proteins. The HSV-2 genome is also GC-rich (69%) and is highly co-linear with the HSV-1 genome. The HSV genome consists of two larger unique domains [unique long (U_L) and unique short (U_S)], flanked by short directed repeat domains (Fig. 1-1). Through homologous recombination, this arrangement allows inversion of the unique domains resulting in four equimolar isomers of viral DNA.

HSV-1 infection occurs normally within epithelial tissues of the eye and mouth. Infection usually occurs early in childhood and, depending on the socio-economic background, 30-90% of the population will possess antibodies directed against HSV-1. Typically, HSV-1 infection does not result in severe disease unless present in immunocompromised patients, in which case infection can result in meningitis or encephalitis. Destruction of the central nervous system has been reported in rare cases of otherwise normal patients. HSV-2 infects epithelial tissue of the genitalia. Because HSV-2 is sexually transmitted, individuals are infected later in life and fewer of the population will possess antibodies directed against it. Both viruses undergo productive infection at the initial site of contact and within 2-4 weeks (in animal models) can be found within the sensory ganglia innervating the primary site of infection.

Latency (reviewed in ref. 74,94,98), a process which is still not well understood, occurs within the neuronal cell body. The latent viral genome has no free DNA ends,

suggesting that it is present in the form of a closed circle. Within the HSV-infected neuron, no viral proteins have been consistently detected and only one abundant viral RNA transcript has been observed. For reasons still unknown, various stimuli including physical or emotional stress (sunburn, anxiety, etc.), tissue damage at the initial site of infection, and/or the intake of certain drugs or hormones, will result in productive infection within the neuron (25). Infectious virus then travels down the sensory axon to tissues at or near the initial site of infection resulting in lytic infection of the surrounding epithelial cells. The fate of the neuron in which reactivation occurs is contraversial and uncertain. It has been suggested that from current available observations, virus multiplication in the neuron results in its destruction (74).

As productive infection occurs efficiently in tissue culture, the lytic cycle of HSV has been extensively examined (reviewed in ref. 74). Although many questions still remain, it is possible to describe the basic "life-cycle" of HSV. Viable progeny can be detected as early as eight hours post infection in permissive cell lines, but peak virus production requires approximately 18-20 hours. The viral life-cycle consists of several steps. These include attachment, entry, release of viral DNA, expression of viral genes, viral DNA replication and packaging, and finally, release of infectious virions.

Upon addition of the virus to a cell culture, HSV-1 virions attach to the host cell. Attachment and entry of the virus are complex processes likely involving multiple cell-surface components. To date, no one specific host cell receptor-viral glycoprotein interaction has been identified. However, binding of both HSV-1 and HSV-2 can be facilitated by the interaction of viral glycoproteins (for example, gB, gC and gD) with glycosaminoglycan chains (GAGs) of cell surface proteoglycans (reviewed in ref. 51,74,87). After attachment, the virion envelope fuses in a pH-independent fashion with the cell plasma membrane or an early endosome (101). Attachment of the virion to the cell surface is not sufficient for viral penetration. Several viral glycoproteins have been identified that are required for entry, including gB, gD, gH and gL (74). Recently, a

novel mediator of virion entry has also been identified. HVEM (herpesvirus entry mediator), a member of the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor family, mediates the entry of several wildtype (WT) strains of HSV (52). It is unknown if HVEM aids in membrane fusion or in release and transport of virion components once within the cell. Other mediators of viral entry are anticipated but have yet to be identified. After penetration, the viral capsid and components of the tegument are released into the cytoplasm. The capsid, along with some of the tegument proteins are transported to the nuclear pore complex.

Once at the pore, the viral DNA genome is released into the nucleus where it rapidly circularizes. VP16 (also referred to as α-TIF) is a major component of the tegument and a strong transcriptional activator of immediate-early viral genes (reviewed in ref. 57,74). After entering the nucleus, VP16, along with cellular transcription factors, recruits the host cell RNA polymerase II to transcribe the viral genome (12). Viral gene expression occurs in several temporal waves (10,31; reviewed in ref. 74).

The first set of viral genes to be transcribed are referred to as alpha (α) or immediate-early (IE) genes (reviewed in ref. 74). These genes do not require any *de novo* viral protein synthesis for their expression (37). Their promoters contain elements similar to those found in cellular genes such as a TATA box, cap site, and other cellular *cis*-acting elements. These viral genes also possess conserved transcription factor binding sites called "TAATGARAT" sequences upstream of their initiation sites. In conjunction with sequences flanking this region, the TAATGARAT sequence is recognized and bound by the cellular transcription factor Oct1 in association with VP16, resulting in the expression of the IE genes (reviewed in ref. 57,74). Immediate-early gene expression peaks roughly two to four hours post-infection (hpi), although some can still be detected late in infection. Five IE genes are expressed during infection: ICP0, ICP4, ICP22, ICP27, and ICP47 (ICP = infected cell protein or polypeptide). ICP0, ICP4, ICP22 and

ICP27 all encode regulatory proteins, while ICP47 encodes an immunological modulator. The products of these genes are discussed in more detail below.

The second set of genes to be transcribed are referred to as beta (β), delayed-early (DE) or early (E) genes (reviewed in ref. 74). These genes require IE gene products for efficient expression (19,32,64,95). Their promoters possess binding sites for cellular transcriptional factors as well as TATA boxes and cap sites. Delayed-early genes exhibit peak rates of synthesis five to seven hours post-infection. This class can be divided into two sub-classes, β_1 and β_2, as some DE genes such as ICP6 and ICP8 are observed earlier than five hours post-infection yet still belong to this kinetic class. The DE genes mostly encode nucleic acid metabolism and DNA replication enzymes. Their expression is down-regulated by the progression into the final wave of expression as noted by enhanced levels of DE products when viral DNA synthesis is inhibited.

The final kinetic class of genes to be expressed is the gamma (γ) or late (L) gene class (reviewed in ref. 74). γ genes are subdivided into γ_1 (leaky-late) and γ_2 (true-late) genes. Leaky-late genes (e.g., gB) are expressed relatively early during this phase and are enhanced by but do not require viral DNA synthesis for expression. True late-genes (e.g., gC) are expressed later in infection and absolutely require viral DNA synthesis for their expression. The exact structure of γ gene promoters is undetermined. For some late genes, such as US11, the only regulatory element detectable within the promoter is the TATA box/cap-site (34). Other late genes appear to require regions within the 5' untranslated region of their transcripts for appropriate expression (reviewed in ref. 74). Late genes encode structural and other virion component proteins, including VP16 and *vhs*.

Viral DNA replication and capsid assembly occur within the infected nucleus (reviewed in ref. 74). Replication is thought to proceed by a rolling circle mechanism. Long concatamers of DNA are produced which are then cleaved and packaged into preformed capsids. Intact capsids are believed to bud through the inner lamellae of the

nuclear membrane and proceed through the cytoplasm to be released into the extracellular space. The exact mechanism of virion translocation out of the nucleus and through the cytoplasm is undetermined. By electron microscopy, both enveloped and unenveloped capsids are seen within the cytoplasm. It is possible that a progression of enveloping and de-enveloping steps occur as the capsid moves to the cellular membrane, resulting in its ultimate release from the host cell.

III. Immediate-early Gene Products

The IE proteins, as discussed previously, are expressed immediately after infection through the action of the viral transactivator VP16 and the host RNA polymerase II complex. Four of the five proteins (ICP0, ICP4, ICP22, and ICP27) possess gene regulatory roles during infection. The fifth IE protein, ICP47, has no known effect on virus transcription or replication in cell culture.

ICP0: ICP0 (also referred to as Vmw110 or IE110), although predicted to be 80 kDa, migrates between 110 and 124 kDa on SDS polyacrylamide gels (reviewed in ref. 74). This protein is found in both the nucleus and cytoplasm of the cell depending on the time of infection and the presence of other immediate-early proteins (54,106). Observations from transient expression assays suggest that ICP0 enhances the transcription of several viral and heterologous promoters in cooperation with ICP4 (reviewed in ref. 74). This protein is non-essential for productive infection at high multiplicities of infection but is required for effective growth when lower concentrations of virus are used. Although ICP0 is not essential, its deletion from or mutation within the HSV-1 genome results in a delay in IE and DE gene expression and an impairment of viral replication (5,9). ICP0 also appears to play a role in initiating reactivation of the virus from its latent state in mouse models (6,39).

ICP4: ICP4 (also referred to as Vmw175 or IE175) is an essential nuclear phosphoprotein of approximately 175 kDa. Three species of ICP4 (59) ranging from 160

- 170 kDa can be resolved on SDS-PAGE. These various forms, which possibly represent multiple phosphorylation states (reviewed in ref. 74), may possess different functional roles. ICP4 is the major transcriptional trans-activator of HSV-1. Mutational analysis of this protein demonstrates that it is required for viral DE and L gene expression (16,17). ICP4 also has an auto-regulatory effect during infection, resulting in its own down-regulation (reviewed in ref. 74), and it may repress some other viral IE genes (41). It possesses several functional domains, including a DNA-binding domain, a multimerization domain, at least one transactivation domain (TAD), and a nuclear localization signal (NLS) sequence. The DNA-binding domain, TAD, and the NLS are required for transactivation of certain viral gene promoters (18,83,102), suggesting interaction with DNA is required for gene activation. To further support this hypothesis, several viral genes possess ICP4 DNA-binding sites in their promoter regions. The exact mechanism by which ICP4 transactivates viral genes remains unknown. ICP4 can form a tripartite complex with the cellular transcription factors TFIIB and TBP, and facilitate the binding of TFIID to the TATA box in the presence of TFIIB (84). It interacts with TFIID via another cellular transcription factor, TAF250 (7). It is possible that ICP4 enhances the recruitment of the cellular RNA polymerase II complex to the viral genes resulting in the expression of the viral genome. The mechanism by which ICP4 negatively regulates viral genes is partially understood. Binding of ICP4 to promoters of genes inhibited by ICP4 is required but not sufficient to inhibit transcription of these genes (24,41,72,83). Recently, another function of ICP4 was reported. Along with its transcription regulatory role, it was found that ICP4 can suppress HSV-1-induced apoptosis (40).

ICP22: ICP22 is a 68 kDa nuclear phosphoprotein which is non-essential for productive infection in some lines of tissue culture cells (reviewed in ref. 74). In those cell lines in which it is required, ICP22 is necessary for the optimal expression of ICP0 and a subset of γ proteins. This protein may be phosphorylated by the viral UL13 gene product, as ICP22 is hypophosphorylated in UL13 mutant infected cells (reviewed in ref.

74). ICP22 is involved in the modification of host RNA polymerase II (69). When ICP22 is present, RNA polymerase II is found in a phosphorylation state which is intermediate between its normal hyperphosphorylated II_o state and its non-phosphorylated II_a state. It has been hypothesized that this modification of phosphorylation state may help recruit the cellular RNA polymerase II to the viral genome for transcription (69).

ICP27: ICP27 (also referred to as Vmw63 or IE63) is a 63 kDa essential nuclear phosphoprotein. As it is the focus of this thesis, it is discussed in greater detail below.

ICP47: ICP47 is a 12 kDa protein which aids in virus evasion of the host immune system (reviewed in ref. 74). It is non-essential in tissue culture. This protein inhibits presentation of MHC class I molecules on the surface of the cell thereby effectively preventing the destruction of infected cells mediated by $CD8^+$ T-cells (103). ICP47 interacts with the TAP transporter system in the endoplasmic reticulum to ultimately prevent antigen presentation (2,22,30,90).

IV. Infected Cell Protein 27

Physical characteristics. ICP27 is the gene product of the UL54 locus which is the only immediate-early gene located entirely within a unique region of the HSV genome (Fig. 1-1). This polypeptide consists of 512 amino acid residues. ICP27, when analyzed by SDS-PAGE, migrates with a calculated molecular weight of approximately 63,000, which is slightly higher than its theoretical molecular weight of 55,000. This is believed to be due in part to a highly acidic region near the N-terminus of the protein which may cause aberrant migration on SDS-PAGE (Fig. 1-1). It is also possible that post-translational modifications of ICP27 may contribute to the observed higher molecular weight. Phosphate molecules have been demonstrated to cycle on and off of ICP27 (99) although the exact site(s) of phosphorylation has yet to be mapped. Several consensus casein kinase II phosphorylation sites are present within the protein sequence

and are considered possible modification sites. Nucleotidylation of ICP27, in the form of the addition of an adenosine or guanine nucleotide, has also been documented (3). The specific role of these modifications is not yet known. ICP27 also posesses a cysteine rich region within in its C-terminal half (residues 483 to 508; 27,93; Fig.1-1) which bears similarity to zinc finger nucleic acid binding domains. Using metal chelate chromatography, Vaughan *et al.* (93) presented evidence that ICP27 binds to zinc *in vitro*. The C-terminal 105 amino acid residues of the protein are required for this interaction (93). Together, these observations suggest that this region may act similarly to other zinc finger domains found in a variety of transcription factors.

ICP27 is predominantly localized to the cell nucleus during infection (1). Both in infected cells and cells transiently expressing ICP27, this protein possesses a somewhat diffuse nuclear staining pattern, but demonstrates more intense regions of nuclear staining that do not seem to correspond to the nucleolar structures of the cell (38,68). This "patchy" staining may result from colocalization of ICP27 with snRNP complexes (63,81). Due to its large size, it is highly likely that ICP27 possesses a nuclear localization signal (NLS) sequence to assist its entry into the nucleus. Analysis of truncation and linker insertion mutants suggested that this signal may be present within the first 263 amino acid residues of the protein (27,67,68).

Functions during lytic infection. All sub-families of the *Herpesviridae* include viruses which possess a recognizable homolog of ICP27 (Table 1-1). This homology suggests a significant and possibly conserved role for ICP27 during infection. Indeed, from the analysis of temperature-sensitive (ts) mutants mapped to the UL54 locus, it was shown that ICP27 is essential for productive lytic infection (76). Further studies using deletion mutants confirmed this observation (44,67). Genetic studies of HSV-1 ICP27 mutants demonstrates that ICP27 is required for: i) the down-regulation of immediate-early genes, ii) the enhancement of viral DNA replication, iii) the expression of some late

genes, and iv) the efficient shut-off of host cell protein synthesis at later times during infection (44,67,76).

ICP27 appears to carry out two distinct functions that contribute to the progression of viral infection into the late phase (67). First, ICP27 increases the level of viral DNA replication up to 10-fold, most likely by enhancing the expression of a subset of DE gene products that are involved in DNA replication (47,77,91). The N-terminal region of ICP27 (amino acid residues 12 to 64) is required for stimulation of viral DNA replication (65). However, analysis of truncation mutants has also implicated amino acid residues 406 to 504 in this function (67). ICP27 may also affect DNA replication by modifying the formation of replication compartments. This hypothesis is supported by recent work which investigated the location of viral DNA replication proteins within the infected cell (13). ICP27 was found to affect both the intranuclear localization of ICP8, a viral encoded ssDNA binding protein required for DNA replication, and the assembly of other replication proteins into active replication sites (13). In further support of this hypothesis, ICP27 is required for the efficient formation of replication foci at late times during infection (62), suggesting that ICP27 may indirectly affect DNA replication. Second, ICP27 stimulates expression of L gene mRNA's by a process which appears to be independent of viral DNA replication (29,65,67,76). Identification of those regions of ICP27 which are required for this DNA replication-independent stimulation of L gene expression is complex. Domains located both in the N- and C-terminal halves of ICP27 can affect the induction of L genes. Within the C-terminal half of ICP27, stimulation of L genes requires the C-terminal eight codons of ICP27 (67). It was also shown that point mutations within the C-terminal region of ICP27, resulting in amino acid substitutions at codons 340 and 341, 465 and 466, or 488, produced mutant ICP27 molecules deficient in this function (65). In the N-terminal region of the ICP27, deletion of codons 103 to 178 also resulted in deficient L gene expression (29). However, a slight decrease in the efficiency of viral DNA replication was also observed with this mutation.

Regulation of gene expression by ICP27. Evidence indicates that ICP27 can both enhance or repress co-transfected reporter genes. In general, ICP27 appears to modify the actions of the viral transactivators ICP4 and ICP0 (20,23,66,82,89), although there is evidence that ICP27 can transactivate both viral and cellular genes independent of any other viral proteins (4,66).

The results from these experiments indicate that ICP27's effects on viral genes are complex. Some reporter genes, driven from viral DE and L gene promoters, have been found to be repressed by ICP27, while others have been found to be activated (27,50,66,68,82,89). Adding to the complexity is the fact that some groups have reported that a particular HSV-1 promoter-driven reporter gene is activated by ICP27 in combination with ICP4 and ICP0, while other groups have reported a repressive effect on the same promoter-reporter gene combination (27,50,66,68,82,89). These discrepancies, however, appear to be explained by differences in RNA processing signals in the various reporter gene constructs (8,79). Expression of reporter genes possessing intron sequences is down-regulated by ICP27, while expression of reporter genes possessing certain poly(A) signals is up-regulated. These results have led to the belief that ICP27 regulates gene expression at a post-transcriptional level (discussed below).

Synergism between ICP27, ICP4, and ICP0. The co-operative role of ICP27 with ICP4 and ICP0 is complex. Transient expression assays have been used extensively to study how these proteins synergistically regulate gene expression. In these assays, ICP27 represses the expression of some reporter genes which are activated by ICP4 and ICP0, and enhances the expression from others. The reporter genes used consisted of a variety of viral DE and L gene promoters as well as different downstream mRNA processing signals. As mentioned above, it appears that the mRNA processing signals within the reporter genes dictate whether ICP27 represses or enhances expression from these genes, suggesting that ICP27 may post-transcriptionally regulate genes that are transcriptionallly induced by ICP4/ICP0. However, this view may be simplistic as a

variety of evidence, discussed below, suggest complex interactions between ICP27 and ICP4 and ICP0.

First, ICP27 may modify the actions of ICP4 and ICP0 by affecting the cellular localization of these proteins. During WT HSV-1 infection, ICP0 is localized both to the nucleus and cytoplasm of the infected cell. Localization in the nucleus, as observed by indirect immunofluoresence, is diffuse with a more intense punctate speckled pattern throughout the nucleus. Punctate cytoplasmic staining is also present. However, when ICP27 is absent, ICP0 localizes almost exclusively to the infected nucleus in a distinct, highly punctate fashion (106). ICP27 also affects ICP0 localization in transiently transfected cells. When ICP27 and ICP0 are expressed transiently, ICP0 is highly cytoplasmic; in the absence of ICP27, ICP0 is predominantly nuclear. The amount of ICP27 plasmid which is transfected into cells inversely correlates with the extent of nuclear localization of ICP0. From this work, it appears that ICP27 inhibits ICP0 from localizing in the nucleus, although it does not completely prevent its accumulation there during infection. A similar study involving the localization of ICP4 (105) indicates that ICP27 similarly affects the nuclear localization of ICP4, preventing it from obtaining a completely nuclear localization. Thus, ICP27 may indirectly affect the ability of ICP4 and ICP0 to affect gene expression by sequestering ICP4 and ICP0 in the cytoplasm. The C-terminal region of ICP27 is required for the cytoplasmic localization of both ICP0 and ICP4, suggesting that ICP27 may affect the localization of both ICP0 and ICP4 by a common mechanism.

ICP27 also effects the biochemical state of ICP4. In the absence of ICP27, more slowly migrating forms of ICP4 are observed on SDS-PAGE (50,66,89). This difference may be due to a change in phosphorylation state of ICP4. The various phosphorylated forms of ICP4 possess different DNA binding abilities (reviewed in ref. 74), suggesting that modification of ICP4 by ICP27 may regulate its function. As phosphorylation state may also affect the nuclear import of proteins (reviewed in ref. 56), ICP27-associated

modification of ICP4 could explain ICP27's affect on ICP4's cellular localization. To date, no kinase or phosphatase activites have been associated with ICP27. Therefore, if ICP27 does alter phosphorylation of ICP4, it is likely to do so indirectly.

Finally, evidence has recently been presented that demonstrates *in vivo* and *in vitro* physical interactions between ICP4 and ICP27. Using co-immunoprecipitation and mobility shift assays, Panagiotidis *et al.* (58) found that ICP27 binds preferentially to the less modified forms of ICP4. In addition, ICP27 was found to be associated in protein-DNA complexes involving ICP4 and the ICP4 binding site in the HSV-1 thymidine kinase promoter. It is thus possible that ICP27 modulates ICP4's DNA binding activity through a direct physical interaction (58).

Domains within ICP27 that are required for activation and repression. Initial studies on ICP27 using transient expression assays identified, although not unambiguously, regions of ICP27 that are required for the repression and activation functions observed. This evidence is summarized below. However, it should be noted that the data collected to date regarding the repression and activation domains in ICP27 are complex and often contradictory.

Some evidence suggests that the C-terminus of the protein is required for repression of ICP4/ICP0-induced reporter genes. One study localized the repressor region to the C-terminal 80 amino acid residues of ICP27 (27), the final 62 residues being very important for this activity. In this study, linker insertions and deletions in the N-terminal 406 amino acid residues had no affect on ICP27's ability to repress induction of pTK-CAT expression in the presence of ICP4 and ICP0. McMahan and Schaffer (50), however, identified two repression domains in the C-terminus. One of these domains falls between amino acid residues 465 to 511, supporting Hardwicke *et al.*'s conclusion (27). This region (amino acid residues 465-511) contains the putative zinc finger motif described earlier (Figure 1-1) and is conserved among ICP27 homologs. Rice *et al.* (68), however, determined that an ICP27 molecule truncated at amino acid residues 406 or 504

possessed WT repression activity, suggesting that the C-terminal end of the molecule is not required for repression. Although this appears to contradict other findings, it is consistent with the existence of a second repression domain identified by McMahan and Schaffer (50) which was mapped to amino acid residues 327 to 406. To further complicate the situation, it was found that the region between amino acid residues 407 and 464 interferes with repression when the protein is truncated just after amino acid residue 464 (50). As well, when the acidic region of ICP27 (amino acid residues 12 to 64) was deleted, the resulting molecule (d1-2) was incapable of efficiently repressing ICP4 induced transactivation, suggesting that the N-terminus of the protein also contributes to repression (70). These results do not discount the importance of the C-terminal region for ICP27's repression function, but rather suggest that multiple domains may be involved in this gene regulatory role.

Identification of the activator domain(s) within ICP27 has also proven to be complicated. Two research groups (27,50) have shown that linker insertion mutations, truncations, and deletions within the C-terminal half of ICP27 (amino acid residues 263-512) eliminate the ability of the protein to transactivate promoters induced by ICP4 and ICP0. As mutations in the N-terminal half of the gene did not cause any loss of activation function, it was concluded that the activator domain was within the C-terminal half of the protein (27,50). Indeed, truncation of the molecule at amino acid residue 504 eliminates the ability of ICP27 to activate gene expression, suggesting that the final 8 amino acid residues are required for this function (70). Point mutations within the C-terminal half of the gene, which result in the alteration of only one or two amino acid residues, also eliminate the ability of the protein to transactivate (65). However, a truncated ICP27 molecule consisting of the N-terminal 263 amino acid residues demonstrated partial activation of ICP4/ICP0 induced promoters (68). This suggests that an activator domain is present within the N-terminal half of the protein. Once again, it is

very difficult from the data collected to date to define the exact region(s) of ICP27 which are responsible for its activation function.

Analysis of *trans*-dominant mutants of ICP27 has provided additional clues to ICP27's function. Mutations that cause defects specifically in ICP27's activator function were found to be *trans*-dominant in both transient expression assays and during infection (86). Mutations that eliminated both activation and repression functions, however, were not dominant. From these findings, it was suggested that the repressor region described above (amino acid residues 434-512) might define an important binding domain, possibly involving protein-protein or protein-nucleic acid interactions (86). Mutations in this domain would eliminate putative functional interaction thereby affecting both activation and repression. Mutations in the activator domain (excluding the region that overlaps the repressor domain) should be able to bind but would not trans-activate. These mutants would be expected to compete for binding with any WT protein present resulting in the *trans*-dominant phenotype observed.

V. Mechanisms By Which ICP27 May Control Gene Regulation

ICP27 plays several essential roles during lytic infection. It is required for the transition from the DE to L phase of infection by inducing viral DNA synthesis and the expression of some L genes. ICP27 also possesses a negative regulatory function, down-regulating its own expression as well as some of the other IE genes (23,67). Finally, ICP27 plays a role in the shut-off of host protein synthesis during infection.

The exact mechanism by which ICP27 regulates gene expression is unknown. Although it is possible that this regulation could be at the level of transcription, no direct transcriptional role for ICP27 has been reported. Considerable research, however, suggests that ICP27 regulates gene expression via one or more post-transcriptional effects. This evidence is summarized below.

Control of gene expression at the post-transcriptional level. Much research regarding ICP27 has focussed on its ability to post-transcriptionally affect gene expression. From these studies, it appears that ICP27 may regulate gene expression at the level of mRNA processing (such as polyadenylation or splicing), mRNA stability, or even mRNA translation. It is also possible that ICP27 may affect the cellular location of viral RNA transcripts. Indeed, evidence for all of these roles has been observed.

Evidence has suggested that ICP27 can negatively effect the translation of ICP4 mRNA (67). When ICP27 is not present or is non-functional, there is an increase in ICP4 protein levels within the cell but no effect on the overall level of ICP4 mRNA. This suggests that ICP27 affects the expression of ICP4 post-transcriptionally, possibly at the level of translation.

Some of the strongest evidence for post-transcriptional regulation by ICP27 has come from co-transfection experiments. Chapman *et al.* (8) observed that ICP27 activates the expression of a reporter gene under the control of a retroviral promoter. However, activation was independent of the viral promoter sequence. Instead, it was dependent upon the sequences 3' to the reporter gene which correspond to the 3' end of Moloney murine leukemia virus and a 150 bp internal region upstream of the 3' LTR. This sequence contains a functional poly(A) site. Several other studies have since shown that ICP27 regulation of reporter genes is dependent on the presence of specific mRNA processing signals (47,48,79). Further evidence for post-transcriptional regulation by ICP27 has come from studies in infected cells. Cells infected with ICP27 temperature-sensitive mutants at the permissive temperature and then shifted to the non-permissive temperature for several hours do not accumulate late gene transcripts, although transcription of late mRNAs is observed to continue (85). This suggests that ICP27 affects the processing of pre-mRNA or the stability of the processed mRNA transcripts.

Possible inhibition of splicing by ICP27. Almost all cellular genes possess intron sequences within their transcripts which must be removed to produce the functional

mRNA. However, only four HSV-1 genes expressed during lytic infection require splicing of their transcripts in order to produce functional protein (11,60,71,96). Of these intron-containing transcripts, three are transcribed from the IE genes ICP0, ICP22, and ICP47. A fourth intron-containing transcript is produced at late times from the UL15 gene.

ICP27 appears to inhibit the removal of intervening sequences from cellular and viral pre-mRNA transcripts (reviewed in ref. 78). The first evidence for this came from transient expression assays. Expression of ICP4/ICP0-transactivated CAT reporter genes containing 5' and 3' introns are reduced 5- to 10-fold in the presence of ICP27 (79). More recent studies further support the hypothesis that ICP27 inhibits splicing. First, in HSV-1-infected cells, the levels of accumulated mRNA of several cellular genes were reduced when functional ICP27 was present, but not when it was absent (26). Data from nuclear run-off analysis indicated that these decreases occurred post-transcriptionally. Second, precursor RNA from viral intron-containing transcripts accumulate in the presence of functional ICP27 (28). Finally, *in vitro* splicing extracts prepared from HSV-1 infected cells are less efficient than uninfected extracts at splicing a cellular β-globin pre-mRNA transcript. This is not observed if the extracts are prepared from cells infected with ICP27 mutant virus (28). From these studies, it appears that ICP27 inhibits pre-mRNA splicing, possibly resulting in the down-regulation of both cellular and viral intron-containing genes.

The mechanism by which ICP27 affects splicing is unknown. However, it is interesting that HSV-1 infection causes a reorganization of antigens associated with snRNP complexes (43). In uninfected cells, snRNP complexes display a nuclear punctate pattern. Shortly after infection, the individual complexes appear to become slightly larger and relocalize to the periphery of the nucleus. ICP27 is sufficient and necessary for this redistribution (63,81). A redistribution of splicing factors is also observed during adenovirus infection (33). It is possible that snRNP reorganization induced by ICP27

results in the sequestering of splicing factors away from cellular transcripts, thereby preventing their splicing. Repression of intron-containing genes could be a mechanism by which HSV-1 down-regulates host cell protein synthesis later during infection.

Some evidence suggests that ICP27 may inhibit splicing and effect snRNP distribution via a direct physical interaction. ICP27 can be co-immunoprecipitated from infected cells using an antisera (termed anti-Sm antisera) from patients with systemic lupis erythematosus (80). These patients possess autoantibodies that are directed against several of the common cellular snRNP proteins. Co-immunoprecipitation of ICP27 with snRNP proteins suggests a physical interaction between ICP27 and components of the splicing complex. The C-terminal region of ICP27 (amino acid residues 450 to at least 504) is required for this interaction (80). The C-terminal regions of ICP27 is also required for the redistribution of snRNP complexes described above (81), and possesses at least one domain responsible for the repression of ICP4/ICP0-induced intron-containing genes (27,50,68). These results suggest that the interaction between the C-terminal region of ICP27 and cellular snRNP's could result in the inhibition of splicing, possibly by sequestering snRNP components away from active sites of pre-mRNA processing.

Modification of poly(A) site utilization by ICP27. HSV-1-infected cells possess a heat labile activity, termed late processing factor (LPF), which increases the processing efficiency at HSV-1 late gene poly(A) sites both *in vitro* and *in vivo* (47,49). ICP27 is required for induction of LPF, which occurs early during infection (48). Although processing of at least two L poly(A) sites is increased by ICP27, processing of a variety of IE and E poly(A) sites is not affected (47,48). In *in vitro* polyadenylation assays using extracts from mock-infected cells, the processing of the two L poly(A) sites was less efficient than that of several IE or E gene poly(A) sites (47). These results suggest that ICP27 enhances the processing of transcripts possessing inherently weak poly(A) sites. Further supporting this hypothesis, ICP27 was found to enhance the mRNA accumulation

of a reporter gene lacking a G/U box near its poly(A) site (79). The G/U box, at the RNA level, defines a U- or GU-rich region downstream of the consensus hexanucleotide (AAUAAA) poly(A) signal. Although it is not essential for polyadenylation, it increases the efficiency of this process (42,96). Recently, it was demonstrated that HSV-1 infection results in the enhanced *in vitro* binding of known polyadenylation factors to RNA substrates containing poly(A) sites. Functional ICP27 is required for this effect (47). Together these results suggest that ICP27 may activate the expression of some HSV-1 late genes by activating the usage of their inherently weak poly(A) sites.

ICP27 may affect mRNA transport and stability. HSV-1 immediate-early proteins can up-regulate the expression of reporter genes that contain the 3' UTR regions of the β-interferon and c-myc genes (4,53). ICP27 itself is sufficient to increase the half-life and steady state levels of mRNA from a reporter gene possessing the human β-interferon (IFN) open reading frame and natural 3' processing signals (4). ICP27 also up-regulates the expression of CAT activity from reporter genes containing 3' processing signals from either the β-IFN or c-myc genes. Brown *et al.* (4) determined that many of the conserved cysteine and histidine residues near the C-terminus of ICP27 (Fig. 1-1) are essential for the up-regulation of these reporter genes. Interestingly, both the β-IFN and c-myc mRNAs are inherently unstable and contain an AU-rich sequence in the 3' end of their transcripts which has been implicated in mRNA destabilization. Brown *et al.* (4) have suggested that ICP27 may activate these genes post-transcriptionally by increasing their mRNA stability, perhaps through an interaction with the AU-rich region.

Recently, it was found that ICP27 can affect the cellular localization of various viral transcripts during infection (61). Using *in situ* hybridization techniques, Phelan *et al.* (61) observed that viral intron-containing transcripts are increasingly retained within the nucleus as WT HSV-1 infection proceeds. These transcripts are not retained within the nucleus when cells are infected with an ICP27 deletion virus. At the same time, intronless transcripts are rapidly exported from the nucleus regardless of the presence of

ICP27. It is likely that intron-containing transcripts retained within the nucleus would be degraded, ultimately resulting in the down-regulation of their expression. In this way, ICP27's ability to inhibit splicing of pre-mRNA combined with its retention of intron-containing transcripts could post-transcriptionally down-regulate the expression of cellular genes, aiding in host protein synthesis shutoff at later times during infection.

VI. Thesis Objectives

From the evidence collected to date, ICP27 appears to be an essential, multi-functional regulatory protein required for the progression of HSV-1 infection into the late phase. It acts, at least in part, through post-transcriptional mechanisms resulting in the down-regulation of intron-containing genes and the activation of genes possessing weak poly(A) signal sequences (such as some HSV-1 late genes). It may also stabilize various mRNA transcripts and play a role in mRNA transport from the nucleus. Although much has been deciphered over the years, there is still much remaining to be determined. Further characterization of ICP27 at the molecular level could elucidate basic aspects of ICP27 which would further advance our understanding of this protein. With this background, several projects were undertaken.

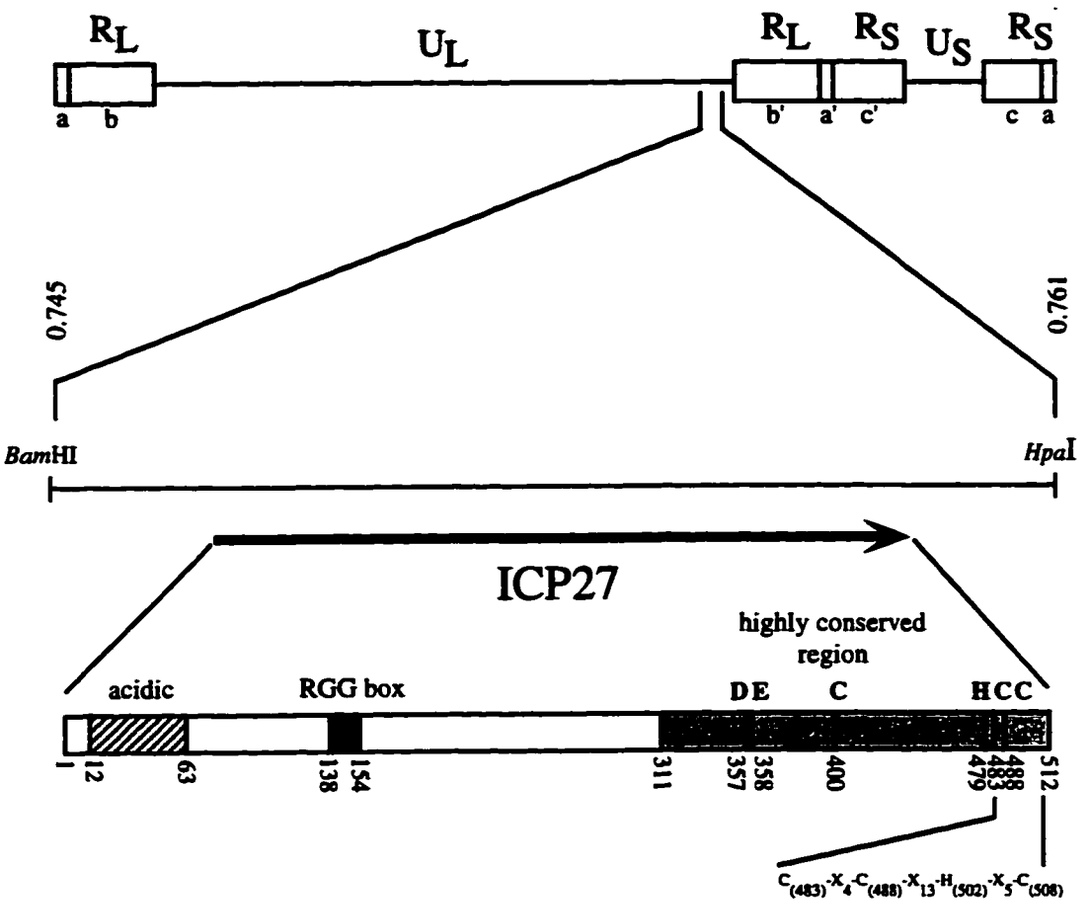
(1) ICP27 is a nuclear protein with functions that would be expected to occur within the nucleus. Due to its size, it is likely that ICP27 requires active import to gain entry into the nucleus. This suggests that it possesses a nuclear localization signal (NLS). ICP27 is also found within the cytoplasm of some cells, although to a lesser degree. It is unknown whether cytoplasmic ICP27 is merely lingering after translation or whether ICP27 has a functional role within the cytoplasm of infected cells. To determine if nuclear localization is important for ICP27's function, we performed studies to identify ICP27's NLS.

(2) ICP27 plays a role in viral gene expression via post-transcriptional mechanisms. As discussed previously, several features of this post-transcriptional control

suggest that ICP27 interacts with the host cell splicing and polyadenylation machinery. It is possible that this involves a direct interaction between ICP27 and RNA. In this light, the interaction between ICP27 and RNA was investigated. Does ICP27 bind to RNA and if so, does this interaction occur with any sequence-specificity? It was also of interest to identify the region of ICP27 which is required for this potential interaction, as two putative nucleic acid binding domains are evident within ICP27's sequence.

(3) Using transient expression assays, ICP27 has been shown to down-regulate intron-containing transcripts and to up-regulate genes containing specific polyadenylation signal sequences. We questioned if this regulation occurs upon genes contained within the viral genome itself. To investigate ICP27's role upon genes contained within its own genome, a model system utilizing recombinant HSV-1 viruses was devised. It was anticipated that this system would provide insight into ICP27's *in vivo* effects on gene expression and could be used to elucidate the regions of ICP27 required for these function(s).

FIG. 1-1. Structure of the HSV-1 ICP27 gene and its encoded protein. Shown at the top is a representation of the prototype arrangement of the HSV-1 genome. Shown immediately below is a *Bam*HI-*Hpa*I restriction fragment from the HSV-1 genome. The narrow lines depict unique (U) regions of the viral genome, the open bars denote repeat regions (R), the lower case letters designate the components of both the long and short repeat regions, and the bold arrow depicts the location and direction of transcription of the ICP27 gene within the HSV genome. The numbers above the *Bam*HI-*Hpa*I restriction fragment denote the map coordinates corresponding to this region of the genome. Shown below the *Bam*HI-*Hpa*I restriction fragment is a representation of the ICP27 protein. The hatched box depicts a highly acidic region in the N-terminus, the black box depicts a highly basic region consisting almost entirely of arginine and glycine residues, and the grey box depicts the C-terminal portion of the protein which is highly conserved among ICP27 homologs of a variety of herpesviruses (4). The letters in bold are single letter amino acids representations (C=cysteine, D=aspartate, E=glutamate, H=histidine) which are conserved among all eight herpesviruses (4) listed in Table 1-1. The numbers below the protein representation correspond to the amino acid residues within ICP27. Shown immediately below the conserved C-terminus of ICP27 is the putative zinc finger nucleic acid binding domain (27,93).


RL
UL
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a
b
b'
a'
c'
c
a
0.745
0.761
BamHI
HpaI
ICP27
highly conserved region
acidic
RGG box
DE
C
HCC
1
12
63
138
154
311
357
358
400
479
483
488
512
C(483)-X4-C(488)-X13-H(502)-X5-C(508)

Table 1-1

Proteins with sequence homology to HSV-1 ICP27 are found in all classes of herpesviruses

Virus Name	Homolog Name	Virus Class	Reference
HSV-1	ICP27	α	46
HSV-2	ICP27	α	45
VZV	ORF4	α	14
HCMV	UL69	β	100
EBV	BMLF1	γ	15
EHV-1	UL3	α	104
BHV-1	bie27	α	21
HVS	IE52	γ	55

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CHAPTER 2

NUCLEAR AND NUCLEOLAR LOCALIZATION SIGNALS WITHIN ICP27

Chapter Two - Nuclear and Nucleolar Localization Signals within ICP27[1]

INTRODUCTION

ICP27 is localized to the cell nucleus during infection, as determined by both cell fractionation (1) and immunofluorescence (26) studies. ICP27 is also nuclear when it is expressed in uninfected cells (48). These studies suggest that, like most other nuclear proteins, ICP27 possesses one or more nuclear localization signals (NLS's). In addition, some evidence suggests that ICP27 interacts with cell nucleoli. First, although WT ICP27 protein is not preferentially found in the nucleoli, it does show significant accumulation in these structures (26,44,48). Second, certain mutant ICP27 polypeptides show altered nucleolar localization, as demonstrated by immunofluorescence analysis of cells expressing ICP27 nonsense mutants (44,48). The *n*263R protein, which consists of the N-terminal half of ICP27 (263 out of 512 amino acid residues), is preferentially found in nucleoli, while the *n*406R protein, which consists of the N-terminal ~80% of ICP27 (406 amino acid residues), is localized to the nucleus but is largely excluded from nucleoli.

It is likely that ICP27 function depends on its correct intranuclear localization. As described previously, there is much evidence suggesting a post-transcriptional role by ICP27 in gene regulation, including effects on splicing and polyadenylation of pre-mRNA. ICP27's intra-nuclear localization may influence these post-transcriptional effects. For example, ICP27 can cause the re-distribution of snRNP complexes (39,54). Not only can it co-localize with these complexes (39) but it is also capable of interacting with protein(s) within these complexes, based on the results of co-immunoprecipitation assays (53). Mullen *et al.* (35) observed that a truncated form of ICP27, transiently expressed, colocalizes with pre-existing nuclear structures referred to as ND10 or PML oncogenic domains. Although the exact function of these punctate structures is unknown, it has been argued that they may represent transcriptional domains (64,69).

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[1] A version of this chapter has been published: Mears, W.E., Lam, V., and Rice, S.A. (1995). *J. Virol.* 69:935-947. The data in Figure 2-10 and Table 2-1 was collected by V. Leong (nee: Lam).

These examples suggest that ICP27's location within the cell could be related to its role during infection.

The objective of this study was to identify the polypeptide determinants within ICP27 which influence its ability to localize to, and within, the nucleus. This was done using a procedure similar to that utilized to delineate the SV40 large T antigen NLS. Proteins consisting of segments of ICP27 fused to chicken pyruvate kinase (PK), a cytoplasmic protein, were expressed in Vero cells and their cellular localization was visualized by indirect immunofluorescence.

MATERIALS AND METHODS

Cells, viruses and infections. All infections and transfections were carried out in Vero (African green monkey kidney) cells or in V27 cells (44), a derivative of Vero cells which contain an integrated copy of the ICP27 gene. Vero cells were originally obtained from the American Type culture collection (Rockville, Md.). The cells were propogated in Dulbecco modified Eagle medium (DMEM) plus 10% heat-inactivated fetal bovine serum (GIBCO). The viruses used for infections were the WT strain KOS1.1 (21 originally obtained from M. Levine, University of Michigan, Ann Arbor), and the ICP27 mutants *d*27-1 (44), *d*3-4, and *d*4-5 (both described below). Infections were carried out at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell and infected cells maintained in 199V media (199 media (GIBCO) + 5% heat inactivated fetal bovine serum (GIBCO)).

Construction of plasmids. To begin these studies, we constructed a vector, ppAPKX, which could be used to engineer ICP27-pyruvate kinase (PK) fusion genes. This was accomplished in several cloning steps. First, a functional poly (A) signal was cloned in pUC19. This was done by isolating a 100 bp *Xba*I-*Sal*I fragment containing the ICP27 gene poly (A) site from the plasmid ptkCAT27 (described in chapter 5) and cloning it into the *Kpn*I site of pUC19 using *Kpn*I linkers. Next, a 1.8 kb *Bgl*II fragment containing the chicken PK coding region (codons 17-529) was isolated from the plasmid PK10b (14) and cloned into the *Bam*HI site of pUC19-polyA. This generated the plasmid pUC19-polyAPK. Finally, the *Xba*I site upstream of the PK coding region in pUC19-polyAPK was changed to an *Xho*I site. To do this, pUC19-polyAPK was digested with *Xba*I, the 3' recessed ends were filled in using the Klenow fragment of *E. coli* DNA polymerase I, and an 8 bp *Xho*I linker was inserted. This plasmid was designated ppAPKX.

ppAPKX was used to engineer constructs in which N-terminal segments of the ICP27 coding region were fused to the PK coding region. To do this, we utilized the ICP27 plasmid mutants pM1 through pM16, which contain engineered *Xho*I restriction

sites in the ICP27 gene (45). The *Xho*I sites are spaced at numerous sites throughout the ICP27 coding region and occur in a common reading frame. To construct hybrid genes, pM mutants were doubly-digested with *Hind*III and *Xho*I. The small *Hin*dIII-*Xho*I restriction fragments were then cloned into ppAPKX that had been digested with *Hind*III and *Xho*I. Ligation of the *Xho*I ends results in the in-frame fusion of the ICP27 and PK coding regions. The constructs were named according to the pM plasmid which was used in each construction, e.g. pM4PK was derived using pM4. pMSPK was constructed by taking advantage of the naturally occuring *Sal*I site in the ICP27 gene. To construct pMSPK, the *Hind*III-*Sal*I fragment from pM27 (45) was cloned into *Hind*III-*Xho*I-digested ppAPKX.

To engineer PK fusion genes containing C-terminal segments of ICP27, we modified pM1PK by converting its *Xho*I site to a *Cla*I site. To do this, pM1PK was linearized with *Xho*I, the 3' recessed ends were filled in, and an 8 bp *Cla*I linker was inserted. The resulting plasmid was designated pMCPK. Next, pMCPK was linearized with *Bst*XI, which cuts near the 3' end of the PK open reading frame. The DNA ends were made blunt using T4 DNA polymerase, and an 8 bp *Xho*I linker was inserted. The resulting plasmid was designated pMCPKX. To construct hybrid genes, members of the pM1-M16 series were doubly-digested with *Eco*RI and *Xho*I. The small *Eco*RI-*Xho*I restriction fragments were then cloned into pMCPKX via its *Eco*RI and *Xho*I sites. Ligation of the *Xho*I ends results in the in-frame fusion of ICP27 coding segments to codon 523 of PK. As a consequence, the C-terminal six residues of PK are replaced by C-terminal portions of ICP27. The C-terminal constructs were named according to the pM mutants used, e.g. pMPK3 was derived using pM3. To study the effect of simply deleting the C-terminal six residues of PK, we constructed pMPKΔ, which contains nonsense mutations after codon 523. To make pMPKΔ, pMCPKX was linearized with *Xho*I, the 3' recessed ends were filled in, and a 12 bp *Nhe*I linker containing stop codons in all three reading frames (New England Biolabs) was inserted.

Plasmids encoding hybrid genes which contained internal portions of the ICP27 coding region were made using two different strategies. To make pM3-SPK, pM4-SPK,

pM5-SPK, pM6-SPK, and pMS-16PK, the plamids pM3, pM4, pM5, pM6, and pM16, respectively, were digested with *Xho*I and *Sal*I, and the small *Xho*I-*Sal*I restriction fragments were cloned into the unique *Xho*I site of pM1PK. Restriction enzyme analysis was used to identify those clones which had the insert in the desired orientation. The remaining hybrid plasmids were constructed by a multiple-step procedure. In the first step, pM1PK was altered by the insertion of a *Bgl*II linker immediately downstream of its unique *Xho*I site. This plasmid was designated pMXBPK. Next, oligonucleotide-directed mutagenesis (Altered Sites System, Promega) was used to engineer *Bgl*II sites into pM2, pM3, pM4, and pM5. This resulted in the plasmid derivatives pM2-4B, pM2-5B, pM3-4B, pM3-5B, pM3-6B, and pM4-6B, which contain engineered *Bgl*II sites at ICP27 codons 138/139, 153/154, 138/139, 153/154, 173/174, and 173/174, respectively. Finally, the small *Xho*I-*Bgl*II fragments from the above plasmids were cloned into pMXBPK via its *Xho*I and *Bgl*II sites. This resulted in plasmids pM2-4PK, pM2-5PK, pM3-4PK, pM3-5PK, pM3-6PK and pM4-6PK.

Two additional plasmids were constructed. The first was pMPK3-4, which encodes a hybrid protein in which amino acid residues 110-137 of ICP27 are tagged to the C-terminus of PK. To construct pMPK3-4, pM3-4PK was modified by the insertion of a 12 bp *Nhe*I stop codon linker at its *Bgl*II site. The resulting plasmid, pM3-4NPK, was digested with *Xho*I and *Eco*RI, and the 2.0 kb *Xho*I-*Eco*RI fragment was cloned into *Xho*I-*Eco*RI digested pMCPKX. This generated pMPK3-4. The second plasmid construct was pMd3-4, which encodes an ICP27 molecule lacking amino acid residues 109-139. To engineer this construct, pM3 and pM4 were digested with *Xho*I and *Eco*RI. The 1.6 kp *Xho*I-*Eco*RI fragment from pM4 was then ligated to the 6.4 kb *Eco*RI-*Xho*I fragment of pM3, generating pMd3-4.

Construction of HSV-1 recombinants. The recombinant viruses *d*3-4 and *d*4-5, bearing engineered deletions in the ICP27 gene, were generated by Vivian Leong (nee: Lam) utilizing a strategy involving *in vivo* homologous recombination as previously described (44,47). The plasmids which served as the source of the mutant ICP27 alleles were pMd3-4 (described above) and pMd4-5. pMd4-5 was constructed in a manner

analogous to pMd3-4, except that pM4 and pM5 were the parental plasmids. The *d*3-4 and *d*4-5 ICP27 alleles encode ICP27 molecules that lack amino acid residues 109-139 and 138-154, respectively. For each mutant, two independent viral isolates, designated a and b, were obtained. The genomic structures of all four viral isolates were confirmed by diagnostic Southern blotting (46).

Transfections. For transient transfections, Vero cells were plated on glass coverslips in 3.8 cm^2 wells of 12-well tissue culture plates. The next day, cells in individual wells were transfected with one μg of an ICP27-PK hybrid plasmid, using the calcium phosphate precipitation procedure as described previously (18,43). Plasmid DNA (pUC19) was used as the carrier DNA. Sixteen to eighteen hours later, the cells were mock-infected, or infected with *d*27-1. At 6 hpi, the cells were fixed and processed for immunofluorescence as described below.

For stable transfections, Vero cells plated in 25 cm^2 flasks were transfected with 0.5 μg of pSV2neo, which encodes the neomycin resistance gene (62), and a 5-fold molar excess of ICP27-PK hybrid plasmid (~2.5 μg). Sheared salmon sperm DNA was used as carrier DNA. Approximately 18 hours after transfection, the cells were subjected to glycerol-shock (14) for 2 min. The following day, the cells were trypsinized and ~1 x 10^6 cells were plated in a 75 cm^2 flask in media containing 300 μg/ml (active concentration) G418 (Geneticin, GIBCO). Colonies were allowed to develop over the next 2-3 weeks, during which time the media was changed every 3-4 days. After approximately 3 weeks, the colonies (~25-75 per flask) were trypsinized and pooled. For immunofluorescence experiments, the cell pools were plated on coverslips as described above, infected with *d*27-1, and processed for immunofluorescence at 6 hpi.

Immunofluorescence and immunoblot analyses. Cells were fixed with 3.7% formaldehyde, permeabilized with acetone, and processed for single or double indirect immunofluorescence as described by Quinlan et al. (42). The following primary antibodies were used: H1113 (1) and H1119, mouse monoclonal antibodies specific for ICP27 (Goodwin Institute for Cancer Research, Plantation, Florida); anti-PK, a

polyclonal rabbit antisera raised against chicken pyruvate kinase (14), and ANA-N, human antisera specific for a nucleolar antigen (Sigma). H1113, H1119, anti-PK, and ANA-N were diluted 1:200, 1:600, 1:50 and 1:2, respectively, in phosphate-buffered saline (PBS). The secondary antibodies used for indirect immunofluorescence were tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse IgG, dichlorotriazinyl amino fluorescein (DTAF)-conjugated donkey anti-rabbit IgG, and DTAF-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., Mississauga, Ontario), diluted 1:200, 1:150, and 1:200, respectively, in PBS. The cells were visualized with a Zeiss Axioskop 20 fluorescence microscope equipped with a Plan-Neofluar 63X objective lens.

To assess the integrity of the hybrid proteins in the stably transfected cells, immunoblot analyses were carried out. Cells were grown to confluence in 25 cm^2 flasks and infected with *d*27-1. At six hpi, total proteins were harvested and subjected to immunoblot analysis as described previously (48). The antibodies used were H1113, H1119 , and anti-PK, diluted 1:1000, 1:1800, and 1:1500, respectively. Immunoreactive proteins were detected by enhanced chemiluminescence using commercially available reagents (ECL detection kit, Amersham).

RESULTS

A stable transfection assay for protein localization. The objective of this study was to identify the polypeptide determinants of ICP27 which mediate its nuclear and nucleolar localization. To do this, we asked which portions of ICP27 can direct a cytoplasmic protein, chicken pyruvate kinase (PK), into the cell nucleus or to nucleoli. Specifically, our experimental strategy involved the construction of hybrid ICP27-PK genes, the introduction of these genes into mammalian cells, and the localization of the expressed proteins by immunofluorescence microscopy.

We first carried out experiments to find optimal conditions for the immunofluorescent localization of proteins expressed from a transfected gene under the control of the ICP27 promoter. pM27, which contains the WT ICP27 gene (45), was used as a test plasmid. In the first set of experiments, pM27 was transfected into Vero cells, and ICP27 localization was assessed one day after transfection by indirect immunofluorescence. We found that the percentage of cells expressing detectable ICP27 was quite low, <0.5% of the total (data not shown). However, additional experiments showed that the problem of low expression could be overcome by infecting the cells one day after transfection with an HSV-1 ICP27 deletion mutant, *d*27-1 (44), and performing immunofluorescence at 6 hpi. HSV-1 infection has been previously shown to enhance the expression of transiently transfected IE promoters, through the action of the virion transactivator protein VP16 (37,41) and perhaps other viral transactivators such as ICP0. After infection, a significant fraction (>2%) of the cells showed readily detectable expression of ICP27. However, ICP27 localization in many of the positive cells appeared aberrant. In these cells, ICP27 was distributed about equally in both the nuclear and cytoplasmic compartments (Fig. 2-1, A and B). This pattern was in contrast to ICP27's nearly exclusive nuclear localization in WT HSV-1-infected cells (Fig. 2-1, E and F). In addition, the fluorescent signal in many of the transfected cells was so strong that it was difficult to discern ICP27's intra-nuclear distribution.

The strong signals and unusual localization of ICP27 in the transiently-transfected, infected cells might have resulted from non-physiologically high expression of ICP27 due to multiple, unintegrated plasmid templates present in the cells. We therefore tested a stable transfection assay, in which the gene copy number would be expected to be lower. In this case, pM27 was co-transfected into Vero cells with pSV2neo (62), a plasmid conferring resistance to the drug G418. G418-resistant colonies were allowed to develop over several weeks. The colonies (~100) were then pooled and tested for ICP27 expression by immunofluorescence. It was found that the pooled transfectants failed to express detectable levels of ICP27. This was not surprising, as we have previously observed that a stably transfected ICP27 gene is not constitutively expressed in Vero cells, but can be induced by HSV-1 infection (44,46). When the pooled transfectants were infected with *d*27-1, ~25% of the cells expressed ICP27 at 6 hpi (Fig. 2-1, C and D). Significantly, ICP27 was localized nearly exclusively in nuclei and in a pattern that was very similar to that seen in the WT infection. The stable transfection assay therefore more accurately reproduces the normal physiological localization of ICP27. For this reason, we used this method in further experiments to assess the cellular localization of ICP27-PK hybrids.

Identification of an NLS in the N-terminal region of ICP27. We next constructed a set of hybrid genes in which N-terminal coding segments of the ICP27 gene were fused to the PK open reading frame (Fig. 2-2). The fusion genes were under the control of the ICP27 gene promoter and carried a downstream poly (A) signal also derived from the ICP27 gene. The plasmids were introduced into Vero cells by stable transfection, and the G418-resistant colonies from each transfection (ranging from ~25-100 colonies) were pooled. To assess the localization of the hybrid proteins, the transfectants were infected with *d*27-1 and processed for immunofluorescence at 6 hpi. Immunofluorescent staining was performed with anti-PK, a polyclonal antiserum specific for chicken PK (14), and H1119, a monoclonal antibody (MAb) directed against ICP27. It was found that H1119 reacted strongly with all of the hybrids, including M1PK, a protein which contains only the N-terminal 11 amino acid residues of ICP27 (Fig. 2-2).

This result demonstrates that the epitope recognized by H1119 is contained within the ICP27's N-terminal 11 amino acid residues. This was confirmed in a separate study in which it was shown that ICP27 amino acid residues 1-12 could confer H1119-immunoreactivity to an unrelated poxviral protein (66). We consistently found that immunofluorescent staining with H1119 resulted in significantly less background staining than did staining with anti-PK, although H1119 did show a low-level of cross-reaction with a cellular protein localized at points of cell-cell contact. For this reason, the photographic documentation of hybrid protein localization in this study was routinely done on cells using the H1119 MAb. All results, however, were confirmed by staining with the anti-PK antibody.

The cellular localizations of the the N-terminal ICP27-PK hybrids is summarized in Fig. 2-2. Hybrid proteins possessing 11, 63, or 108 N-terminal amino acid residues of ICP27 did not show significant accumulation in the cell nucleus. An example of such cytoplasmic localization is shown for the M3PK protein in Fig. 2-3A. Fusion of the N-terminal 137 amino acid residues of ICP27, however, resulted in a PK hybrid that localized very efficiently to the cell nucleus. Additional constructs containing larger N-terminal portions of ICP27 were also efficiently nuclear-localized. In this and all further localization experiments, control immunoblots using either anti-ICP27 or anti-PK antibodies confirmed that the expressed fusion proteins were of the approximate predicted molecular sizes and were not detectably degraded (data not shown). From these data, we conclude that the N-terminal 137 amino acid residues of ICP27 contains a strong NLS.

Although all constructs possessing 137 or more N-terminal amino acid residues of ICP27 localized to nuclei, there were some striking differences in nucleolar localization (Fig. 2-3, B-D). The conclusions concerning nucleolar localization were made by visually comparing the fluorescence signal with the phase-contrast image of the cells (the cell nucleoli were readily visible at this stage of infection). Three general patterns of nucleolar localization were observed, and are summarized in Fig. 2-2. First, the M4- and M16PK hybrids were largely excluded from nucleoli, as shown for M16PK in Fig. 2-3B.

Second, the M5- and M6PK proteins localized throughout the nucleus, but showed some preferential accumulation in nucleoli, particularly around the peripheral regions. This type of staining is shown for M5PK in Fig. 2-3C. Finally, the MS- and M10PK fusion proteins showed more or less even localization throughout nuclei, including significant, but not preferential, localization in nucleoli. This staining pattern is shown for MSPK in Fig. 2-3D.

Identification of additional weak NLS's in ICP27. The above experiments demonstrated that ICP27's N-terminal 137 amino acid residues contain a strong NLS. However, if sequences C-terminal to amino acid residue 137 contain additional NLS's, they would not have been identified. Therefore, we engineered additional genes in which C-terminal portions of the ICP27 coding region were fused to the C-terminus of the PK gene (Fig. 2-4). At their N-termini, all of the hybrids possessed the N-terminal 11 amino acid residues of ICP27, providing a convenient epitope-tag for staining with the H1119 antibody. Because all of the hybrid gene constructions resulted in the replacement of PK's six C-terminal amino acid residues, we constructed an additional control plasmid, pMPKΔ, which encodes a similarly truncated PK molecule not containing attached ICP27 sequences.

The localization results for the C-terminal ICP27 fusions are summarized in Fig. 2-4. Truncation of PK's C-terminus (MPKΔ) led to a very low but detectable amount of nuclear localization compared to M1PK, although cytoplasmic localization was still very much predominant (Fig. 2-5A). This fusion protein should possess a molecular mass of roughly 57 kDa and as such may be able to freely diffuse through the nuclear pore. It is therefore possible that small amounts of this fusion protein are able to diffuse through the nuclear pore complex. Fusion of ICP27 amino acid residues 489-512 had no further effect, but fusion of ICP27 amino acid residues 400-512 or 306-512 (MPK13 and MPK10, respectively) led to a slight increase in nuclear localization, as shown for MPK10 in Fig. 2-5B. Hybrid proteins containing ICP27 amino acid residues 202-512, 175-512, or 155-512 (MPK7, MPK6, and MPK5, respectively), on the other hand, showed significant localization in nuclei, being distributed approximately equally

between the nucleus and cytoplasm. This staining pattern is shown for MPK5 in Fig. 2-5C. The addition of ICP27 amino acid residues 140-512 led to a PK hybrid (MPK4) that was predominantly nuclear, although nearly all cells still showed significant cytoplasmic localization (Fig. 2-5D). Complete nuclear localization was not achieved until ICP27 amino acid residues 110-512 or 13-512 had been attached to PK (MPK3 and MPK5, respectively), as shown for MPK3 in Fig.2-5E. In contrast to the N-terminal ICP27-PK hybrids, all of the nuclear-localized PK hybrids in this series showed a similar nucleolar localization phenotype, being largely excluded from nucleoli.

Two conclusions concerning ICP27's NLS's can be drawn from this experiment. First, the C-terminal region of ICP27, from amino acid residues 140-512, appears to contain several weak NLS's which function additively. However, by itself, this C-terminal portion of ICP27 is unable to confer complete nuclear localization to PK. Second, since the MPK3 hybrid localized completely to the nucleus, amino acid residues 110-512 contain a strong NLS .

Mapping of ICP27's strong NLS. Together, the above experiments suggest that ICP27 contains a strong NLS in its N-terminal half. To more precisely map this signal, we engineered additional constructs in which internal portions of the ICP27 coding region were fused to the PK gene. The internal ICP27 sequences were linked to the H1119 epitope tag and positioned at the N-terminus of the PK moiety (Fig. 2-6). The hybrid genes were then tested in the stable transfection assay. Hybrid proteins possessing ICP27 amino acid residues 110-261, 65-137, 65-152, 110-152, or 110-172 all localized efficiently to the nucleus, although M3-5PK, possessing amino acid residues 110-152, showed some cytoplasmic localization. None of the other constructs, however, including one that contained most of the C-terminal half of ICP27, showed significant localization in the nucleus.

The data summarized in Figs. 2-2, 2-4 and 2-6 demonstrate that all ICP27-PK fusion proteins which contain amino acid residues 110-137 of ICP27 localize efficiently to the nucleus. To test directly whether these amino acid residues define ICP27's strong NLS, we constructed two hybrid genes in which the sequence encoding these amino acid

residues was fused to an epitope-tagged PK gene (Fig. 2-7A). One hybrid, M3-4PK, encoded a protein in which amino acid residues 110-137 were positioned near the N-terminus of PK. The other, MPK3-4, encoded a protein in which amino acid residues 110-137 were tagged to the C-terminus of PK. Both gene constructs were tested in the stable transfection assay, together with their appropriate control plasmids. The M3-4PK protein was cytoplasmic, indicating that amino acid residues 110-137 do not function as an NLS in this context. In contrast, the MPK3-4 protein showed strong, although not exclusive, nuclear localization (Fig. 2-7B), demonstrating that amino acid residues 110-137 can function as an efficient NLS in this protein context. These data, combined with the results summarized in Figs. 2-2, 2-4, and 2-6, demonstrate conclusively that amino acid residues 110-137 define a strong NLS, capable of mediating the complete nuclear localization of an unrelated protein.

Identification of a nucleolar localization sequence. The data summarized in Figs. 2-6 and 2-7A provide further information regarding the ICP27 sequences which govern nucleolar localization. Although several proteins were excluded from the nucleolus, the hybrid proteins M2-5PK, M3-5PK, and M3-6PK, which contain ICP27 amino acid residues 65-152, 110-152, and 110-172, respectively, all showed preferential localization in nucleoli. This staining pattern is shown for the M3-6PK hybrid in Fig. 2-8, A and B. Double immunofluorescence labeling with a nucleolar-specific antiserum demonstrated that the observed preferential localization was indeed nucleolar (Fig. 2-8C).

These data demonstrate that a relatively short sequence in ICP27, from amino acid residues 110-152, can confer preferential nucleolar localization to PK. This sequence therefore defines a nucleolar localization signal, or NuLS. Consistent with this conclusion is the fact that all of the ICP27-PK hybrids which showed some degree of nucleolar localization contained amino acid residues 110-152 (Figs. 2-2, 2-4 and 2-6). However, three proteins which did contain these amino acid residues, M16PK, MPK3, and MPK1, were excluded from nucleoli (Figs. 2-2 and 2-4). Therefore, the function of ICP27's NuLS appears dependent on protein context.

ICP27's strong NLS and NuLS function in their natural context. To see if ICP27's strong NLS functions in its natural setting, we engineered a mutant ICP27 gene, designated Md3-4, in which the sequences encoding the NLS (codons 109 - 139) were deleted. The mutant and WT genes were then tested in the stable transfection assay. As expected, WT ICP27 localized nearly exclusively in the cell nucleus (Fig. 2-9A). In contrast, the d3-4 protein was found in both the nucleus and cytoplasm (Fig. 2-9B). In some cells, the mutant protein was distributed approximately equally between the nucleus and cytoplasm, while in others nuclear localization was predominant. Therefore, deletion of ICP27's strong NLS leads to a significant defect in its nuclear accumulation. This demonstrates that ICP27's strong NLS functions in its natural context. However, a substantial fraction of the d3-4 protein was still able to enter the nucleus, indicating that other sequences in ICP27 must have NLS activity. This is consistent with our PK-targeting studies, wherein we found that amino acid residues 140-512 were able to mediate significant, but not complete, nuclear localization of PK. Together, these results indicate that ICP27 contains multiple NLS's that function with differing efficiencies.

To extend these results, we engineered two HSV-1 recombinants, *d*3-4 and *d*4-5, which had targeted disruptions in the ICP27 gene. The *d*3-4 mutant contains the d3-4 allele described above, and thus encoded an ICP27 molecule lacking the strong NLS (amino acid residues 109-139). The *d*4-5 mutant, on the other hand, contains an in-frame deletion which removed codons 138-154. This latter alteration leaves ICP27's strong NLS intact, but removes a portion of the NuLS. Both HSV-1 mutants were isolated and propagated in V27 cells (44), a derivative of Vero cells which contain a stably transfected ICP27 gene and therefore complement the growth of viral ICP27 mutants. For each mutant, two independent isolates, designated a and b, were obtained and analyzed in subsequent experiments.

To see if the *d*3-4 and *d*4-5 mutants were defective for growth in Vero cells, a single-cycle growth experiment was performed. Vero cells were infected at an MOI of 10 (using mutant virus titers determined on V27 cells), and the cultures were incubated for 24 hours. As a control, the same inocula were used to infect cultures of V27 cells.

The yield of infectious virus was then determined by plaque assay of the harvested cell lysates on V27 cells. The results, shown in Table 2-1, demonstrate that both *d*3-4 and *d*4-5 are significantly defective for growth in Vero, but not V27, cells. The *d*3-4 mutant showed a 9 to 34 -fold defect in growth in Vero cells compared to its growth in V27 cells. The *d*4-5 mutant was even more deficient, replicating 58 to 84 -fold less efficiently in Vero than V27 cells. In contrast, the WT parent, strain KOS1.1, showed nearly equivalent growth in Vero and V27 cells (less than a 2-fold difference). Therefore, the sequences deleted in *d*3-4 and *d*4-5, which encode important localization signals, are required for optimal growth of the virus in Vero cells.

We next used immunofluorescence to examine the cellular localization of the ICP27 polypeptides encoded by *d*3-4 and *d*4-5 (Fig. 2-10). As predicted, the *d*3-4 protein (panel C) showed a significant defect in nuclear localization compared to the WT protein (panel B), although nuclear localization was still predominant. The staining pattern was very similar to that seen earlier when the Md3-4 protein was expressed in stably transfected, *d*27-1-infected cells (Fig. 2-9B). The *d*4-5 ICP27 molecule, in contrast, was not deficient in its ability to localize to the cell nucleus (panel D). This was consistent with expectation, since this mutant ICP27 still contained the strong NLS. However, the intranuclear localization of the *d*4-5 protein differed from the WT, in that the *d*4-5 protein was largely excluded from nucleoli (compare panel D to B). This was confirmed by double immunofluorescence labeling using nucleolar-specific antisera (not shown). These results demonstrate that both the strong NLS and the NuLS function during the HSV-1 infection to help mediate the nuclear and intra-nuclear localization of ICP27.

Mapping of an ICP27-specific MAb epitope. As demonstrated earlier, the H1119 MAb maps to the N-terminal 11 amino acid residues of ICP27, since these amino acid residues alone can can confer immunoreactivity (this study and ref. 66). We used our collection of ICP27-PK hybrids to map the epitope for a second ICP27 MAb, H1113 (1). Key data are summarized in Table 2-2. The M3PK protein, possessing the N-terminal 108 amino acid residues of ICP27, did not react with H1113, but M4PK, possessing the N-terminal 137 amino acid residues, did react. MPK4, a hybrid

containing ICP27 amino acid residues 140-512, was unable to bind to H1113, but MPK3, possessing amino acid residues 110-512, did bind. These data suggest that the epitope for H1113 maps to amino acid residues 110-137. Consistent with this hypothesis, we found that MPK3-4, which contains amino acid residues 110-137 tagged to the C-terminus of PK, also reacted with H1113. These data do not formally exclude the possibility that amino acid residues 1-11 contribute to the H1113 epitope. However, we have recently engineered a mutant ICP27 mutant gene, designated M1X, which contains a stop codon inserted after codon 12 (46). This mutant gene expresses a truncated ICP27 protein, most likely by re-initiating translation at codon 50, a downstream AUG in a favorable translation context (27). Consistent with this, the M1X-encoded protein fails to react with the N-terminal specific H1119. However, it does react with H1113 (46). Together, these data demonstrate that the epitope for MAb H1113 maps to amino acid residues 110-137.

Amino acid residues 110-137 also define ICP27's strong NLS. It was therefore of interest that M3-4PK, the hybrid protein which contained the NLS sequence but failed to localize to the nucleus (Fig. 2-7A), was not recognized by H1113 (Table 2-2). Therefore, amino acid residues 110-137 likely have an altered conformation in this protein, or are buried internally and thus are not accessible. Either possiblity would explain the failure of the NLS to function in this protein context.

DISCUSSION

Identification of multiple NLS's in ICP27. Proteins enter the nucleus via large proteinaceous structures roughly 125 MDa and consisting of over 100 proteins. These nuclear pore complexes provide aqueous channels across the nuclear envelope (reviewed in refs. 12,17,36). Although it is possible for small proteins to diffuse through the pore complexes, the import of large proteins (>40-60 kDa) is a pore-mediated, energy-dependent process (reviewed in refs. 12,15,17,57). In nearly all cases, large nuclear proteins possess one or more NLS's, which are required for import. Although there is no universal consensus for NLS's, many are short sequences which contain a high proportion of basic amino acid residues. These "classical" NLS sequences are divided into two related types. First, there are signals which consist of a single short basic sequence. This region is usually less than 10 residues in length and is often flanked by a proline or glycine residue. The NLS of simian virus 40 (SV40) large T antigen, PKKKRKV, is the prototype for this type of signal (23,24,28). Second, there are bipartite NLS's which are composed of two clusters of basic residues separated by a 10-12 amino acid residue linker sequence: the first cluster contains two basic amino acid residues, while the second cluster consists of five amino acid residues, of which at least three must be basic (10). Bipartite NLS's were first characterized in the *Xenopus laevis* nucleoplasmin protein (50), and appear to be the most common type of NLS (10). The basic region of these NLS signals interact in the cytosol with a soluble cellular protein termed importin α (previously described as the NLS receptor) which recruits the NLS protein to the cytoplasmic face of the nuclear pore complex (reviewed in refs. 12,17). After docking with the pore via importin β, the importin-NLS complex transverses the nuclear envelope. The NLS protein is released and the transport components eventually return to the cytoplasm.

In addition to these two well-characterized signals, a few other types of NLS's have been identified. For example, a relatively long (48 amino acid residues) and weakly basic sequence serves as the NLS of the type 1 human T-cell leukemia virus (HTLV-1)

Tax protein (61) while a sequence rich in aromatic and glycine residues (termed M9) is essential for import of hnRNP A1 (58,68). It is suspected that proteins containing these non-classical NLS sequences enter the nucleus using the same nuclear pore complexes but different specific "receptor" factors. Indeed, recently a novel cytosolic protein, transportin, has been identified which binds the M9 sequence and actively transports M9-containing proteins across the nuclear envelope (40).

Although the predicted sequence of ICP27 contains a relatively long arginine-rich basic region (see below) and several smaller basic regions, it does not contain sequences which are closely related to the SV40 T antigen NLS, the hnRNP A1 M9 NLS, or which fit the bipartite NLS consensus. It was thus impossible to predict, *a priori*, which portions of ICP27 might function as NLS's. Therefore, to map NLS's, we identified the regions of ICP27 which can direct a cytoplasmic protein, PK, into the nucleus. We found that ICP27 contains multiple signals which can mediate nuclear localization, and that these signals function with differing efficiencies. First, ICP27 contains a strong NLS, mapping to amino acid residues 110-137, which can mediate complete nuclear localization. Second, ICP27 contains one or more weak signals, mapping to amino acid residues 140-512, which can mediate substantial nuclear localization, but which are incapable of mediating complete nuclear localization. It is relevant to point out that all of our experiments were performed in the context of HSV-1-infected cells. Thus, it is possible that other viral components may contribute to the function of ICP27's NLS's, and that the nuclear localization of ICP27 may differ in uninfected cells. A future experiment to test ICP27's NLS's in the absence of other viral protein would be to place the NLS fusion genes under the control of a stronger promoter such as the cytomegalovirus minimal promoter or the SV40 promoter. With such a modification, it is likely that efficient transient expression of these genes in Vero cells would then be possible.

The conclusion that amino acid residues 110-137 define ICP27's strong NLS comes from the PK-targeting studies which show that these residues are necessary for complete nuclear localization, and that alone they can target PK into the nucleus. It is

relevant to point out that, although the PK fusion proteins containing amino acid residues 110-137 (or 110-152) did exhibit predominant nuclear localization, some cytoplasmic localization was still evident. Thus, while amino acid residues 110-137 appear to define the minimal extent of the strong NLS, flanking sequences may influence its efficiency. Indeed, Hibbard and Sandri-Goldin (20) demonstrated that the arginine-rich regions C-terminal to amino acid residue 137 were able to enhance but were not sufficient for complete nuclear localization.

The results of our PK localization experiments do not formally rule out the possibility that ICP27's N-terminal 11 amino acid residues, which were included in all constructs as an epitope tag, also contribute to nuclear localization. This is very unlikely, however, for several reasons. First, the N-terminal amino acid residues did not mediate any nuclear localization on their own. In addition, an effect of the N-terminal amino acid residues on nuclear localization in our experiments would require that they interact in multiple circumstances with distal protein sequences. Finally, we found that a mutant ICP27 protein which lacks its normal N-terminus is still capable of efficiently localizing to the cell nucleus (46).

To verify that ICP27's strong NLS functions in its natural context, we engineered an ICP27 gene, designated Md3-4, in which the sequences encoding the strong NLS were deleted (codons 109-139). The Md3-4 protein, expressed from either a stable transfected gene or an HSV-1 recombinant, showed significantly enhanced cytoplasmic localization. This demonstrates that the strong NLS is required for highly efficient nuclear localization. However, a substantial fraction of the Md3-4 ICP27 was still able to localize to the nucleus. This suggests that ICP27's weak NLS's also function in the context of the WT protein. Our genetic analysis of the *d*3-4 mutant demonstrated that, in Vero cells, the sequence encoding the strong NLS is required for optimal virus growth. Further work will be required to see if this growth deficiency results directly from the reduced level of nuclear-localized ICP27.

The predicted sequence of ICP27's strong NLS, i.e. amino acid residues 110-137, is ARRPSCSPERHGGKVARLQPPPTKAQPA (33,46). Inspection of this sequence

reveals a potentially significant similarity to the consensus sequence of bipartite NLS's, as exemplified by the *X. laevis* nucleoplasmin protein (Fig. 2-11A). ICP27's sequence, from amino acid residues 111-127, matches the bipartite consensus, with the exception that the second cluster of basic amino acid residues, KVARL, contains only two of five, rather than three of five, basic amino acid residues. It is noteworthy that both basic clusters, but not much of the linker sequence, is conserved in the corresponding region of the HSV-2 ICP27 (Fig. 2-11A). It seems likely, therefore, that ICP27's strong NLS is a member of the bipartite family. If so, it suggests that not all bipartite NLS's need contain three basic amino acid residues in their second basic cluster.

The function of ICP27's strong NLS is dependent on its protein context. This was demonstrated by the fact that the NLS did not function in the M3-4PK protein, in which it was positioned near the N-terminus of PK. Several previous studies have shown that NLS's can be context-dependent (reviewed in ref. 15). For example, the NLS of SV40 large T antigen is not functional when inserted into a hydrophobic region of PK (51). The dependence of NLS's on protein context is likely based on the necessity for these sequences to fold into specific three-dimensional structures which are exposed on the protein surface. Although the three-dimensional structure of bipartite NLS's is not known, it has been proposed that the linker region loops out, juxtaposing the two clusters of basic residues. This may create a single basic surface that functions like the SV40 T antigen class of signals (10). We found that H1113, a MAb which recognizes amino acid residues 110-137, did not bind to the M3-4PK protein. This suggests that the NLS may be aberrantly folded in M3-4PK and therefore unable to interact productively with the nuclear transport machinery.

Our experiments further suggest that the C-terminal region of ICP27, from amino acid residues 140-512, contains multiple weak NLS's. The evidence for this comes from the analysis of PK hybrids which contain various C-terminal regions of ICP27. By increasing the extent of the attached ICP27 sequences, we were able to increase PK nuclear localization in a stepwise fashion (Figs. 2-4 and 2-5). Fusion of amino acid residues 400-512 led to a low level of nuclear localization, fusion of amino acid residues

202-512 led to ~50% nuclear localization, and fusion of amino acid residues 140-512 led to predominant, but incomplete, nuclear localization. The simplest explanation of these results is that amino acid residues 400-512, 202-399, and 140-201 each contain a weak NLS, and that the effects of these NLS's are additive. In this regard, it is noteworthy that the largest effect on nuclear localization in this C-terminal fusion series resulted from the addition of amino acid residues 140-155 (construct MPK4). Therefore, amino acid residues 140-155, which include multiple arginine residues (see below), may be the most potent of ICP27's weak NLS's. This finding is supported by that of Hibbard and Sandri-Goldin (20). However, it is possible to interpret our data in terms of a single weak NLS. In this scenario, the very C-terminal region of ICP27 could have a functional but weak NLS which is stabilized or made more efficient by adjoining ICP27 sequences. It is relevant to point out that several of the PK fusion proteins which contained the putative weak signals (e.g. M4-SPK, M5-SPK, M6-SPK, and MS-16PK) did not exhibit any nuclear localization. Therefore, protein context also appears to affect the function of ICP27's weak NLS's.

Inspection of amino acid residues 140-512 reveals a few basic sequences which could conceivably function as minor NLS's, but no sequences which are obviously related to known NLS's. One possibility is that one or more of the weak signals are not *bona fide* NLS's, but instead mediate an interaction with another protein which does possess an NLS. This would allow the co-import of ICP27. Such a mechanism has been proposed for the nuclear entry of several proteins, including the adenovirus DNA polymerase (71) and the *S. pombe* cdc2 kinase (4). Since our experiments were performed in the context of HSV-1-infected cells, a putative co-importer of ICP27 could be either a viral or a cellular protein.

The presence of multiple NLS's within a single protein has been observed for several other proteins (19,49,65). Given the efficiency of ICP27's strong NLS, it is not clear why or if ICP27 requires secondary NLS's. One possible explanation for multiple NLS's in ICP27 is that one or more of these signals do not function in some of the cell types in which HSV-1 naturally replicates, such as neurons. Alternately, the optimal

nuclear localization of ICP27 in a given cell type may require the combined efficiency of multiple NLS's. This hypothesis is consistent with studies that show the nuclear localization of proteins can be made more efficient by increasing the number of NLS's (11,51, reviewed in ref. 15). One model of nuclear import (reviewed in ref. 17) suggests that transport across the nuclear envelope involves a series of docking, undocking, diffusion and re-docking steps. Multiple NLS's could aid this translocation by maintaining a processive transport of the molecule along the "stationary" pore complex.

Identification of a nucleolar targeting signal in ICP27. Our experiments demonstrate that ICP27 possesses a relatively short sequence, mapping to amino acid residues 110-152, which can mediate the preferential nucleolar localization of PK. This NuLS, appears to function in its natural context, since WT ICP27 shows significant, although not preferential, localization in nucleoli in both infected and uninfected cells (this study, 26,44,48). Furthermore, when a portion (amino acid residues 138-154) of the NuLS was deleted in the context of a recombinant virus , ICP27 was found to be excluded from nucleoli (Fig. 2-10D).

Similar to its NLS's, the function of ICP27's NuLS is dependent on protein context. This was demonstrated by the fact that several ICP27-PK hybrid proteins which possessed the NuLS were either excluded from nucleoli (e.g. MPK1), or showed an unusual localization around the nucleolar peripheries (e.g. M5PK). It is interesting to note that fusion protein length appears to inversely correlate with the ability of the hybrid proteins to localize in nucleoli. Thus, it is possible that large proteins are less efficient at entering nucleoli under the conditions of our experiments. Protein context may also affect the function of the NuLS in other ways. For example, the NuLS may be non-functional in some contexts due to aberrant folding or to additional ICP27 sequences which are inhibitory to nucleolar localization.

Several studies have attempted to define protein sequences which are involved in the specific localization of proteins to nucleoli. For a few viral proteins, including HTLV-1 Rex and human immunodeficiency virus type 1 (HIV-1) Rev and Tat, relatively short NuLS's have been identified (8,9,59). Although a consensus sequence is not

evident, two common features have been noted in these signals: the sequences are extremely rich in basic amino acid residues, in particular arginine residues, and they contain a functional NLS. It has been suggested that these NuLS's are modified or extended NLS's which additionally function to target proteins to nucleoli (15). However, the nucleolar localization of several cellular proteins does not appear to be mediated by such short targeting signals (29,38,56,70). Rather, nucleolar localization in these cases is dependent both on an NLS and on fairly large protein regions which in some cases correspond to known functional domains. For example, the nucleolar localization of nucleolin is dependent on RNA-binding motifs which mediate its interaction with pre-rRNA (56), and the nucleolar localization of the transcription factor mUBF depends on functional DNA-binding domains and correlates with rDNA-binding (29). These results have led to the proposal that the nucleolar localization of proteins is not mediated by a signal-dependent targeting mechanism, but rather by stable binding of nuclear proteins to protein or nucleic acid constituents of nucleoli (29,56,70). The short viral NuLS's described above can be accomodated by this model if these sequences mediate a stable interaction with nucleolar components. This may be the case, since the NuLS's of HIV-1 Rev and Tat have been shown to possess RNA-binding activities (3,7,30,67). Although the natural targets of Rev and Tat are believed to be specific sequences in viral pre-mRNAs, it has been suggested that related RNA sequences or structures might be present in pre-ribosomal or ribosomal RNA, leading to the nucleolar accumulation of these proteins when they are expressed in cells at high levels (7,56).

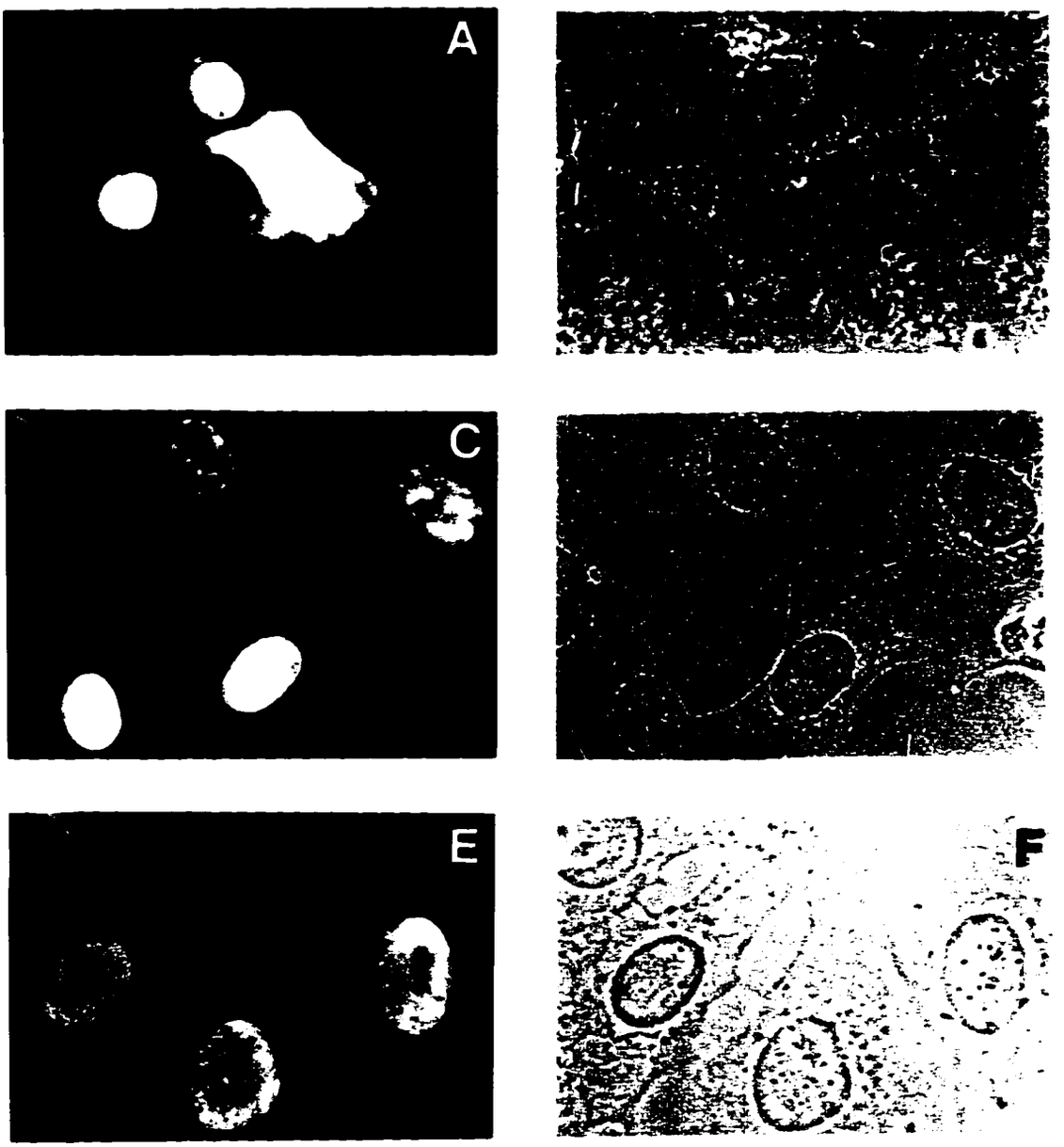
ICP27's NuLS appears similar in kind to the short basic NuLS's of Rex, Rev and Tat in that it is composed of an NLS (amino acid residues 110-137) and a contiguous arginine-rich basic region (amino acid residues 138-152). Interestingly, the arginine-rich subdomain of ICP27's NuLS is also very rich in glycine residues and bears similarity to a recently identified sequence motif found in a number of cellular proteins (Fig. 2-11B). This motif, termed the "RGG box" (25) or "GAR (for glycine-arginine-rich) domain" (16), is found in many proteins involved in nuclear RNA processing, particularly in nucleolar proteins involved in pre-ribosomal RNA processing or in heterogeneous

nuclear RNA-binding proteins (hnRNPs) involved in mRNA maturation (31) and is one of several known RNA-binding domains (5, 25, 31).

Two arguments suggest that ICP27's RGG box is important in its biological functions. First, the RGG box is highly conserved in HSV-2 ICP27 (Fig. 2-11B), even though the overall sequence identity between the two proteins is only 67% in their N-terminal halves. Second, the *d*4-5 viral mutant, which encodes an ICP27 molecule lacking the RGG box, is unable to replicate efficiently in Vero cells (Table 2-1). Deletion of amino acid residues 104-178 (which includes the RGG box) results in deficient late gene expression independent of viral DNA synthesis (20). However, the direct effect of specifically deleting ICP27's RGG box on viral gene expression remains to be elucidated. Further phenotypic analysis of the *d*4-5 mutant, currently in progress, should help to elucidate the biological role of the RGG box *in vivo*.

As reviewed earlier, studies of HSV-1 mutants suggest that ICP27 affects HSV-1 DNA replication and mRNA production, neither of which are thought to occur in nucleoli. Given additional evidence which suggests that ICP27 interacts with the cellular mRNA splicing and/or polyadenylation machinery (34,39,55), it is intriguing to note that a number of the cellular RGG proteins are involved in pre-mRNA maturation or processing. In fact, one of the best matches with ICP27's RGG box is a sequence from the SmD protein (52), a constituent of the spliceosomal U snRNPs (Fig. 2-11B). Based on the above considerations, we hypothesize that that ICP27's RGG box mediates, or helps to mediate, a specific interaction with pre-mRNA or small nuclear RNAs involved in mRNA processing. We speculate, that as a secondary consequence of this RNA-binding activity, ICP27 is able to interact to some extent with ribosomal RNA, leading to the low level of nucleolar localization that is seen for the WT protein. The strong preferential nucleolar localization that is exhibited by the truncated ICP27 molecule *n*263R (44,48) or by certain ICP27-PK hybrids (this study) might result from the deletion of specificity determinants elsewhere in the ICP27 molecule. This might relax the requirements for RNA-binding by the RGG box, leading to enhanced ribosomal RNA binding and nucleolar accumulation.

FIG. 2-1. ICP27 localization in transfected and infected Vero cells. (A-D) Vero cells were transiently (A and B) or stably (C and D) transfected with pM27, a plasmid encoding WT ICP27. The transfected cells were infected with *d*27-1, an HSV-1 ICP27 deletion mutant, and processed for immunofluorescence at 6 hpi. (E and F) Vero cells were infected with WT HSV-1 and processed for immunofluorescence at 5 hpi. Immunofluorescent staining was performed with H1119, an ICP27-specific MAb. Panels A, C and E are immunofluorescence images; panels B, D and F are the corresponding phase-contrast images.


A
C
E
F

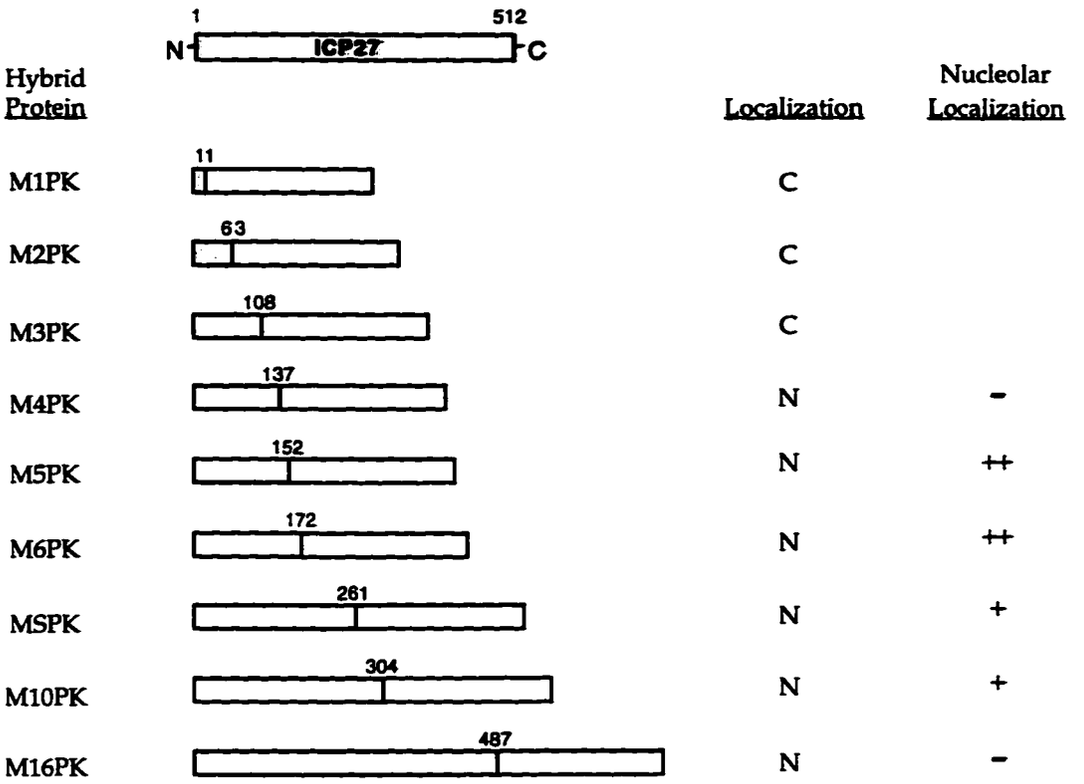
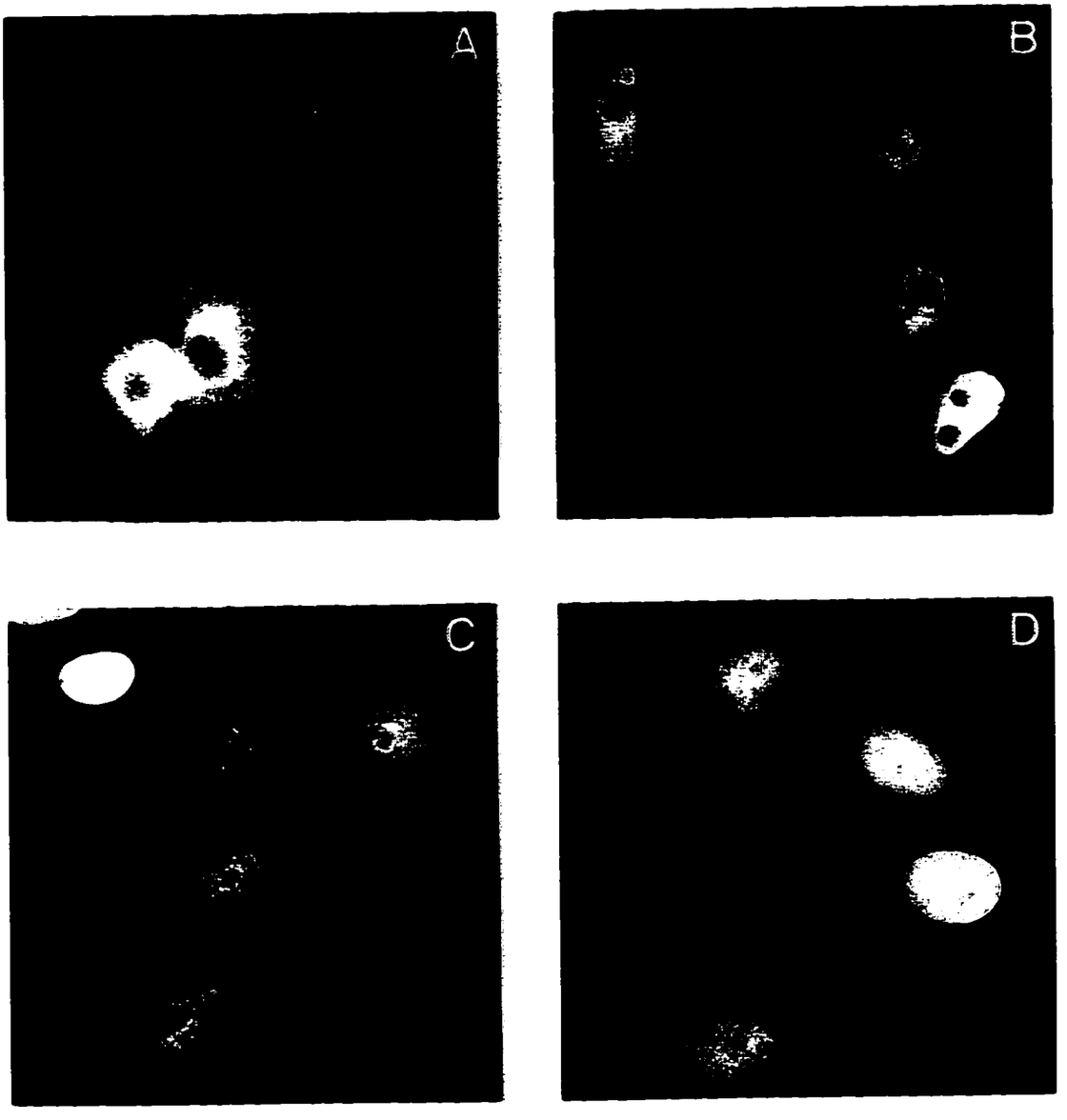


FIG. 2-2. Localization of PK fusion proteins containing N-terminal portions of ICP27. Plasmids were constructed which encode the PK fusion proteins illustrated above. Protein sequences are represented as bars, with the stippled bars representing N-terminal ICP27 sequences and the open bars representing the PK moiety (amino acid residues 17-529; not to scale). The numbers above the bars denote the extent of ICP27 amino acid residues. For comparision, the WT ICP27 molecule is shown at the top. The plasmids were introduced into Vero cells by stable transfection, fusion protein expression was induced by infection with *d*27-1, and protein localization was determined at 6 hpi by immunofluorescent staining with H1119, an ICP27-specific mouse MAb, and anti-PK, a polyclonal rabbit sera. Abbreviations: C= cytoplasmic localization; N= nuclear localization; + = low level of nucleolar localization; ++ = moderate nucleolar localization, with preferential staining of nucleolar peripheries; - : exclusion from nucleoli.

FIG. 2-3. Immunofluorescent staining of PK fusion proteins containing N-terminal portions of ICP27. Stably-transfected Vero expressing various fusion proteins were processed for immunofluorescence as described in the text. The photographs show immunofluorescent staining with H1119, an anti-ICP27 MAb which recognize the N-terminal 11 amino acid residues of ICP27. Note that H1119 also shows a low level of cross-reactivity with a cellular antigen which is found at or near areas of cell-cell contact, allowing the outlines of most cells to be discerned. The panels show expression of the following proteins: A: M3PK ; B: M16PK; C: M5PK; D: MSPK


A
B
C
D

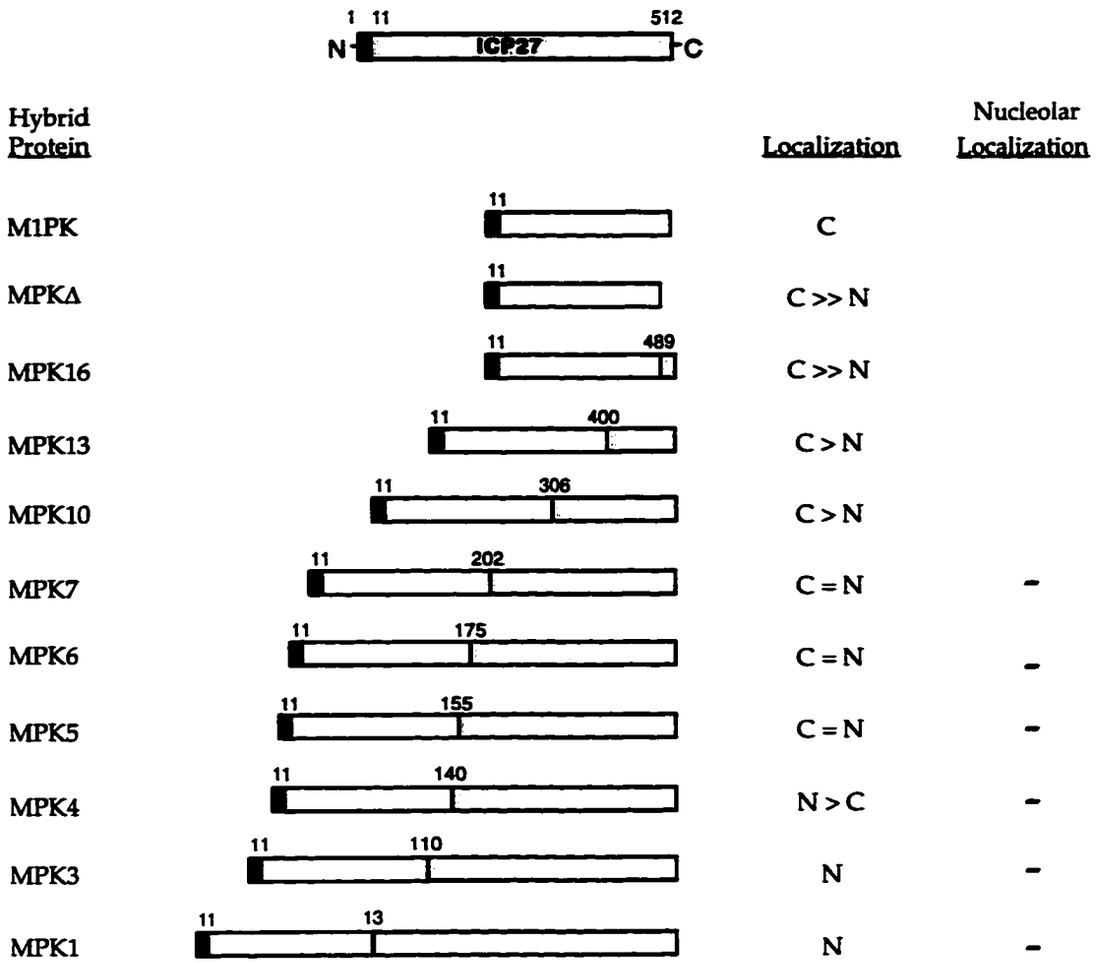
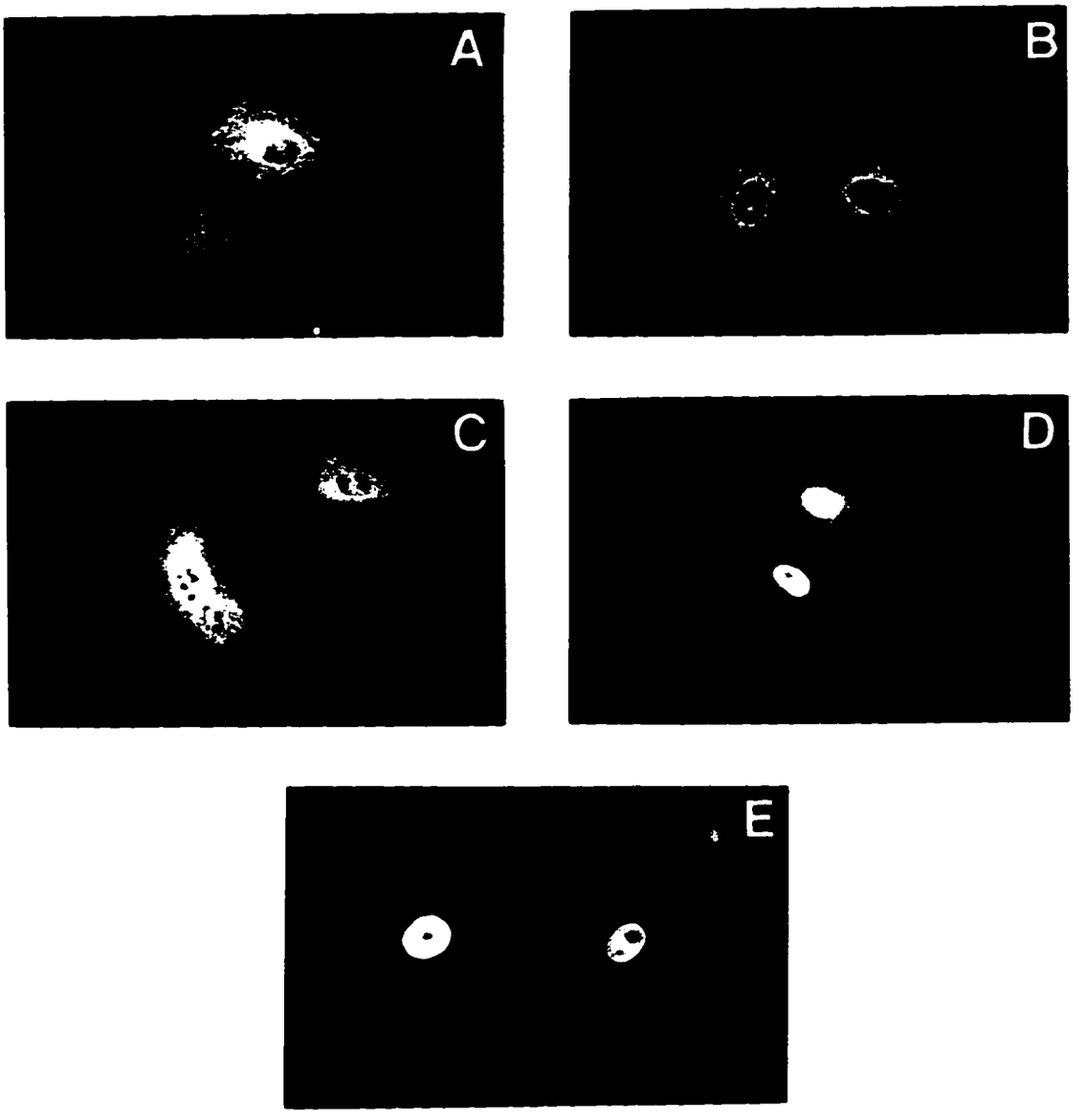


FIG. 2-4. Localization of PK fusion proteins containing C-terminal portions of ICP27. Plasmids were constructed which encode the ICP27-PK fusion proteins illustrated above. Dark-stippled bars represent the N-terminal 11 amino acid residues of ICP27, open bars represent the PK moiety (amino acid residues 17-523; not to scale), and light-stippled bars represent C-terminal ICP27 sequences. The numbers above the bars denote the extent of C-terminal ICP27 amino acid residues. For comparision, the WT ICP27 is illustrated at the top. Protein localization was determined by the stable transfection assay as described in the text. Abbreviations: C= cytoplasmic localization; N= nuclear localization; - : exclusion from nucleoli.

FIG. 2-5. Immunofluorescent staining of PK fusion proteins containing C-terminal portions of ICP27. Stably-transfected Vero cells expressing various fusion proteins were processed for immunofluorescence using the H1119 antibody. The panels show expression of the following proteins: A: MPKΔ ; B: MPK10; C: MPK5; D: MPK4; E: MPK3.


A
B
C
D
E

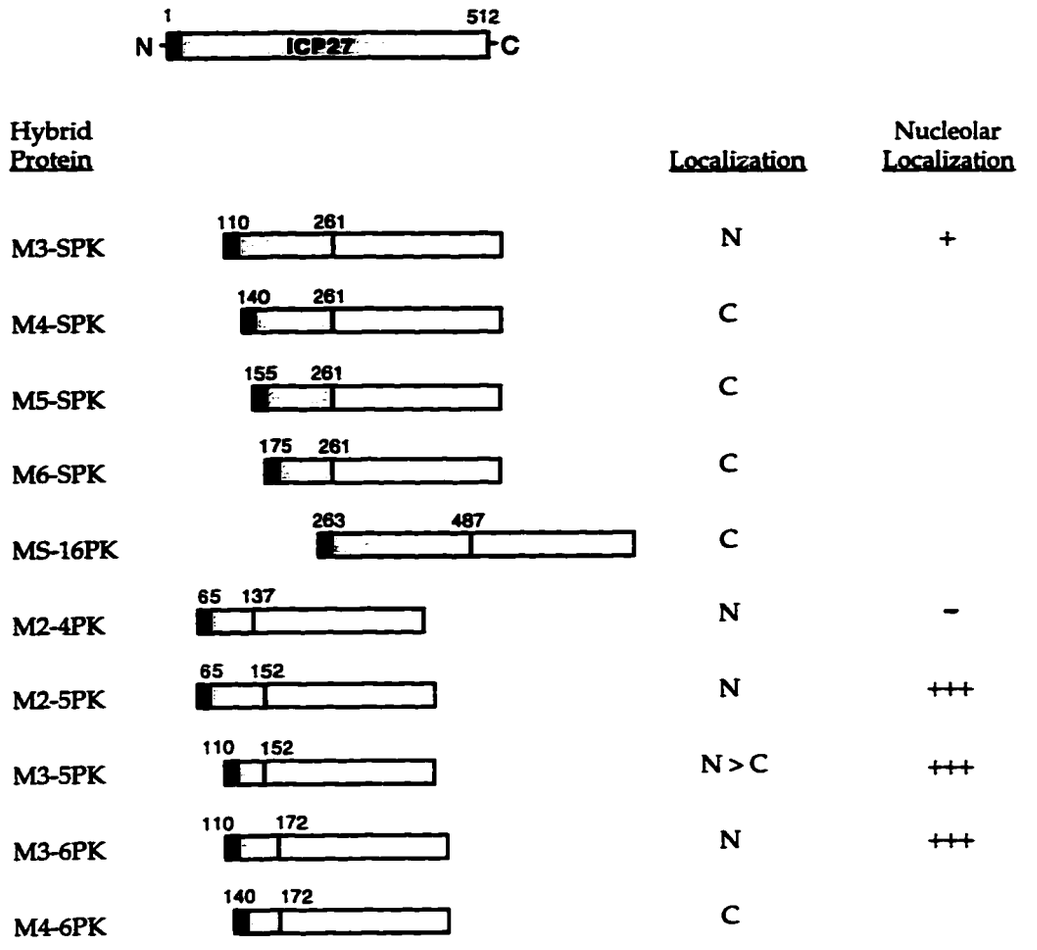


FIG. 2-6. Localization of PK fusion proteins containing internal portions of ICP27. Plasmids were constructed which encode the ICP27-PK fusion proteins illustrated above. Dark-stippled bars represent the N-terminal 11 amino acid residues of ICP27, light-stippled bars represent internal sequences of ICP27, and the open bars represent the PK moiety (amino acid residues 17-529; not to scale). The numbers above the bars denote the extent of internal ICP27 amino acid residues. For comparision, the WT ICP27 is shown at the top. Protein localization was determined by the stable transfection assay as described in the text. Abbreviations: C= cytoplasmic localization; N= nuclear localization; + = low level of nucleolar localization; - : exclusion from nucleoli; +++ = preferential staining throughout nucleoli.

FIG. 2-7. Amino acid residues 110-137 define ICP27's strong NLS. (A) Schematic representation of ICP27-PK fusion proteins and summary of the localization results. Dark-stippled bars represent the N-terminal 11 amino acid residues of ICP27, light-stippled bars represent ICP27 amino acid residues 110-137, and the open bars represent PK protein sequences. The localization of the fusion proteins was determined by the stable transfection assay as described in the text. Abbreviations: C= cytoplasmic localization; N= nuclear localization; - : exclusion from nucleoli. (B) Immunofluorescent staining of MPK3-4 using the H1113 antibody. (C) Immunofluorescent staining of MPKΔ using the H1119 antibody.

A.

Hybrid Protein		Localization	Nucleolar Localization
M1PK	PK	C	
M3-4PK	110 137 PK	C	
MPKΔ	PK	C >> N	
MPK3-4	110 137 PK	N > C	-

FIG. 2-8. Identification of a nucleolar localization signal in ICP27. Vero cells were stably transfected with pM3-6PK, which encodes a PK fusion protein containing ICP27 amino acid residues 110-172 (Fig. 3-6). The cells were infected with *d*27-1 and fixed at 6 hpi. The figure shows a single field of cells processed for double-immunofluorescence microscopy. (A) Phase-contrast image. (B) Immunofluorescent staining with H1119. (C) Immunofluorescent staining with ANA-N, an antisera specific for nucleoli.

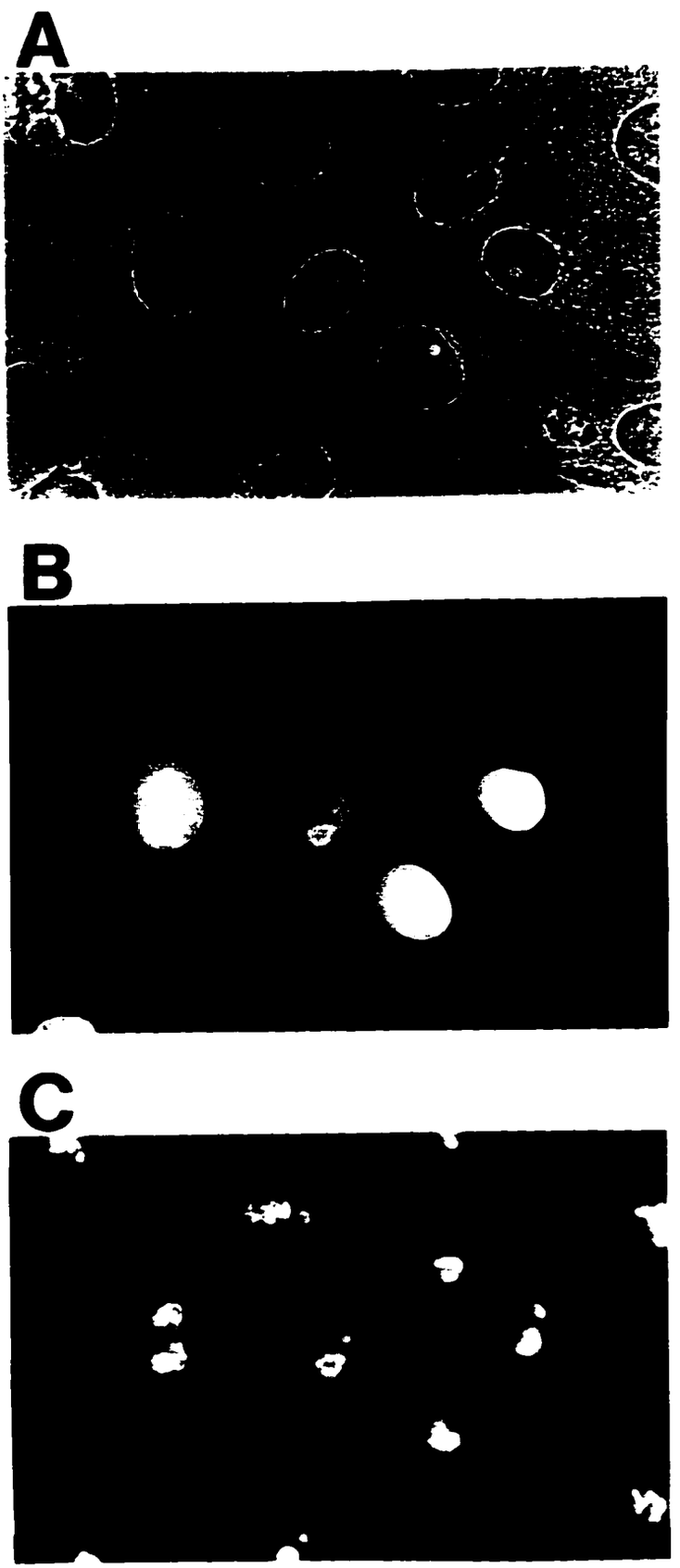

A
B
C

FIG. 2-9. Deletion of ICP27's strong NLS leads to a partial defect in nuclear localization. Vero cells were stably transfected with pM27, which encodes WT ICP27, or pMd3-4, which encodes an ICP27 molecule lacking amino acid residues 109-138. The cells were infected with *d*27-1, and processed for immunofluorescence at 6 hpi using the H1119 antibody. (A) WT ICP27 localization from pM27. (B) Md3-4 localization.

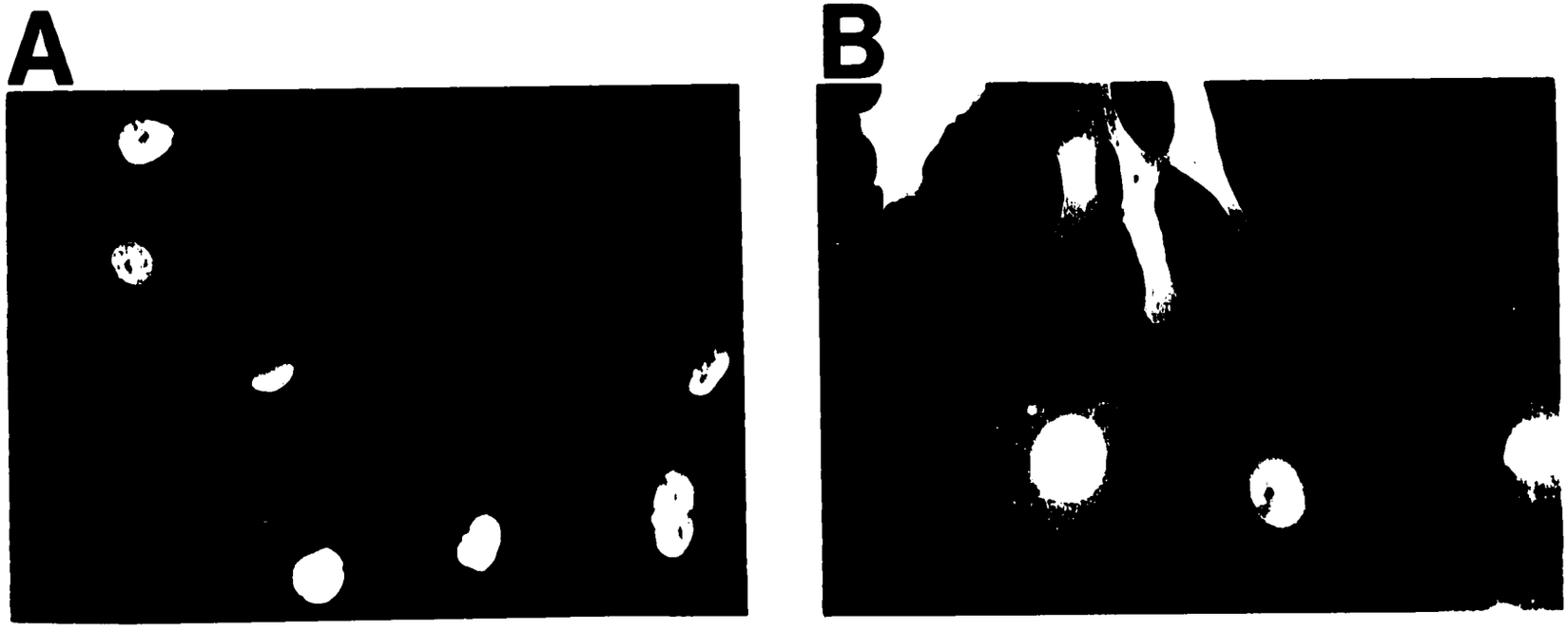
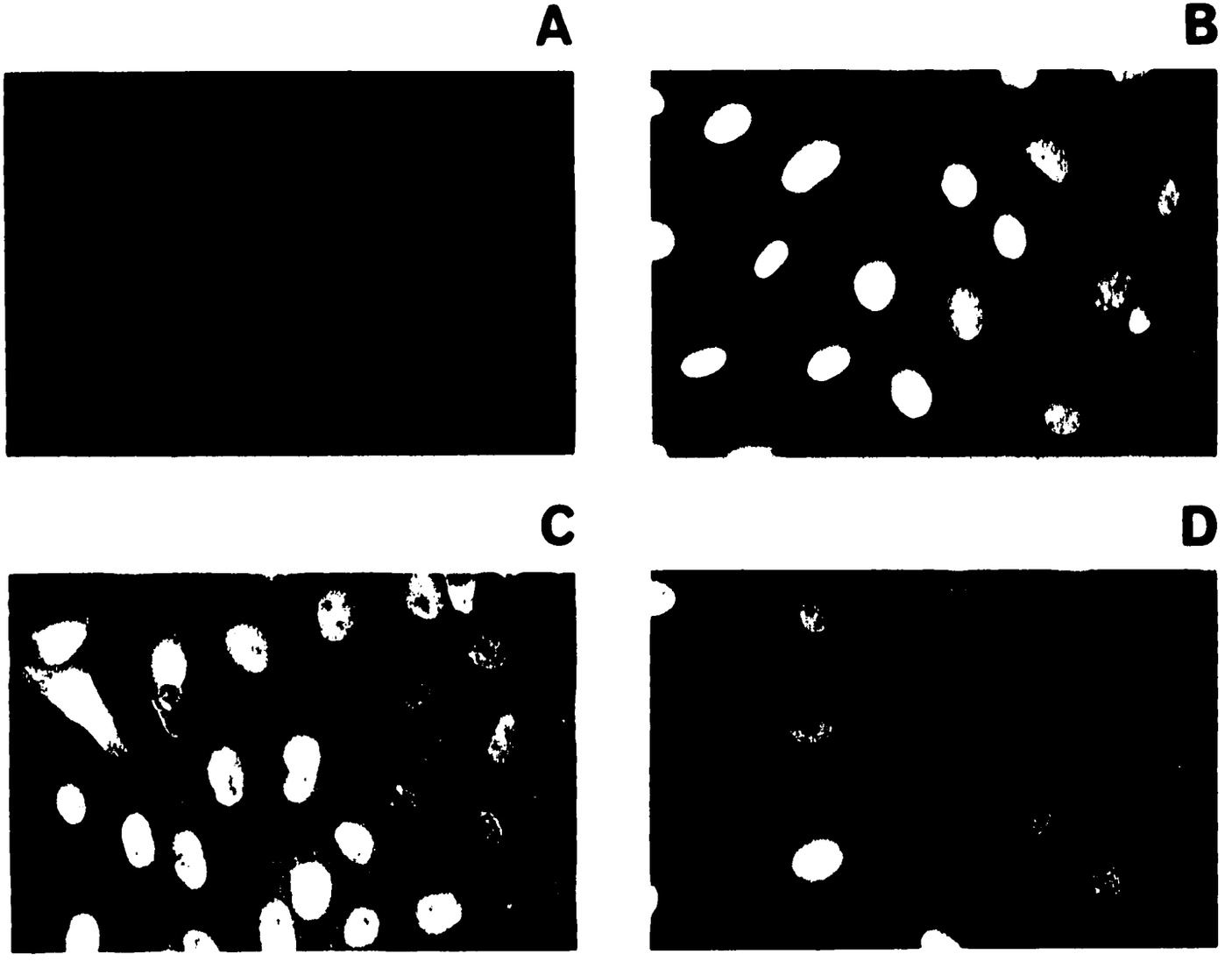

A
B

FIG. 2-10. Localization of mutant ICP27 molecules expressed from recombinant viruses. Vero cells were mock-infected (A), or infected with WT HSV-1 (B), *d*3-4a (C), or *d*4-5a (D). At 5 hpi, the cells were fixed and processed for immunofluorescence using the H1119 antibody. All four micrographs were done under the same conditions of infection, staining, exposure, and printing to allow for direct comparison.


A
B
C
D

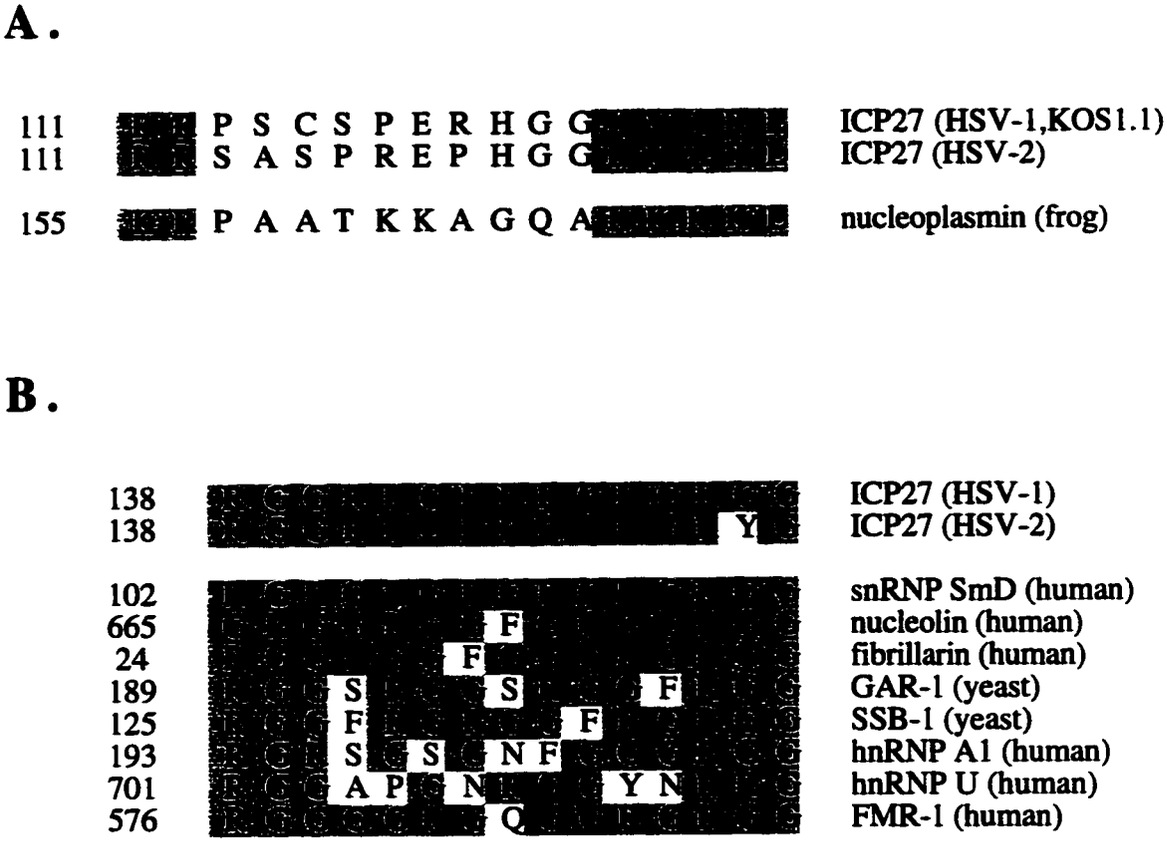


FIG. 2-11. Nuclear and nucleolar localization signals in ICP27. Protein sequences are shown in the single letter code. The position in the primary sequence of the first amino acid residue in each line is shown at the left. (A) Similarity of ICP27's NLS to bipartite NLS's. A portion (amino acid residues 111-127) of ICP27's strong NLS is shown. Note that amino acid residue 119 is arginine (R) in HSV-1 strain KOS1.1 (46); this differs from the sequence of HSV-1 strain 17, in which amino acid residue 119 is a glutamine (Q) (33). The corresponding sequence from HSV-2 ICP27 (32) is shown immediately below. At the bottom, the bipartite NLS of the *Xenopus laevis* nucleoplasmin protein (50) is shown. The two clusters of basic amino acid residues which define the bipartite NLS motif are highlighted. (B) Comparison of ICP27's RGG box region, amino acid residues 138-152, with corresponding sequence from HSV-2 ICP27 and with sequences found in cellular proteins. Arginine and glycine residues are highlighted. The sequences of the cellular proteins were taken from references 2, 6, 16, 22, 25, 52, 60, and 63.

TABLE 2-1

Growth properties of HSV-1 ICP27 deletion mutants

Virus	Virus Yield (PFU/cell)[a] in cell line:		Ratio, Yield V27/ Yield Vero
	Vero	V27	
*d*3-4a	7.0	62	8.9
*d*3-4b	1.7	58	34
*d*4-5a	0.57	33	58
*d*4-5b	0.43	36	84
KOS1.1	170	104	0.61

[a] 2.3 x 10^6 Vero cells were infected at a multiplicity of infection of 10 PFU per cell. The same inocula were used to infect parallel cultures of 2.6 x 10^6 V27 cells. The cultures were incubated at 37° C for 24 h and harvested. Virus yield was determined by plaque assay on V27 cells.

TABLE 2-2.

Mapping of the H1113 epitope

Protein	ICP27 amino acid residues	H1113 reactivity	Nuclear localization[a]
M3PK	1-108	-	C
M4PK	1-137	+	N
MPK4	1-11; 140-512	-	N>C
MPK3	1-11; 110-512	+	N
MPK3-4	1-11; 110-137	+	N
M3-4PK	1-11; 110-137	-	C

[a] C, cytoplasmic; N, nuclear.

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CHAPTER 3

THE RGG BOX MOTIF OF ICP27 MEDIATES RNA-BINDING ACTIVITY AND DETERMINES *IN VIVO* METHYLATION

Chapter Three - The RGG Box Motif of ICP27 Mediates RNA-Binding Activity and Determines *In vivo* Methylation[2]

INTRODUCTION

The evidence of post-transcriptional gene regulation by ICP27 suggests the possibility that ICP27 interacts with RNA *in vivo*. Consistent with this possibility, ICP27 can be retained on ssDNA (calf thymus DNA)-agarose and is eluted at salt concentrations of 0.2 to 0.4 M KCl (30). Due to the variety of messages that ICP27 appears to affect, the exact RNA sequence or structure to which ICP27 might interact is not readily apparent. Based on the sequence of ICP27, two regions with the potential to be involved in nucleic acid binding can be identified. First, we noted that ICP27 possesses a sequence which resembles an "RGG box", a putative RNA-binding motif found in a number of cellular nuclear proteins involved in messenger and ribosomal RNA metabolism (3,12,17). ICP27's RGG box sequence is composed of fifteen consecutive arginine and glycine residues, and maps to amino acid residues 138-152. It is required for ICP27's nucleolar localization, possibly reflecting an *in vivo* RNA-binding activity (Chap. 2; 18). It is also essential for its biological function, as a recombinant HSV-1 which encodes an RGG box-minus ICP27 fails to replicate efficiently in cultured cells (Chap. 2; 18). Second, a region located within the C-terminus of the protein which is highly conserved among ICP27 homologs consists of several cysteine and histidine residues similar to nucleic acid binding zinc finger domains. As the C-terminus of ICP27 is required for various functions including the ability to bind ssDNA (30), this region may also possess RNA binding acitivity.

The goal of this study was to ask whether ICP27 can physically interact with RNA *in vitro*, and to explore the possible role of the RGG box motif and the C-terminal

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cysteine-rich domain of ICP27 in RNA-binding. To study ICP27's interaction with RNA, we utilized a homopolymeric RNA binding assay and northwestern blotting analyses.

MATERIALS AND METHODS

Plasmids. Plasmid constructs for *in vitro* transcription/ translation of ICP27 were constructed using the vector pCITE-1 (Novagen). In order to clone the WT ICP27 gene, pCITE-1 was first modified by the introduction of an *Sst*I site into its polylinker sequence. This was done by cleaving pCITE-1 with *Acc*I, filling in the DNA ends using the Klenow fragment of *E. coli* DNA polymerase, and inserting an *Sst*I linker. This plasmid was termed pCITE-*Sst*I. To generate pCITE-27, encoding WT ICP27, the following steps were performed. A plasmid containing the ICP27 gene, pM27 (24), was digested with *Drd*I, which cuts just upstream of ICP27's initiation codon. The resulting ends were made blunt using T4 DNA polymerase. The DNA was then digested with *SstI*, which cleaves downstream of ICP27's termination codon. The 2.0 kb *Drd*I-*Sst*I fragment containing the ICP27 gene was then cloned into *BalI*/*Sst*I-digested pCITE-SstI. An analogous procedure was used to engineer pCITE-d4-5, in this case utilizing the plasmid pMd4-5 (chap. 2; 18) instead of pM27. pCITE-d4-6 was constructed by ligation of the 1.5 kb *XhoI*/*SstI* ICP27 gene-containing fragment from pM6 (24) to the 4.4 kb *Xho*I/*Sst*I vector fragment of pCITE-d4-5. pCITE-d5-6 was constructed by ligating the 1.6 kb *Dra*III/*Sst*I fragment of pMd5-6 (13) to the 4.0 kb vector fragment of pCITE-d4-6 which is obtained after complete *Sst*I digestion and partial *Dra*III digestion. pCITE-M11, pCITE-M15 and pCITE-M16 were constructed by substitution of the 1.2 kb *SalI*/*Sst*I ICP27 gene fragments of pM11, pM15 and pM16 (24) into pCITE-27 in place of the WT ICP27 gene fragment. The ICP27 molecules encoded by pCITE-27 and the derivatives described above have three additional N-terminal amino acid residues (Met-Ala-Val) compared to WT ICP27. To construct pCITE-262C, pBS27 (25) was digested with *Sal*I, and the DNA ends were made blunt using the Klenow enzyme. After cleavage with *Sst*I, the 1.2 kb fragment was cloned into *BalI*/*Sst*I-cleaved pCITE-SstI. pCITE-262C encodes a protein having an N-terminal methionine residue followed by amino acid residues 262-

512 of ICP27. HnRNP C1 was produced by *in vitro* transcription/ translation using the plasmid pHC12 (29), obtained from G. Dreyfuss (University of Pennsylvania).

Plasmids for bacterial expression of glutathione-S-transferase (GST)-ICP27 fusion proteins were constructed using the vectors pGEX-*Sst*I and pGEX-*Bgl*II, which are modified forms of pGEX-5X-3 (Pharmacia). To create pGEX-SstI, the *Not*I site in pGEX-5X-3 was converted to an *Sst*I site by digesting pGEX-5X-3 with *Not*I, filling in the DNA ends using the Klenow fragment, and inserting an *Sst*I oligonucleotide linker. pGEX-*Bgl*II was made in a similar fashion, except a *Bgl*II linker was used. To generate pGEX-27, the 2.0 kb *DrdI/Sst*I ICP27 gene fragment obtained from pM27 was cloned into the 4.9 kb *SmaI/Sst*I vector fragment of pGEX-*Sst*I. This plasmid encodes a GST-ICP27 fusion protein consisting of GST, 12 linker amino acid residues, and all 512 amino acid residues of WT ICP27. pGEX-d4-5 was constructed by ligating the 1.7 kb *Dra*III/*Sst*I fragment of pMd4-5 to the 5.2 kb *Dra*III/*Sst*I vector fragment of pGEX-27. The plasmid pGEX-RGG was constructed as follows. First, oligonucleotide-directed mutagenesis (Altered Sites System, Promega) was used to engineer a *Bgl*II site in the plasmid pM4 (24). This resulted in plasmid derivative pM4-5B which contains an *Xho*I site at codons 138/139 of the ICP27 gene and a *Bgl*II site at codons 153/154. pM4-5B was cleaved with *Xho*I and *Bgl*II, and the small *Xho*I-*Bgl*II fragment was cloned into the 4.9 kb *SalI/Bgl*II vector fragment of pGEX-*Bgl*II to produce pGEX-RGG. The polypeptides encoded by pGEX-5X-3 and pGEX-RGG differ only at their C-termini: GST contains the sequence -SSGRIVTD; this is replaced by -GRRGRRRGRGRGGPDLPAAS in GST-RGG (the underlined amino acid residues correspond to amino acid residues 140-152 of ICP27).

The plasmids used to generate RNA probes for the northwestern blotting assays, pBSSV40PA and pBSIFNPA (2), were provided by Charles Brown and L.P. Perera (National Institutes of Health).

Expression of *in vitro*-translated ICP27. For *in vitro* transcription/ translation, plasmids were first linearized at appropriate restriction sites. pHC12 was cleaved with *Nsi*I, whereas pCITE-27, pCITE-d4-5, pCITE-d4-6, pCITE-d5-6, and pCITE-262C were cleaved with *Ecl*136II. C-terminally truncated forms of ICP27 were produced by linearization of plasmids as follows: pCITE-d4-5 was digested with *Xho*I to generate the N137 template; pCITE-27 was digested with *Nco*I to generate the N189 template; pCITE-27 was digested with *Sal*I to generate the N261 template; pCITE-M11 was digested with *Xho*I to generate the N339 template; pCITE-27 was digested with *Stu*I to generate the N405 template; and pCITE-M16 was digested with *Xho*I to generate the N487 template. RNA transcripts were produced by *in vitro* transcription of the linearized plasmids using the RIBOMAX system (Promega), according to the manufacturers directions. Transcription was carried out with T7 RNA polymerase (BRL), with the exception of pHC12 which was transcribed using SP6 RNA polymerase (Promega). After transcription, RNAs were precipitated by the addition of ammonium acetate to 2.5 M, and subsequently resuspended in water. *In vitro* translation was carried out in a rabbit reticulocyte lysate (Promega) in the presence of 800 μCi/ml [^{35}S]-methionine/cysteine (DuPont NEN), following the manufacturer's suggested conditions.

RNA homopolymer binding assays. Binding of *in vitro* translated protein to ribonucleotide homopolymers or ssDNA was carried out as described by Kiledjian and Dreyfuss (12). Briefly, 10^5 counts per minute of trichloroacetic acid (TCA)-precipitatable translated protein was made up to a final volume of 0.5 ml with binding buffer (10 mM Tris pH 7.6, 2.5 mM $MgCl_2$, 0.5% Triton-X-100, and NaCl of the appropriate concentration). To this, 50-60 μl of washed agarose beads containing attached ribonucleotide homopolymer (Pharmacia, Sigma) or ssDNA (Pharmacia) were added. The mixture was incubated for 60 min at 4°C on a rocking platform. The beads were pelleted by centrifugation for 1 min in a microfuge, and washed 5 times with binding buffer. Protein was eluted from the beads by boiling in SDS-PAGE sample

buffer, and analyzed by SDS-PAGE on 12.5% or 15% gels. Radioactive bands were visualized and quantitated by autoradiography and phosphorimaging analysis (Fuji).

Expression and purification of GST fusion proteins. GST fusion proteins were expressed in *E. coli* strain BL21. Individual transformants were grown overnight at 37°C in media containing 100 μg/ml ampicillin. The overnight cultures were diluted 1:10 in a total volume of 100 ml and grown at 37°C until the optical density (A_{600}) reached 1.1-1.4 units. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM, and induction was allowed to proceed for 2 h. The cells were pelleted at 4°C, resuspended in 5 ml PBS, and lysozyme (Sigma) was added to 1 mg/ml. Lysis was allowed to proceed on ice for 60 min. The cell lysate was spun at 5000 x g for 10 min at 4°C, and the resulting pellet was quick frozen in a dry ice-isopropanol bath. After thawing, the pellet was resuspended in 5 ml PBS containing 0.1% sodium deoxycholate (Sigma). After a 10 min incubation on ice, $MgCl_2$, Triton-X-100, and DNase I were added to final concentrations of 8 mM, 1%, and 10 μg/ml, respectively. Incubation was continued for 30 min on ice with gentle agitation. The mixture was then spun at 10,000 x g for 10 min at 4°C and the supernatant was retained. To purify GST and GST-fusion proteins, 5 ml of supernatant was incubated with 100 μL of pre-washed glutathione sepharose 4B beads (Pharmacia; 50% slurry) for 30 min at 4°C. The beads were then washed 3-4 times in PBS. Bound protein was eluted at room temperature with 3-4 50 μl washes of elution buffer (50 mM Tris-HCl pH 8.0, 10 mM glutathione). Purified protein preparations were stored at -70°C until needed. The protein concentration of each preparation was measured using the Bradford assay (Biorad). The preparations were also analyzed by Western blotting using monoclonal antibodies directed against GST (Pharmacia) or ICP27 (H1113, Goodwin Institute for Cancer Research, Plantation, Fla.).

Northwestern blotting assays. ^{32}P-labeled riboprobes for the northwestern blotting experiments were generated by *in vitro* transcription. To generate templates for the β-IFN and SV40 RNA probes, pBSIFNPAand pBSSV40PA were linearized with

*Eco*RI. To generate a template for the β-IFN antisense probe, pBSIFNPA was linearized with *Hin*dIII. One μg of each DNA template was transcribed *in vitro* using T3 RNA polymerase (for the β-IFN and SV40 probes; Promega) or T7 RNA polymerase (for the β-IFN antisense probe) in the presence of 50 μCi [α-^{32}P]CTP (400Ci/mmol; DuPont NEN). After transcription, the samples were phenol/chloroform extracted, and ethanol precipitated twice using 2.5 M ammonium acetate as the precipitating salt. The final RNA pellet was resuspended in RNase-free TE (10 mM Tris-HCl, pH. 8.0; 1 mM EDTA). Approximately 8 x 10^5 cpm of each probe were analyzed by electrophoresis in a 5% denaturing acrylamide-urea gel.

The northwestern blotting assays were carried out as described by Brown *et al.* (2). Briefly, 10 or 40 μg of purified GST (expressed from *E. coli* cells harboring pGEX-5X-3) or GST fusion proteins were electrophoresed on 15% SDS-acrylamide gels, and electrophoretically transferred to nitrocellulose filters. The transferred proteins were renatured *in situ* for 12 h at 4°C in binding buffer (10 mM Tris-HCl, pH 7.6; 50 mM NaCl; 1 mM EDTA; 1x Denhardt's solution). The filters were incubated at room temperature for 8 hours in 15 ml binding buffer containing 1.7 x 10^7 cpm of ^{32}P-labeled RNA probe. The blots were individually washed three times in binding buffer for 10 min at room temperature, and analyzed by autoradiography.

Cells, viruses, and infections. Vero cells were propagated in DMEM containing 5% heat-inactivated fetal bovine serum (GIBCO). HSV infections were carried out at an MOI of 10 PFU/cell as described previously (chap. 2; 18). Strain KOS1.1 was the WT strain of HSV-1 used (10). HSV-2 (strain G) was obtained from the American Type Culture Collection (Rockville, Md.). The HSV-1 ICP27 mutants *d*27-1, *n*263R, *n*406R, *d*1-2, *d*3-4, and *d*4-5 have been described previously (chap. 2; 18,23,25). The HSV-1 mutant alleles *d*5-6 and *d*1-5 were constructed by Vivian Leong (nee: Lam) utilizing engineered *Xho*I sites in the HSV-1 ICP27 gene (24), and recombinant viruses bearing

these alleles were isolated (13). *d*1-5 and *d*5-6 encode ICP27 molecules missing amino acid residues 13-153 and 154-173, respectively.

***In vivo* methylation assay.** Metabolic labeling of methylated proteins was performed as described by Liu and Dreyfuss (16). At 5 hpi, media was removed from the infected cells and replaced with media containing 100 µg/ml cycloheximide and 40 µg/ml chloramphenicol. After 30 min, the cells were rinsed twice with methionine-free DMEM (GIBCO), then incubated for 3 hours in methionine-free DMEM containing 100 µg/ml cycloheximide, 40 µg/ml chloramphenicol and 45 µCi/ml L-[*methyl*-^{3}H]methionine (DuPont NEN). In some experments, 100 µM anisomycin was used in place of cycloheximide. After labeling, the cells were rinsed in PBS containing 50 µg/ml Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) and 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), and lysed in modified RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 50 µg/ml TLCK, 100 µg/ml PMSF] for 30 min on ice. The cell lysate was transferred to a microfuge tube, and insoluble material was pelleted at 10,000 x g for 10 min at 4°C. The supernatant was transferred to a new tube and analyzed immediately or stored at -70°C.

Immunoprecipitations. Immunoprecipitations were carried out using a mixture of H1113 and H1119, two mouse monoclonal antibodies specific for ICP27 (Goodwin Institute for Cancer Research, Plantation, Fla.). To carry out the immunoprecipitations, 0.8 µl of each antibody was added to 400 µl of cell lysate, and the samples were incubated at 4°C on a rocking platform. After 30 min, 80 µl of a 1:40 dilution of rabbit anti-mouse IgG (H+L chains; Jackson ImmunoResearch Laboratories Inc.) was added as a bridging antibody. After a further 30 min incubation, 200 µl of prewashed *Staphylococcus aureus* cells (Pansorbin, Calbiochem), resuspended in modified RIPA buffer (10% w/v), was added, and the incubation was continued for another 30 min. The cells were pelleted and washed four times in modified RIPA buffer. Bound proteins were eluted by heating the cells for 10 min at 85°C in SDS-PAGE sample buffer.

Immunoprecipitates were analyzed by electrophoresis on 12.5% SDS-acrylamide gels. For fluorographic enhancement, gels were impregnated with 1.0 M sodium salicylate before drying. Immunoprecipitates were also analyzed by immunoblotting using a 1:1000 dilution of the H1119 antibody. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL detection kit; Amersham).

***In vitro* methylation assay.** To ask if cellular enzymes could methylate ICP27, an *in vitro* experiment was carried out using rabbit reticulocyte extract (Promega; 16). One μg of GST or each GST-fusion protein (GST-ICP27, GST-d4-5, GST-RGG) was combined with 35 μl rabbit reticulocyte lysate. 8.5 μCi ^{3}H-S-adenosyl-methionine (SAM) was added and the reaction mix made to a final volume of 60 μl. A control reaction was performed in which water was included rather than GST protein samples. After incubation at 37°C for 30 min, the GST proteins were collected using glutathione sepharose beads. The proteins were analysed by fluorography after separation on 15% SDS-PAGE, as described above.

RESULTS

ICP27 binds to poly (G) RNA homopolymers. ICP27's roles in post-transcriptional gene regulation suggest a possible direct association with RNA. To gain insight into ICP27's possible interaction with RNA, we tested its ability to interact with various RNA homopolymers *in vitro* (28). Such homopolymer binding assays have been used by many investigators to characterize the RNA-binding potential and target specificity of putative RNA-binding proteins (1,6,12,20,27). For the binding studies, full-length ICP27 was expressed by *in vitro* translation in a rabbit reticulocyte lysates in the presence of [^{35}S]methionine/cysteine. The radiolabeled protein was incubated with agarose beads to which RNA homopolymers [poly (A), poly (C), poly (G), or poly (U)] were covalently attached. The binding reactions and subsequent washing steps were carried out at several NaCl concentrations ranging between 0.1 M to 1.0 M. After washing, protein was eluted from the beads and analyzed by SDS-PAGE and phosphorimaging analysis. ICP27 bound to poly (G) ribopolymers, with significant binding of the input material occurring up to a NaCl concentration of 0.5 M (Fig. 3-1 A and B). ICP27 also bound weakly to poly (U) homopolymeric RNA at 0.1 M and 0.25 M NaCl. In contrast, ICP27 did not bind to poly (A) or poly (C). In control experiments, *in vitro* translated hnRNP C1 protein bound avidly to poly (U) agarose beads up to a NaCl concentration of 1.0 M (data not shown), consistent with previous results (28). Based on these experiments, we conclude that ICP27 binds with moderate avidity to poly (G) RNA homopolymers, and weakly to poly (U) homopolymers.

Previously, Vaughan *et al.* demonstrated that ICP27 purified from infected cells can bind to a single-stranded DNA-agarose column, and be eluted at relatively high salt (30). We therefore examined ICP27's interaction with ssDNA in our binding assay. To do this, *in vitro* translated ICP27 was incubated with ssDNA- or poly (G)-agarose beads at NaCl concentrations ranging from 0.25M to 2.0 M. As a control, *in vitro* translated

hnRNP C1, previously demonstrated to interact with ssDNA (21), was also tested for ssDNA binding. After binding and washing, protein was eluted from the beads and analyzed by SDS-PAGE and phosphorimaging (Fig. 3-1C). ICP27 bound to poly (G) as before, but demonstrated little if any binding to ssDNA. In contrast, hnRNP C1 protein bound to the ssDNA, up to a NaCl concentration of 1.0 M. We conclude that, under the conditions of this binding assay, *in vitro* translated ICP27 binds to poly (G) RNA but not to ssDNA.

Role of the RGG box in poly (G) binding. In order to identify the sequences in ICP27 which are responsible for poly (G) binding, several N- or C-terminally truncated forms of ICP27 were expressed by *in vitro* translation and tested in the binding assay at a NaCl concentration of 0.25 M NaCl (Fig. 3-2). A fragment corresponding to the C-terminal half of ICP27, 262C, did not bind efficiently to poly (G) (<10% of WT ICP27 binding). This suggests that the N-terminal half of ICP27 is required for strong binding. Several C-terminally truncated forms of ICP27 (N487 to N137) were also studied. Significant poly (G) binding (>50% of WT binding) was observed for the mutants N487, N405, N339, N261, and N189. Interestingly, the N189 protein showed enhanced binding compared to the WT protein. The only mutant in this series which did not bind efficiently to poly (G) was N137, which was similar to 262C in its low binding (~10% of WT). Overall, these data demonstrate that the major determinant of poly (G) binding maps to the N-terminal 189 amino acid residues of ICP27. Additionally, the data suggest that sequences important for RNA-binding are located between amino acid residues 137 and 189, since N189 but not N137 bound strongly to poly (G). This region of ICP27 contains the RGG box motif, which maps to amino acid residues 138-152.

To determine whether the RGG box or nearby sequences are required for ICP27's interaction with poly (G), three ICP27 deletion proteins (Fig. 3-3A) were expressed by *in vitro* translation and tested in the poly (G) binding assay. The d4-5 mutant lacks amino acid residues 138-152, thus containing a precise deletion of the RGG box sequence. The

d5-6 mutant lacks amino acid residues 153-174; this deletion removes a second arginine-rich sequence designated "R2" by Hibbard and Sandri-Goldin (9). The mutant d4-6 lacks both the RGG box and R2 sequences. The poly (G) binding activity of these mutants, as well as WT ICP27, was measured at 0.1, 0.25, and 0.5 M NaCl (Figs. 3-3 B and C). Both the d4-5 and d4-6 proteins were defective in poly (G) binding, showing less than 5% of the WT level of binding at 0.25 M and 0.5 M NaCl. These two mutants differed slightly at 0.1 M NaCl, in that d4-5 mutant bound weakly, whereas d4-6 did not bind to an appreciable extent. In contrast to d4-5 and d4-6, the d5-6 mutant bound to poly (G) almost as efficiently as the WT protein, although its binding was reduced approximately two-fold compared to WT at 0.5 M NaCl. Together, these data indicate that the RGG box motif, but not the arginine-rich sequence immediately C-terminal to it (R2), is required for efficient poly (G) binding.

Direct interaction of ICP27's RGG box with RNA. After our studies had been initiated, Brown *et al.* (2) used a northwestern blotting assay to demonstrate that ICP27 is an RNA-binding protein. These investigators showed that a bacterially-expressed GST-ICP27 fusion protein can bind to >600 nucleotide-long RNA probes corresponding to sequences from the 3' ends of the c-myc or β-IFN genes. The binding appeared to be target-specific, as GST-ICP27 did not bind to a similar-sized RNA corresponding to sequences from the 3' end of the SV40 early region. To determine whether ICP27's RGG box plays a role in this RNA interaction, we tested several GST fusion proteins for their ability to bind to the β-interferon RNA probe used by Brown *et al.* (2). The proteins tested were (i) GST; (ii) GST-ICP27, a fusion having all of ICP27 tagged to GST; (iii) GST-d4-5, a fusion identical to GST-ICP27 but lacking the RGG box; and (iv) GST-RGG, a fusion containing only the RGG box sequence (amino acid residues 140-152) tagged to GST. The proteins were expressed in *E. coli*, purified by glutathione-sepharose affinity chromatography, and analyzed by SDS-PAGE and Coomassie blue staining (Fig. 3-4A). Although the GST and GST-RGG preparations yielded major bands of the

expected molecular size (lanes 2 and 5, respectively), much of the protein in the GST-ICP27 and GST-d4-5 preparations (lanes 3 and 4) migrated as bands that were smaller than expected, possibly due to proteolysis during expression and/or purification. However, there was some apparently full-length GST-ICP27 and GST-d4-5 in each preparation, migrating at the expected molecular mass of ~95 and ~93 kDa, respectively (denoted by the circles alongside lanes 3 and 4). Western blot analysis of the GST-ICP27 and GST-d4-5 preparations demonstrated that the putative full-length molecules as well as most of the smaller species reacted with both an anti-GST monoclonal antibody and H1113, a monoclonal antibody directed against an N-terminal epitope in ICP27 (data not shown).

For the northwestern blotting assay, GST or the GST fusion proteins were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose filters, and renatured *in situ*. Identical filters were incubated with three different ^{32}P-labeled RNA probes. One probe was the ~650 nucleotide β-IFN gene probe previously shown to form a complex with GST-ICP27 (2)(Fig. 3-4B, lane 2). As controls for specificity, two other probes were used: an anti-sense version of the β-IFN probe (lane 1), and an ~840 nucleotide RNA corresponding to the 3' end of the SV40 early region (lane 3), previously shown not to form a complex with GST-ICP27 (2). After binding, the northwestern filters were washed and analyzed by autoradiography. The results (Fig. 3-4C) indicate that the RGG box is both required and sufficient for interaction with RNA, as both GST-ICP27 and GST-RGG, but not GST or GST-d4-5, efficiently bound to RNA molecules. However, we did not observe the specificity reported by Brown *et al.* (2), as all three probes bound comparably to GST-ICP27 and GST-RGG. Although the β-IFN probe appeared to bind slightly more efficiently to GST-ICP27 in this experiment than did the SV40 probe, this was not observed in a repeat experiment (data not shown).

Two major bands in the GST-ICP27 preparation formed complexes with RNA (Fig. 3-4C, lane 2). These did not correspond to full-length GST-ICP27, but to the two

truncated forms of GST-ICP27 indicated by the black squares in Fig. 3-4A. However, a repeat experiment in which 40 μg of protein, rather than 10 μg, was analyzed on the northwestern blot demonstrated that the full-length GST-ICP27, but not full-length GST-d4-5, could also bind to the β-IFN and SV40 RNA probes (data not shown).

Two important conclusions can be drawn from these experiments regarding the role of ICP27's RGG box in RNA binding. First, the RGG box is required, in the context of both intact and truncated GST fusion proteins, for interaction with RNA molecules. Second, the RGG box sequence alone can mediate RNA binding, since GST-RGG efficiently binds to RNA.

***In vivo* methylation of ICP27.** The experiments described above suggest that ICP27 is an RGG-box type RNA-binding protein, since its RGG box sequence can mediate RNA-binding. A characteristic of many cellular RGG box proteins is that they are targets for post-translational modification by protein arginine methyltransferases, which dimethylate arginine residues to produce N^G-N^G dimethylarginine (16,19). The function of this unusual modification is not known. To determine whether ICP27 is methylated *in vivo,* we carried out a metabolic labeling experiment to specifically identify methylated proteins (16). The assay is based on the fact that the methyl donor for protein methylation, S-adenosyl methionine, is derived from free methionine in the cell. Thus, if translation is blocked, methylated proteins can be specifically labeled by incubating cells in L-[*methyl*-^{3}H]methionine. To carry out the methylation assay, Vero cells were mock-infected, infected with WT HSV-1, or infected with d27-1, an ICP27 deletion mutant (23). At 5 hpi, the cells were treated with cycloheximide and chloramphenicol to inhibit protein synthesis. Thirty min later, the cells were labeled with L-[*methyl*-^{3}H]methionine for 3 h in the presence of the translation inhibitors. The labeled proteins were examined by SDS-PAGE and fluorography (Fig. 3-5). Three lines of evidence indicate that the majority of labeling is due to *in vivo* methylation, not incorporation of methionine via protein synthesis: (i) the pattern of labeled proteins is dramatically different from that

obtained when cells are labeled with [^{35}S]-methionine in the absence of translation inhibitors (23,25); (ii) control experiments demonstrated that the cycloheximide/ choramphenicol treatment inhibited overall translation levels approximately 20-fold (not shown); and (iii) treatment with a more stringent inhibitor of protein synthesis, anisomycin, led to a nearly identical pattern of labeled proteins (not shown).

The pattern of protein methylation in WT HSV-1 infected cells was quite distinct from that of the mock-infected cells (Fig. 3-5, lanes 1 and 2). First, the labeling of several cellular proteins was markedly reduced after HSV-1 infection. In particular, the labeling of histones was significantly inhibited. Moreover, this effect requires ICP27, as histone methylation was not inhibited in the d27-1 infection (lane 3). Second, several methylated proteins were either induced or enhanced in WT-infected cells. We noted two prominent bands, labeled "a" and "b" in Fig. 3-5. These proteins may correspond either to viral proteins which are methylated, or to cellular proteins whose methylation is induced upon infection.

Although the identity of band "b" was not investigated further, we suspected that band "a" might correspond to ICP27, since it has approximately the same apparent molecular mass (~63 kDa), and is absent from the ICP27 null mutant infection (Fig. 3-5, compare lanes 2 and 3). To determine whether band "a" corresponds to ICP27, a second experiment was performed. Vero cells were mock-infected, or infected with WT HSV-1, WT HSV-2, d27-1, or d4-5 (chap. 2; 18). d4-5 is a defective HSV-1 ICP27 mutant which contains the same RGG box deletion shown in Fig. 3-3A. The infected cells were labeled with L-[*methyl*-^{3}H]methionine as before, and the cell lysates were subjected to immunoprecipitation using a mixture of two anti-ICP27 monoclonal antibodies. Analysis of the fluorograph (Fig. 3-6A) indicated that both HSV-1 and HSV-2 ICP27 are methylated proteins, since they efficiently label with L-[*methyl*-^{3}H]methionine. Furthermore, comparison of the immunoprecipitated HSV-1 ICP27 with the total labeled lysate (not shown) demonstrated that ICP27 corresponds to band "a" seen in Fig. 3-5.

Interestingly, no labeled ICP27 was present in the d4-5 immunoprecipitate (Fig. 3-6A, lane 5), suggesting that the RGG box is required for *in vivo* methylation of ICP27. To demonstrate that the d4-5 protein was present in the immunoprecipitate, we subjected the immunoprecipitates to Western blot analysis (Fig. 3-6B). The d4-5 protein was indeed present, in amounts comparable to the WT protein (compare lanes 2 and 5). Therefore, the RGG box is required for ICP27's *in vivo* methylation. Moreover, this experiment confirms the validity of the methylation assay, as the WT and d4-5 ICP27 proteins contain equal numbers of methionine residues, yet label distinctly with L-[*methyl*-^{3}H]methionine.

Requirement for the RGG box in *in vivo* methylation. The above experiment demonstrates that the RGG box sequence is required for ICP27's methylation, but does not exclude the possibility that other regions also are involved. To systematically determine which portions of ICP27 are required for *in vivo* methylation, we utilized several HSV-1 ICP27 mutants which contain nonsense or in-frame deletion mutations in the ICP27 gene (Fig. 3-7A). Infected Vero cells were labeled with L-[*methyl*-^{3}H]methionine, and ICP27 proteins were subjected to immunoprecipitation. To confirm that the ICP27 mutant proteins were expressed and immunoprecipitated, the samples were first subjected to Western blot analysis (Fig. 3-7B). With one exception, all of the mutant proteins were present in quantities similar to that of WT ICP27. The exception was the 406R protein, which was present in somewhat lesser amounts. To determine whether the proteins were methylated, the immunoprecipitates were analyzed by SDS-PAGE and fluorography (Fig. 3-7C). Although the 406R protein was present in lower amounts, it was clearly methylated (lane 9), as was the 263R protein (lane 8). This indicates that methylation occurs predominantly or exclusively on the N-terminal half of ICP27. Examination of several N-terminal in-frame deletion mutant proteins gave further information about the sequences in ICP27 required for methylation. The d1-2 and d5-6 proteins were efficiently methylated (lanes 3 and 6, respectively), indicating that

amino acid residues 12-63 and 154-173 are not required. The d3-4 protein (lane 4) was weakly labeled, indicating that the engineered deletion in d3-4 lowers the efficiency of, but does not completely prevent, ICP27 methylation. The only two mutant proteins which did not appear to be methylated to any appreciable extent were those encoded by d4-5 and d1-5, both of which lack the RGG box motif. These data demonstrate that the RGG box is a critically important determinant of ICP27's *in vivo* methylation.

***In vitro* methylation of ICP27's RGG box.** To investigate if viral proteins were required for the *in vivo* methylation of ICP27 observed in infected cells, we asked whether the GST-ICP27 fusion proteins described above could be methylated *in vitro* in a rabbit reticulocyte extract. Liu and Dreyfuss (16) reported that rabbit reticulocyte lysates possess an RGG-specific methyltransferase activity capable of methylating hnRNP A1. GST or the GST-ICP27 fusion proteins were added to aliquots of rabbit reticulocyte lysate containing ^{3}H-S-adenosyl-methionine (SAM). As mentioned previously, SAM serves as methyl donor for known protein methyltransferases. After incubation, the GST proteins were purified from the rabbit reticulocyte lysate using glutathione sepharose beads. The collected proteins were analysed on 15% SDS-PAGE and the labeled proteins were visualized by fluorography (Fig. 3-8). Both GST-ICP27 and GST-RGG but not GST or GST-d4-5 were labeled in this experiment. A low molecular weight band was observed in all lanes. This band is likely due to methylation of a rabbit reticulocyte protein or represents residual SAM. As with the northernwestern analyses, a truncated version of GST-ICP27 was observed whereas the full length molecule was not apparent. The absence of the full length molecule may have been a result of the low concentration (1 μg) of protein used in this assay. Thus, it appears that ICP27 can be methylated *in vitro* by a cellular enzyme or enzymes and this methylation does not occur when its RGG box is deleted. Furthermore, ICP27's RGG box was methylated specifically *in vitro* suggesting that it is this region of ICP27 that is methylated *in vivo* as well.

DISCUSSION

ICP27's RGG box and RNA-binding. In this work, we show that ICP27 binds to RNA homopolymers composed of poly (G) and that it is therefore an RNA-binding protein. While this study was in progress, two other groups also demonstrated that ICP27 can interact with RNA *in vitro* (2,11).

In our work, we used both the poly (G)-binding assay and the northwestern blotting assay described by Brown *et al.* (2) to identify the sequences in ICP27 which mediate RNA-binding. Both sets of experiments implicate ICP27's RGG box motif, which maps to amino acid residues 138-152. In the poly (G) binding assay, there is a strict correlation between the presence of the RGG box and RNA-binding. In addition, the RGG box is required in the context of an otherwise intact ICP27 molecule for association with poly (G). In the northwestern assay, the RGG box is required for RNA interaction, and alone can mediate RNA-binding when attached to a heterologous protein. Together, our data demonstrate that ICP27's RGG box is an RNA-binding domain that can mediate interaction with both poly (G) RNA homopolymers and more complex RNA sequences.

Our results differ from those of Brown *et al.* (2) in that we do not observe the same RNA target specificity in the northwestern blotting assay. Brown *et al.* found that GST-ICP27 could associate with RNAs derived from the 3' ends of the β-IFN and c-myc genes, but not with an RNA derived from the 3' end of the SV40 early region. In contrast, we find that GST-ICP27 fusion binds efficiently to both the β-IFN and SV40 probes, as well as to an anti-sense version of the β-IFN probe. We note that Ingram *et al.* also failed to find evidence for sequence-specific RNA-binding by ICP27 (11). A complicating factor in our experiments is that the major RNA-binding polypeptides in our GST-ICP27 preparation are approximately 25-30 kDa smaller than full-length. However, truncation of our GST-ICP27 is unlikely to explain the conflicting results, as

the GST-ICP27 fusion protein of Brown *et al.* (2) is also ~30 kDa smaller than expected, and thus likely corresponds to a similar proteolytic fragment. The discrepancies in binding results thus leave open the question of ICP27's RNA target specificity. However, the fact that ICP27 binds efficiently to poly (G) RNA raises the possibility that G-rich sequences play a role in the recognition of natural RNA targets by ICP27.

The RGG box motif is found in a number of cellular RNA-binding proteins. It is defined as an arginine- and glycine-rich sequence that usually contains closely spaced RGG repeats interspersed with other, often aromatic, amino acid residues (3). Most of the RGG box proteins are known or suspected to bind RNA (8,12,17,27). In some cases the RGG box defines a functional domain which mediates, or helps to mediate, RNA-binding (3,12,17). However, for most of the RGG box proteins, the RGG box is unlikely to be the sole determinant of RNA binding, since many also possess one or more well-characterized ribonucleoprotein (RNP) RNA-binding motifs. It has been proposed that the RGG box of nucleolin interacts relatively non-specifically with pre-rRNA and helps to unfold it, allowing the more specific RNP domains to gain access to their specific target sequence (7). Thus, RGG boxes may work in concert with other protein domains to mediate RNA-binding.

The minimal size of a functional RGG box RNA-binding sequence is unknown, but most are larger than the fifteen amino acid residues which comprise ICP27's RGG box. In this work, we have shown that thirteen amino acid residues of ICP27's RGG box, GRRGRRRGRGRGG, are sufficient to mediate RNA-binding when attached to a heterologous protein, GST. To our knowledge, this is the smallest RGG-like sequence which has been shown to bind to RNA. The GST-RGG molecule may therefore be a useful tool for defining the specific amino acid residues which interact with RNA.

Two observations suggest that the C-terminus of ICP27 may inhibit RNA-binding by the RGG box. First, the C-terminally truncated N189 polypeptide displays significantly enhanced poly (G) binding compared to the WT protein. Second, C-

terminally truncated forms of GST-ICP27 appear to be more efficient RNA binding proteins than full-length GST-ICP27, although the full-length molecule binds RNA to some extent. These observations raise the possibility that the C-terminal region of ICP27 may negatively regulate the RNA-binding activity of the RGG box. However, it is also possible that enhanced RNA-binding by truncated ICP27 molecules may represent an artifactual effect of mutagenesis.

ICP27's RGG box and protein methylation. Several cellular proteins involved in nuclear RNA metabolism, including fibrillarin (15), nucleolin (14), and hnRNP A1 (22,31), are methylated on arginine residues. The predominant modified amino acid residue in these proteins is the asymmetrically dimethylated derivative N^G-N^G dimethylarginine (DMA). The function of this protein modification is unknown. Recently, Liu and Dreyfuss found that, in addition to hnRNP A1, many other hnRNP proteins are methylated *in vivo* (16). In addition, these investigators partially purified a protein arginine N-methyltransferase from HeLa cells, using unmodified recombinant hnRNP A1 as a substrate. The enzyme activity appeared to be specific for RGG box sequences, and modified arginine residues to DMA and N^G-monomethylarginine. Moreover, *in vitro*, the enzymatic activity methylated the same spectrum of hnRNP proteins that are methylated *in vivo*. These results suggest that many hnRNP proteins, and possibly other RGG box proteins, are methylated on their RGG box motifs by a common cellular enzyme.

Based on the above information, we investigated the methylation status of ICP27 in HSV-1-infected cells. Our results demonstrate that ICP27 is one of the major methylated proteins in infected cells, at least under the conditions of our labeling assay. Furthermore, ICP27's methylation correlates with, and requires the presence of the RGG box sequence. Amino acid residues 109-137, just N-terminal to the the RGG box, may also play a role in methylation, as the d3-4 protein was only weakly labeled in the *in vivo* methylation assay. However, it is worth noting that the d3-4 mutation, in addition to

deleting codons 109-137, also alters the first two codons of the RGG box, changing them from RG to LE. This modification to the RGG box could also explain the reduced methylation of the d3-4 protein. Overall, our results indicate that the RGG box is critical for methylation, and suggest that it is the RGG box motif itself which is the target of methylation. If so, then the modified amino acid residue is arginine, as this is the only amino acid residue in the RGG box sequence known to accept methylation (4). However, further experiments will be required to directly map the site(s) of methylation and to identify the modified amino acid residue(s).

What role, if any, could RGG box methylation play in the biological function of ICP27? An intriguing possibility is that methylation regulates ICP27's association with RNA. Methylation of arginine's guanidinium group would not alter the strong positive charge of the arginine side chain, but the bulky methyl groups could sterically affect RNA binding. In addition, arginine methylation might alter hydrogen bonding interactions between arginine residues and RNA. It is thus conceivable that arginine methylation could alter the overall level, or target specificity, of ICP27's binding to RNA. It is noteworthy that the enzymatic activity thought to be responsible for RGG box methylation is not found in *E. coli* (16). Since bacterially-expressed GST-ICP27 and GST-RGG bind RNA, methylation is probably not required for RNA-binding. In this work we demonstrate that rabbit reticulocyte lysates possess an activity capable of specifically methylating ICP27's RGG box (16). Thus, it is possible that if sufficient SAM is available during the *in vitro* translation, the ICP27 used in our poly (G) binding assays could be methylated to some extent. However to date, atttempts to directly demonstrate that *in vitro* translated ICP27 is methylated have led to inconclusive results. If ICP27 produced by *in vitro* translation is methylated, it may be possible to block this process with methylation inhibitors. In this way, both methylated and unmethylated ICP27 could be compared in RNA-binding assays.

Possible *in vivo* RNA-binding by ICP27's RGG box. We have previously shown that the RGG box is important for ICP27's biological functions, as an HSV-1 mutant which encodes an RGG box-minus ICP27 fails to replicate in Vero cells (Chap. 2; 18). The studies described herein demonstrate that ICP27's RGG box motif can function *in vitro* as an RNA-binding domain. It is tempting to speculate that ICP27's RGG box also mediates RNA-binding activity *in vivo*. Previously, we suggested that the nucleolar localization of ICP27, which depends on the RGG box, reflects an *in vivo* association with abundant RNAs in the nucleolus (chap. 2; 18). However, the physiologically relevant RNA target, or targets, of ICP27 are not necessarily nucleolar. Given ICP27's effects on pre-mRNA splicing, possible targets might be one or more of the snRNA's associated with spliceosomal snRNP's. In this regard, it is interesting to note that at least a small fraction of ICP27 interacts with U snRNPs during infection (26). Another possibility is that ICP27 might function as an hnRNP protein, binding to nascent pre-mRNA in the nucleus (5). Indeed, our work shows that ICP27 shares characteristics with several cellular hnRNP proteins, in that it contains an RGG box RNA-binding domain and is methylated. In this scenario, ICP27's association with pre-mRNA's, perhaps in combination with cellular hnRNP proteins, could lead to modified hnRNP complexes on viral or cellular transcripts. Such novel complexes might determine alternate pathways of mRNA polyadenylation, splicing, or export from the nucleus. This model could explain how a single viral protein can affect multiple and seemingly diverse aspects of pre-mRNA metabolism.

FIG. 3-1. Binding of *in vitro* translated ICP27 to RNA homopolymers. (A and B) Binding of ICP27 to RNA hompolymers. ICP27, produced by *in vitro* translation in the presence of [^{35}S]-methionine/cysteine, was incubated with RNA homopolymers [poly (A), poly (C), poly (G), or poly (U)] attached to agarose beads. Binding and washing were carried out at various NaCl concentrations ranging from 0.1 to 1.0 M. Bound protein was eluted and analyzed by SDS-PAGE, autoradiography, and phosphorimage analysis. (A) Binding of ICP27 to poly (G) and poly (U) ribopolymers. The lane labeled "input" contains an aliquot of the translation mixture used in the binding reaction. The other lanes show protein bound at various salt concentrations. Equivalent fractions of the input and bound samples were loaded on the gel to facilitate the determination of percent binding. The positions of molecular weight markers are indicated at the left (molecular mass in kilodaltons). (B) Quantitation of ICP27 binding to RNA homopolymers at different NaCl concentrations. The percentage of input ICP27 bound at each NaCl concentration was determined by phosphorimage analysis. (C) Comparison of ICP27's binding to poly (G) RNA and ss DNA. *In vitro* translated ICP27 or hnRNP C1 protein were bound at various NaCl concentrations to agarose beads containing either poly (G) RNA or ss DNA. Analysis and quantitation of binding were carried out as in panels (A) and (B).

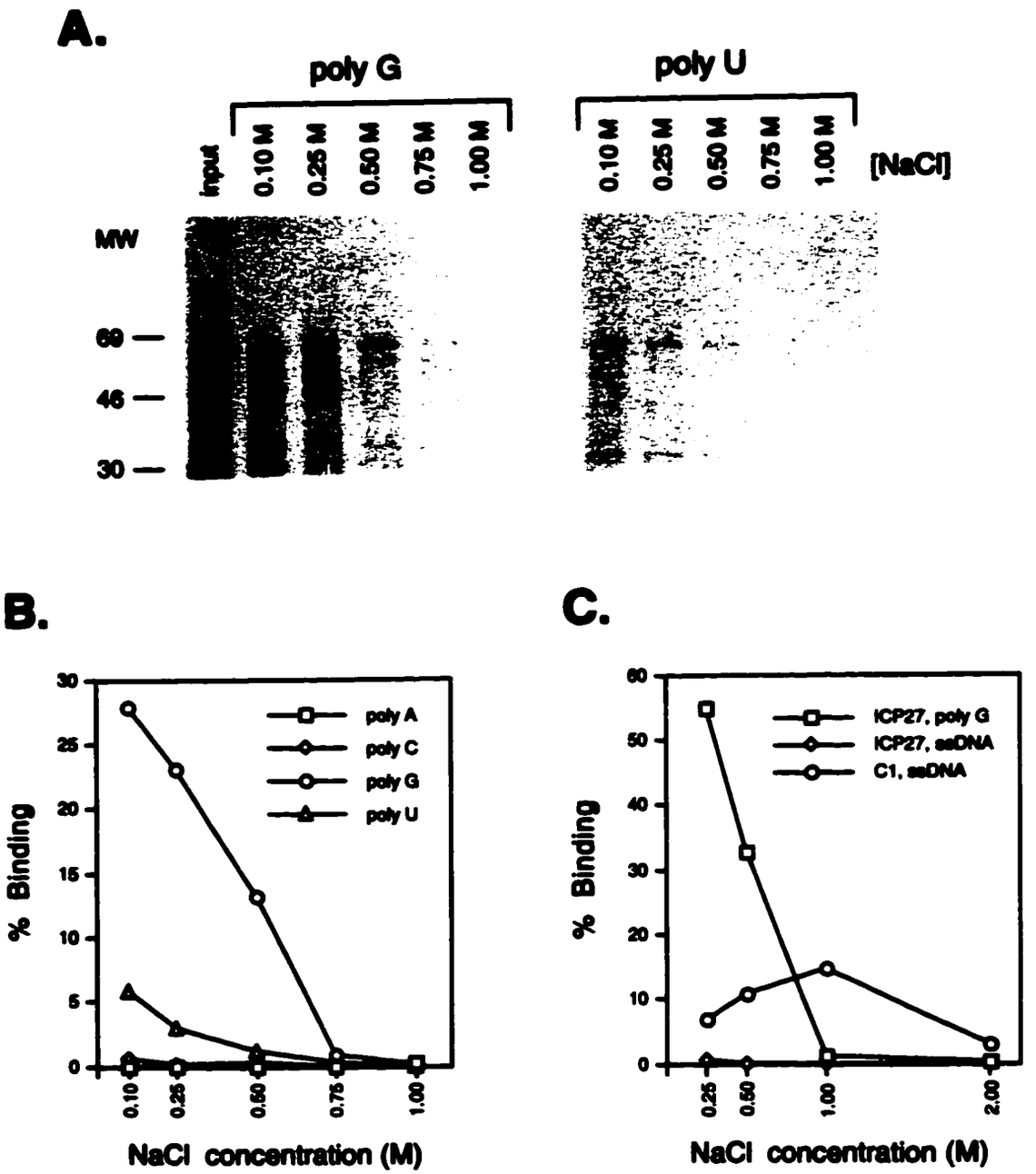

A.
poly G
poly U
Input
0.10 M
0.25 M
0.50 M
0.75 M
1.00 M
[NaCl]
MW
69
46
30
B.
% Binding
poly A
poly C
poly G
poly U
NaCl concentration (M)
C.
% Binding
ICP27, poly G
ICP27, ssDNA
C1, ssDNA
NaCl concentration (M)

FIG. 3-2. Poly (G) binding by ICP27 truncation mutants. (A) Schematic diagram of ICP27 N- and C-terminal truncation mutants produced by *in vitro* translation. The RGG box sequence is indicated. (B) Binding of truncation mutants to poly (G). The truncation mutants were tested in the poly (G) binding assay at a NaCl concentration of 0.25 M. The values given are the percent binding relative to a WT ICP27 control. The values for each mutant are means of 4-9 independent binding assays; the error bars indicate standard deviations. The mean value of WT ICP27 binding to poly (G) in these experiments was 37.5%.

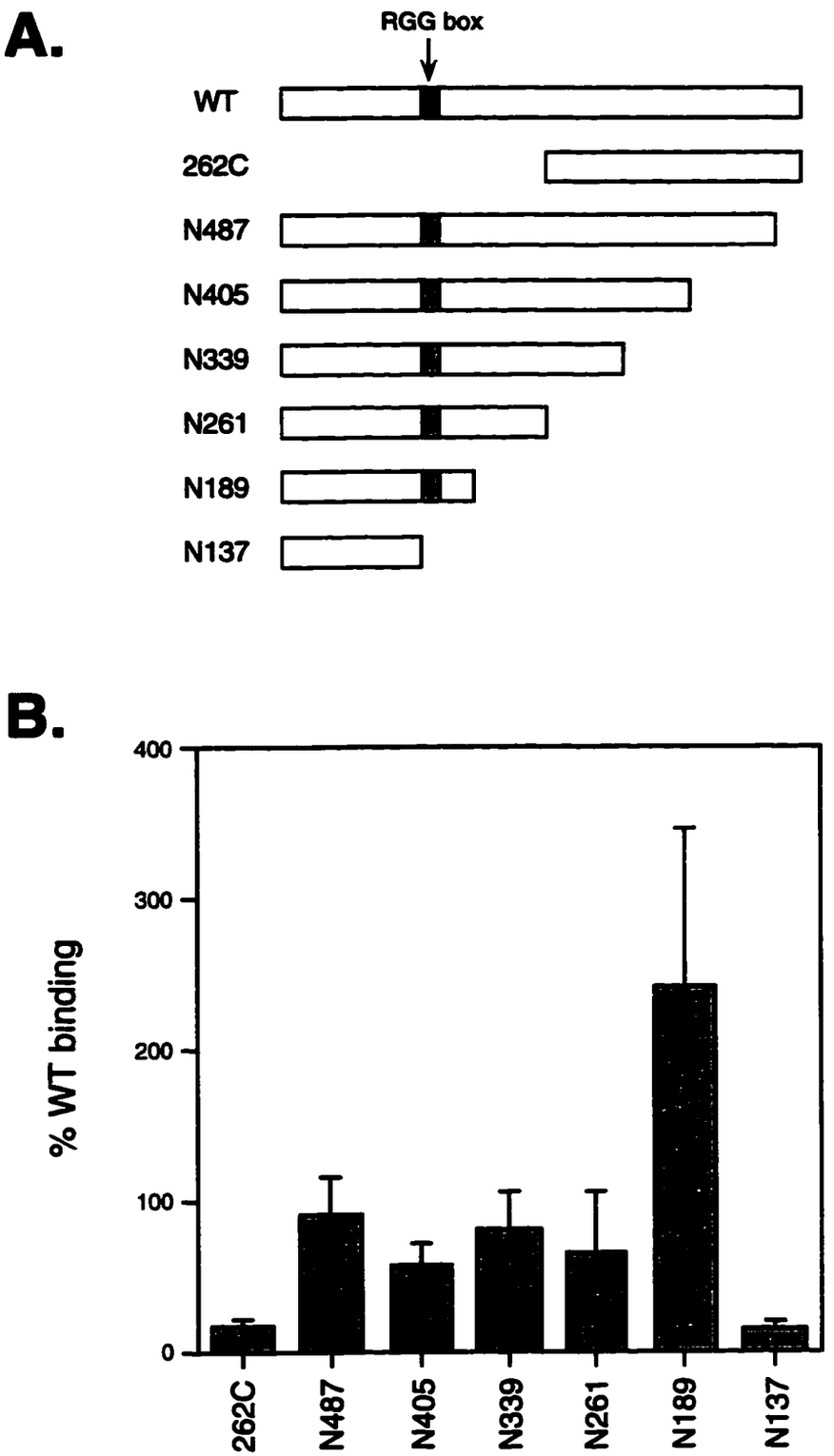

A.
RGG box
WT
262C
N487
N405
N339
N261
N189
N137
B.
% WT binding
400
300
200
100
0
262C
N487
N405
N339
N261
N189
N137

FIG. 3-3. Poly (G) binding by ICP27 in-frame deletion mutants. (A) Schematic diagram of ICP27 in-frame deletion mutants. The sequence of amino acid residues 138-174 of ICP27 is shown at the top. Below, the open bars represent ICP27, while the cross-hatched and gray bars represent the RGG box and R2 regions, respectively. The lines connecting bars indicate in-frame deletions. (B) Poly (G) binding by in-frame deletion mutants. WT ICP27 or in-frame deletion mutants were expressed by *in vitro* translation and tested in the poly (G) binding assay at the NaCl concentrations indicated. Equivalent fractions of the input and bound fractions were analyzed by SDS-PAGE and autoradiography. (C) Quantitation of poly (G) binding by ICP27 deletion mutants. The data from (B) were quantitated by phosphorimaging analysis. Poly (G) binding of each deletion mutant is presented as a percentage of WT ICP27 binding at the same NaCl concentration.

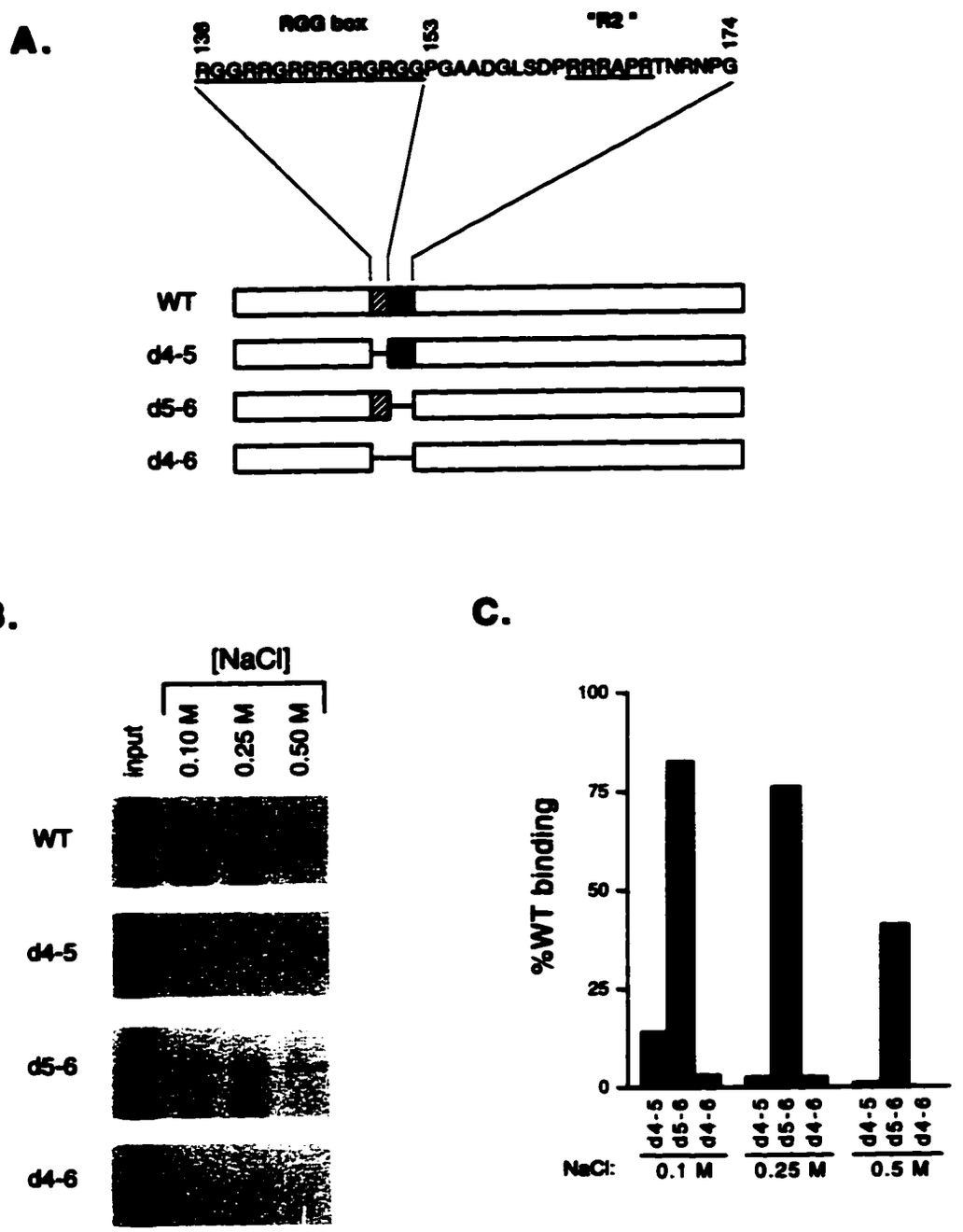

A.
RGG box
"R2"
138
153
174
RGGRRGRRRGRGRGGPGAADGLSDPRRRRAPRTNRNPG
WT
d4-5
d5-6
d4-6
B.
[NaCl]
input
0.10 M
0.25 M
0.50 M
WT
d4-5
d5-6
d4-6
C.
%WT binding
100
75
50
25
0
d4-5
d5-6
d4-6
NaCl:
0.1 M
0.25 M
0.5 M

FIG. 3-4. RNA binding by GST-ICP27 fusion proteins. (A) Purification of GST fusion proteins. GST or GST-ICP27 fusion proteins were expressed in *E. coli* and purified by affinity chromatography on glutathione sepharose. Ten μg of each preparation were analyzed by SDS-PAGE and Coomassie blue staining. The molecular mass of each marker protein is indicated at the left, in kilodaltons. The open circles to the right of lanes 3 and 4 indicate the positions of full length GST-ICP27 and GST-d4-5. The closed squares to the right of lane 3 indicate truncated GST-ICP27 products which interact strongly with RNA. (B) ^{32}P-labeled RNA probes. RNA probes for the northwestern assay were generated by *in vitro* transcription in the presence of [α-^{32}P]CTP. An aliquot of each probe was analyzed by 5% urea-acylamide gel electrophoresis and autoradiography. Lane 1: antisense version of β-IFN gene 3' end probe; lane 2: β-IFN gene 3' end probe; lane 3: SV40 early region 3' end probe. The positions and nucleotide sizes of markers are indicated at the right. The expected size of the β-IFN probes is 650 nucleotides; that of the SV40 probe is 840 nucleotides (3). (C) Binding of GST-ICP27 and GST-RGG to RNA. Ten μg of GST fusion preparations were electrophoresed on 15% SDS-PAGE, blotted to nitrocellulose, and incubated with ^{32}P-labeled RNA probes as described by Brown *et al.* (3). An autoradiograph of the washed blot is shown. The molecular masses of the protein standards are indicated at the left, in kilodaltons.

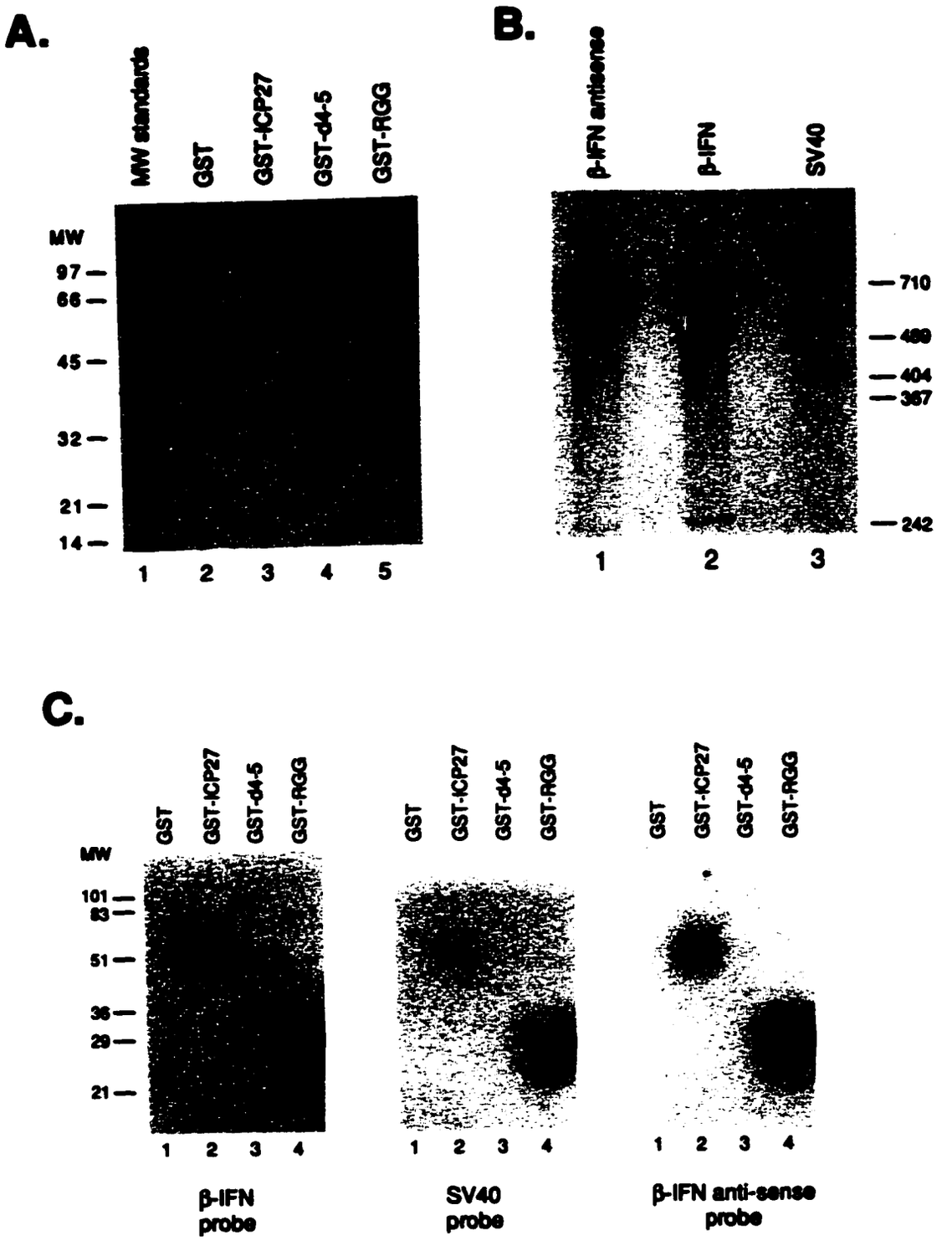

A.
MW standards
GST
GST-ICP27
GST-d4-5
GST-RGG
MW
97
66
45
32
21
14
1 2 3 4 5
B.
β-IFN antisense
β-IFN
SV40
710
404
367
242
1 2 3
C.
GST
GST-ICP27
GST-d4-5
GST-RGG
MW
101
83
51
36
29
21
1 2 3 4
β-IFN probe
SV40 probe
β-IFN anti-sense probe

FIG. 3-5. Protein methylation in HSV-1 infected Vero cells. Mock- or HSV-1-infected Vero cells were treated with translation inhibitors (100 μg/ml cycloheximide and 40 μg/ml chloramphenicol) at 5 hpi. Thirty min later, the cells were labeled for 3 h with 45 μCi/ml L-[*methyl*-^{3}H]methionine in the presence of the same translation inhibitors. The cells were scraped and lysed in RIPA buffer, and the lysates were electrophoresed on a 15% SDS-polyacrylamide gel. A fluorograph of the gel is shown. Lane M: protein standards; lane 1: mock-infected cells; lane 2: WT HSV-1-infected cells; lane 3: d27-1-infected cells. At the left, the molecular mass of the protein standards are indicated (in kilodaltons). At the right, bands corresponding to the two major methylated proteins in WT HSV-infected cells are indicated (a and b), as well as the position of cellular histones.

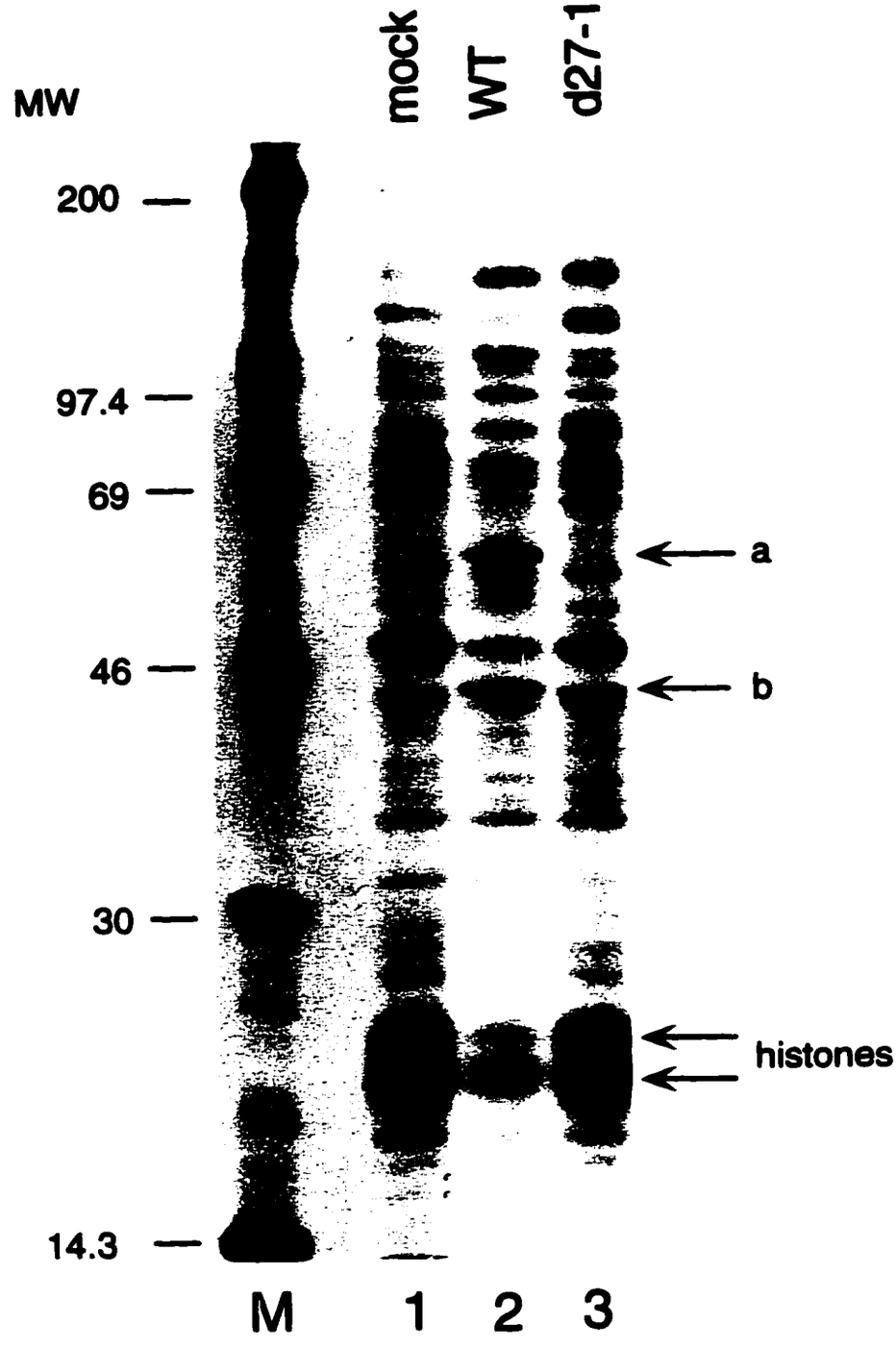

MW
mock
WT
d27-1
200
97.4
69
46
30
14.3
a
b
histones
M
1
2
3

FIG. 3-6. *In vivo* methylation of ICP27. Vero cells were mock-infected (lane 1) or infected with WT HSV-1, WT HSV-2, d27-1, or d4-5 (lanes 2-5, respectively). The cells were labeled from 5.5 - 8.5 hpi with L-[*methyl*-^{3}H]methionine as described in the text. Cell lysates were subjected to immunoprecipitation using a mixture of two monoclonal antibodies specific for ICP27 (H1113 and H1119). (A) Labeling of ICP27 with L-[*methyl*-^{3}H]methionine. Aliquots of the immunoprecipitates were run on 15% SDS-PAGE and analyzed by fluorography. A 40 h exposure is shown. The molecular mass of the protein standards are shown in lane M (in kilodaltons). (B) Presence of ICP27 in the immunoprecipitates. Aliquots of the immunoprecipitates were run on 15% SDS-PAGE, and the proteins were electrophoretically transferred to nitrocellulose. The filter was subjected to Western blot analysis using a mixture of H1113 and H1119 antibodies. Antigen detection was performed using an enhanced chemiluminescence detection system; a 1 sec exposure is shown.

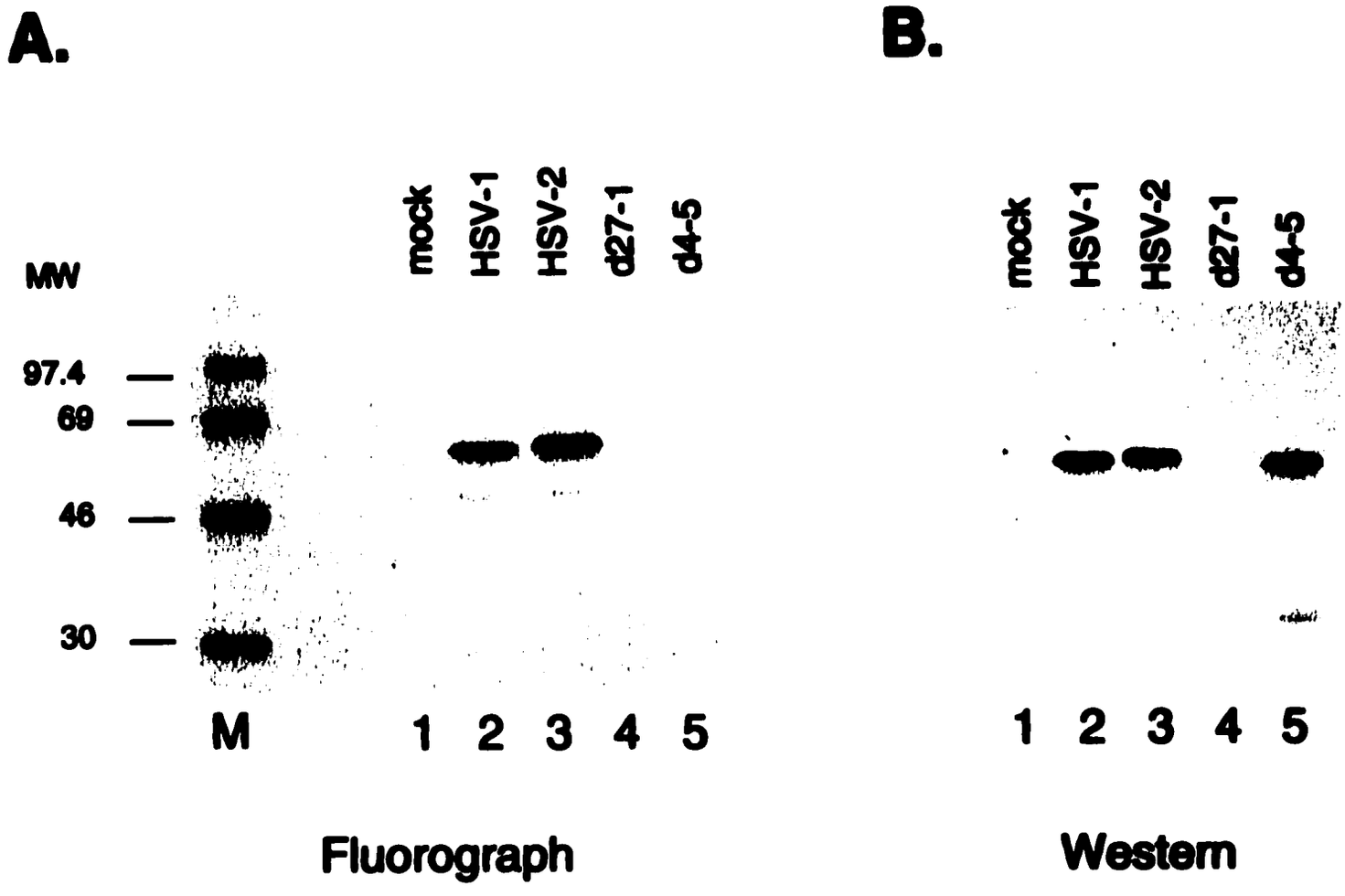
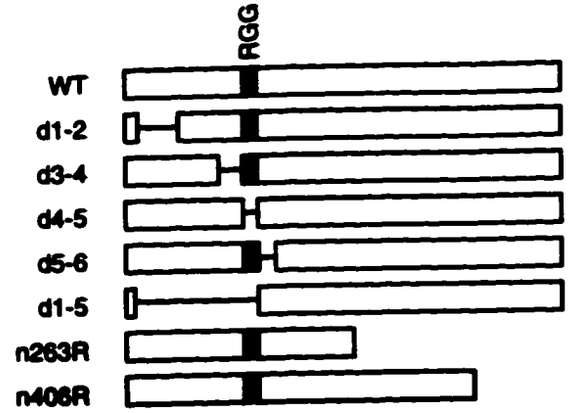
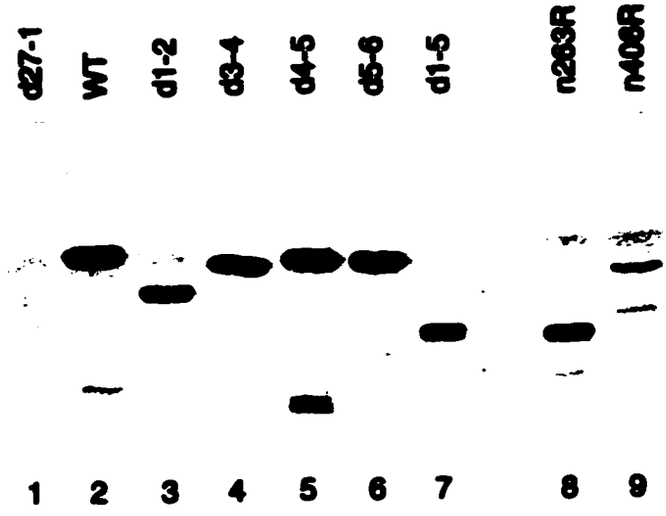

A.
B.
MW
97.4
69
46
30
mock
HSV-1
HSV-2
d27-1
d4-5
M
1
2
3
4
5
Fluorograph
Western

FIG. 3-7. The RGG box is required for ICP27 methylation. (A) ICP27 proteins expressed by HSV-1 ICP27 mutants. Bars represent ICP27 coding segments; lines connecting bars indicate in-frame deletions. The RGG box is indicated by a stippled box. (B) and (C) *In vivo* methylation assay. Cells were infected with WT HSV-1, d27-1, or the ICP27 mutants shown in panel A. Cells were labeled with L-[*methyl*-^{3}H]methionine and anti-ICP27 immunoprecipitations were performed as described in the text. (B) Western blot detection of ICP27 molecules in immunoprecipitates. Aliquots of the immunoprecipitates were subjected to Western analysis using ICP27-specific monoclonal antibodies. Antigen detection was by enhanced chemiluminescence; a 5 sec exposure is shown. (C) Labeling of ICP27 with L-[*methyl*-^{3}H]methionine. Aliquots of the immunoprecipitates were subjected to SDS-PAGE and fluorography. A 6 day exposure is shown. The positions of molecular mass standards are shown at the right, in kilodaltons.

A.


RGG
WT
d1-2
d3-4
d4-5
d5-6
d1-5
n263R
n406R

B.


d27-1
WT
d1-2
d3-4
d4-5
d5-6
d1-5
n263R
n406R
1 2 3 4 5 6 7 8 9

C.

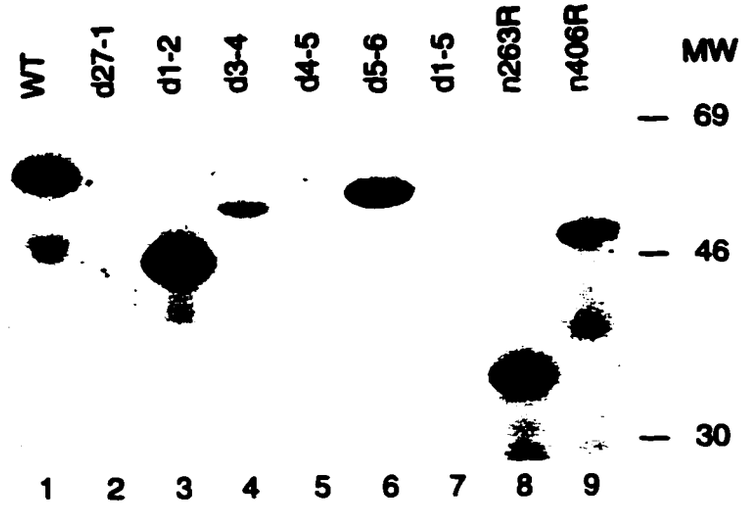
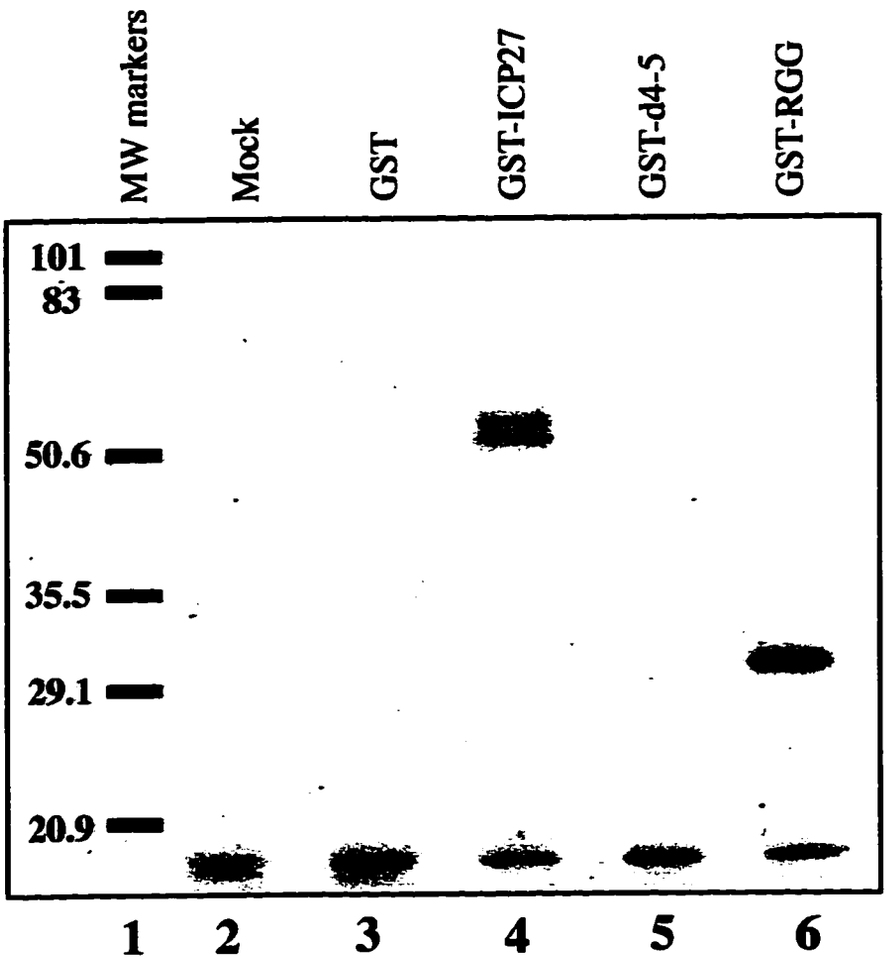

WT
d27-1
d1-2
d3-4
d4-5
d5-6
d1-5
n263R
n406R
MW
69
46
30
1 2 3 4 5 6 7 8 9

FIG. 3-8. *In vitro* methylation of GST-ICP27 fusion proteins. One microgram of each GST protein was added to rabbit reticulocyte lysate containing ^{3}H-SAM and allowed to incubate for 30 minutes. Proteins were collected, separated on 15% SDS-PAGE, and their radioactive signals enhanced using fluorography. Shown is a 35 day exposure of the enhanced gel. The protein standards and their molecular masses are indicated on the left in kilodaltons (lane 1). Lanes 2-6 show the results from each incubation in which the following proteins were added to the reticulocyte extract: Lane 2 - water (no GST protein), Lane 3 - GST protein, Lane 4 - GST-ICP27 protein, Lane 5 - GST-d4-5 protein, and Lane 6 - GST-RGG protein. Note that the labeled protein present in lane 4 corresponds to the truncated GST-ICP27 species observed in Figure 3-4.


MW markers
Mock
GST
GST-ICP27
GST-d4-5
GST-RGG
101
83
50.6
35.5
29.1
20.9
1
2
3
4
5
6

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CHAPTER 4

ICP27 IS A NUCLEAR SHUTTLING PROTEIN

Chapter Four - ICP27 is a Nuclear Shuttling Protein[3]

INTRODUCTION

Considerable research suggests that ICP27 regulates gene expression via one or more post-transcriptional mechanisms. ICP27 appears to enhance the efficiency of pre-mRNA polyadenylation (24,25,43), alter the nuclear distribution of splicing factors (34,44), inhibit splicing itself (13,14), and affect the stability (2) and nucleocytoplasmic transport of some mRNAs (35). Consistent with a possible role in post-transcriptional gene regulation, recent studies have shown that ICP27 can bind to RNA (2,18,Chap. 3; 26).

Recently, several proteins implicated in post-transcriptional gene regulation, including several cellular hnRNP proteins, have been shown to be nuclear shuttling proteins. That is, although they are localized predominantly to the cell nucleus, they can be demonstrated to move continually between the nuclear and cytoplasmic compartments of the cell. We have previously observed that ICP27 has several characteristics that are similar to some of the cellular hnRNP proteins. These include post-translational methylation, presence of an RGG box and interaction with RNA (2,15,18,chap. 2; 27, chap. 3; 26). Based on these similarities, we were interested to see if ICP27 is a nuclear shuttling protein. Utilizing an interspecies heterokaryon assay, WT and mutant forms of ICP27 were tested for their ability to traverse the nuclear pore bi-directionally.

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[3]A version of this chapter is being submitted to *Virology*. Mears, W.E., and S.A. Rice. (1997). "The herpes simplex virus immediate-early protein ICP27 shuttles between nucleus and cytoplasm."

MATERIALS AND METHODS

Cells, viruses and infections. Vero and 3T6 (mouse fibroblast) cells were obtained from the American Type Culture Collection (Rockville, Md.) and were propagated in DMEM containing 5% heat-inactivated fetal bovine serum (GIBCO). HSV-1 infections were carried out at an MOI of 10 PFU/cell as described in Chapter 2. The HSV-1 strain KOS1.1 (17) was the WT strain used in these experiments. The HSV-1 mutants *d*1-2, M11, M15, M16, and *n*504R have been described previously (38,39,40).

Interspecies heterokaryon assay for nuclear shuttling. The interspecies heterokaryon assay is a modifed version of previously published procedures (1,36). Vero cells were grown on round coverslips in 12-well trays (1.5 x 10^5 cells/well) and infected with WT HSV-1. Four hpi, 3T6 cells were plated on the infected Vero cells (3.5 x 10^5 cells/well) in 199V media containing 50 μg/ml cycloheximide and 0.15% human immune globulin (Armour Pharmaceutical Company, Kankakee, Ill.). Four hours later, the cycloheximide concentration of the media was increased to 100 μg/ml. After 30 minutes, the cells were washed in PBS. Cell fusion was performed by removing the media and adding 50% polyethylene glycol (w/w) in Hank's Balanced media (GIBCO) to each coverslip for two minutes. After fusion, the coverslips were washed in PBS four times. All reagents and solutions involved in the fusion process were prewarmed to 37°C. After washing, the coverslips were returned to the media containing 100 μg/ml cycloheximide for 60 minutes, after which time cells were fixed for immunofluorescence.

In some experiments, the level of ICP27 in infected cells was increased by performing a "cycloheximide release" (16). This protocol was performed in the following manner. Vero cells were infected with HSV-1 in the presence of 50 μg/ml cycloheximide. Four hpi, the cells were washed repeatedly with media to remove the cycloheximide and infection was allowed to proceed for two hours. At this time, 3T6

cells were added as described above. The remainder of the assay was identical to that described above.

To examine the shuttling of ICP27 in the absence of other viral proteins, we expressed ICP27 in Vero cells by transient transfection. The plasmid used for transfection was pC27, which contains the open reading frame of the ICP27 gene under the control of a minimal CMV promoter. To construct pC27, the ICP27 open reading frame was obtained from the plasmid pBS27 (39) in the following manner. pBS27 was first cleaved with *Drd*I, and the ends were made blunt with T4 DNA polymerase. After ligation of *Eco*RI linkers, the resulting DNA was treated with *Eco*RI, releasing a 2 Kbp *Eco*RI-ICP27 containing fragment. This fragment was cloned into pUHD10-3 (11) which had been previously linearized with *Eco*RI and treated with calf alkaline phosphatase. The orientation of the ICP27 gene in the resulting clone, pC27, was verified by restriction enzyme digestions. Shuttling analysis of ICP27 expressed in transfected cells was performed using a modification of the protocol of Michael *et al.* (31). Vero cells grown in 25-cm^2 flasks were transfected with 8 μg of pC27 using the calcium phosphate precipitation procedure as described previously (Chap. 2; 27). Twenty-four hours later, the transfected Vero cells were transferred to round coverslips in 12-well trays (2 x 10^5 cells/well). Approximately 12 hours later, 3T6 cells in the presence of 50 μg/ml cycloheximide were plated (3 x 10^5/well) on the transfected Vero cells. After four hours to allow the cells to adhere to the coverslips, the cycloheximide concentration was increased to 100 μg/ml and the fusion assay was performed as described above.

Immunofluorescence. Immunofluorescence was performed as described previously (chap. 2; 27). The primary antibodies used were H1119, H1115, and H1114, which are mouse monoclonal antibodies specific for HSV-1 ICP27, ICP8, and ICP4, respectively. All three were obtained from the Goodwin Institute for Cancer Research, Plantation, Florida. These antibodies were diluted 1:600, 1:1000 and 1:800 respectively, in PBS. The secondary antibody used for indirect immunofluorescence was tetramethyl

rhodamine isothiocyanate-conjugated anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories Inc., Mississauga, Ontario), diluted 1:200 in PBS. Cells were also stained, at the time of secondary antibody addition, with 0.5 μg/ml Hoechst #33258 dye (Sigma) and Bodipy-FL Phallocidin (Molecular Probes Inc., Eugene, Oregon) diluted 1:100. The cells were visualized with a Zeiss Axioskop 20 fluorescence microscope equipped with a PlanNeofluar 63X objective lens. Photographic images were constructed using the ArcusII desktop scanner (AGFA), a Power Macintosh 8500/150 and the Photo Shop program version 3.0 (Adobe). Black and white negatives were scanned individually and combined to obtain Figures 2, 3, 5, and 6.

RESULTS

Intracellular movement of ICP27 in interspecies heterokaryons. To see if ICP27 is a nuclear shuttling protein, we modified an interspecies heterokaryon assay that has been used previously to study the shuttling of nuclear and nucleolar proteins (1,29,36,37). To apply this assay to HSV-1 nuclear proteins, HSV-1 infected Vero (monkey) cells are fused with uninfected 3T6 (mouse) cells using polyethylene glycol (Fig. 4-1). Fusion is performed in the presence of cycloheximide, to inhibit protein synthesis, and human immune globulin, to prevent infection of the mouse cells by residual virus. One hour after fusion, the localization of viral nuclear proteins in interspecies heterokaryons is determined by indirect immunofluorescence. Since *de novo* protein synthesis is inhibited before and after cell fusion, the appearance of a viral nuclear protein in the mouse nucleus of an interspecies heterokaryon suggests that it has been exported from the infected monkey nucleus and been imported into the uninfected mouse nucleus, i.e. that it has shuttled.

In the first experiment, the potential of ICP27 to shuttle in interspecies heterokaryons was examined (Fig. 4-2). Heterokaryons were identified by both phase microscopy (Fig. 4-2A) and the staining of actin filaments using fluorescent phallocidin (not shown). To differentiate monkey nuclei from mouse nuclei, cells were also stained with Hoechst dye. Monkey nuclei stain relatively diffusely with Hoechst while mouse nuclei exhibit a characteristic speckled pattern (Fig 4-2B; mouse nuclei in heterokaryons are indicated by the arrows). Examination of numerous interspecies heterokaryons indicated that nearly all that contained ICP27 in their monkey nuclei also displayed ICP27 in their mouse nuclei (Fig. 4-2C). As expected, ICP27 was not present in unfused mouse cells. These results suggest that ICP27 is capable of nuclear shuttling.

To see if other HSV-1 nuclear proteins behave similarly to ICP27 in this assay, we carried out another experiment in which the viral nuclear proteins ICP4 and ICP8

were examined in addition to ICP27. ICP4, an IE protein, is the major transcriptional transactivator of HSV-1, while ICP8 is a DE DNA replication protein with ssDNA binding activity (42). For each protein, numerous (>100) interspecies heterokaryons were examined. The degree of viral protein staining within the mouse nuclei of heterokaryons was compared to the staining observed in the monkey nuclei within the same heterokaryon. The results are tabulated in Table 4-1. For ICP27, the protein was found in both the monkey and the mouse nuclei of 100% (114/114) of the interspecies heterokaryons that contained viral protein staining. A representative heterokaryon is shown in Fig. 4-3 panels A - C. In contrast, ICP4 and ICP8 were detected in a minority of mouse nuclei in interspecies heterokaryons and in these, staining was weak. A typical example of ICP4 staining is shown in Figure 4-3 panels D - F.

It should be noted that we observed that a fraction of infected cells, both fused and unfused, exhibited low levels of ICP27 staining in the cytoplasm in addition to nuclear staining. Such cytoplasmic staining was not generally observed for ICP4 and ICP8. Heterokaryons that contained significant levels of cytoplasmic staining were not included in the analysis presented in Table 4-1 as it was impossible to determine if the accumulation of ICP27 within the mouse nuclei of these heterokaryons was due to export of ICP27 from the monkey nucleus or due to uptake of some of the cytoplasmic ICP27. The observation of cytoplasmic ICP27 in some cells complicates the interpretation of our experiments, as it suggests the possibility that the ICP27 found in mouse nuclei may have originated from the cytoplasm rather than infected monkey nuclei. However, experiments presented below strongly suggest that ICP27 does shuttle from monkey to mouse nuclei.

Mutations in ICP27 inhibit its nucleocytoplasmic transport. We attempted to use a number of ICP27 viral mutants to further investigate the potential shuttling of ICP27. However, under the conditions of our interspecies heterokaryon assay, several mutant ICP27 molecules were expressed at significantly reduced levels compared to WT

ICP27 (data not shown), perhaps as a result of reduced protein stability. In an attempt to increase the levels of ICP27 present within the infected monkey cells prior to fusion, we used a "cycloheximide release" protocol to enhance the expression of IE proteins (16). This was done by infecting Vero cells in the presence of cycloheximide to allow immediate-early transcripts to accumulate to high levels. The cycloheximide was then washed out, allowing for a burst of IE protein synthesis. Uninfected mouse cells and cycloheximide were then added and heterokaryons formed as previously described. The cycloheximide reversal protocol resulted in comparable levels of WT and several mutant forms of ICP27 (see Fig. 4-5 A,E,I).

Five mutant forms of ICP27, all defective for lytic growth, were analyzed using the "cycloheximide release" interspecies heterokaryon assay (Fig. 4-4). For each mutant form of ICP27, numerous interspecies heterokaryons were examined and the results tabulated as before (Table 4-2). For WT ICP27 the results were similar to before, with strong staining seen in >90% of mouse nuclei of the interspecies heterokaryons (Table 4-2; Fig. 4-5 B,C,D). The mutant ICP27 molecules exhibited several different phenotypes in this assay. One mutant protein, the n504R form of ICP27, appeared to be similar to the WT (Table 4-2; Fig. 4-5 J,K,L). The remaining mutant forms of ICP27 showed modest to dramatic alterations in their ability to be transported into mouse nuclei. The mutants d1-2, M11, and M16 showed a modest defect with regard to their nuclear trafficking in this assay (Table 4-2). They were present in >80% of mouse nuclei within positive interspecies heterokaryons. However, for these mutants the intensity of ICP27 staining in mouse nuclei was generally weaker than for the WT protein. One mutant, M15, was dramatically different in the interspecies heterokaryon assay. In one experiment, M15 was completely absent from mouse nuclei within heterokaryons containing positive monkey nuclei. In another experiment, M15 stained only weakly in a minority (11%) of mouse nuclei (Table 4-2; Fig. 5 F,G,H).

As discussed previously, the ICP27 protein found within the mouse nuclei of interspecies heterokaryons might have been derived, at least in part, from a cytoplasmic pool of protein. However, under the cycloheximide reversal conditions, none of the mutants examined here displayed visible cytoplasmic staining immediately before fusion. Moreover, under normal conditions of infection, the 504R protein was not observed in the cytoplasm, in contrast to the WT protein. The 504R protein though is as efficient as WT ICP27 in localizing to mouse nuclei in interspecies heterokaryons. Since the 504R protein was localizated to mouse nuclei, yet exhibited no cytoplasmic staining, its origin must have been the infected Vero nucleus. Therefore, we interpret these findings to indicate that 504R and by extension WT ICP27, shuttles between the monkey and mouse nuclei. The M15 protein is defective for shuttling, while the d1-2, M11, and M16 proteins are partially deficient in this process.

Nuclear shuttling of ICP27 in the absence of other viral proteins. To determine if viral infection or other viral proteins are required for ICP27 shuttling, Vero cells were transiently transfected with a plasmid containing the ICP27 open reading frame under the control of a CMV-derived promoter. The transfected Vero cells were fused to 3T6 cells in the presence of cycloheximide and the location of ICP27 in interspecies heterokaryons was examined. ICP27 was found to localize in both the monkey and mouse nuclei of interspecies heterokaryons, but was otherwise not seen in mouse cells (Fig. 4-6C). In some transfected cells, a significant level of ICP27 was observed within the cytoplasm of the cell, similar to what is observed in infected cells. It appears that the intracellular movement of ICP27 observed under conditions of transient expression mimics the movement observed during infection. Thus, the ability of ICP27 to shuttle between nucleus and cytoplasm appears to be an inherent property of the protein.

DISCUSSION

It was demonstrated nearly 40 years ago that some nuclear proteins move back and forth across the nuclear membrane (9). This movement was referred to as "shuttling", and recently several nuclear proteins have been characterized as shuttling proteins. These include some of the heterogenous nuclear RNP (hnRNP) proteins (36,37), several nucleolar proteins (1,28), the human immundeficiency virus type 1 (HIV-1) Rev protein (19,29), U1 small nuclear RNP (snRNP)-specific protein U1A (20), steroid hormone receptors (12), heat shock-related proteins (23), and protein kinase inhibitor (PKI) (5). Although the precise function of shuttling is not yet known, some shuttling proteins appear to be involved in the nucleocytoplasmic transport of mRNA. hnRNP A1 is associated with pre-mRNA in the nucleus and mRNA in the cytoplasm (36) suggesting that it may directly mediate the transport of cellular mRNAs (31,37). HIV Rev is involved in the nuclear export of viral intron-containing mRNA via binding to the Rev-response element (RRE) within these messages (3,7).

ICP27 is a nuclear shuttling protein. The HSV-1 nuclear protein ICP27 is capable of binding to RNA, and has a number of post-transcriptional effects on gene expression, including effects on the nucleocytoplasmic transport of mRNA (34). We therefore asked whether ICP27 is a shuttling protein. Using an interspecies heterokaryon assay, we demonstrate here that ICP27 is capable of nuclear shuttling. No other viral proteins are required for this activity as ICP27 was observed to shuttle in cells transiently transfected with the ICP27 gene. ICP4 and ICP8 were also examined using the interspecies heterokaryon assay. Unlike ICP27, neither of these two viral proteins demonstrated any significant level of shuttling, indicating that not all HSV-1 nuclear proteins move constitutively between nucleus and cytoplasm. It is possible that the low levels of ICP4 and ICP8 that were observed in some of the mouse nuclei of interspecies

heterokaryons resulted not from shuttling, but from low levels of residual cytoplasmic protein present at the time of fusion.

If the ability of ICP27 to shuttle is an important biological property, then it might be expected that one or more ICP27 mutants would be unable to shuttle. Consistent with this, we found that the C-terminal codon substitution mutant, M15 ($P_{465}G_{466} \rightarrow$ LD), is severely deficient at shuttling, while the mutants M11, M16, and d1-2 are partially deficient. In contrast, the C-terminal truncation mutant 504R efficiently shuttles between nucleus and cytoplasm. Several other deletion mutants were examined using the "cycloheximide release" interspecies heterokaryon assay (not shown). However, in these cases, significant amounts of these proteins were observed within the cytoplasm of the interspecies heterokaryons. This made it impossible to draw conclusions regarding the shuttling ability of these mutants.

Unfortunately, one mutant that was impossible to study due to its cytoplasmic location was the deletion mutant d4-5. This mutant is of particular interest as we have previously demonstrated that the arginine/glycine-rich region removed in d4-5 mediates ICP27's RNA binding ability (Chap.3, 26). Since Rev, and possibly hnRNP A1, is exported from the nucleus in association with RNA, it is interesting to speculate that a putative interaction between ICP27 and mRNA could result in the co-export of ICP27 and mRNA. However, recent analysis of Rev has indicated that its nuclear export signal (NES) is located within the C-terminal "activation domain". This region is not involved with interactions with the Rev responsive element (RRE) found in RNA molecules that are actively transported from the nucleus by Rev. If ICP27 is similar to Rev, it is possible that ICP27's RGG box is not required for its nuclear export.

It was surprising to find that M11, M15, M16 and *n*504R displayed different shuttling abilities in the interspecies heterokaryon assay. Previously, characterization of these mutants had shown that all exhibit similar phenotypes (38,39). All are WT for viral DNA replication but fail at late times to downregulate both IE and DE genes and are

unable to induce certain L genes. Although the M15 viral mutant exhibited the most severe reduction in viral growth in Vero cells (38,39), this difference does not readily explain why M15 might behave differently than M11 and M16 in the interspecies heterokaryon assay. Further studies are required in order to determine the significance of this difference in shuttling ability and how it affects ICP27's function.

ICP27 may possess sequences responsible for its nuclear export. Although much has been elucidated with respect to nuclear protein import, the process of nuclear protein export is not well understood (for reviews see: 4,8,10,32). Two pathways for nuclear protein export have been proposed. First, it has been suggested that nuclear protein shuttling can be a passive process and potentially occurs for all nuclear proteins that do not possess a nuclear retention signal (22,45). Second, nuclear protein export could be an active process, mediated by specific NES sequences. Such sequences have been identified in Rev (6,30,46), PKI (46), hnRNP A1 (31), RANBP1 (41), and HTLV-1 Rex (21). The Rev, Rex, RanBP1, and PKI NES's consist of leucine-rich segments within the protein while the hnRNP A1 NES, termed M9, contains no leucine residues and is glycine-rich. All of these proteins exhibit rapid export from and re-import to the nucleus (within one hour).

We were able to detect nucleocytoplasmic transport of ICP27 one hour after fusion of both infected and uninfected cells. It is possible that ICP27 contains an NES that mediates active export. ICP27 does not contain any significant homology to the hnRNP A1 NES. It does, however, possess a variety of leucine-rich segments including the regions between amino acid residues 8 to 20, 215 to 223, and 387 to 419. Amino acid residues 215 to 223 (PPPLMTLAI) perhaps bear the strongest similarity the Rev NES (LPPLERLTL). However, since there are several leucine-rich regions throughout ICP27, it is difficult to speculate, by sequence analysis alone, where a putative NES might reside within the protein. It is noteworthy that the deletion in the d1-2 protein (amino acid residues 12-64) removes all but two leucine residues from the most N-terminal leucine-

rich region, yet this mutant is able to shuttle, albeit less efficiently. In contrast, the C-terminal codon substitutions of M15 completely inhibit ICP27's nucleocytoplasmic transport capabilities. These findings suggest that the N-terminal leucine-rich region of ICP27 is an NES and limited export is able to occur with only two leucine residues or that there are multiple NES sequences present in the protein. Obviously, further studies are required in order map the NES in ICP27. As multiple NES sequences may be present and multiple NLS's are known (15,chap. 2; 27), heterologous protein fusion studies may be required in order to specifically map the NES's of ICP27.

It is possible that ICP27 does not contain a nuclear export signal, but rather that it is actively exported via an interaction with other actively exported components. For example, the M15 point mutation may not affect an NES but may instead affect ICP27's interaction with other exported proteins thereby rendering it non-shuttling. It is noteworthy that the amino acid residues mutated in the M15 protein are highly conserved in ICP27 homologs (2) and are required for WT function of the protein (39).

It is also possible that ICP27 shuttling is a passive process. That is, it is not retained within the nucleus nor does it require active export to leave the nucleus to re-enter the cytoplasm. The M15 mutation might cause or enhance an interaction with non-shuttling components of the nucleus thereby trapping it within the nucleus and preventing it from shuttling.

The biological role of ICP27's shuttling capability remains to be elucidated. The discovery of a biologically defective point mutant which is unable to shuttle lends support to the hypothesis that shuttling is associated with an important function. It is interesting to note that the ICP27 homolog of Varicella-Zoster virus (VZV), the ORF4 protein, has a cytoplasmic cellular localization (33). It is possible that HSV-1 ICP27 also possesses a cytoplasmic function and as such may require the ability to traverse the nuclear pore complex in both directions. It has been proposed that ICP27 may play a role in RNA stability (2). Although the cellular location of this effect has not been identified, it is not

unreasonable to speculate this might occur in the cytoplasm, prolonging the half-lives of several mRNA species. Other cytoplasmic functions could involve mRNA localization or translation as suggested for hnRNP proteins (reviewed in ref. 37). Finally, it is possible that ICP27's shuttling ability relates to its ability to regulate RNA processing and transport. Research has demonstrated that ICP27 can affect the location of viral intron-containing messages within the cell. ICP27 is required for the expression of the gC transcript (38) apparently at the post-transcriptional level. Perhaps ICP27's shuttling may indicate a role in mRNA transport of viral messages such as gC. Further work needs to done to determine the role of nuclear shuttling with respect to ICP27 function and to identify an NES, if one is present.

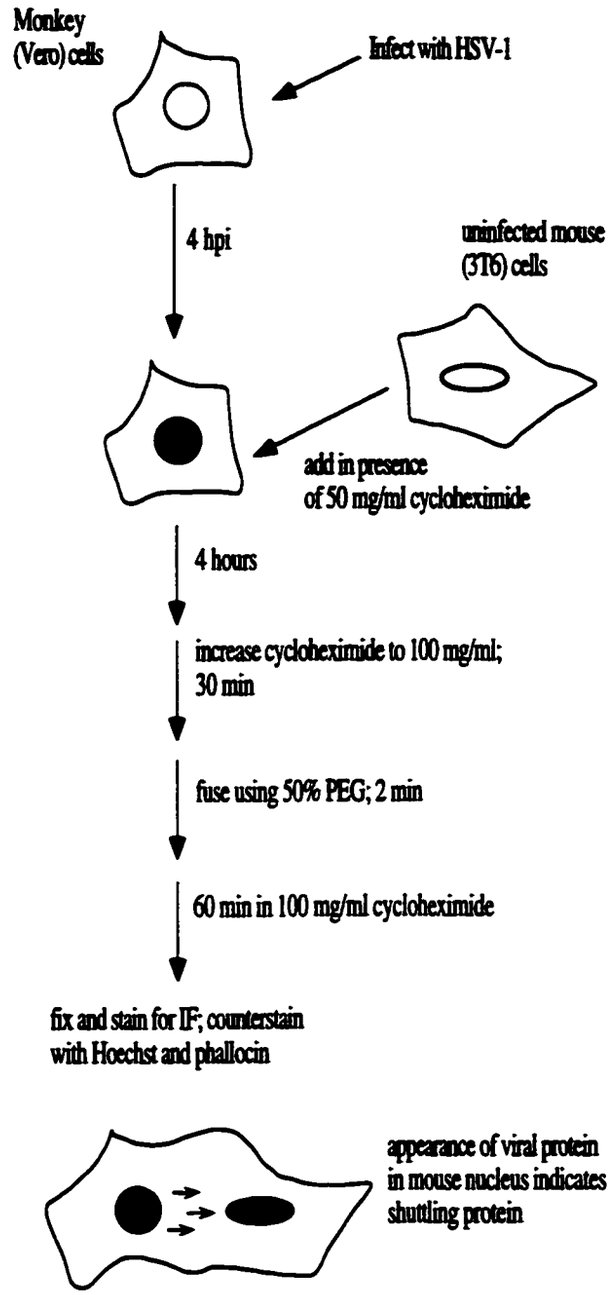

FIG. 4-1. Summary flowchart of the interspecies heterokaryon assay to examine nuclear shuttling of HSV-1 nuclear proteins.

FIG. 4-2. Visualization of ICP27 in interspecies heterokaryons. A, B, C: photomicrographs of a field of cells containing interspecies heterokaryons. A) Phase contrast image. B) Hoechst staining. Note that 3T6 nuclei are distinguishable from Vero nuclei as they display an intense nuclear speckled pattern. C) ICP27 staining using mouse monoclonal antibody H1119. Arrows indicate ICP27-positive 3T6 nuclei in interspecies heterokaryons. Bar = 20 μm.

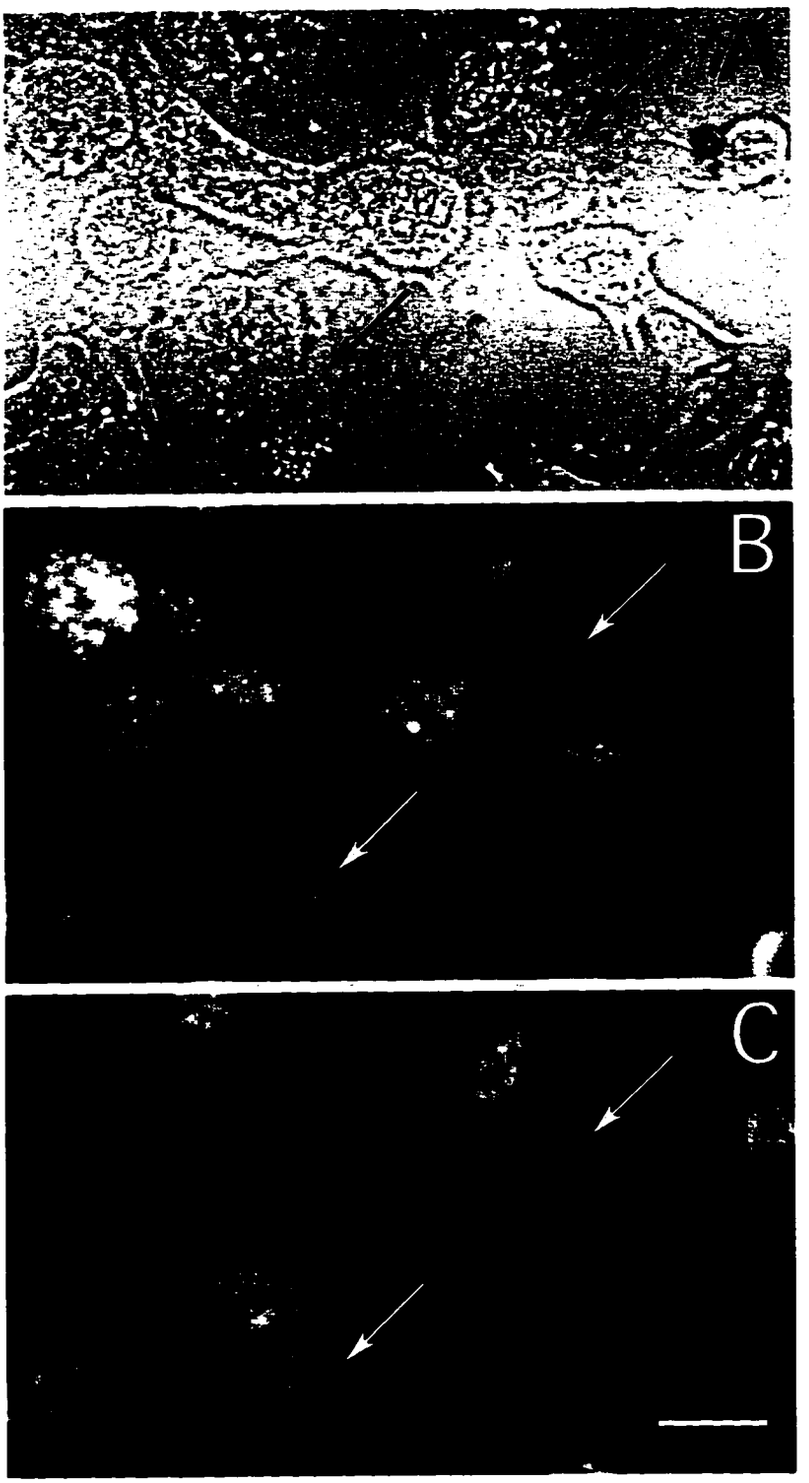
B
C

FIG. 4-3. Nucleocytoplasmic trafficking of ICP27 and ICP4 in interspecies heterokaryons. The photomicrographs show results of an interspecies heterokaryon assay using WT HSV-1. Panels A, B, and C show a field of cells stained for ICP27, while D, E, and F show a field of cells stained for ICP4. A, D) Phase contrast image. B, E) Hoechst staining. C) ICP27 staining with mouse monoclonal antibody H1119. F) ICP4 staining with mouse monoclonal antibody H1114. Arrows indicate mouse nuclei within heterokaryons. Bar = 20 μm.

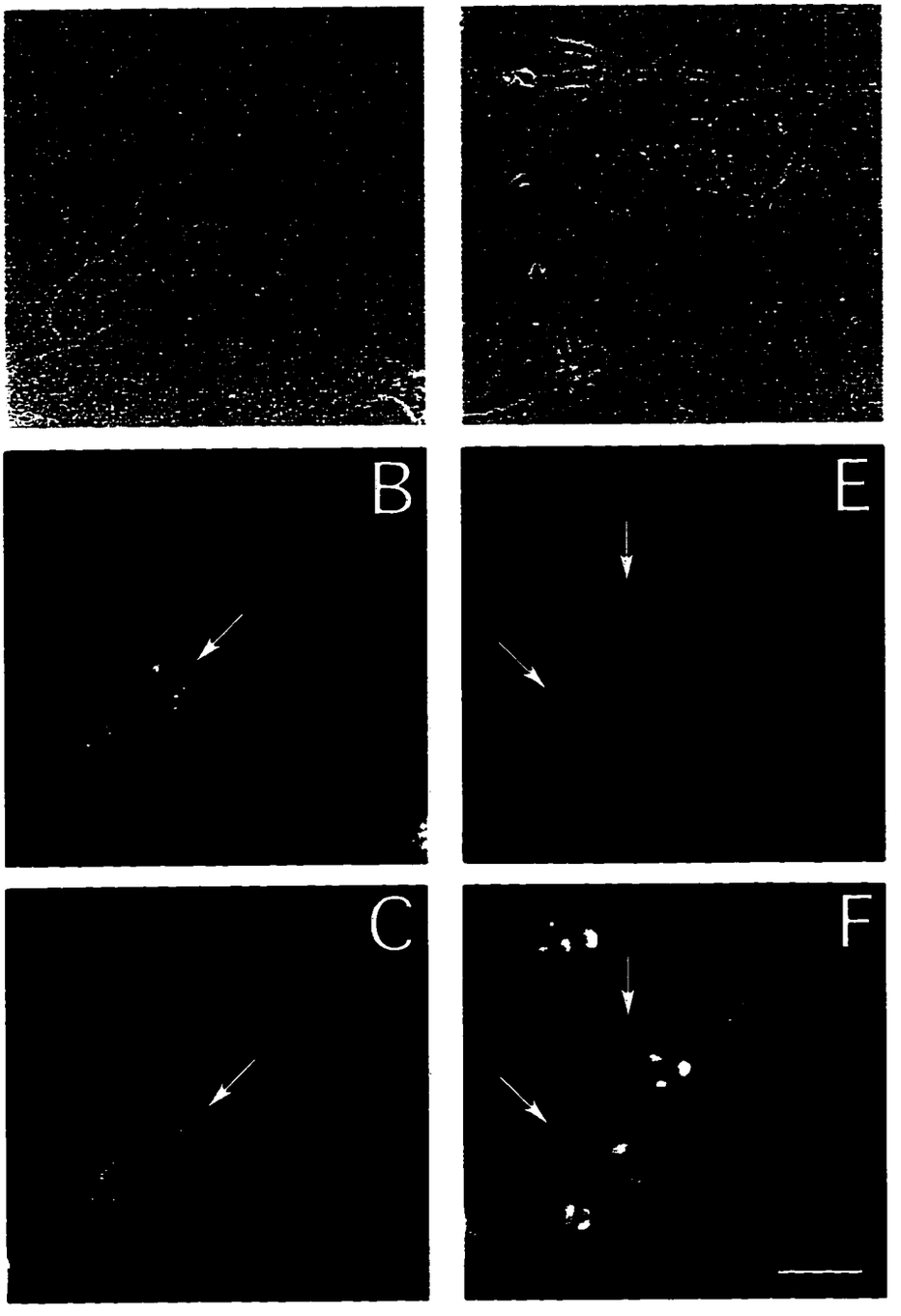
B
E
C
F

FIG. 4-4. Schematic diagram of HSV-1 ICP27 mutants used in this study. The bar at the top represents the intact WT ICP27 protein. The horizontal line indicates WT sequences of ICP27 that have been removed. The vertical lines represent the relative position of the amino acid residues that have been changed due to point mutations of the ICP27 gene. The resulting changes in amino acid sequence are identified below each mutation using the single letter code. d1-2 is a deletion mutant in which an N-terminal acidic region has been removed (amino acid residues 12 to 64) (37). M11, M15 and M16 contain point mutations resulting in the modification of either one or two amino acid residues (36). 504R is a truncation mutant that results due to the insertion of a stop codon containing linker at amino acid 505 (35). Four non-ICP27 amino acid residues are translated after amino acid residue 504, indicated by the hatched box.

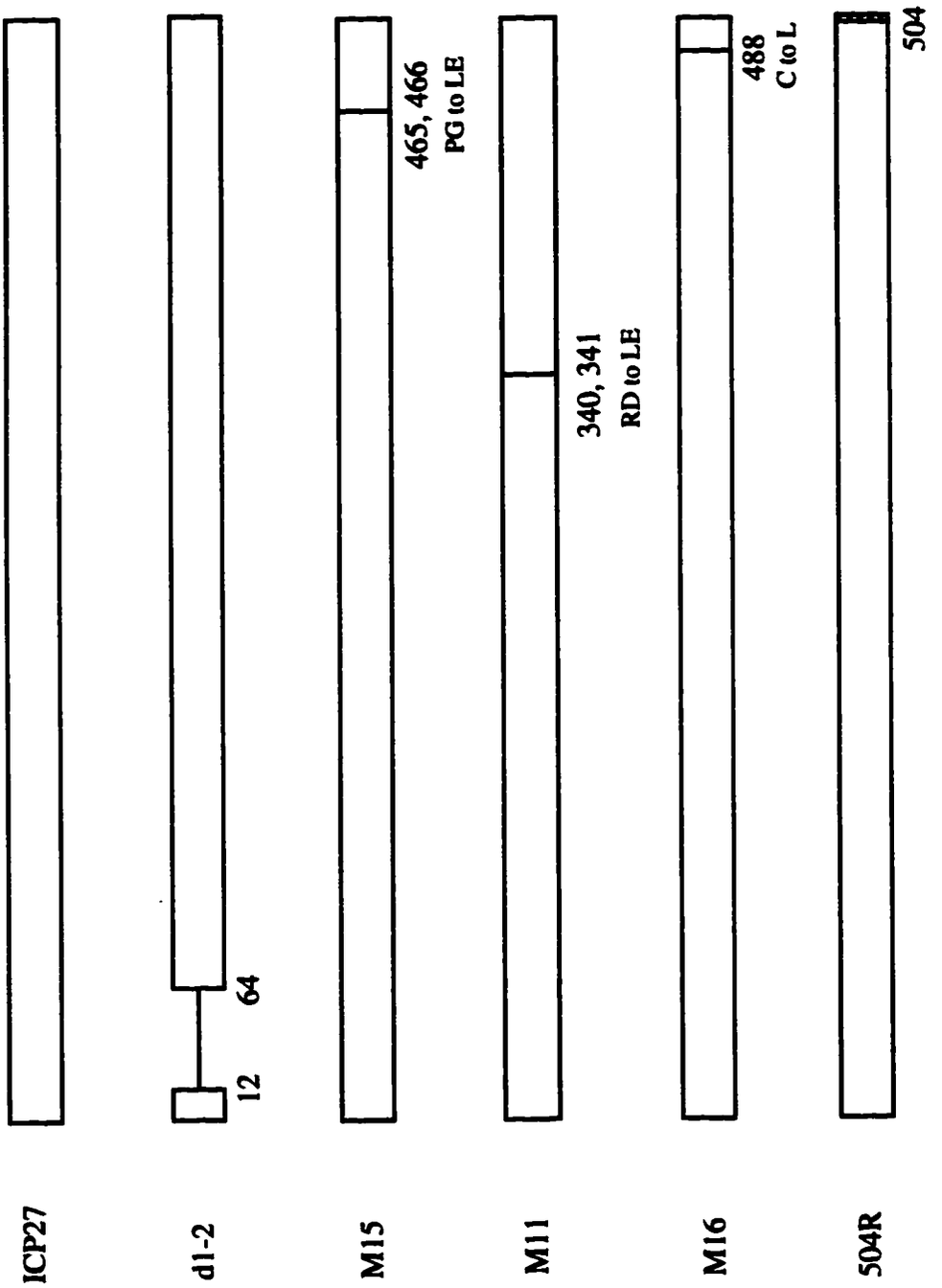
ICP27
d1-2
12
64
M15
465, 466
PG to LE
M11
340, 341
RD to LE
M16
488
C to L
504R
504

FIG. 4-5. Nuclear trafficking of HSV-1 ICP27 mutants. Vero cells were infected with either WT HSV-1 or the mutants M15 or *n*504R and fused to uninfected 3T6 cells using the "cycloheximide release" protocol described in the text. The photomicrographs show cells from the interspecies heterokaryon assays before or after fusion. Panels A, B, C, and D - WT infected cells. E, F, G, and H - M15 infected cells. I, J, K, and L - *n*504R infected cells. A, E, and I show infected Vero cells and uninfected 3T6 cells 30 minutes before fusion, stained for ICP27 using H1119. (B, C, & D), (F, G, & H), and (J, K, & L) each show the same field of cells one hour after fusion. B, F, and K) Phase contrast image. C, G, and K) Hoechst staining. D, H, and L) ICP27 staining using H1119 antibody. The arrows in these pictures indicate 3T6 nuclei within heterokarons. Bar = 20 μm.

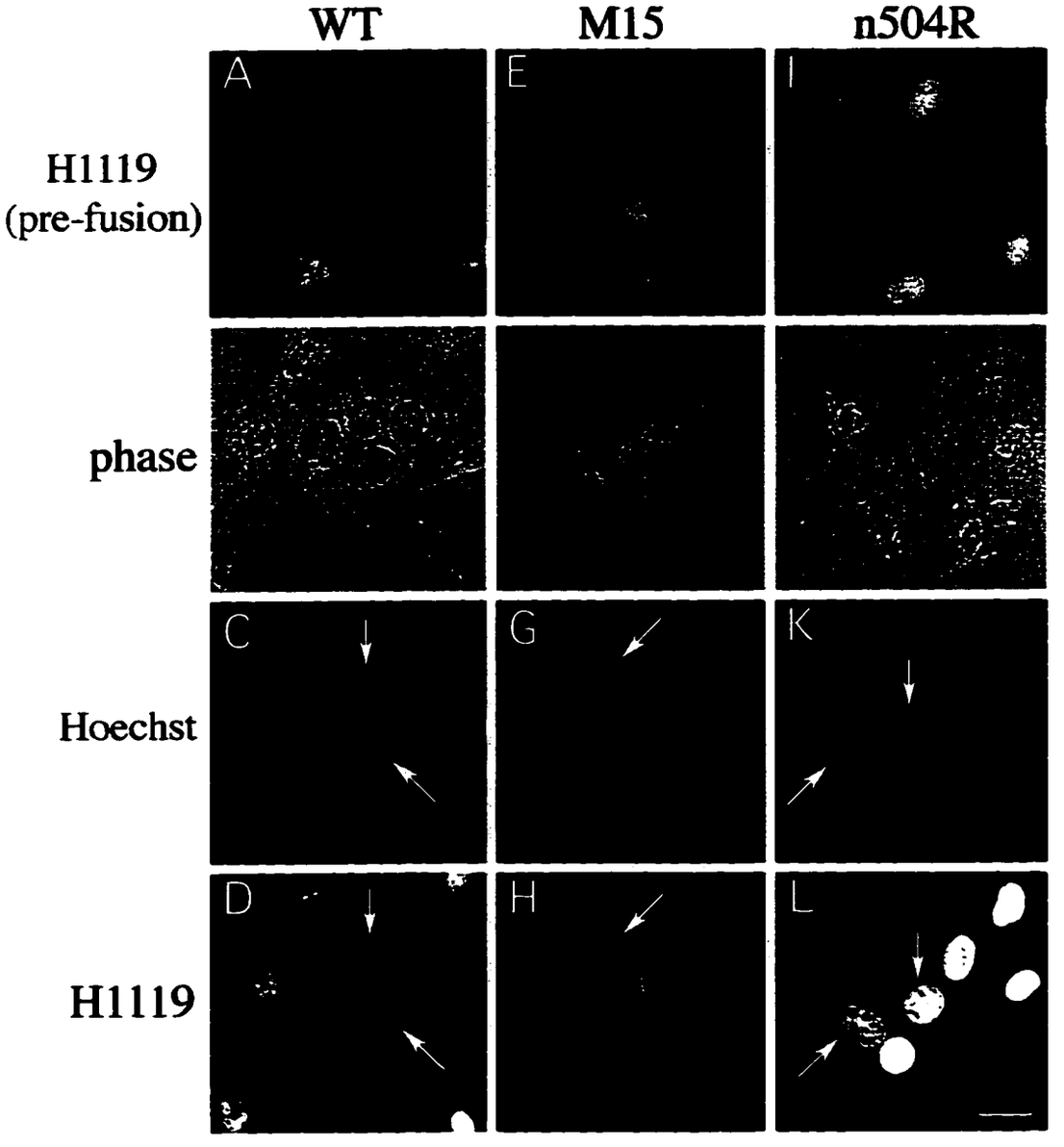
WT
M15
n504R
H1119
(pre-fusion)
phase
Hoechst
H1119
A
E
I
C
G
K
D
H
L

FIG. 4-6. ICP27 is capable of nuclear shuttling in the absence of other viral proteins. Vero cells, transiently transfected with an ICP27 gene-containing plasmid, were used in the interspecies heterokaryon assay. Shown are photomicrographs of the same field of transiently transfected Vero cells one hour after fusion to untransfected 3T6 cells. A) Phase contrast image. B) Hoechst staining. C) ICP27 staining with H1119. Arrow indicates 3T6 nucleus. Bar = 20 μm.

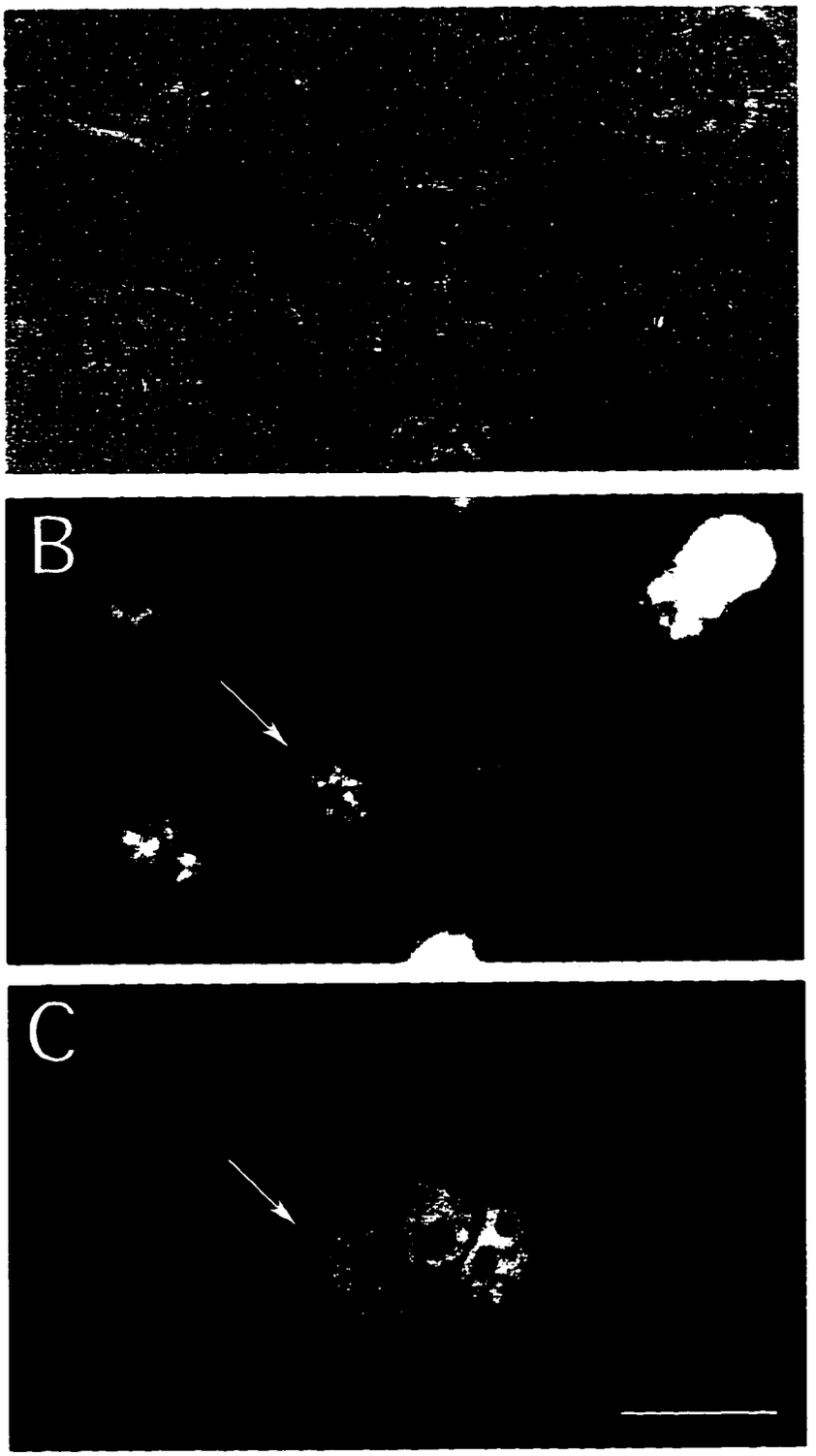
B
C

Table 4-1

Trafficking of viral nuclear proteins within interspecies heterokaryons

viral protein	# of heterokaryons examined	Staining of mouse nuclei in interspecies heterokaryons		
		% positive		% negative
		++ [a]	+ [b]	-
ICP27	114	94.7%	5.3%	0%
ICP4	114	0%	14.9%	85.1%
ICP8	115	0.9%	31.3%	67.8%

a - ++ indicates strong ICP27 staining within 3T6 nuclei (visually comparable in intensity to that observed in Vero nuclei of same heterokaryon)

b - + indicates weak ICP27 staining within 3T6 nuclei (visually < 50% of that seen in Vero nuclei)

Table 4-2

Trafficking of ICP27 within interspecies heterokaryons

	virus	# of heterokaryons examined	ICP27 staining within mouse nuclei of interspecies heterokaryons: % positive ++[a]	% positive +[b]	% negative -
Expt. 1					
	KOS1.1(WT)	220	85.0%	8.6%	6.4%
	M15	254	0.4%	0.8%	98.8%
	*d*1-2	231	19.6%	60.9%	19.6%
Expt. 2					
	KOS1.1	254	83.5%	14.6%	1.9%
	*n*504R	247	93.5%	5.3%	1.2%
	M15	287	0%	11.5%[c]	88.5%
	M11	281	33.1%	58.7%	8.2%
	M16	249	32.1%	62.7%	5.2%

a - ++ indicates strong ICP27 staining within 3T6 nuclei (visually comparable in intensity to that observed in Vero nuclei of same heterokaryon)

b - + indicates weak ICP27 staining within in 3T6 nuclei (visually < 50% of that seen in Vero nuclei)

c - ICP27 was detectable in the mouse nuclei in trace amounts (just above background staining)

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CHAPTER 5

EVIDENCE FOR TRANSCRIPTIONAL GENE REGULATION BY ICP27

Chapter Five - Evidence for Transcriptional Gene Regulation by ICP27[4]

INTRODUCTION

Both transient expression assays and studies of HSV mutants have indicated that ICP27 plays an essential role in viral gene regulation. Late gene expression is up-regulated by ICP27 both due to an enhancement of viral DNA replication and through a mechanism which is independent of DNA synthesis. At the same time, ICP27 also down-regulates some immediate-early gene expression. The mechanism(s) by which these actions are performed has not been elucidated.

Regulation of gene expression, in general, can occur at several stages including transcription, pre-mRNA processing, mRNA transport, mRNA degradation, and translation. The strong transcriptional transactivator ICP4 can function through promoter sequences to stimulate or repress transcription. ICP27 can modify the effects of ICP4 and ICP0 (another viral transactivator) either directly or indirectly, resulting in a further enhancement or a repression of the induction observed (7,8,27,37,42). ICP27's co-operative role with ICP4 and ICP0 suggests that ICP27 may function at the transcriptional level. However, evidence from transient expression assays indicates that regulation by ICP27 can occur independently of promoter sequences (3,34,39), instead depending on downsteam mRNA processing signals. Messages containing intron sequences are down-regulated in the presence of ICP27 (13,14,34), while messages containing certain poly(A) signal sequences (apparently weak poly(A) signals) are up-regulated (18,21,34).

The synergistic effect of ICP4/ICP0 with ICP27 on viral gene regulation could be explained as follows. ICP4 and/or ICP0 (or other transcriptional activators) induce transcription of the gene of interest after which ICP27 can affect processing of the

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[4]The data presented in Figure 5-9 were collected by Dr. C. Spencer.

message affecting its expression co-ordinately with these proteins. As most cellular genes possess intron sequences, their down-regulation would aid the virus in host protein synthesis shut off. As well, it has been suggested that some viral late genes possess weak polyadenylation signals which under the conditions of the transient assay are upregulated by ICP27 expression (18,34).

Transient expression experiments are useful for intial studies, however, these assays are limited. Since the gene of interest is present on plasmid DNA molecules, the regulation observed does not necessarily represent the regulation which occurs on viral genes within the viral genome itself. In order to further study ICP27's gene regulatory effects, a model system utilizing the HSV-1 genome was devised. Construction of recombinant viruses which contain a reporter gene inserted into the ICP27 locus was intended to provide a valid system to study ICP27's effect on gene regulation during viral infection. The construction and initial characterization of this model system are described. Unexpectedly, our results indicate that ICP27 can induce viral gene expression at the level of transcription.

MATERIALS AND METHODS

Cells, viruses and infections. All infections and transfections were carried out in Vero or V27 cells (28). Cells were maintained as described in Chapter 2. KOS1.1 (15) was the WT strain and *d*27-1 (28) was the ICP27 deletion mutant used in these experiments. The chloramphenicol-acetyltransferase (CAT)-gene containing viruses were derived from *d27-lacZ1*, a β-galactosidase (*lacZ*)-containing variant of KOS1.1 (28) and are described below. Single virus infections were carried out at an MOI of 10 PFU/cell. Co-infections were performed at an overall MOI of 10 PFU/cell meaning each virus was used at an MOI of 5 PFU/cell.

Construction of plasmids. In order to construct recombinant viruses containing the CAT gene in place of the ICP27 gene, a plasmid which contains the ICP27 gene and flanking HSV sequences was modified in the following way. The starting plasmid was pBH27 (27), which contains a *Bam*HI-*Hpa*I fragment (2.4 kbp) of HSV-1 DNA. The entire ICP27 open reading frame along with small amounts of 5' and 3' flanking sequences were removed by complete digestion with *Nae*I. *Xho*I linkers were added by T4 DNA ligase and excess linkers removed by subsequent *Xho*I digestion. The resulting vector, containing roughly 600 bp of HSV-1 DNA was termed pBHΔ27X. In order to enhance the recombination of the modified viral sequences into the viral genome, the amount of HSV-1 DNA flanking the reporter gene insertion was increased utilizing the plasmid pPs27pd1 (30). pPs27pd1 contains the 6 kbp *Pst*I HSV-1 DNA fragment containing the ICP27 gene. The *Bam*HI-*Sst*I fragment (600 bp) of pBHΔ27X was cloned into the 6.4 kbp *Bam*HI-*Sst*I fragment of pPs27pd1. The resulting vector, pPsΔ27X, extends the amount of HSV-1 DNA flanking the *Xho*I site to 0.9 and 3.4 Kbp, respectively. The CAT reporter genes were then cloned into the unique *Xho*I site, as described below.

The parental CAT reporter gene, ptkCAT27, was constructed by V. Leong and S. Rice (29) and consists of the CAT gene under the control of the HSV-1 thymidine kinase (TK) promoter and the ICP27 polyadenylation signal sequence. The TK promoter (-105 to +51 relative to the initiation site) was obtained from pBLCAT2 (17). The 800 bp CAT open reading frame was originally obtained from pBLCAT3 (17) by digestion with *Bam*HI and *Cla*I, while ICP27's polyadenylation signal sequence (90 bp) was isolated from of pBH504R (30) by *Nae*I digestion.

To produce the plasmids required to construct the recombinant viruses, ptkCAT27 was cleaved with *Sst*I and 3' overhanging ends were blunted using T4 DNA polymerase. *Sal*I linkers were added to the blunt DNA ends using T4 DNA ligase. The resulting DNA was cleaved with *Sal*I. The 1.1 Kbp DNA fragment that contained the TK promoter, CAT gene, and ICP27 poly(A) signal was isolated and ligated to pPsΔ27X cleaved with *Xho*I. Before transformation, the ligated DNA was cleaved with *Xho*I to reduce the amount of religated vector. After transformation, the orientation of the CAT gene in transformants was identified using various restriction enzymes. The resulting plasmids were termed ppstkCAT27 (the CAT gene in orientation opposite to that which the ICP27 gene normally resides) and ppstkCAT27rev (the CAT gene in the same orientation as that which the ICP27 gene normally resides).

Isolation of CAT-containing viruses. The isolation of HSV-1 viruses containing the CAT gene in place of the ICP27 gene was performed using a strategy that has been previously described (Chap. 2; 23,27). The plasmids ppstkCAT27 and ppstkCAT27rev were cleaved with *Hind*III and *Eco*RI to release the fragment containing the HSV-1 DNA from plasmid DNA sequences. After phenol/choloroform-isoamylalcohol extraction, the cleaved products (1 μg) were individually co-transfected into V27 cells with 4 μg of infectious *d27-lacZ1* DNA (28). Transfection was by calcium phosphate technique as previously described (Chap. 2; 23). Four to five days later, cells and virus were collected and subjected to several freeze-thaw cycles at -70°C. The resulting viral progeny were

plated at various dilutions on V27 cells and maintained in 199O media (Chap. 2; 23). Three days later, plaques were overlaid with 0.5% agarose, 199V media and 300 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Clear plaques were isolated and treated to three cycles of freezing and thawing. High titre preliminary stocks were produced by infecting V27 cells with virus from the isolated plaques. Total genomic DNA was collected from infected V27 cells and viral genomes analyzed by Southern blot analysis. DNA was blotted to nylon (GeneScreenPlus) in 10x SSC (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate) and UV-crosslinked to the filter. A ^{32}P-labeled DNA probe containing ICP27 sequences was hybridized to the filter at 65°C overnight. After washing, the hybridized radioactive bands were visualized by autoradiography. After verification that the viral isolates possessed the expected genomic structures, stocks were made from two plaque-purified isolates of each virus and stored at -70°C. The isolates which possess the CAT gene in an orientation opposite to that which the WT ICP27 gene is normally transcribed were termed KATa and KATb. Viruses possessing the CAT gene in the same orientation as WT ICP27 gene were termed KATreva and KATrevb.

CAT assays. Vero or V27 cells in 25 cm^2 flasks were infected either in the presence or absence of 400 µg/ml phosphonoacetic acid (PAA), 20 mM HEPES pH 7.0. At various times post-infection, monolayers were rinsed with PBS and collected in TNE (10 mM Tris pH 7.8, 150 mM NaCl, 1 mM EDTA) using a cell scraper (Falcon). After centrifugation, cells were resuspended in 250 µl of 0.25 M Tris-HCl pH 7.8. Cells were disrupted by three cycles of freeze-thawing. Cell debris was removed by centrifugation and lysates were stored at -20°C. Relative CAT activities were measured by enzyme assay of appropriate dilution of the cell extract (10,27). CAT assays were performed by the addition of acetyl CoA (final concentration = 0.5 mM) and ^{14}C-labeled chloramphenicol (final concentration = 0.25 µCi) to 50 µl of cell extract, with a final buffer concentration of 0.15 M Tris-HCl pH 7.8. The degree of enzymatic activity contained within each extract was assessed by the extent of conversion of

chloramphenicol. After 1 hour incubation at 37°C, reactions were stopped by the addition of ethyl acetate (1 ml) and vigorous vortexing. The ethyl acetate layer was removed to a clean tube and the products within it were dried in a Speed-Vac (about 30 minutes). Reaction products were resuspended in 30 µl chloroform and spotted onto Silica-Gel 250 µm TLC plate with plastic backing. Plates were developed by ascending chromatography in 95% chloroform-5% methanol, dried and radio-labeled products were visualized by autoradiography. Extent of conversion was quantitated using phosphoimaging analysis. To obtain the relative CAT activity, the extent of conversion was normalized according to the protein content of each extract, as determined with a Bio-Rad protein assay kit, which utilizes the Bradford assay (1).

Northern analysis. Vero cells were infected with KOS1.1 alone or coinfected with KOS1.1 and KAT either in the presence or absence of 400 µg/ml PAA, 20 mM HEPES pH 7.0. All solutions used for RNA isolation were RNase-free. At various times post-infection, cells were trypsinized, spun down at 4°C, washed with 10 ml of chilled PBS and then resuspended in 400 µl chilled PBS. To each sample, five volumes of 4M guanadinium isothiocynate were added and samples were stored at -70°C. After thawing, one gram of CsCl was added to each of the samples and mixed. The homogenate was layered onto a 1.75 ml CsCl cushion and spun at 35,000 rpm, 20°C overnight in a swinging bucket rotor. The RNA pellet was isolated, resuspended in 200 µl TE, and cleaned by phenol and chloroform/isoamyl alcohol extractions. RNA was precipitated in ethanol, resuspended in TE, and stored at -20°C.

Ten micrograms of each sample of RNA were run on a large formaldehyde-1.2% agarose gel and then blotted overnight to nylon filters (GeneScreenPlus) in 20x SSC. The filter was then subjected to either UV-crosslinking or baked for two hours under vacuum at 90°C. The blots were hybridized with ^{32}P-labeled DNA probes at 65°C. After extensive washing, the filters were visualized using autoradiography. In some cases, the

hybridized probe was removed by boiling the blots in 1% SDS and 0.1x SSC for 30 minutes.

Nuclear run-on analysis. Vero cells were mock-infected, singly-infected with KOS1.1 or *d*27-1, or co-infected with KOS1.1 and KAT or *d*27-1 and KAT. Infections were performed in the presence of 400 μg/ml PAA, 20 mM HEPES pH 7.0. Eight hpi, nuclei were isolated and assays were performed as previously described (Rice *et al.*, 1995). Run-on transcription was allowed to occur in the presence of ^{32}P-UTP (specific activity > 3000 Ci/mmol) in a buffer containing 150 mM KCl. After a 30 minute incubation, RNA products were isolated. Single-stranded DNA probes of various viral and cellular genes were produced from M13 vectors. These were slot-blotted, UV-crosslinked, and baked to nylon membranes (Genescreen). The various ^{32}P-labeled RNA products were hybrized to the blots for 48 hours at 65°C. Following hybridization, the blots were treated with RNase A to reduce background binding of RNA and then washed extensively. The blots were visualized by autoradiography.

The ssDNA probes used were such that both sense and anti-sense transcription would be detected from the genes of interest. These probes were constructed by cloning gene regions of interest into either M13mp18 or M13mp19. The TK probes consist of an 840 bp fragment containing the TK open reading frame from codons 50 to 360 cloned into M13. To make the TK probes, pGEM-HSVTK (generously provided by Dr. C. Rasmussen) was cleaved with *Acc*I and *Sau*3AI. Overhanging ends were blunted using the Klenow fragment and *Bam*HI linkers were added. After cleavage with *Bam*HI, the 840 bp fragment was inserted into M13mp18 linearized with *Bam*HI. The CAT probes consist of a 780 bp fragment containing the Tn9 chloramphenicol acetyltransferase gene open reading frame cloned into M13. The CAT cartridge was originally isolated from the pCM4 plasmid (Pharmacia). The cellular γ-actin 3' probes were produced by Alison Kilvert. The γ-actin probes and the probes for viral genes ICP27, ICP4, ICP8 and gC have been previously described (6,31). Rates of transcription of the tkCAT27 gene were

determined using the Imagemaster VDS Video Imager system and corresponding software (Pharmacia).

RESULTS

Construction of the HSV-1 mutant viruses, KAT and KATrev. A model system was devised to study the effect of ICP27 on viral gene regulation within the context of the HSV-1 genome itself. The strategy was to insert model reporter genes into the viral genome and to compare their expression in the presence and absence of ICP27. The bacterial chloramphenicol-acetyl transferase (CAT) gene was chosen as the reporter gene since it is not normally expressed in eukaryotic cells and the enzymatic activity of the CAT gene product can readily be assayed (10).

Our long-term goal was to study the effect of various RNA processing signals, such as introns and poly(A) signals, on the expression of viral genes. However, first it was necessary to delineate the baseline expression of a parental reporter gene inserted into the HSV-1 genome. The parental reporter gene we designed contained three elements: i) the HSV-1 TK gene promoter (-105 to +51), ii) the CAT open reading frame, and iii) a polyadenylation signal sequence derived from the ICP27 gene. We note here that the region of the TK promoter encompassing nucleotides -105 to +51 with respect to the transcription start site was previously determined to contain the minimal sequences required for efficient basal transcription in both non-viral and viral systems (12,17,19,20).

The CAT gene was inserted into the viral genome in the place of the ICP27 gene. The reasons for this were two-fold: i) most importantly, this genetic replacement allows the study of the reporter gene in the absence of ICP27, and ii) the existence of V27 cells, which complement the growth of ICP27 mutants, allows the ICP27 genetic locus to be used as a convenient cloning site. Manipulating the ICP27 locus would also hopefully result in minimal disrupting effects on the rest of the HSV-1 genome.

To engineer a CAT-encoding virus, genetic alterations were first performed in the context of recombinant plasmids. The ICP27 coding region along with its TATA box and polyadenylation signal were eliminated by removal of the region encompassed by the

*Nae*I restriction enzyme sites illustrated in Figure 5-1. Deleting this region maintained the UL52/UL53 polyadenylation site found upstream of the ICP27 gene and the UL55 TATA box and initiation site downstream of the ICP27 gene. The reporter gene was inserted in two orientations with respect to the normal orientation of the ICP27 gene itself (Fig. 5-2).

DNA molecules containing the CAT reporter gene flanked by HSV-1 sequences upstream and downstream of the *Nae*I sites were recombined into the HSV-1 genome by homologous recombination (28). This resulted in two viruses in which the ICP27 gene was replaced by the CAT gene. The first virus contained the reporter gene inserted into the ICP27 locus in the opposite orientation to that which the ICP27 gene normally resides. In this way, any remaining upstream ICP27 promoter sequences or downstream regulatory elements would not be expected to affect expression of the CAT gene. This virus was referred to as KAT, indicating the presence of the CAT gene within the ICP27 locus. The second virus possessed the CAT reporter gene inserted in the same orientation as the ICP27 gene. In this orientation, the reporter gene may be subject to regulatory sequences which remain upstream of the ICP27 locus. This virus was referred to as KATrev to distinguish the reporter gene orientation from that of the first virus. Comparison between the expression of the reporter gene from these two viruses might indicate if sequences external to the reporter gene were responsible for affecting its expression.

To verify that the CAT gene was inserted into the proper location in the KAT and KATrev viral isolates, Southern blot analysis was performed. Total genomic DNA was collected from infected V27 cells and subjected to digestion with *Pst*I. The resulting blot was probed with ^{32}P-labeled DNA corresponding to the 6.0 Kbp *Pst*I WT HSV-1 DNA fragment containing the ICP27 locus (Fig. 5-2). The results are shown in Figure 5-3. As expected, all viral DNAs possessed a hybridizing band of roughly 5.5 Kbp, corresponding to the *Pst*I fragment from the U_L - R_L junction at the opposite end of the U_L region of the

genome. Excluding this common band, both WT HSV-1 and *d*27-*lac*Z1 DNA possessed single hybridizing bands of the expected size. As there is a novel *Pst*I site introduced into the genome by the addition of the reporter gene, two hybridizing bands are expected for both KAT and KATrev viruses. The size of these bands, shown in Figure 5-2, are diagnostic for the orientation of the inserted gene. As expected, KAT and KATrev DNA contained the anticipated 3.6 and 1.7, and 4.3 and 1.0 Kbp bands, respectively. Genomic DNA from the second isolate of KAT, KATb, also displayed DNA fragments of the anticipated size when subjected to Southern analysis (data not shown). As expected, none of the CAT-containing viruses were capable of growing on Vero cells as demonstrated by plaque assays (data not shown). Thus, we were able to isolate HSV recombinant viruses in which the ICP27 gene was replaced with the desired CAT reporter genes.

ICP27 induces CAT expression from the KAT virus. We decided to initially focus on expression of the CAT gene in the KATa virus. As the expression of the endogenous TK gene is not believed to be dependent on ICP27 activity, it was anticipated that expression of the CAT gene in KAT would not depend on ICP27. To test this hypothesis, expression of the gene was compared in the presence and absence of ICP27. ICP27 can be provided in *trans* from a co-infected WT virus. Vero cells were co-infected with the KAT virus and either WT HSV-1 (strain KOS1.1) or the ICP27 deletion virus, *d*27-1. Co-infection with *d*27-1 acts as a control to account for the presence of a second HSV-1 genome in the co-infected cells. Eight hpi, cell lysates were collected and assayed for CAT enzymatic activity (Fig. 5-4A; 10). Unexpectedly, it was found that the level of CAT activity was significantly higher in the presence of ICP27 (cells co-infected with KOS1.1). In fact, in the absence of ICP27 (cells co-infected with *d*27-1), CAT expression was quite low (close to background levels in the enzyme assay) suggesting that the tkCAT27 gene is relatively inactive in the absence of ICP27.

To further investigate the effect of ICP27 on induction of the reporter gene, lysates from cells co-infected with KOS1.1 or *d*27-1 were collected at various times post-infection and assayed for CAT activity (Fig. 5-4B). No significant activity was observed for either infection up to 4 hpi. As before, enzymatic activity was observed 8 hpi in KOS1.1 co-infected cells and continued to increase at 12 hpi. *d*27-1 co-infected Vero cells possessed no significant CAT activity up to 8 hpi but by 12 hpi did possess enzymatic activity. Therefore, although the CAT gene was expressed in the absence of ICP27 late in infection, ICP27 was required to stimulate the expression of this gene at early times post-infection.

As discussed previously, viral gene expression occurs in a temporally-regulated fashion in three waves of expression termed IE, DE, and L. The IE and DE genes are expressed before viral DNA synthesis, while the L genes require viral DNA synthesis for their efficient expression (reviewed in ref. 32). The promoter which drives the expression of the tkCAT27 gene is derived from the HSV-1 DE TK gene promoter. This promoter possesses regulatory elements required for transcription of the endogenous TK gene and as such is anticipated to exhibit DE gene expression kinetics (4). Viral DNA replication and subsequent progression into the last wave of viral gene expression results in down-regulation of DE genes. The results shown in Figure 5-4B indicate that the tkCAT27 gene may not be behaving as a typical DE gene since its mRNA is present in higher amounts at L times, suggesting that its expression seems to be highly activated at these times.

To investigate the expression of the tkCAT27 gene in more detail, we studied its expression in the presence of a specific inhibitor of viral DNA replication, phosphonoacetic acid (PAA). The expression of most DE genes is enhanced by inhibiting viral DNA replication, which acts to halt the infection in the DE phase. Vero cells were infected in the presence or absence of PAA and lysates were collected at 8 hpi and assayed for CAT activity (Fig. 5-5). In the absence of PAA, ICP27 stimulated CAT

expression as before. Interestingly, this effect was much more dramatic in the presence of PAA. Thus, it appeared that halting infection at the DE phase greatly magnified the stimulatory effect of ICP27 on the CAT gene. It is noteworthy that the presence of PAA efficiently blocked expression of the tkCAT27 gene when ICP27 was absent. These results suggest that the tkCAT27 gene in KAT is positively regulated by two factors: the presence of ICP27 and the process of viral DNA replication. When viral DNA synthesis is prevented, the tkCAT27 gene becomes absolutely dependent on ICP27 for its expression.

To further investigate the role of ICP27 in activating the CAT gene, we decided to provide ICP27 to the system in another manner. To do this, the KAT virus alone was used to infect either Vero or V27 cells. V27 cells are a stably transfected Vero cell line that possess approximately one copy of the ICP27 gene per haploid cell. Although the ICP27 gene is not constitutively active in these cells, infection with HSV-1 results in the expression of the stably transfected ICP27 gene. KAT-infected Vero and V27 cell lysates were compared with cell lysates from Vero cells which had been co-infected with KAT and either KOS1.1 or *d*27-1 (Fig. 5-6A). All co-infections and infections were performed in the presence of PAA and cell lysates were collected 8 hpi. It was found that ICP27 was required to observe significant levels of CAT activity regardless of whether it was provided by co-infection with KOS1.1 or by infection of V27 cells (Fig. 5-6A). However, the effect was much more dramatic when ICP27 was supplied by co-infection.

One potential explanation for the above result is that less ICP27 is supplied by the V27 cells than by co-infection with KOS1.1, thereby resulting in less CAT expression. Consistent with this observation, indirect immunofluorescence of V27 cells infected with KAT virus displayed lower levels of ICP27 staining than did Vero or V27 cells co-infected with KAT and KOS1.1 (data not shown). It is also of interest to note that the passage number of the V27 cells used significantly affected the degree of CAT activity observed. Figure 5-6B illustrates the CAT activity observed when late passage (between

passage 52 and 55) V27 cells, similar to those used in Fig. 5-6A, or early passage (approximately passage 20) V27 cells were infected with the KAT virus. The early passage V27 cells had significantly increased levels of CAT activity compared to that observed in later passage cells. As well, early passage V27 cells (passage 20) infected with *d*27-1 were found, by indirect immunofluorescence, to possess a higher percentage of nuclei which stained positively for ICP27 than did late passage V27 cells (passage 53) similarly infected (S.A. Rice, unpublished results).

From these results, it would appear that ICP27 induces the expression of the CAT reporter gene regardless of whether it is provided via co-infection or from a stable cell line. The differences in the level of induction observed are probably related to the relative levels of ICP27 provided by the co-infecting virus or age of the cell line.

The tkCAT27 and endogenous HSV TK gene are expressed dissimilarly. The above results suggest that ICP27 is required for expression of the tkCAT27 gene prior to viral DNA replication, and under conditions where DNA replication is inhibited. This was surprising as the endogenous TK gene is not known to be similarly dependent upon ICP27 expression. To investigate this apparent difference, RNA from the endogenous TK gene and the tkCAT27 gene were compared by Northern blot analysis. Vero cells were co-infected with KAT and KOS1.1 in the presence or absence of PAA and total cellular RNA was collected at various times post-infection. The blotted RNA was first hybridized with an 800 bp ^{32}P-labeled DNA probe directed against the CAT coding region (Fig. 5-7). As expected, no CAT RNA was observed in mock-infected cells (lanes 1 and 8) or in Vero cells infected with KOS alone (lanes 2 and 9). In the absence of PAA (Fig. 5-7A), heterogenously sized RNAs were observed, suggesting that non-specific intiation and/or 3' end formation was occuring at late times. In the presence of PAA (Fig. 5-7 B and C), low levels of a specific CAT-containing RNA were observed at 6 and 8 hpi (lanes 5 and 6) and this level increased by 12 hpi (lane 7).

Next, we compared the expression pattern observed for tkCAT27 gene to that of the endogenous TK gene. Different blots of the same RNA preparations used above were hybridized first with a 840 bp ^{32}P-labeled DNA probe directed against the open reading frame of TK (Fig. 5-8 And B). Since the TK gene (UL23) overlaps with the UL24 open reading frame, we used a probe that corresponds to sequences unique to the TK open reading frame (roughly codons 50 to 360). DE genes are expressed early during infection, peaking between 4-6 hpi and diminishing as viral DNA synthesis increases, roughly 8-12 hpi. As expected, the endogenous thymidine kinase gene was expressed with typical DE gene kinetics (Fig. 5-8A). Expression peaked at around 4 hpi (lane 4) then steadily decreased as infection proceeded. By 12 hpi, very little TK message was observed in either KOS1.1 infected (lane 9) or KOS1.1/KAT co-infected cells (lane 7). The TK message observed was a single major RNA species. In the presence of PAA (Fig. 5-8B), TK RNA levels from KOS1.1/KAT co-infected cells peaked at 4 hpi (lane 4) and remained relatively constant through-out the remainder of the infection. Cells infected with KOS1.1 alone possessed a similar high level of TK RNA at 12 hpi (lane 9). In the presence of PAA, a single major TK RNA species was observed, similar to that observed in the absence of PAA. From these results it appeared that the endogenous TK gene was being expressed as anticipated for a DE gene.

The blots in Figure 5-8 A and B were stripped and reprobed for CAT RNA (data not shown). Similar results to that observed in Figure 5-7 were found. As before, in the absence of PAA, KOS1.1/KAT co-infected cells expressed heterogenously sized CAT RNA species from 6 - 12 hpi. Very low levels of a single major CAT RNA message were observed in the presence of PAA. As a single major species of TK RNA was observed on the same filter, it is unlikely that RNA degradation caused the multiple CAT RNA species observed in the absence of PAA. Thus, it would appear that the tkCAT27 gene but not the endogenous TK gene produces heterogenous RNA species under

conditions where DNA replication occurs. As well, the tkCAT27 gene is not expressed with kinetics similar to the endogenous TK gene or other known DE genes.

Transcriptional induction of the CAT gene by ICP27. Although ICP27 increased the level of CAT gene expression from the KAT virus, it was unknown at what level this might be occurring. To determine if ICP27 enhances the transcription of the tkCAT27 gene, nuclear run-on analysis was performed. The level of transcription measured using the nuclear run-on assay is directly related to active transcription and is not dependent on post-transcriptional effects such as mRNA processing or stability. As initiation of transcription cannot occur under the conditions of this assay, only those genes which possess RNA polymerase II molecules actively engaged in their transcription at the time of sample collection will produce radiolabeled transcripts.

Vero cells were mock-infected or infected with *d*27-1, KOS1.1, KOS1.1 plus KAT, or *d*27-1 plus KAT. All infections were carried out in the presence of PAA to inhibit viral DNA replication. The reasons for this were two-fold. First, the effect of ICP27 on induction of the tkCAT27 gene in the KAT virus is much more dramatic when DNA replication is inhibited by PAA (Fig. 5-5). Second, the results of nuclear run-on assays of HSV-1 infections are not easily interpretable unless viral DNA replication is inhibited. This is because, under conditions of viral DNA replication, nuclear run-on assays demonstrate high levels of transcription throughout the HSV-1 genome in HSV-1 infected cells (9,44). It is unknown if this observation represents ongoing transcription during viral infection or is due to non-productive interactions of RNA polymerase II complexes with the viral genome.

Nuclei were isolated 8 hpi, and subjected to nuclear run-on transcription analyses (performed by Dr. C. Spencer). The radiolabeled RNA was then hybridized to sense or anti-sense CAT ssDNA probes (Fig. 5-9). Additional ssDNA probes corresponding to all three viral temporal gene classes were also included, along with cellular γ-actin and M13 probes as controls.

Consistent with its low expression, tkCAT27 transcription was barely detectable in Vero cells co-infected with *d*27-1 and KAT. However, this gene was transcribed at a significant rate (approximately 6 fold higher) in Vero cells co-infected with KOS1.1 and KAT. In contrast, the endogenous TK gene was transcribed in the absence of ICP27, and transcription was not significantly enhanced by ICP27. The above results strongly suggest that ICP27 can transcriptionally transactivate the tkCAT27 gene. However, it should be noted that this run-on experiment yielded two unexpected results. First, mock-infected cells showed hybridization to the ICP4 sense probe. This may result from hybridization with a cellular message. Second, it was surprising that the viral gC gene was transcribed in all infections. Since this message is derived from a true late gene, it was not expected to be transcribed when viral DNA synthesis was inhibited. One possible explanation for the gC transcription observed is that the treatment with PAA did not sufficiently inhibit viral DNA synthesis allowing expression of this late gene. Transcription of the anti-sense strand of several viral genes tested was also observed which further supports the possiblity that DNA replication was not completely inhibited.

In summary, the results from the nuclear run-on analysis suggest that ICP27 stimulates the expression of the tkCAT27 reporter gene at the transcriptional level. However, as this analysis consists of only one experiment, further nuclear run-on experiments will be required to confirm this conclusion.

The orientation of the CAT gene affects its basal activity but not its response to ICP27. To determine if the genomic orientation of the CAT reporter gene influences its expression and its response to ICP27, CAT expression from the KATrev virus was compared to that from the KAT virus. Vero cells were co-infected with KOS1.1 and KAT, KOS1.1 and KATrev, *d*27-1 and KAT, or *d*27-1 and KATrev in the presence of PAA, and cell lysates were collected 8 hpi. Unlike cells infected with KAT, cells infected with KATrev possessed significant levels of CAT activity in the absence of ICP27 (Fig. 5-10). Therefore, ICP27 is not required for CAT expression from KATrev

under the conditions where DNA replication is inhibited. However, when Vero cells were co-infected with KOS1.1 and KATrev, the levels of CAT activity were greatly increased demonstrating that ICP27 further enhances the level of CAT expression from this reporter gene. Thus, although the orientation of the reporter gene within the ICP27 locus affects the CAT gene's basal level of expression, ICP27 enhances this expression regardless of gene orientation.

DISCUSSION

A model system to investigate regulation of viral genes by ICP27. In the past few years, research has revealed several post-transcriptional effects of ICP27 on gene expression. In transfection assays, genes possessing certain (perhaps weak) poly(A) signals are induced by ICP27, while those possessing intron sequences are repressed (18,21,34). Direct evidence that ICP27 may inhibit mRNA splicing comes from the work of Sandri-Goldin and colleagues. These investigators showed that the levels of specific cellular mRNA's are reduced in the presence of ICP27 and that this reduction appears to be caused by a post-transcriptional effect (13). In addition, they demonstrated that *in vitro* extracts prepared from HSV-1 infected cells are deficient in splicing and that this effect requires ICP27 (14). Possibly related to its role in splicing regulation, ICP27 is required for the HSV-induced redistribution of snRNP complexes within the nucleus (26,36), and can physically interact with these complexes (35). ICP27 can also interact with RNA (2,16,Chap. 3; 22) and can affect the stability of some cellular messages (2,24). Consistent with a post-transcriptional regulatory role, ICP27 also appears to affect the cellular transport of viral intron-containing messages, causing them to be retained within the nucleus (14,25).

Despite the above insight into possible modes of action by ICP27, the actual mechanism(s) by which ICP27 regulates viral genes during infection is still unknown. In part, this is because it is difficult to examine systematically which *cis*-element [i.e., promoter, intron, poly(A) site] in viral genes confers their regulation by ICP27. The model system described here, in which a non-essential manipulatable model gene is inserted into the HSV-1 genome, could provide a very useful means to study the effect of ICP27 *in vivo*. This approach can be used to delineate the sequences in genes which mediate their regulation by ICP27 within the context of infection. In addition, since

mutant as well as WT ICP27 molecules can be supplied by co-infection, the model system can be used to identify which regions of ICP27 are responsible for this regulation.

Transactivation of the tkCAT27 gene by ICP27. Although the ultimate intent of our model system was to test the effect of various post-transcriptional signals, it was first necessary to construct a virus possessing a "parental" CAT gene and to characterize its expression and possible regulation by ICP27. To construct the parental reporter gene, we utilized a promoter from the HSV-1 TK gene (containing sequences from -105 to + 51 relative to the initiation start site). Studies have established that this promoter sequence possesses four regulatory elements required for basal expression of the TK gene in a non-viral system (19,20). Subsequent research suggests that the same regions are required for basal expression within the HSV-1 genome (4,12). As expression of the endogenous TK gene is not dependent on the presence of ICP27 (S. Rice, unpublished observations), expression of the tkCAT27 reporter gene was also anticipated to be independent of ICP27.

Unexpectedly, we discovered that the tkCAT27 reporter gene of the KAT recombinant virus was dependent on ICP27 for its expression. Some expression of tkCAT27 was observed in the absence of ICP27 when DNA synthesis was allowed. However, when viral DNA synthesis was inhibited, the gene became totally dependent upon ICP27 for its expression. Moreover, Northern analysis demonstrated that the tkCAT27 and endogenous TK genes were expressed in quite different manners. Thus, the tkCAT27 gene behaved quite differently than was expected, and quite distinctly from the endogenous TK gene.

Nuclear run-on analysis demonstrated that ICP27 significantly enhances the transcription from the tkCAT27 reporter gene. In contrast, ICP27 does not appear to affect the transcription of the endogenous TK gene. These results strongly suggest that ICP27 can stimulate a viral gene by enhancing its transcription. It is noteworthy that three groups have recently demonstrated that during infection ICP27 transactivates a sub-

set of DE genes, some of which encode DNA replication factors (18,33,43). However, the level at which these genes are stimulated by ICP27 was not identified. It is interesting to speculate that we have artificially constructed a gene, tkCAT27, which is regulated similarly to the sub-set of naturally occuring DE genes which are trans-activated by ICP27. This model would predict that ICP27 stimulates these DE genes by enhancing their transcription. The ability of ICP27 to affect transcription is supported by the recent finding that ICP27 is required for the transcriptional shutoff of certain cellular genes after infection (41).

There are several possible explanations for why the tkCAT27 gene in the KAT virus is regulated so differently from the endogenous TK gene. One possible explanation relates to differences between the endogenous TK promoter and the minimal TK promoter used in tkCAT27. Although genes possessing the "minimal TK promoter" are expressed in both non-viral and viral systems (4,12,19,20), other regions of the HSV TK promoter may be required for optimal expression of the TK gene from the genome (5,38,45). It is possible that the minimal TK promoter used in the tkCAT27 gene lacks flanking elements required for its efficient expression in the absence of ICP27. It is also possible that other regions within these genes result in their different expression patterns. The HSV-1 TK gene coding region has been implicated in regulation of this gene after transcription initiation (11). Possibly, the CAT open reading frame possesses similar regulatory elements. Finally, the differences between the tkCAT27 and TK expression could result from their different poly(A) sites. It is interesting to speculate that ICP27's poly(A) signal sequence, which is part of the CAT reporter genes described here, may have a role in the induction of tkCAT27 by ICP27. Previously it was shown that ICP27 can stimulate a co-transfected reporter gene bearing ICP27's poly(A) site by approximately four-fold (34). This finding suggests that this poly(A) signal may be involved in the ICP27-induced expression of tkCAT27. However, another group has recently found that the *in vitro* 3' processing efficiency of a reporter gene possessing

ICP27's poly(A) signal was unaffected by ICP27 (18). From these reports, it is uncertain if ICP27's poly(A) signal could result in the differences in expression observed between the TK and tkCAT27 genes.

Expression of tkCAT27 in the absence of ICP27 was higher from the KATrev virus than from KAT, indicating that the orientation of the reporter gene within the HSV-1 genome affects its basal level of expression. The ICP27 promoter region possesses four consensus binding sites for the virion transactivator, VP16, as well as other uncharacterized sequences important for WT ICP27 expression (40). In the KATrev virus, these sequences are upstream of the tkCAT27 gene, similar to how they are oriented with respect to the WT ICP27 gene. Thus, it is not unreasonable to expect that these upstream regulatory sequences might stimulate basal expression of the tkCAT27 gene in the KATrev virus.

Although the basal level of CAT gene expression was affected by the orientation of the tkCAT27 reporter gene, the gene in either orientation was induced by ICP27. This orientation-independent induction of tkCAT27 suggests that the element(s) responsible for ICP27 enhancement is contained within the reporter gene itself, although it remains possible that an enhancer is present flanking the reporter gene. The model system we have developed here should provide a means to map the *cis*-element(s) necessary for the induction of viral gene expression by ICP27.

We observed that the KAT virus expressed heterogeneously-sized RNA from the tkCAT27 gene region under conditions of viral DNA replication. As the Northern blot probe we used was double-stranded, it is impossible to say whether some or all of this RNA corresponds to CAT "sense" RNA. If these multiple species correspond to aberrantly initiated or processed transcripts, this might explain the reduced levels of CAT activity observed when DNA synthesis is allowed to proceed. It is not known why progression of infection should result in the production of multiple RNA species. However, nuclear run-on analysis of HSV-1 infected cells, under conditions of viral DNA

replication, results in apparently high levels of transcription for nearly all regions of the viral genome, including both sense and anti-sense strands of known genes (9,44). Our results suggest that this result may not be an artifact of the nuclear run-on assay, but may reflect bona-fide transcription. Analysis of the RNA produced from the KATrev virus may provide insight into the nature of these heterogeneously-sized CAT containing RNA species. Further study, such as primer extension and S1 analyses will be required to decipher the molecular species which make up these heterogeneous RNAs and to determine their significance to HSV-1 gene expression.

In summary, our results provide some of the first evidence that ICP27 can induce viral genes at the level of transcription. Preliminary results from additional recombinant viruses have confirmed that ICP27 is required for the transcription of a tkCAT27 reporter gene which additionally contains an intron (data not shown). However, when an intron is present, CAT enzyme levels are greatly decreased in the presence of ICP27 (data not shown). These results suggest that ICP27 also possesses a means of negatively regulating genes at a post-transcriptional level. However, the effect of ICP27 on transcription will need to be delineated before this model sytem can be used to study ICP27's post-transcriptional effects.

FIG. 5-1. Schematic representation of the *Pst*I HSV-1 genomic DNA fragment containing the ICP27 locus. Shown is a scale representation of the 6.0 Kbp *Pst*I genomic DNA fragment (1cm = 250 bp). Expanded above the *Pst*I fragment is the region located between the unique restriction enzyme sites *Bam*HI and *Sst*I. TATA box sequences (TATAA) and polyadenylation signals (A) are indicated. The bent arrow represents ICP27's transcription initiation site. The large arrows indicate the position and orientation of each gene's open reading frame. Bold lines indicate regions surrounding the ICP27 open reading frame that are maintained in the recombinant reporter viruses. Restriction endonuclease sites are indicated by single letter code: B - *Bam*HI, N - *Nae*I, P - *Pst*I, and S - *Sst*I. This *Pst*I fragment also contains a portion of the L-component HSV-1 genomic repeat region, indicated by the open box.

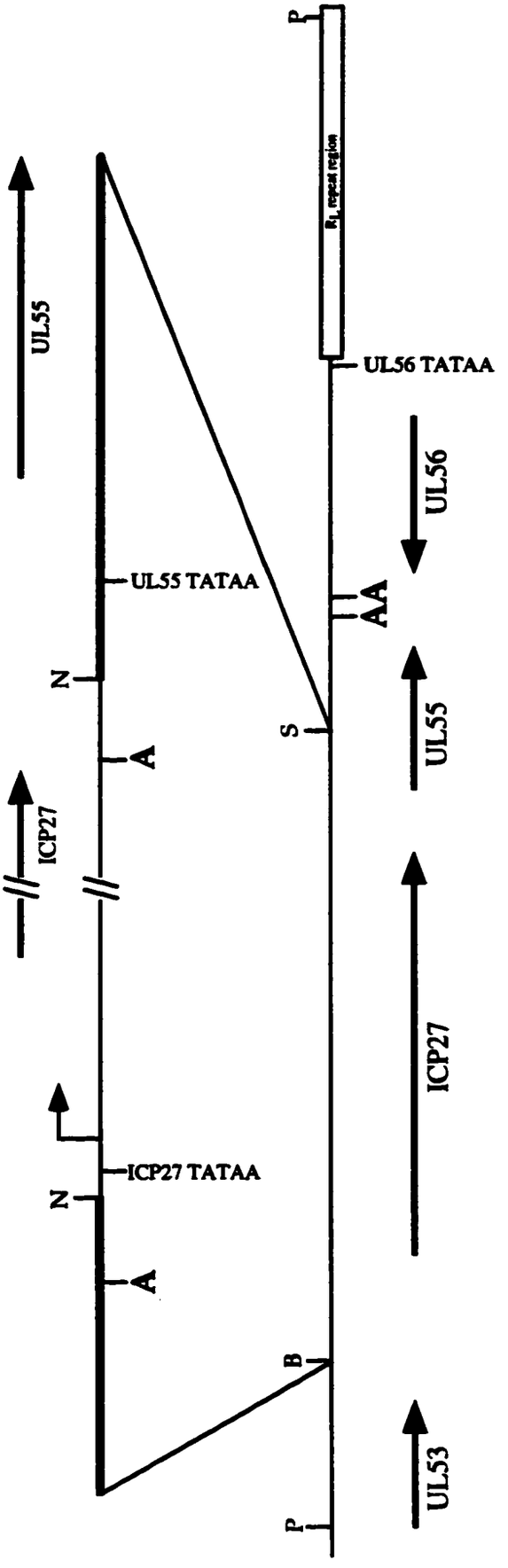

P
UL55
UL56 TATAA
UL56
UL55 TATAA
AA
N
S
A
UL55
ICP27
ICP27
ICP27 TATAA
N
A
B
UL53
P

FIG. 5-2. Structure and orientation of WT ICP27, β-galactosidase, and CAT reporter genes. Illustrated are the *Pst*I ICP27 locus containing DNA fragments expected for each virus. Lengths are in Kbp. Restriction endonuclease sites are indicated by single letter code: B - *Bam*HI, P - *Pst*I, and S- *Sst*I. Arrows indicate the orientation of each gene of interest within the ICP27 locus. The gray box indicates the ICP27-β-galactosidase fusion gene. The open boxes represent the chloramphenicol acetyltransferase open reading frame. The black boxes represent ICP27's polyadenylation signal sequence and the hatched boxes represent the minimal thymidine kinase promoter from HSV-1.

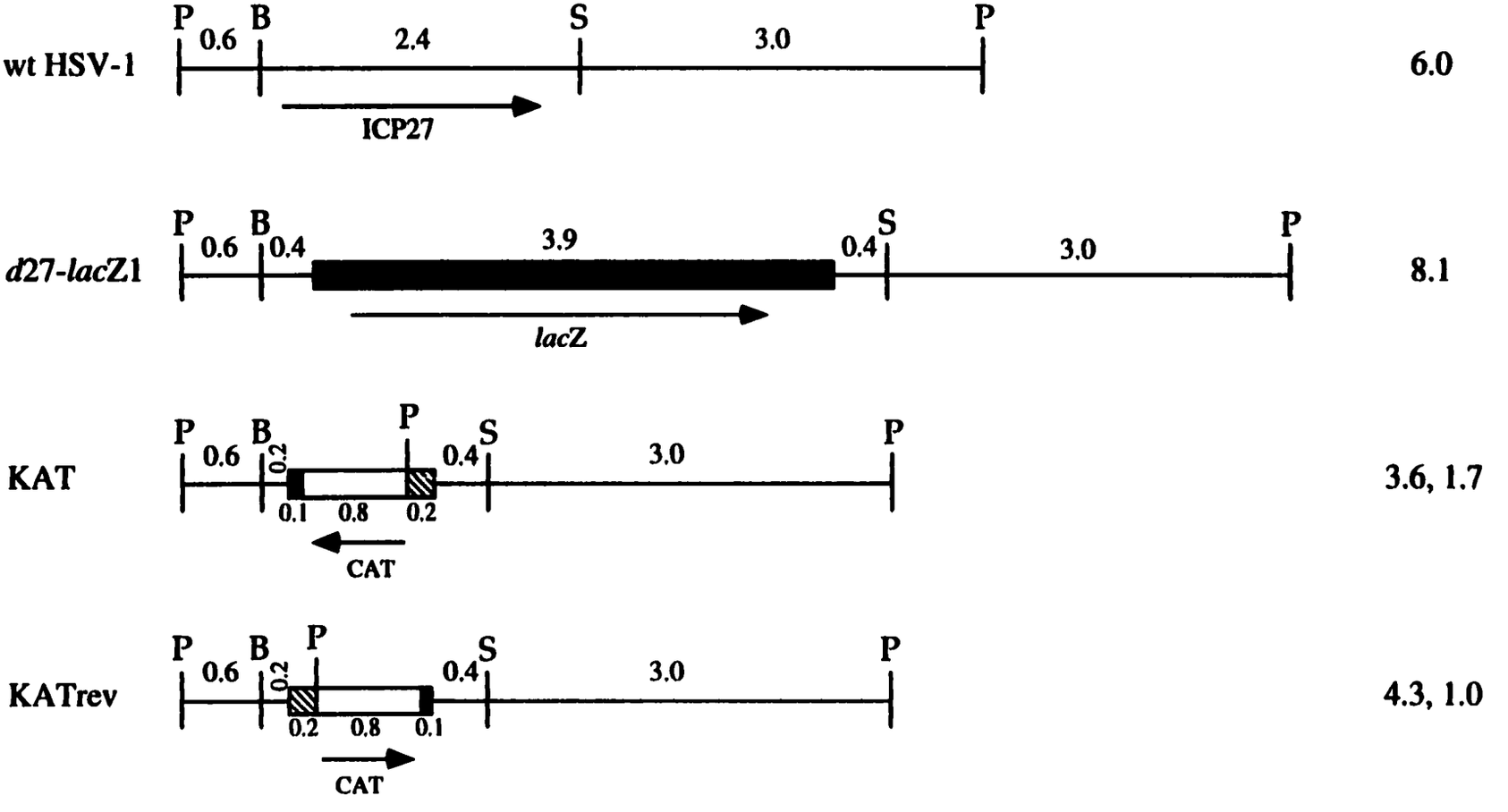

Calculated Size of
PstI fragments
wt HSV-1
P
0.6
B
2.4
S
3.0
P
ICP27
6.0
d27-lacZ1
P
0.6
B
0.4
3.9
0.4
S
3.0
P
lacZ
8.1
KAT
P
0.6
B
0.2
P
0.4
S
3.0
P
0.1
0.8
0.2
CAT
3.6, 1.7
KATrev
P
0.6
B
0.2
P
0.4
S
3.0
P
0.2
0.8
0.1
CAT
4.3, 1.0

FIG. 5-3. Southern analysis to verify the genomic structure of the KAT and KATrev viruses. HSV-1 genomic DNA collected from infected V27 cells was cleaved with *Pst*I. After transfer to a nylon filter, the DNA was probed with ^{32}P-labeled plasmid DNA possessing the 6.0 Kbp *Pst*I ICP27 gene-containing HSV-1 DNA fragment (Fig. 5-2). The positions of the various DNA size standards, represented in Kbp, are indicated along the left side of the scanned image. The asterix indicates the common *Pst*I viral DNA fragment corresponding to the U_L - R_L junction at the opposite end of the U_L region of the genome.

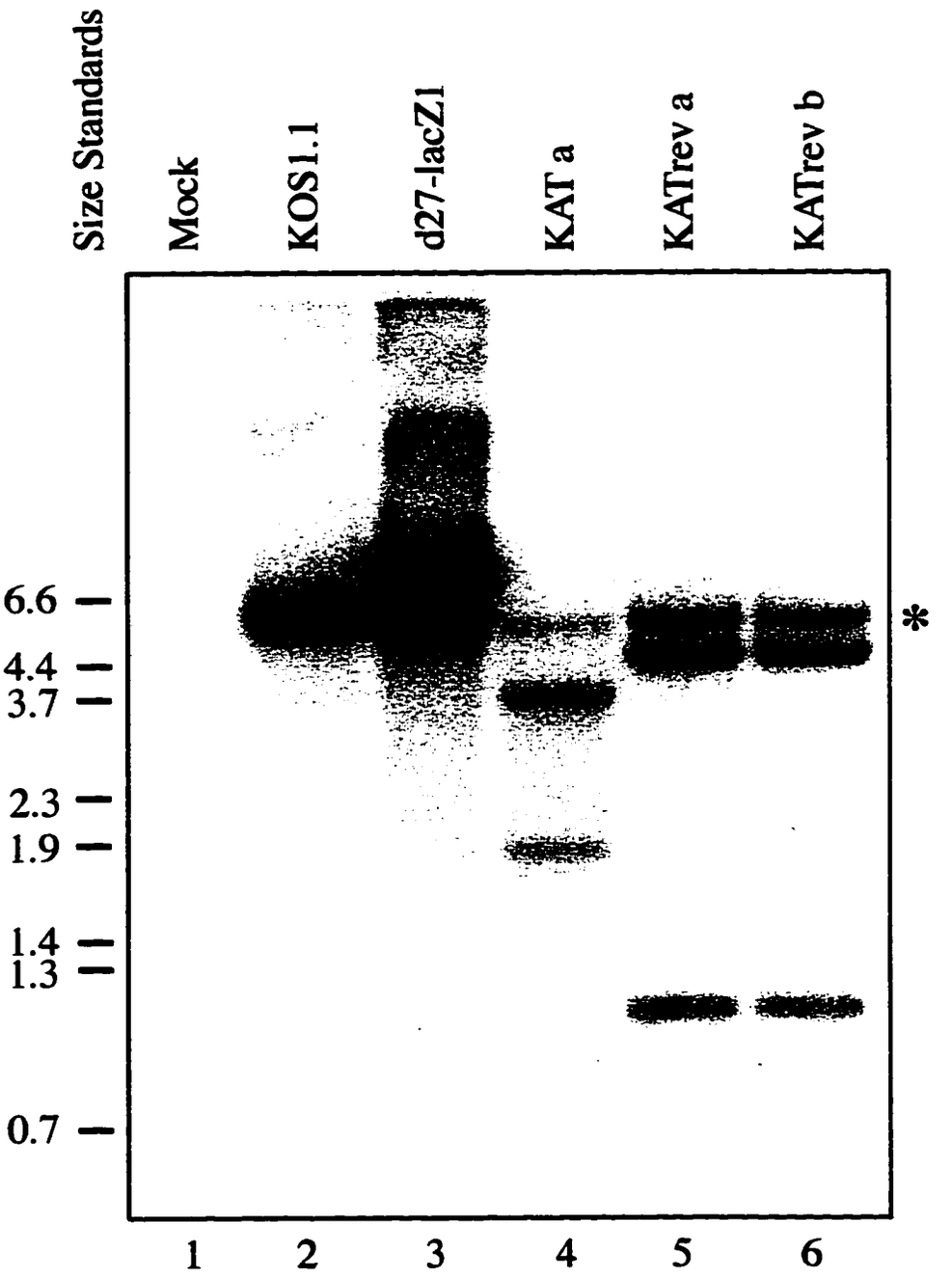
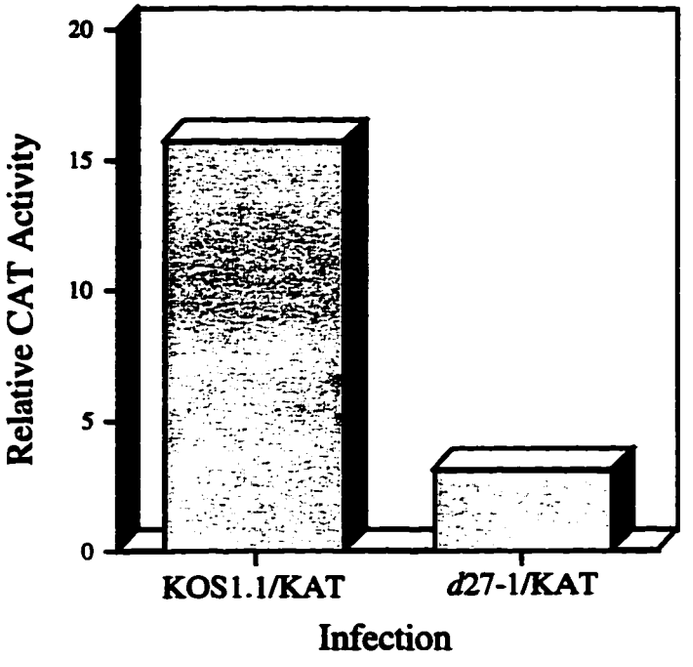
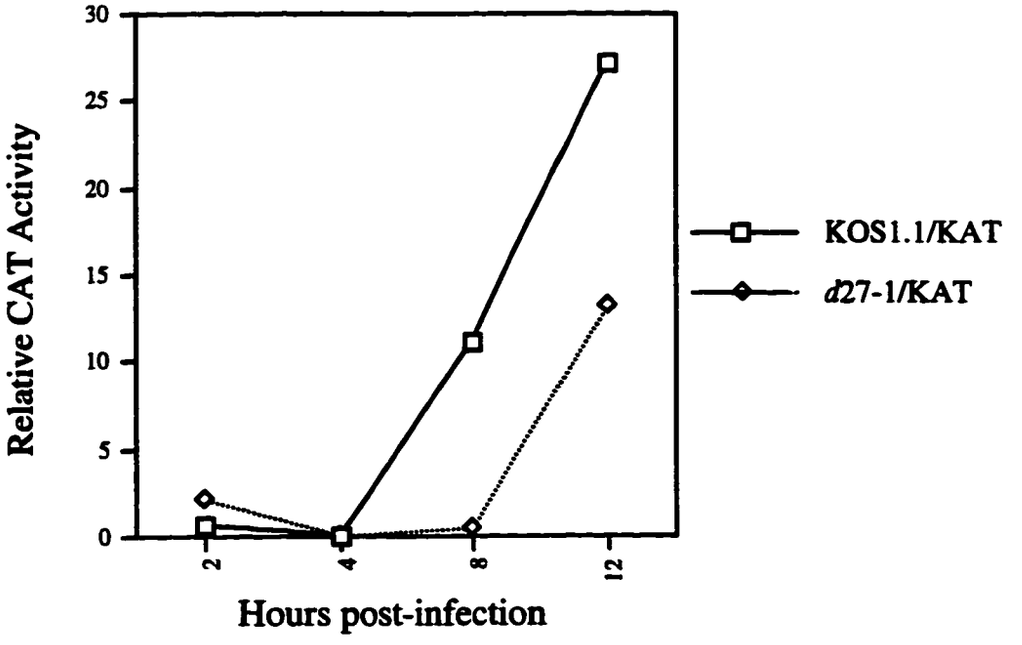

Size Standards
Mock
KOS1.1
d27-lacZ1
KAT a
KATrev a
KATrev b
6.6
4.4
3.7
2.3
1.9
1.4
1.3
0.7
*
1
2
3
4
5
6

FIG. 5-4. Levels of CAT activity in Vero cells co-infected with KAT virus and KOS1.1 or *d*27-1. (A) Graphic representation of levels of CAT activity from cell lysates of Vero cells co-infected with KAT and either KOS1.1 or *d*27-1. Samples were collected eight hpi. (B) Time course of CAT expression in KAT co-infections with KOS1.1 or *d*27-1. The open boxes represent activity observed in KOS/KAT co-infected cell lysates while the open diamonds represent the activity observed in *d*27-1/KAT co-infected cell lysates.

A.


20
15
10
5
0
Relative CAT Activity
KOS1.1/KAT
d27-1/KAT
Infection

B.


30
25
20
15
10
5
0
Relative CAT Activity
2
4
8
12
Hours post-infection
KOS1.1/KAT
d27-1/KAT

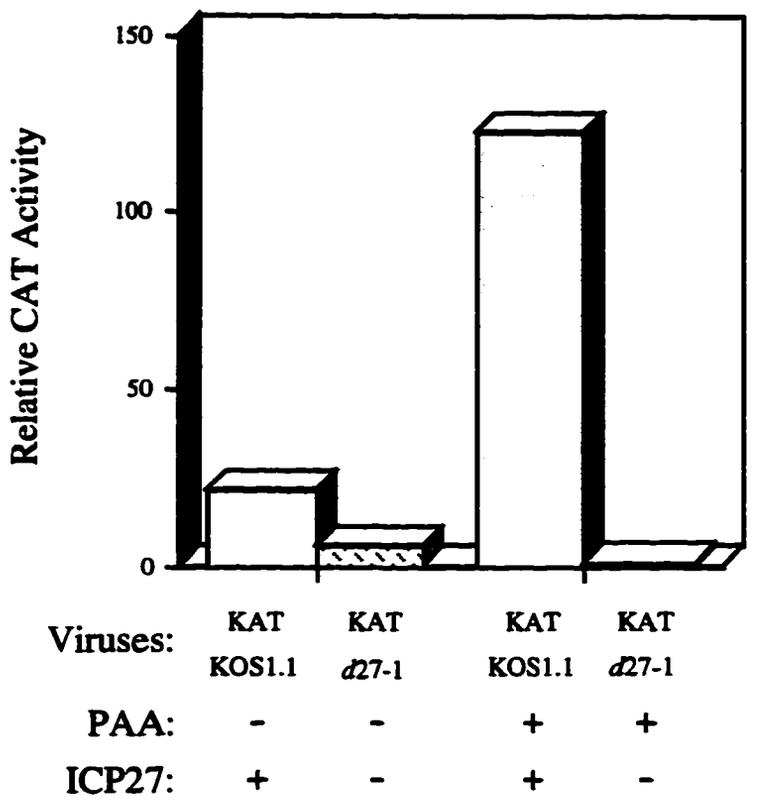
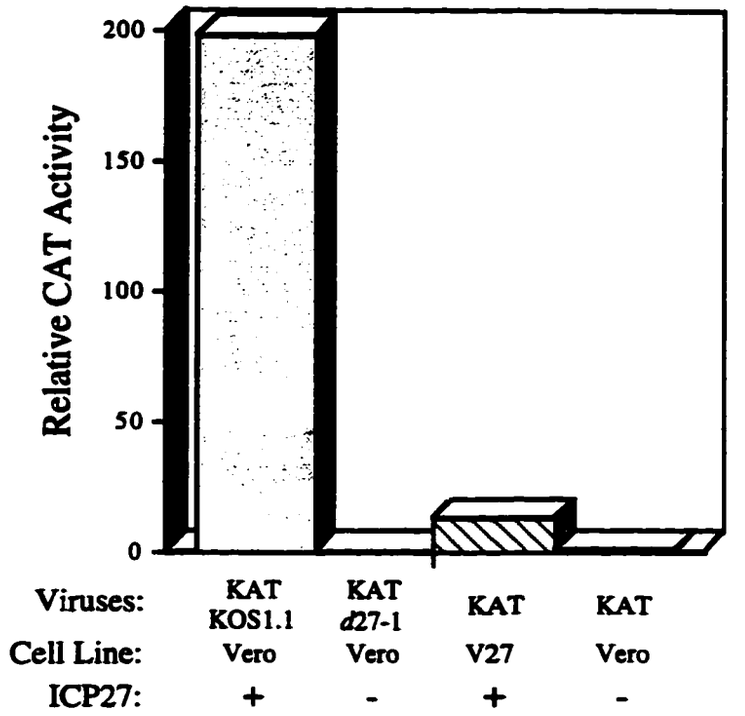


FIG. 5-5. Effect of PAA on CAT expression from the KAT virus. Vero cells were co-infected with KAT and either KOS1.1 or *d*27-1 in the presence or absence of 400 µg/ml PAA. Cell lysates were collected eight hpi and assayed for CAT activity.

FIG. 5-6. ICP27 enhances CAT activity regardless of whether it is expressed from the viral genome or from a stable cell line. (A) Relative CAT enzymatic activities observed in infected cell lysates. Infections were allowed to proceed for eight hours in the presence of 400 μg/ml PAA before collection of cell lysates. (B) Comparison of CAT enzymatic activity observed when ICP27 is provided via late passage (approximately passage 50-55) or early passage (approximately passage 19-24) V27 cells. Vero and V27 cells were infected with KAT virus in the presence of PAA. Eight hours post-infection, relative CAT activities were measured.

A.


Relative CAT Activity
200
150
100
50
0
Viruses:
KAT KOS1.1
KAT d27-1
KAT
KAT
Cell Line:
Vero
Vero
V27
Vero
ICP27:
+
-
+
-

B.

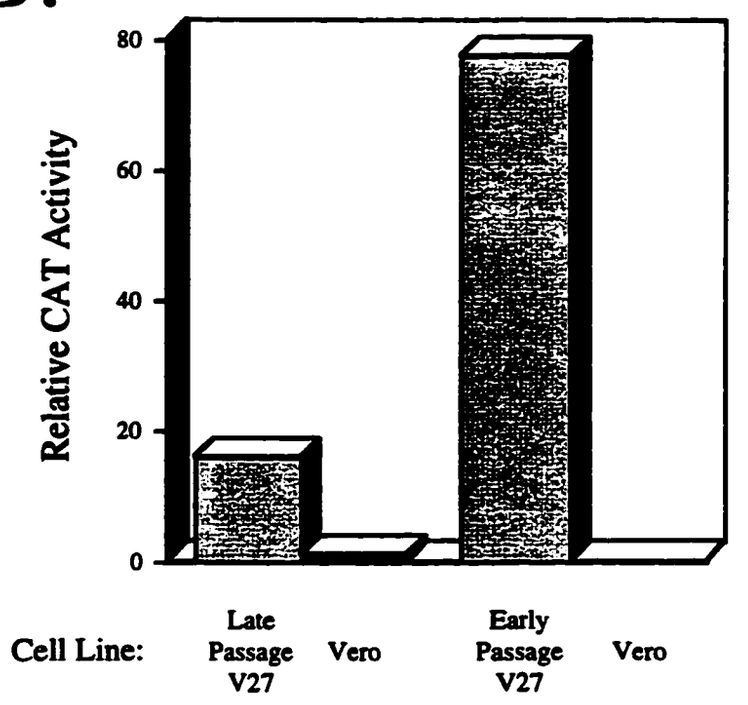

Relative CAT Activity
80
60
40
20
0
Cell Line:
Late Passage V27
Vero
Early Passage V27
Vero

FIG. 5-7. Expression of CAT RNA from the KAT recombinant virus. Vero cells were mock-infected, KOS-infected or KOS/KAT co-infected, and RNA samples were collected at various times post-infection. After separation on formaldehyde-1.2% agarose gels and transfer to nylon, the RNAs were fixed to the filters by UV-crosslinking. The blots were probed with an 800 bp ^{32}P-labeled DNA fragment consisting of the open reading frame of the CAT gene. Shown are scanned images of autoradiographs of two different northern blots of RNA obtained from Vero cells infected in the presence or absence of PAA. Lanes 1 and 8: mock-infected; lanes 2 and 9: KOS-infected; lanes 3 - 7: KOS/KAT co-infected. (A) Cells infected in the absence of PAA. A 41 hour exposure is shown. (B and C) Cells infected in the presence of PAA. Panel B represents a 41 hour exposure while panel C represents a 72 hour exposure of the same blot.

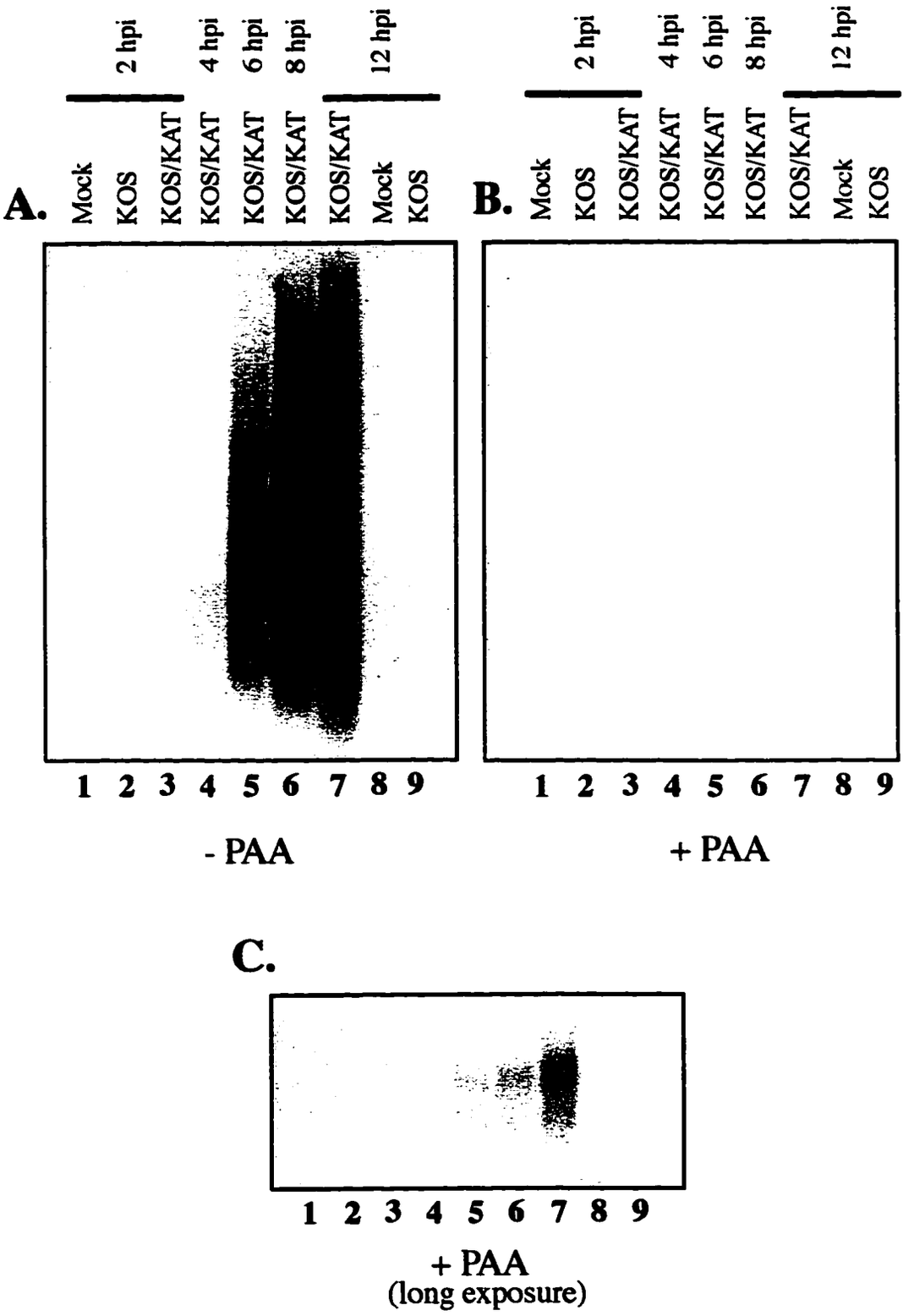

A.
B.
2 hpi
4 hpi
6 hpi
8 hpi
12 hpi
Mock
KOS
KOS/KAT
KOS/KAT
KOS/KAT
KOS/KAT
KOS/KAT
Mock
KOS
1 2 3 4 5 6 7 8 9
- PAA
+ PAA
C.
1 2 3 4 5 6 7 8 9
+ PAA
(long exposure)

FIG. 5-8. The tkCAT27 gene behaves differently than the endogenous TK gene. The same RNA preparations used in Figure 5-7 were analyzed by Northern analysis for expression of both the endogenous TK gene and the tkCAT27 gene. After transfer to nylon filters, the RNA was fixed by baking under vacuum. Filters were hybridized with a ^{32}P-labeled DNA probe directed against the TK open reading frame. Shown are scanned images of the resulting autoradiographs. Lanes 1 and 8: mock-infected; lanes 2 and 9: KOS-infected; lanes 3 - 7: KOS/KAT co-infected. (A) RNA obtained from Vero cells infected in the absence of PAA. (B) RNA obtained from Vero cells infected in the presence of PAA. The exposure time of these autoradiograms was 31 hours.

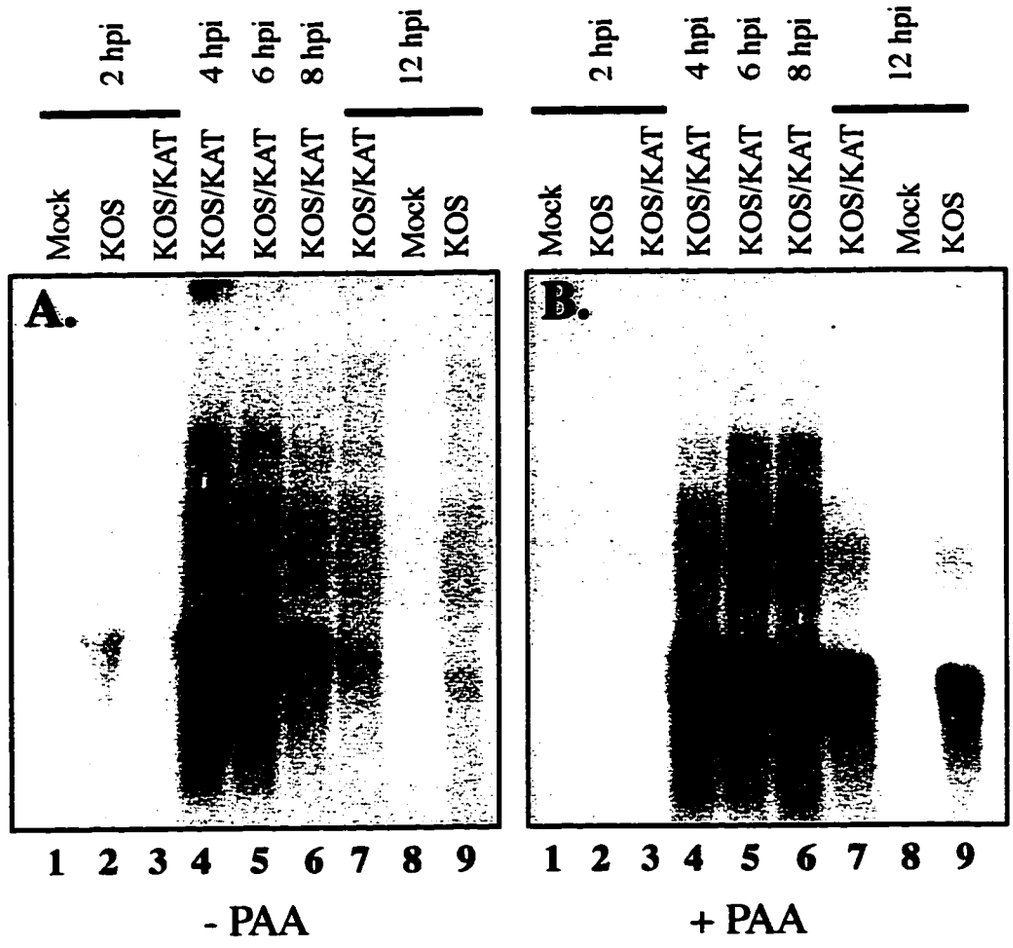
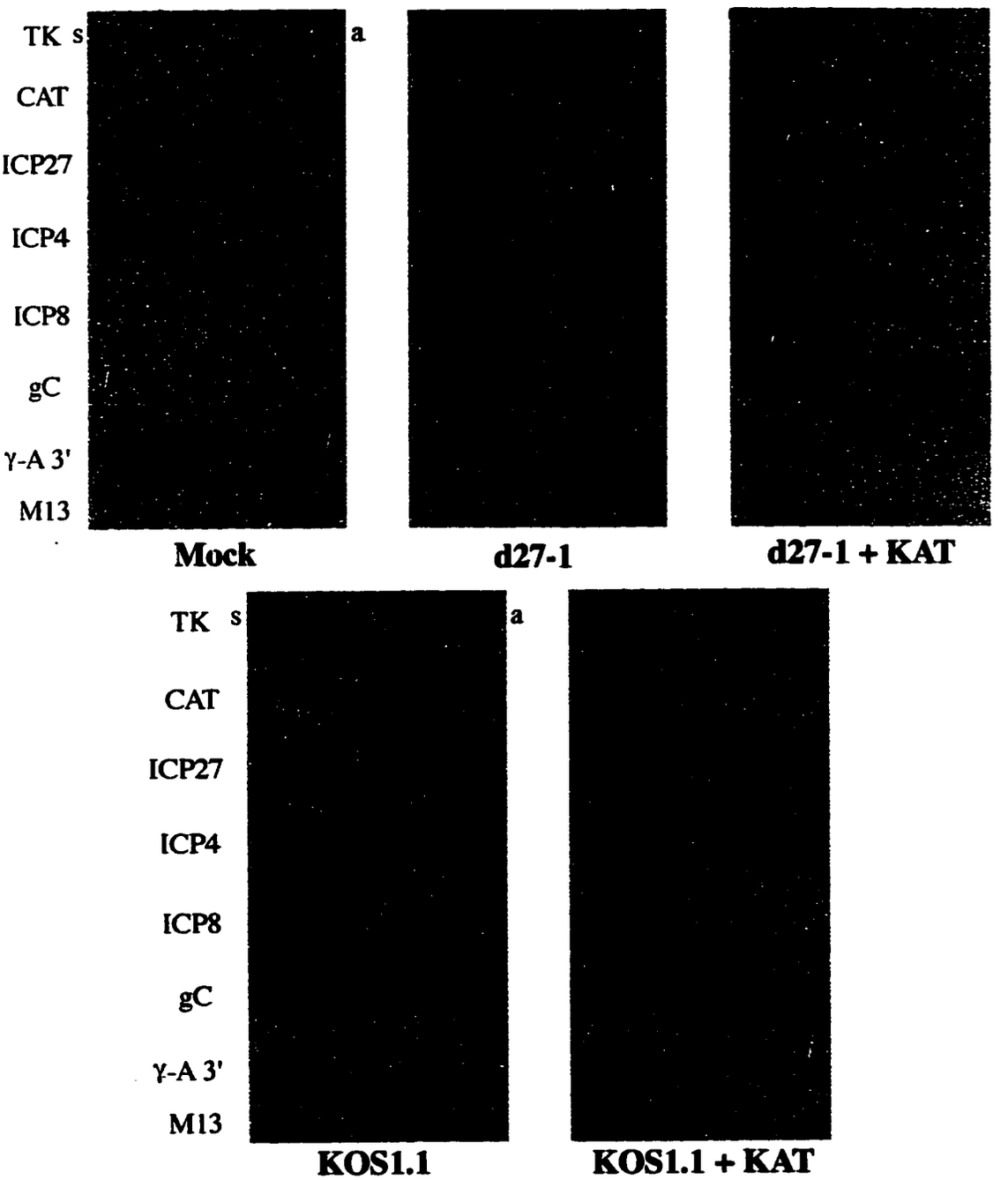

2 hpi
4 hpi
6 hpi
8 hpi
12 hpi
Mock
KOS
KOS/KAT
KOS/KAT
KOS/KAT
KOS/KAT
KOS/KAT
Mock
KOS
A.
B.
1 2 3 4 5 6 7 8 9
- PAA
+ PAA

FIG. 5-9. Nuclear run-on analysis indicates that ICP27 enhances CAT reporter gene expression at the level of transcription. Vero cells were mock-infected, infected with KOS1.1 or *d*27-1, or co-infected with KAT and KOS1.1 or *d*27-1 in the presence of 400 μg/ml PAA. Nuclei were collected eight hpi. Run-on transcription in the presence of ^{32}P-labeled UTP was performed and the resulting RNA transcripts were hybridized to nylon filters containing various single stranded DNA probes. Shown are scanned images of the autoradiographs of hybridized filters. The probes used are listed along the left side of scanned image. The left-hand side of each panel corresponds to ssDNA probes which detect sense strand transcription, while the right-hand side of each panel contain ssDNA probes which detect the corresponding anti-sense strand transcription.


TK s
a
CAT
ICP27
ICP4
ICP8
gC
γ-A 3'
M13
Mock
d27-1
d27-1 + KAT
TK s
a
CAT
ICP27
ICP4
ICP8
gC
γ-A 3'
M13
KOS1.1
KOS1.1 + KAT

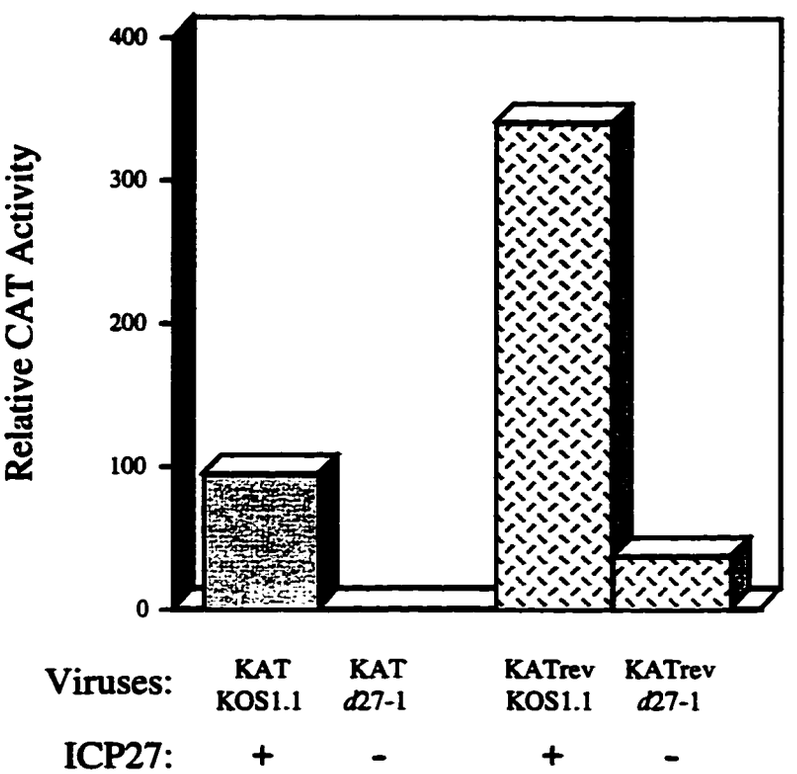


FIG. 5-10. The orientation of the tkCAT27 gene affects its basal expression but not its induction by ICP27. Vero cells were co-infected with the viruses shown in the presence of 400 μg/ml PAA. Eight hpi, cell lysates were collected and assayed for CAT activity.

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CHAPTER 6

FINAL DISCUSSION

Chapter Six - Final Discussion

The work done during the course of this thesis has demonstrated that ICP27 is a methylated RNA-binding protein which is capable of nuclear shuttling activity. We also found that it possesses a strong NLS and a NuLS in its N-terminal half. The region of the protein containing the NuLS sequence includes a stretch of arginine and glycine residues. This region is responsible for ICP27's RNA-binding ability and is required for its post-translational methylation. In addition, we have demonstrated that ICP27 can affect the expression of a viral genome based reporter gene by inducing its transcription.

It is apparent that ICP27 controls gene expression both transcriptionally (shown here) and post-transcriptionally (1,18,19,39). The data collected in this thesis provide us with the opportunity to speculate on how ICP27 may perform both transcriptional and post-transcriptional roles during infection.

I. ICP27's Novel Properties: Mechanistic Implications and Further Experiments

ICP27 directly interacts with RNA. Research done in the course of this thesis demonstrates that ICP27 interacts with RNA *in vitro*. While these studies were in progress, two other groups also demonstrated interactions between ICP27 and RNA (1,10). We determined that ICP27's RGG box can mediate RNA-binding, similar to the RGG box sequence of the cellular hnRNP U protein (15). We also found that the C-terminal zinc finger-like domain in ICP27, which is capable of binding ssDNA (43), is not involved in the RNA-binding that we observe.

It is not yet known if ICP27 binds to a specific RNA sequence or structure. It has been suggested that the mRNA stability determining AU-rich 3' UTR regions of some cellular genes are ICP27 RNA-binding targets (1). Other work argues against this hypothesis, suggesting that there may not be a highly specific nucleic acid sequence with

which ICP27 interacts (10, Chap. 3;21). If ICP27 is a hnRNP-like protein (discussed below), we speculate that it may interact relatively non-specifically with RNA.

Further studies to identify nucleic acid sequences or structures to which ICP27 might preferentially bind would be of benefit. There are several possible strategies to determine RNA-protein interactions. For example, a variety of RNA transcripts can be transcribed *in vitro*, in the presence of biotinylated rCTP. The resulting biotinylated transcripts can be combined with *in vitro* translated ^{35}S-labeled ICP27 and interactions between the two can be observed utilizing pull-down assays. It may also be possible to utilize mobility shift analysis using different classes of cellular RNA species, (e.g. snRNA, pre-mRNA, tRNA, rRNA) combined with recombinant ICP27 to narrow down possible binding targets of ICP27. Analysis involving UV-crosslinking between recombinant ICP27 and RNA transcripts has already been used by Ingram *et al.* (10) to investigate ICP27 interactions with viral mRNA sequences encoding poly(A) sites, introns, and coding regions of particular genes. In this study (10), ICP27 interacted with all species of RNA tested, further supporting the idea that ICP27 may interact relatively non-specifically with RNA.

ICP27 is methylated *in vivo*. We discovered that ICP27 is post-translationally modified at one or more amino acid residues by the addition of methyl groups. Such modification is seen in several hnRNP proteins, including hnRNP A1. HnRNP A1 is methylated on arginine residues located within its RGG box domain (16,32,45). Similarly, we observed that the *in vivo* methylation of ICP27 requires ICP27's RGG box. Our data indicate that a cellular protein methyltransferase is likely responsible for methylation of ICP27, as ICP27's RGG box can be methylated *in vitro* in a rabbit reticulocyte extract. It should be possible to use peptide mapping to determine the specific amino acid residue or residues in ICP27 which are methylated. As arginine is the only amino acid residue in ICP27's RGG box which is known to accept methylation (5), it is quite likely that one or more arginine residues are the target for methylation. It may be

possible to identify which amino acid residue(s) is methylated by mutating each of the arginine residues in this region, for example, to a glycine or lysine residue. If we can identify the exact amino acid residue(s) that is methylated, and prevent ICP27 methylation by mutation of that codon, it may be possible to demonstrate the function of ICP27 methylation *in vivo*.

The function of this post-translational modification of ICP27 remains uncertain. In fact, the role of arginine methylation in any protein is unknown. As it appears to be the RNA-binding domain of ICP27 which is modified, perhaps this methylation is a means by which RNA-binding can be regulated. Binding studies using recombinant unmethylated versus recombinant methylated hnRNP A1 protein did not demonstrate a dramatic difference in RNA-binding ability between the two types of molecules (33). However, in this work, the methylated protein fraction was a mixture of methylated and unmethylated protein, possibly obscuring an effect of methylation. We anticipate that it will be possible to use our rabbit reticulocyte-derived ICP27 to produce methylated and unmethylated ICP27 for use in *in vitro* RNA-binding experiments. This could be done by translating ICP27 or various ICP27 mutants in the presence or absence of L-homocysteine, an analog of SAM, which is a potent inhibitor of protein methyltransferases. Preliminary analysis indicates L-homocysteine inhibits all protein methylation in our rabbit reticulocyte lysates (not shown), but does not affect protein synthesis.

ICP27 is a nuclear shuttling protein. We have demonstrated that ICP27 is a nuclear shuttling protein, similar to several other nuclear proteins, including hnRNP proteins. HnRNP A1 and several other shuttling proteins possess specific NES sequences required for their shuttling ability (24). It is not known yet whether ICP27 has a similar NES. To determine if ICP27 possesses an NES, it may be helpful to utilize some of the ICP27-PK fusion genes originally constructed to delineate ICP27's NLS. Those constructs possessing ICP27's strong NLS could be used in our interspecies heterokaryon

assay to map the region of the ICP27 required for nuclear export. NES sequences could also be delineated using the "NES mapping" strategy developed by Michael *et al.* (24). These investigators have developed plasmids expressing the nucleoplasmin NPc domain coupled to either a bipartite NLS or the SV40 large T antigen NLS. These proteins cannot shuttle, allowing the vectors to be used for the identification of NES's.

Additional experiments could be done to further characterize ICP27's nuclear export activities. As active export requiring an NES is a temperature-dependent process (24), it will be of interest to determine if this process is temperature-dependent. Preliminary experiments (not shown) involving interspecies heterokaryons maintained at reduced temperatures have provided ambiguous results in regard to this question. It is also interesting to note that the nuclear import of hnRNP A1 and some other hnRNP proteins is transcription-dependent (28). By inhibiting *in vivo* transcription with actinomycin D, the nuclear shuttling of hnRNP A1 can be easily observed as the protein is exported but cannot be reimported and thus accumulates in the cytoplasm. Treatment of cells with actinomycin D also leads to the cytoplasmic accumulation of the HIV Rev protein, demonstrating its shuttling capacity (12,23). However, preliminary experiments using actinomycin D suggest that ICP27 does not require trancription for its entry into the nucleus (not shown).

ICP27 localizes to the nucleolus of the cell. Our laboratory has intriguing results with respect to the intranuclear localization of various ICP27 mutants. While WT ICP27 exhibits staining throughout the nucleus, including nucleoli, several ICP27 mutant proteins, including 406R, M11, M15, M16, *d*1-2, and *d*4-5 (33, Chap. 2; 22, personal observation), exhibit nucleoplasmic but non-nucleolar staining. Two other ICP27 mutant proteins, n263R (35) and M16R (S. Bunnell, personal communication), display the opposite phenotype, that is, they are found throughout the nucleoplasm but are preferentially localized to the nucleoli.

Interestingly, the nucleolar localization of ICP27 correlates with its function, in that proteins that localize to the nucleolus appear to be more functional than those that do not. The evidence for this is two-fold. First, nucleolar-localized versions of ICP27 result in more gene expression and virus growth in the context of recombinant viruses. For example, the *n*263R virus mutant, although deficient for growth, expresses viral genes better than *n*406R, which express a longer but non-nucleolar version of ICP27 (36). The M16R virus mutant, which only expresses the first 217 amino acid residues of ICP27, is able to grow in Vero cells (S. Bunnell, personal communication). These mutants suggest that the N-terminal region of ICP27 can supply sufficient function to allow virus growth. The preferential nucleolar localization of these N-terminal truncation mutants may be related to this function.

The second line of evidence that indicates a possible important role of nucleolar localization relates to shuttling. The efficiency with which ICP27 is able to shuttle appears to correlate with its ability to localize to the nucleolus. To date, all mutant ICP27 polypeptides which are partially or wholly deficient in shuttling tend to be excluded from nucleoli, whereas the WT protein and mutant polypeptides which are localized throughout the nucleus are capable of shuttling. It would be interesting to see if the d4-5 protein is capable of nuclear shuttling as this protein is non-nucleolar *in vivo* and does not bind RNA *in vitro*. However, since the d4-5 protein possesses a high level of cytoplasmic staining, we are unable to test it in the interspecies heterokaryon assay, preventing any conclusions with respect to its shuttling ability. It will also be interesting to see if the 263R and the M16R proteins are able to shuttle, as these proteins preferentially localize to nucleoli. Together, the data indicate that ICP27's nucleolar localization correlates with its shuttling ability, suggesting a possible role for the nucleolus in this biological phenomenon.

It is interesting to speculate that the nucleolar localization of WT ICP27 and truncated versions of ICP27 which locate preferentially to the nucleolus is due to specific

interactions between ICP27 and components of the nucleolus. It is possible that ICP27's nucleolar localization results from binding of its RGG box to rRNA or pre-rRNA. Ribosomal RNA is a major component of the nucleolus as this is where its transcription, processing, and incorporation into ribosomal sub-units occurs (reviewed in ref. 41). We found that the *in vitro* translated ICP27 truncation mutant 189R possesses enhanced poly (G) binding activity. If ICP27 can interact with rRNA, we speculate that 189R could have enhanced binding to rRNA and might therefore demonstrate a preferential nucleolar localization. Conversely, it is interesting to speculate that the M16R protein, which demonstrates preferential nucleolar localization, may also display enhanced RNA-binding activity *in vitro*.

Another HSV-1 RNA-binding protein (37,38), US11, also localizes to the nucleoli of infected cells (17). This late gene product (11) enters the cell with the virion (39) and appears to post-transcriptionally affect viral gene expression (6,7,38). It possesses characteristics similar to the retroviral post-transcriptional gene regulators Rex and Rev, including the ability to bind to Rex- and Rev-responsive elements and transactivate retroviral envelope glycoprotein expression (7). The exact function of US11 is unknown. However, it is possible that it may work in concert with the ICP27 at late times during infection to regulate gene expression.

ICP27 regulates transcription. We have demonstrated that ICP27 can induce the transcription of a reporter gene incorporated into the HSV-1 genome. Thus, our data indicate that ICP27 exhibits both post-transcriptional and transcriptional activities. Our results, however, do not provide evidence as to whether ICP27 directly affects transcription or if it acts indirectly. These two possibilities are considered below.

It is possible that ICP27 induces the expression of a sub-class of DE genes by directly interacting with the cellular transcription machinery or with other viral transcriptional activators. Recently, it was shown that ICP27 can be found in a complex with ICP4 and the ICP4 consensus DNA-binding site (26). This interaction suggests the

possibility that ICP27 may be present on ICP4-containing promoter complexes on viral genes. Recent studies directed at whether ICP27 interacts with components of the cellular transcriptional machinery provide evidence in support of this model. Preliminary data reported at the 21st Herpesvirus Workshop suggest that ICP27 interacts with the cellular transcription factors TFIIB and TBP in both *in vitro* pull-down assays and *in vivo* yeast two-hybrid assays (2).

It is also possible that ICP27 indirectly affects the transcription of viral genes. As previously discussed, ICP27 affects the cellular location of ICP4 and ICP0 (46,47). It also affects the post-translational modification of ICP4 (20,26,34,42). It is therefore possible that ICP27 indirectly modulates transcription by affecting the intracellular localization or post-translationally modified state of ICP4 or other transcriptional activators.

Continued research into the necessary *cis*-elements, *trans*-factors, and the regions of ICP27 responsible for ICP27's regulation of gene transcription is required. The model system we have constructed will be useful to help determine which *cis*-elements are required for ICP27-induced transcription. Manipulation of the tkCAT27 reporter gene promoter, coding region, and poly(A) site will help delineate the relevant nucleic acid sequences responsible for ICP27's transcriptional effects. Transcriptional control may also result from genomic sequences which flank the reporter gene. Using S1 and RNase protection analysis, the reporter gene transcripts produced in the presence and absence of viral DNA synthesis could be compared. Such analysis would provide insight into the nature of those CAT-containing messages induced by ICP27 early in infection versus those heterogeneous species apparently produced at later times. The potential differences between these messages may provide evidence, for example, as to whether ICP27 is required for proper intiation of transcription of these reporter genes. Finally, by co-infecting Vero cells with the parental reporter gene viruses and the variety of ICP27 mutant viruses previously produced by our laboratory, it should be possible to identify

which region(s) of ICP27 is required for this transcriptional regulation of gene expression.

II. Models of ICP27 Function

Much research has focussed on ICP27's ability to post-transcriptionally regulate gene expression, down-regulating intron-containing genes while up-regulating genes possessing certain poly(A) sites. However, the exact mechanism by which ICP27 performs these functions remains unknown. Two of the most intriguing findings in this thesis are that ICP27 possesses a RNA-binding domain within its N-terminal region, and that ICP27 is a nuclear shuttling protein. From the work done here, in conjunction with results from other research groups, we propose two possible mechanisms of post-transcriptional action by ICP27.

A. Model 1 - ICP27 is an hnRNP protein

Similarities between ICP27 and cellular hnRNP proteins. As discussed previously, ICP27 displays many characteristics in common with several cellular hnRNP proteins. These include the ability to directly interact with RNA, the possession of an RGG box domain, post-translational modification by methylation, and nuclear shuttling (reviewed in ref. 3,9,30). In addition, ICP27's ability to affect gene expression post-transcriptionally suggests that ICP27 may directly interact with the pre-mRNA machinery or with pre-mRNA itself. Based on these similarities to cellular hnRNP proteins, we propose that ICP27 may be a viral hnRNP protein.

Cellular hnRNP proteins. HnRNP proteins are those proteins that form complexes with heterogeneous nuclear RNAs (hnRNAs) in the nucleus. Nascent pre-mRNA, post-chromatin nucleoplasmic pre-mRNA, splicing intermediates, and mRNA are all sub-populations of hnRNA with which hnRNP proteins associate. At least 20 different cellular hnRNP proteins, designated A1 to U, have been identified (reviewed in ref. 9,30). These highly abundant proteins, ranging in size from 34-120 kDa, demonstrate

a general nucleoplasmic localization excluding the nucleoli under normal cellular conditions. Together with snRNPs, hnRNP proteins participate in pre-mRNA splicing, as demonstrated by studies using monoclonal antibodies directed against the hnRNP C proteins (reviewed in ref. 9). From electron microscopy studies, some hnRNP proteins appear to be exported across the nuclear membrane in association with mRNA (29). It is known that some hnRNP proteins remain associated with mRNA while in the cytoplasm of the cell. Recently, a *C. tentans* protein related to the mammalian hnRNP A1 protein was found to be transported across the nuclear pore in association with mRNA and to remain associated in a mRNP complex within the cytoplasm of the cell (44). From all of these findings, it has been speculated that some hnRNP proteins may be directly involved in the nuclear export of mRNA into the cytoplasm. Several roles for these proteins once they are in the cytoplasm have been proposed. These include activities associated with mRNA stability, mRNA localization, and/or regulation of mRNA translation (reviewed in ref. 30,44).

ICP27 could act as a viral hnRNP-like protein. Based on the physical and functional similarities between the hnRNP proteins and ICP27, we speculate that ICP27 may be a component of hnRNP complexes, either replacing a cellular component necessary for cellular transcript processing or biasing the complex in some way to promote viral gene expression. It is also possible that ICP27 disrupts the cellular hnRNP complex resulting in preferential viral gene expression. One way to test these hypotheses is to determine directly if ICP27 is associated with hnRNP complexes in infected cells. Isolation of cellular hnRNP protein complexes can be accomplished by immunopurification of labeled cellular nuclear fractions with a monoclonal antibody directed against hnRNP A1 or other hnRNP proteins (30). The proteins within this complex can be separated and identified using conventional two-dimensional SDS-PAGE. In fact, this type of analysis has helped lead to the identification of over 20 hnRNP proteins (4,31). To see if ICP27 is a part of the cellular hnRNP complex, a

variety of monoclonal antibodies directed against the hnRNP proteins can be used to immunoprecipitate the hnRNP complex from nuclear fractions of cells infected with HSV-1. If ICP27 is a component of the cellular hnRNP complex, one would expect it to be isolated from infected cell lysates along with the other hnRNP proteins.

B. Model 2 - ICP27 regulates translation

A possible role for ICP27 in the cytoplasm. As discussed above, ICP27 shuttles between the nucleus and cytoplasm of the cell. In recent years, some hnRNP proteins which are known to shuttle have been suggested to aid in the transport of mature transcripts to the cytoplasm to be translated. Although several observations have suggested nuclear roles for ICP27, it is also possible that ICP27 possesses a specific role in the cytoplasm, other than as a transporter of mRNA. We have observed by indirect immunofluorescence that as WT HSV-1 infection proceeds, the level of ICP27 within the cytoplasm of infected cells increase. It is interesting that the ICP27 homolog of VZV, ORF4, exhibits a predominantly cytoplasmic localization in infected cells (25), suggesting that ORF4 and perhaps ICP27 have a cytoplasmic function.

To investigate potential roles for ICP27 within the cytoplasm, it would be useful to construct a non-nuclear version of ICP27. Although we found that ICP27 possesses a strong NLS similar to classical bipartite signals (reviewed in ref. 8), elimination of this signal does not completely eliminate nuclear localization. This suggests that ICP27 possesses multiple NLS sequences. For this reason, a non-nuclear version of ICP27 could not be constructed, preventing conclusions as to whether nuclear localization is required for any or all of ICP27's effects during infection. However, data discussed below provide several pieces of evidence that suggest that ICP27 possesses a significant role within the cytoplasm.

Several years ago, Rice and Knipe (35) observed that ICP4 protein synthesis was elevated at late times in cells infected with ICP27 deletion and truncation mutants. However, the levels of ICP4 mRNA were equivalent to the levels in WT-infected cells.

From these results, it was concluded that ICP27 negatively regulates ICP4 expression. We hypothesize, as originally suggested by Rice and Knipe (35) that ICP27 may affect the translation of the ICP4 and possibly other viral transcripts. Although evidence of gene regulation at a translational level by HSV-1 has not been documented, translational regulation of viral gene expression was recently reported in the HCMV virus (14). Given ICP27's RNA-binding activity, it is interesting to speculate that ICP27 may interact with the ICP4 transcript or mRNP complex diverting it away from the ribosomes or preventing its translation in some way.

Possible interaction with proteins associated with translation. Recently, ICP0 was observed to interact with the translation elongation factor EF-1δ, suggesting that it may possess a role in regulating the translation of viral mRNA (13). Although it is unknown if ICP27 and ICP0 directly interact, ICP27 does affect the nuclear localization of ICP0. In the presence of ICP27, ICP0 possesses a cytoplasmic localization which is not apparent in the absence of ICP27. ICP27 could indirectly affect translation of viral and/or cellular genes by affecting the location of ICP0.

The mechanism by which ICP27 affects ICP0 localization has not yet been determined. It is unknown if ICP0 possesses shuttling activity. However, as ICP27 affects ICP0 location, it is possible that ICP0 shuttles in association with ICP27. Investigation of ICP0 shuttling in the presence of ICP27 is not feasible using our cell fusion technique since ICP0 is found within the cytoplasm of cells when ICP27 is present. However, ICP0 shuttling in an ICP27 deletion mutant or shuttling of transiently expressed ICP0 can be tested using the interspecies heterokaryon assay, as under these conditions, ICP0 is expected to be nuclear. If ICP0 is unable to shuttle in the absence of ICP27, this suggests that ICP27 may be responsible for ICP0's export from the nucleus. We speculate that ICP27 may indirectly affect translation elongation via modification of ICP0.

ICP27's RGG box is both a significant part of ICP27's NuLS sequence and is required for ICP27's RNA-binding activity. From these observations, we speculate that ICP27 may interact with rRNA (discussed previously) or with other components of the translational machinery, such as ribosomal subunits. Perhaps as infection proceeds, these interactions result in the export of ICP27 from the nucleus with the ribosome. An interaction of ICP27 with cytoplasmic ribosomes may effect their protein synthetic capacity thereby affecting the translation of mRNAs within the cell.

To determine if ICP27 associates with cellular ribosomes during infection, sedimentation assays of HSV-1 infected cell lysates, similar to that done for the HSV-1 US11 protein (6), can be performed. If ICP27 does interact with components of the ribosome, it may be possible to identify the region of ICP27 required for this interaction using the array of ICP27 mutant viruses constructed in our laboratory. These experiments could help determine if ICP27 plays a role in translation through interaction with components of the ribosome.

C. Summary of post-transcriptional models.

The two models presented above, that ICP27 is a viral hnRNP protein, and that ICP27 regulates viral gene expression at the level of translation, are not mutually exclusive. Some hnRNP shuttling proteins are believed to possess functional roles within the cytoplasm (reviewed above and in ref. 9,30,44). These putative functions include roles in mRNA localization, stability, and translation. In fact, it is possible that an hnRNP protein could help direct specific mRNA transcripts to the ribosomes. It has been demonstrated that ICP27 has a role in the retention of viral intron-containing transcripts within the nucleus (27). Interestingly, the *in situ* hybridzation data presented in that report show an interaction between the RNA probes used and the nucleoli of the infected cells. This interaction could be a non-specific one between rRNA and the riboprobes used. However, it is also possible that the riboprobe binding may indicate the presence of

viral transcripts within the nucleoli of the cell. Possibly, ICP27 is associated with these transcripts regulating their transport.

III. ICP27 is a Complex Multifunctional Regulatory Protein

The findings over the past six years have made it even more apparent that ICP27 is an extremely complex molecule. Although it can affect gene expression by post-transcriptional mechanisms, our findings demonstrate that it can also regulate transcription. The complexities that we have identified in its nuclear and cytoplasmic cellular localization further support the idea that ICP27 possesses many functions. It may be possible to elucidate some of these functions or the mechanisms involved by utilizing a system in which ICP27 expression can be regulated in the absence of other viral proteins. A stable cell line containing ICP27 under the control of a tetracycline-regulatable promoter is currently under construction in our laboratory. Expression of ICP27 could be induced in this cell line, allowing investigation of the effects of ICP27 on the cell itself in the absence of other viral proteins. Such an inducible cell line would be a powerful tool for understanding ICP27's complex effects on the host cell.

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