Effects of HSV-1 Infection on Disparate Cell Signaling Pathways

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Abstract

DEVON ARCH GREGORY: Effects of HSV-1 infection
on disparate cell signaling pathways.
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Herpes simplex virus (HSV) commandeers cellular machinery in order to replicate. Two pathways which are utilized by the virus are the NFκB pathway and the DNA double strand break (DSB) response. The NFκB pathway is activated in a sustained manner following infection. This activation leads to a suppression of apoptosis in certain cell types and may also assist in viral gene expression. The most common route of activation for NFκB is through the IKK complex and IκB. This dissertation examines the NFκB pathway upstream of IκB, characterizing the role of IKKα and IKKβ, the two kinase subunits of the IKK complex. Both were found to be necessary for NFκB activation, though at different junctions in the pathway. Potential upstream effectors of the IKK complex are also characterized. PKR, NIK and Tak1 are kinases that activate the IKK complex following various normal cellular stimuli. None play a role in activation following HSV infection however, suggesting a unique method of activation by HSV.

The cellular DSB response normally recognizes breaks in cellular DNA in order to repair the damage and initiate cell cycle arrest or apoptosis to maintain the integrity of the cell and organism. This dissertation examines the machinery involved in the initial detection and signaling of the DSB response following HSV infection. HSV activates the DSB response dependent upon viral DNA replication. Though downstream signaling in the pathway is not essential for viral replication, it does contribute to the efficacy of replication.

Curiously, Mre11 was seen to be lost following infection. Mre11 is a component of the MRN complex which initially detects DSBs. Its loss suggests that the DSB response is inhibited and so the mechanism for its loss was pursued. Mre11 loss was not dependent upon ICP0, nor the activities of the proteasome or lysosome. Viral DNA replication was required for Mre11 loss, though not late gene expression. Mre11 loss was observed following activation of the DSB response with camptothecin, suggesting that its loss following infection is a result of an endogenous cellular mechanism.

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ActD	Actinomycin D
AT	Ataxia Telangiectasia
ATCC	American Type Culture Collection
A-TLD	Ataxia Telangiectasia-Like Disorder
ATM	Ataxia Telangiectasia Mutated
ATMi	KU-55933, ATM inhibitor
ATP	Adenosine tri-phosphate
ChIP	Chromatin IP
CPT	Camptothecin
CycloX	Cycloheximide
DMEM	Dulbecco's Minimal Essential Medium
DN	Dominant Negative
DNA	Deoxyribonucleic Acid
DNA-PKcs	DNA dependent Protein Kinase catalytic subunit
DNS	Data Not Shown
DSB	Double Strand Break
E	Early
EBV	Ebstein-Barr Virus
eIF2α	eukaryotic translation Initiation Factor 2α
EMSA	Electrophoretic Mobility Shift Assay
EtOH	Ethanol
Fig	Figure

gB, gC, ...	Glycoprotein B, Glycoprotein C, ...
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
HFK	Human Foreskin Keratinocyte
hpi	hours post infection
HR	Homologous Recombination
HSV	Herpes Simplex Virus
HSV-1	Herpes Simplex Virus Type 1
HSV-2	Herpes Simplex Virus Type 2
ICP	Infected Cell Protein
IE	Immediate Early
IF	Immunofluorescence microscopy
IκB	Inhibitor of κB
IKK	Inhibitor of κB Kinase
IL	Interleukin
IP	Immunoprecipitation
KSHV	Kaposi Sarcoma-associated Herpes Virus
L	Late
LTR	Long Terminal Repeat
LPS	Lipopolysaccharide
MEF	Mouse Embryo Fibroblast
MOI	Multiplicity of Infection
MRN	Mre11/Rad50/Nbs1

mRNA	messenger RNA
NBS	Nijmegen Break Syndrome
NFκB	Nuclear Factor κB
NHEJ	Non-homologous End Joining
PAA	Phosphonoacetic acid
PAGE	Polyacrylamide Gel Electophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pfu	plaque forming units
PGA1	Prostaglandin A_1
PKR	double-stranded RNA-depending Protein Kinase
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl sulfate
siRNA	small interfering RNA
ss	single-strand
SV40	Simian Virus 40
TBST	Tris Buffered Saline with Tween-20
TNF	Tumor Necrosis Factor
UL	Unique Long
UNC	University of North Carolina
Us	Unique Small
VP16	Virion associated Protein 16
wt	wild type

CHAPTER 1

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is estimated by the CDC to infect up to 90% of the US population. It is closely related to herpes simplex virus type 2, sharing a homologous genome with 40% sequence identity. Normally, HSV-1 causes oral fever blisters or cold sores, while HSV-2 is associated with genital lesions; however, the viruses are capable of infecting other sites and producing other pathologies. Most common of these are keratitis from ocular infections that can lead to blindness and herpetic whitlow from infection of one's hands or fingers, which is common among health care professionals. The most severe pathologies occur when HSV enters the central nervous system, causing encephalitis that can result in death, and when HSV is contracted by neonates.

Both virus types are spread through direct or indirect contact. The virus will usually initiate a primary infection at the sight of contact, possibly dependent upon pre-existing breaches in the skin, creating a viral lesion. The propagating virus will infect enervating neurons, travel through the axon to the cell body by retrograde transport, and become dormant in the ganglia after the host's immune system has cleared the initial infection. The virus will remain in the neurons for as long as it lives, which can be the life of the host. Periodically, the virus can become active in the neuron, often due to various stresses, and by antegrade axonal transport, spread back to the area of the initial infection. These

reoccurrences are normally mild in comparison to the initial infection and are sometimes even asymptomatic.

Currently there are effective drugs to control HSV, both for initial infection and recurrences; however, there is no method to remove latent virus and resistances to these drugs can develop. There are a number of vaccines against HSV and more in development, however to date none are efficacious enough to be practical. Obviously, by studying the mechanisms of infection of HSV, we hope to develop additional treatments and perhaps eventually even a cure. Better understanding viral infection can also help us better understand the workings of the host as well as help us in developing tools for research and treatment of other diseases.

HSV-1

HSV-1 is one of the eight known human herpesviruses and is the prototypical alphaherpesvirus. The other two known human alphaherpesviruses are HSV-2 and varicella-zoster virus. The betaherpesviruses include human cytomegalovirus (HCMV) and human herpesviruses 6 and 7. The human gammaherpesviruses are Epstein-Barr virus (EBV) and Kaposi sarcoma-associated herpes virus or human herpes virus 8 (KSHV). HSV-1 has a linear double stranded DNA genome of roughly 152kb, which encodes over 80 genes. In the virion, the genome is surrounded by an icosahedral capsid. Surrounding the capsid is the tegument layer, comprised of many proteins encoded by the virus. The envelope, comprised of a lipid bilayer embedded with glycoproteins surrounds the tegument and capsid.

On a cellular level, HSV-1 infection begins when envelope glycoproteins facilitate binding and entry into a host cell. Glycoprotein B (gB) or gC initially interact with heparin sulfate on the cell surface, followed by gD interaction with coreceptors (77, 135, 143). A

number of receptors can interact with gD, including the TNF receptor family member HVEM, immunoglobulin family members HveB (nectin-2), HveC (nectin-1) and HIgB, and 3-O-sulfated heparin sulfate (21, 43, 100, 138). The above has been characterized for cell surface mediated entry, though HSV can also enter cells through an endocytic route (102). Regardless, the tegument and nucleocapsid are eventually released into the cytoplasm. The capsid is then transported via the microtubule network to the nucleus in order to release the viral DNA through a nuclear pore (122, 139).

Once inside the nucleus, the linear genome is circularized, localized to ND10s and gene expression is initiated, though not necessarily in that order (122). Viral gene expression occurs in a temporal cascade, beginning with immediate early (IE) genes, then early (E) genes and finally late (L) genes following viral DNA replication (also known as α, β and γ genes). Virion associated protein 16 (VP16) and host proteins HCF and Oct1 activate IE gene transcription via the TAATGARAT sequence found in all IE gene promoters. There are five IE genes, Infected Cell Proteins (ICPs) 0, 4, 22, 27 and 47. ICP0 is a promiscuous transactivator, important for enhancing viral gene expression and accelerating infection progression (14, 32, 33). ICP0 also possesses E3 ubiquitin ligase activity and is capable of ubiquitinating many proteins, including PML, p53, DNA-PKcs and IkBα (12, 26, 35, 80, 106). In the case of DNA-PKcs and PML, ubiquitination by ICP0 leads to proteasomal degradation in vivo. ICP4 is necessary for expression of E and L viral genes by stabilizing general transcription factors on viral promoters. ICP4 down regulates IE gene expression as well (25). ICP22 has also been implicated in upregulating viral gene transcription, though it does not have transactivation activity itself (115, 121). ICP27 regulates gene expression post transcriptionaly through its interactions with polyadenylation and splicing machinery (53,

112, 120). ICP27 also activates several cellular signal transduction pathways (54, 96, 109). ICP47 is the only IE gene that does not function to regulate gene expression. Instead it blocks peptide loading onto transporter of antigen presentation (TAP) complexes in order to promote immune evasion (39, 59).

Dependent upon IE gene products, expression of the second class of HSV-1 genes occurs. These E genes encode components of the viral DNA replication machinery, including the viral DNA polymerase UL30, single stranded DNA binding protein ICP8 (also called UL29), the processivity factor UL42, origin-binding protein UL9 and the helicase-primase complex consisting of UL5, UL8 and UL52. These localize to so-called prereplicative sites, likely previous ND10 sites, where viral DNA replication is thought to be initiated. Active DNA replication expands these sites, forming replication compartments that fill most of the host nucleus. HSV DNA replication initiates dependent upon UL9, where bi-directional theta replication occurs. At some point however, DNA replication is thought to switch to a rolling circle and/or recombination dependent mechanism (165). The resultant viral DNA is a complex multi-branched concatameric structure.

Following initiation of DNA replication the L genes are transcribed. This class is further divided into leaky late genes that do not require viral DNA replication for expression and true late genes that absolutely require DNA replication for expression. This classification is not entirely accurate however, as all late genes lie on a continuum for the requirement of viral DNA replication (144). Late gene products include the structural components of the virion and machinery necessary for assembling the virion and exporting it out of the cell.

During the course of infection, the host cell has its normal functions modified so that all available resources become available for the virus to replicate itself. ICP27 and the tegument protein vhs (virion host shutoff) inhibit host cell translation by preventing mRNA maturation and by degrading pre-existing mRNA respectively (24, 128). Cellular DNA is condensed and marginalized and cell cycle is halted at G1/S and G2/M (29, 88, 103, 140). HSV-1 also counters the cell's innate defenses against viruses. ICP27 prevents apoptosis through its activation of NFκB and possibly through other mechanisms (49, 50). ICP34.5 counters transcriptional suppression by PKR by directing dephosphorylation of eIF2α (20). Ultimately, viral replication depletes the cell of metabolic resources and causes necrosis.

When HSV-1 infects a neuron, it does not proceed along the lytic course described above, but rather becomes latent. The exact mechanisms by which this manner of infection occurs are not fully understood. It is known that some limited amount of replication occurs within the neuron before the virus becomes silent, meaning that gene expression is curtailed. Though the initial trigger for this switch is unknown, it is thought to be maintained by a chronic immune response (71). Because neurons are generally not turned over, latency persists for the life of the infected individual. The virus may reactivate periodically, often due to stress, possibly because of decreased immune activity during such times. When reactivation occurs, virions are transported antrograde where they infect cells proximal to the original sight of infection. Though the reactivated neuron eventually dies, HSV is maintained in the host till death because not every latently infected neuron reactivates at once and reactivation can lead to infection of previously virus free neurons.

NFκB

All human pathogens must contend with their host's immune response, and HSV is no exception. Central to many of these barriers is nuclear factor Kappa B (NFκB). NFκB is a family of transcription factors that can positively and negatively regulate a number of genes involved in proliferation, apoptosis, inflammation, stress responses, innate and adaptive immune response, oncogenesis and development, all of which contain κB elements, to which NFκB binds, (44, 67, 105, 107). The exact function of NFκB when activated is highly dependent upon the state of other signaling pathways, other transcription factors and the stimuli responsible for activating NFκB (57, 113). The interplay between all these cellular components can determine whether apoptosis or an inflammatory response is initiated.

There are five different NFκB subunits that can dimerize to form a functional transcriptional factor; p65 (relA), RelB, c-Rel, p50 and p52. All the subunits contain a Rel-homology domain which is necessary for DNA binding and dimer formation. Both p50 and p52 can form homodimers or dimerize with any other subunit. The other three subunits only dimerize with p50 or p52 and also contain transactivation domains. Dimers of p50 and p52 can still activate gene expression by interaction with the transcriptional co-activator BCL-3 (10).

Activation of the NFκB pathway normally results from signaling from cell surface or intracellular receptors triggered by cytokines or molecules containing pathogen associated molecular patterns, but various cellular stresses, such as DNA damage, can also lead to activation. TNFα, IL1 and LPS are amongst the best known activators of NFκB, though there are numerous others, some likely yet to be discovered. As there are a number of extracellular and intracellular signals that lead to NFκB activation, there are many different

signaling cascades that can trigger NFκB activation. Normally however, these signaling pathways converge at a signaling bottleneck, the IKK complex. After this point, NFκB activation again is somewhat varied, though there is one pathway that is most frequently observed across many cell types, known as the classical or canonical pathway. Because of its relevance here and the complexity of the many other routes of activation, it will be the only one described.

The classical pathway signals through the IKK complex consisting of inhibitor of κB kinase α (IKKα), IKKβ and IKKγ. IKKα and IKKβ are kinases that target NFκB subunits and a class of regulator proteins known as Inhibitor of κB (IκB). There are 5 IκB proteins; IκBα, IκBβ, IκBγ, IκBε and BCL-3. In the classical pathway, IκBα binds to a p65/p50 heterodimer and masks p65's nuclear localization sequence. Phosphorylation of IκBα by IKK leads to its poly-ubiquitination and proteasome dependent degradation. Once released from IκBα NFκB localizes to the nucleus. It is further regulated by post-translational modifications. Phosphorylation of serine 276 results in a switch of p65's interaction from histone deacetylases such as HDAC to histone acetyl-transferases such as CBP/p300, promoting gene transactivation. Serine 529 and 536 also play a role in promoting transactivation, while serine 311 is important for DNA binding. Acetylation at a number of sites also promotes NFκB transactivation (reviewed in (57, 113, 132, 161)).

Upstream of IKK in the classical pathway are a number of signaling paths. The most commonly studied is the path from TNFα (reviewed in (57)). TNFα is a cytokine that signals through its receptor TNFR1. When TNFR1 binds TNFα, it aggregates and binds adapter molecules to activate various signaling pathways. For NFκB activation, TRADD, RIP and Traf2 or 5 are the necessary adapters. Once these adapters have bound TNFR1, they recruit

the IKK complex and TAK1, which leads to activation of both (136). Once IKK has been activated, NFκB signaling proceeds as described above.

NFκB and viruses

NFκB is very significant for microbial pathogens. Its role in the immune response is a important hurdle to overcome, and many viruses and bacteria have developed mechanisms to interfere with NFκB activation (46, 62). NFκB activation can also serve to benefit viruses however, and several viruses activate and exploit the pathway (61). Perhaps the most well known case is HIV-1. HIV-1 contains κB elements in its LTR that, in conjunction with other transcription elements, are necessary for efficient transcription. HIV-1 can cause NFκB activation through several mechanisms. HIV-1 binding to its receptor, CD4, which can signal to the NFκB pathway when activated, causes induction of NFκB. HIV-1 Tat induces NFκB activation through engagement of cellular signaling cascades involving $p56^{ick}$, while Vpr and Nef induce IL-8 and IL-2 signaling respectively, causing NFκB activation. And finally, activation of the immune response by HIV-1 also results in NFκB activation.

Many other viruses also contain NFκB binding sites, of most relevance here are the herpes viruses. Both EBV and KSHV have NFκB binding sites in the promoters of their major oncogenic proteins, LMP1 and K1 respectively, though it is unclear what role NFκB plays in transcriptional control *in vivo* (7). The HCMV IE1 promoter contains 4 NFκB sites, which may be important for(127) reactivation from latency as well as normal lytic expresion (6, 127). HSV also has NFκB sites in the promoters of ICP0 and VP16, which for the ICP0 gene are capable of binding NFκB (4, 123). Activation of NFκB in all these viruses has been shown to benefit replication (17, 52, 61, 69, 70, 109, 118, 127, 156).

HSV's ability to activate NFκB was first observed in studies looking at HSV-1 activation of HIV provirus (47). The activation is primarily of p65/p50 heterodimers necessary for efficient HSV replication (109, 130). More recently, two phases of NFκB activation have been reported. The initial activation is likely triggered by HSV engagement of receptors capable of signaling to NFκB, and is transient in duration (4, 98, 155). The second activation of NFκB is dependent upon IκB turnover and is sustained for the length of infection (109). Though ICP4 (vi13) and ICP27 (n59r) deficient mutants are both impaired for NFκB DNA biding, n12, a ICP4 mutant, shows only partial impairment of IκB loss while d27-1, a ICP27 null mutant is unable to cause IκB loss (55, 109). Also, an HSV mutant expressing only ICP27, ICP22 and ICP47 (d100) causes IκB degradation by 24 hpi. ICP27's activity that leads to NFκB activation was mapped to a domain within residues 12 to 63 and is also in part dependent upon activation of JNK and p38 signaling pathways, as pharmacological inhibitors of JNK and p38 also reduce IκB loss (55).

As mentioned above, ICP0 and other genes contain κB sites in their promoters (123). For ICP0, NFκB has been shown to bind and contribute to transactivation (4). What role NFκB might play in gene regulation for other HSV genes is not known. NFκB does however appear to inhibit apoptosis in some cell types. HSV infection of HEp-2 cells that either express a dominant negative IκB, or are treated with a pharmacological inhibitor of IκB degradation, induces apoptosis (49, 50). Other reports have suggested that NFκB does not actually play a role in inhibiting apoptosis. SK-N-SH cells infected with R5104, a HSV mutant that aberrantly expresses Us11 at the immediate early stage, fails to cause NFκB activation, but also does not cause apoptosis (147). Mouse embryo fibroblasts (MEFs)

knocked out for p65, p50 or both also do not undergo apoptosis following wt or d120 (a ICP4 deleted mutant) infection, while the latter causes apoptosis in other cell types (148).

The conclusions from the latter experiments are problematic however. R5104 varies from wildtype HSV by several changes, and it is unclear how these may effect both the induction and inhibition of apoptosis separate from NFκB activity (16). It is difficult to interpret the results from the MEFs because while they do not undergo apoptosis following wt infection, as one might expect if NFκB was necessary to prevent apoptosis following infection, they also do not undergo apoptosis following d120 infection. Infection with d120 does not activate NFκB and thus should cause apoptosis regardless of NFκB's presence in the cell. This result suggests that these cell lines have gained a function that either prevents induction of apoptosis due to HSV infection or inhibits apoptosis independent of NFκB. Because these cell lines are sensitive to TNF or sorbitol apoptosis induction, the latter is unlikely (148).

Currently, the pathways leading to NFκB activation are poorly understood and have been part of my dissertation research. IKK is activated following infection, and treatment of infected cells with prostaglandin A_1 (PGA1) inhibited both IKK and NFκB activation (3). In this study, PGA1 also inhibited immediate early gene expression, so it was unclear from these results whether IKK is necessary for NFκB activation during infection, though it would be expected to be. My own work confirmed IKK to be necessary for NFκB activation by HSV and expanded our knowledge for the role of each kinase subunit using MEFs knocked out for one or both of the kinase subunits (50). There are many potential upstream activators of IKK, and which one might target IKK following HSV infection. Taddeo et al reported that PKR is upstream of NFκB activation. However my results do not bear this out (50, 147).

The reason for this discrepancy is unclear. I have also characterized two other potential upstream kinases, TAK1 and NIK, and shown that they are not relevant for HSV activation of NFκB.

Double Strand Break Response

Many types of DNA damage threaten the genomic integrity of a cell. DNA DSBs are one of the more dangerous types of damage, resulting from ionizing radiation, genotoxic chemicals, collapsed replication forks (often due to pre-existing damage in the DNA) and purposeful DNA cleavage for cellular processes, such as V(D)J recombination. If not repaired promptly and properly, DSBs can result in loss of large portions of chromosomes and in translocations and large deletions, potentially causing cell death or deregulation, a worse fate for the organism if cancer results. To avoid these fates, cells have a complex system in place to quickly detect DSB and respond to them.

Primary in the response to DSBs is the MRN complex, comprised of Mre11, Rad50 and Nbs1 (22, 63, 78). The MRN complex is responsible for recognizing free DNA ends and tethering the two broken ends together. The MRN complex also possesses nuclease and helicase activity that process the ends for repair (110, 111, 157). Subsequent to finding the DSB and binding, MRN interacts with ATM, causing ATM to become active through autophosphorylation (9, 15, 79, 160). At this point, signal amplification occurs as ATM phosphorylates a number of components to further repair and alert the cell to the occurrence of the break. At or near the site of the break and to a newly activated ATM are two of its targets, NBS1 and H2A.X (13, 86). H2A.X is a variant of histone H2A, and its phosphorylation allows it to act as a landing pad for MDC1 (170). MDC1 acts as a scaffold to the MRN complex via interaction with NBS1 and aids in further ATM activity (91, 99).

ATM also phosphorylates 53BP1 and BRCA1, which act as additional scaffolding and are important to recruit and aid in activation of some of ATM's other targets, such as p53 and the Chk proteins (38, 41, 94, 99).

In mammalian cells, repair normally occurs via non-homologous end joining (NHEJ), resulting in two broken DNA ends being ligated together regardless of sequence. This non-specificity can lead to small deletions or insertions. For this process of repair, the heterodimeric Ku (Ku70/86) recognizes the DNA ends while the actual rejoining of the ends occurs via the Xrcc4/LigaseIV complex aided by pol μ (63, 92, 108, 158). Though we know these components are necessary for NHEJ, the full details of their interactions are as yet unknown. Another protein that is sometimes involved in NHEJ is the catalytic subunit of DNA dependent protein kinase (DNA-PKcs). DNA-PKcs seems to be necessary for a subset of NHEJ that does not involve the MRN complex.

The other common mechanism by which DSBs are repaired is homologous recombination (HR). This mechanism has been primarily studied in fungi, where it is the primary method of repairing DSB. However, the mechanisms of HR appear to be highly conserved throughout all eukaryotes. HR more accurately repairs DSB and requires a homologous template to the broken duplex, i.e. a sister cromatid. The Rad52 epistasis group (comprised of the MRN complex, RPA, Rad51, Rad52, Rad54, Xrcc2 and Xrcc3) is responsible for the mechanisms of HR. HR has been thoroughly studied and reviewed (142, 167, 168). In simplified summary, a single strand from the broken DNA is bound up by RPA and then Rad54 facilitates loading of Rad52 and Rad51, which form a filament around the single strand (ss) DNA and cause it to invade the double strand (ds) DNA of a homologous duplex. This invasion causes the displacement of one of the intact strands, which can then

form a duplex with the complimentary strand from the broken DNA, all promoted by Rad51 and Rad54. Strands exchange between the homologous DNA occurs and the crossed DNA strands are resolved by being cut and rejoined to the appropriate new dsDNA molecule.

In addition to facilitating repair, ATM also activates cell cycle checkpoints and in extreme cases, apoptosis. ATM phosphorylates ATR, Chk2, Chk1 and p53, all of which can inhibit cell cycle progression (23, 66, 68). ATR can also be activated through interactions with the MRN complex and is a potent activator of Chk1 (94). Chk1 and Chk2 target Cdc25 proteins to inactivate them (42). Normally Cdc25 proteins serve to positively regulate Cdk activity, necessary for cell cycle progression, by removing inhibitory phosphorylations (56, 169). p53 can also inactivate Cdk proteins through its transcriptional regulation of p21, which directly binds to Cdk/cyclin complexes and prevents ATP binding (124). Apoptosis can also be induced by p53, though this requires either additional signals or differential activation as this only occurs in cases of irreparable damage (90).

Viruses and the DNA DSB response

Many previous reports have found interactions between viruses and host cell DNA DSB response machinery. In some instances, cellular DSB machinery is detrimental to a virus, and as such the virus in question has mechanisms to prevent the actions of the cellular response machinery. In other cases, viruses are able to harness the cellular machinery for their own advantage. Whether the cellular machinery is harmful or beneficial for a virus depends on the life cycle of the virus, and in some instances the machinery can be both.

SV40 is a circular dsDNA virus with strong oncogenic properties. The large T antigen of SV40 is a key component for the oncogenic properties of SV40. One transforming mechanism of SV40 large T antigen is to directly bind p53 and prevent its transcriptional

activity that is important in the DNA damage response (76). More recent reports have elucidated a role for SV40 large T antigen in hindering the initial stages of the DNA damage response. Study of cardiomyocytes transformed with SV40 large T antigen found that Mre11 co-immunoprecipitated with SV40 large T antigen and that transformed cell lines had greatly reduced Mre11 protein, though not mRNA. Nbs1 and Rad50 in contrast seemed not to have decreased protein levels (28). In fibroblasts, the presence of SV40 large T antigen was noted to cause a decrease in Mre11 foci formation following IR treatment (166). Unfortunately, none of these studies ventured into actual SV40 infection, so the benefit these actions of large T antigen have on SV40 replication remain in the realm of speculation.

One study of interesting note did find a role for SV40 large T antigen in the context of infection. Wu and colleagues discovered that SV40 large T antigen directly binds to Nbs1. This binding prevents Nbs1, and presumably the entire MRN complex, from preventing refiring of origins of replication that lead to erroneous duplication during S phase. SV40 relies entirely on host DNA replication machinery to duplicate its genome, so loss of replication control allows the SV40 genome to undergo exponential replication (119, 129, 173). Though it is unknown whether this function of the MRN complex is tied to a DNA damage response, these results show an interesting interaction between a virus and DNA damage response machinery.

Like HSV, adenovirus has a linear dsDNA genome. Adenovirus E1B-55K binds and hinders p53 transcriptional activity and in conjunction with E4orf6 causes p53 to be degraded (146). Adenovirus control of p53 allows suppression of apoptosis induced by the virus. It also has two means to inactivate the MRN complex. Late in infection Mre11 and possibly all three components of the MRN complex are degraded dependent upon either E4orf3 or

E1B55K/E4orf6 (31). This late loss of the MRN complex prevents detection of DSB breaks and activation of ATM. For the virus, this failure of the DNA damage response allows its linear progeny genomes to escape concatamerization, which would prevent their packaging. Earlier in infection however, E4orf3 binds the MRN complex and sequesters it to foci throughout the nucleus. This activity of E4orf3 is necessary for viral DNA replication to occur (73).

Recently Kudoh and colleagues characterized components of the DNA damage response during the EBV lytic cycle (95). They found that ATM was activated and phosphorylated Chk2, p53, H2AX and Nbs1. They also observed that activated ATM and MRN complex colocalized with replicating EBV DNA. Interestingly, EBV BZLF-1, a major transcriptional transactivator, counters the down stream effects of cell cycle checkpoint induction by ATM by binding and inhibiting p53 and by activating promoters of the cell cycle, such as E2F-1, cyclin E and Cdc25A (80, 106). These finding indicate that activation of the cell cycle checkpoint by the damage response would be detrimental to EBV replication. However, whether other components such as the MRN complex have any affect on EBV replication is not yet known.

The first reported effect of HSV-1 on the DSB response was the observation that HSV-1 caused the degradation of DNA-PKcs via ICP0 (51). This loss is thought to hinder NHEJ, which could potentially act upon viral DNA (84, 137, 154, 162, 164). Several recent reports have also established a link between viral DNA replication and components of the DSB response. RPA, DNA-PKcs, Rad51, Mre11, Nbs1, ATM, Rad50 and Ku were seen via indirect immunofluorescence microscopy (IF) to localize to replication compartments, and all but Ku and DNA-PKcs also localized to prereplicative sites (154). HSV-1 ssDNA binding

protein, ICP8, which is present at sites of DNA replication, has also been shown to interact with DNA-PKcs, Rad50, Ku, Mre11, RPA and BRCA1 (84, 137, 164). Similar to EBV, HSV-1 also causes activation of many DSB response factors, in this case Nbs1, RPA, ATM, Chk2 and 53BP1 (85). These activation events appear to occur by the MRN complex's activation of ATM, independent of active viral DNA replication. The importance of this activation is underlined by a report by Lilley et al. that demonstrates the inability of virus to replicate in cells deficient for the MRN complex or ATM (137). The importance of ATM has been disputed however, as Shirata et al. reported that while cells from individual with AT or NBS are defective in supporting viral infection, specific knockdown of ATM with siRNA had no effect on viral replication (154). Interestingly, cells deficient in Ku actually facilitate viral replication better than wild type (wt) cells (163). In contrast, HSV inhibits ATR, and thus Chk1, activation (165).

HR is induced by HSV-1 infection as evidenced by recombination during heterotypic coinfection or isomerization of HSV-1's genome during normal infection. HR may also play an important role in viral DNA replication by recovering stalled replication forks (81, 82). Many of the DSB response factors are important components in the HR machinery and thus their importance in HSV-1 replication might be explained. Thus, in one aspect, HSV-1 uses the DSB response machinery to aid in its own replication. However in the case of DNA-PKcs, Ku and perhaps NHEJ in general, HSV-1 seems to antagonize or be antagonized by the DSB response machinery. NHEJ might also be the mechanism of genome circularization during the establishment of latency in neurons. With this paradox it is important to understand the stages of HSV-1's life cycle in which the DSB response interacts with the virus. Currently, evidence suggests a positive role for the DSB response in DNA replication.

As of yet, we do not know if the DSB response machinery might have an effect on late events, such as processing and packaging of replicated DNA.

Materials and Methods

Cells: Spontaneously immortalized mouse embryo fibroblasts (MEFs) derived from normal mice or mice with targeted deletions in IKK α, or IKKβ (172) were obtained form Tal Kafri (UNC-Chapel Hill). MEFs from normal mice or mice with a targeted deletion of the double-stranded RNA-activated protein kinase PKR (37) were obtained from Bryan Williams (Cleveland Clinic). Diploid human embryonic lung fibroblasts (HELs) and HeLa cells were obtained from ATCC. The above cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine calf serum, 100U/ml penicillin, 1% streptomycin, and 1% L-glutamine. Hep2 cells obtained from ATCC were maintained in Minimal Essential Medium supplemented as for DMEM. CV-1 cells were obtained from Saul Silverstein (Saul Silverstein) and grown in DMEM supplemented with 5% newborn calf serum, 100U/ml penicillin, 1% streptomycin, and 1% L-glutamine. Telomerase immortalized human foreskin keratinocytes (HFKs) were a gift from Shannon Kenney (University of Wisconsin-Madison) and Nancy Raab-Traub (UNC-Chapel Hill) with permission from A. J. Klingelhutz who derived the cell line as previously described (104). HFKs were maintained in Keratinocyte-SFM from Gibco supplemented with EGF and bovine pituitary extract.

Viruses: HSV-1 strains KOS1.1 and 17+ were used as the wildtype (wt) virus strains. The following mutants were used: ICP8 mutant d301 (David Knipe, Harvard Univ.); UL30 (polymerase) mutant HP66 (Donald Coen, Harvard Univ.); ICP0 null mutants 7134, d22 and d99 (Priscilla Schaffer, Harvard Univ.); ICP4 mutant n12 (Neal DeLuca, Univ. of

Pittsburgh); UL17 mutant ΔUL17 (Joel Baines, Cornel Univ.); UL25 and UL28 mutants kUL25 and GCB (Fred Homa, Univ. of Pittsburgh); UL15, UL32 and UL6 mutants hr81-1, hr64 and hr74 (Sandy Weller, Univ. of Conneticut) and UL26 mutants dZ and H61E (Prashant Desai, John Hopkins Univ.).

Antibodies: Rabbit polyclonal antibodies against IKKα (H-470, sc-7607), IKKβ (H-744, sc-7218), IκB-α (C-21, sc-371), PARP (H-250, sc-7150) were purchased from Santa Cruz. Polyclonal antibody against p65 (100-4165) was purchased from Rockland. Monoclonal antibodies against Rad50 (13B3) and Mre11 (12D7) and polyclonal antibody against Mre11 (GTX30294) were purchased from Genetex, Inc. Monoclonal antibody against Nbs1 (100-222) and polyclonal antibodies against phospho-Nbs1 (100-284) and phospho-ATM (100-307A2) were obtained from Novus Biologicals. Monoclonal antibody DNA-PKcs (05-423) was purchased from Upstate. Monoclonal antibody against ICP0 (H1A027-100) was purchased from Virusys. Monoclonal antibodies against ICP4 (1101) and ICP27 (H1113) were purchased from Rumbaugh-Goodwin Institute. Polyclonal antibody against ICP8 (3-83) was a gift from David Knipe. Polyclonal antibody against gC (R47) was a gift from Gary Cohen and Roselyn Eisenberg. Polyclonal antibody against VP16 was purchased from Clontech. Goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were purchased from Amersham Biosciences

Infection and preparation of extracts: Near confluent or confluent monolayers seeded one to two days previously were inoculated with virus at the indicated multiplicity of infection (MOI) calculated based on titers corrected for the host cell lines (1 HEL/HFK pfu ~ 4 Vero pfu). Thirty minutes to one hour following inoculation, cells were overlayed with spent media or spent media containing 400 μg/mL phosphonoacedic acid (PAA (an inhibitor

of herpesvirus DNA polymerase)), 10 μg/mL cycloheximide, 5 μg/mL Actinomycin D, 5mM Caffeine, 10μM KU-55933 or, NH_4Cl or MG132 (Sigma) at the indicated concentrations. Monolayers were harvested by washing with cold PBS and then scraping directly into 1X SDS sample buffer [3.85mM Tris-base (pH 6.8), 9.1% β-mercaptoethanol, 1.82% SDS, 4.6% glycerol, and 0.023% bromophenol blue (in 100% EtOH)] and boiling for 5'. In the case of HELs, media from monolayers was collected and the monolayers were treated with trypsin until the cells detached. The cells were then suspended in PBS, combined with the previously collected media and were spun down and washed once with PBS before addition of 1X SDS sample buffer and boiling as above. Fractionated cytoplasmic and nuclear extracts were prepared as described previously (60, 104, 109). Briefly, cells were collected by trypsinization, spun through a cushion of bovine calf serum, washed twice in PBS, resuspended in 3 packed cell volumes of CE buffer (10 mM HEPES, pH 7.8, 1 mM EDTA, 60mM KCl, 1 mM PMSF, 0.1% NP-40, 25% glycerol, 0.4mM NaF, 0.4 mM Na_3VO_4, 10μM pepstatin, and Complete Protease Inhibitor Cocktail [Roche]) and incubated on ice for 10'. Following a 10 sec spin in a bench-top microcentrifuge, the supernatant was collected and the nuclear pellet resuspended in CW buffer (CE buffer without NP40 or glycerol), and repelleted. Nuclei were resuspended in 2 packed cell volumes of NE buffer (20mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 25% glycerol, and phosphatase and protease inhibitors as described above). After incubation on ice for 10', nuclei were pelleted at 60,000 rpm at 4^{o}C for 20' in a Beckman TLA100.3 rotor and the nuclear supernatant recovered.

Western Blotting: Cell or protein equivalents of samples were separated by SDS-PAGE and transferred to PolyScreen polyvinylidene difluoride membranes (Perkin-Elmer

Life Sciences). Ponceau S (Sigma) was used to visualize total protein and insure no discrepancies in protein equivalences. Membranes were blocked in 5% milk in TBST (150mM NaCl, 20mM Tris [pH 7.6], 0.05% Tween 20) then washed and probed with appropriate primary antibodies, followed by further washing and probing with the appropriate secondary antibody, all in TBST. After final washing in TBST, signal was detected using Perkin-Elmer Western Lightening substrate. Multiple exposures were made on Kodak BMR film to insure the linear range was obtained. Images were scanned and processed using Adobe Photoshop.

Electrophoretic mobility shift assay (EMSA): Preparation of nuclear extracts and mobility shift assays for NFκB were performed as described previously (50). Nuclear extract were incubated with a radiolabeled probe containing a κB-binding site TG**GGGATTCCC**CA in buffer containing 10mM Tris-HCl (pH 7.9), 50mM NaCl, 0.5mM EDTA, 10% glycerol, 1 mM DTT, and 2μg poly (dIdC)·(poly dIdC). Following 20' incubation at room temperature, aliquots were fractionated at 4^{o}C on non-denaturing 4% polyacrylamide gels prepared in 0.25X TBE [1X TBE is 10mM Tris base (pH 8.3), 9mM boric acid, and 2mM EDTA]. Gels were then placed on 3MM paper, dried under vacuum and heat, and exposed to BMR film at –70^{o}C.

Plaque assay: Virus was titered as described previously (50). Monolayers of cells in 60mm or 100mm dishes were infected with HSV at a MOI of 5. Cells and medium were harvested at various times pi. and subjected to 4 freeze/thaw cycles in an ethanol and dry ice bath then 37 ^{o}C water bath. Serial 10-fold dilutions of the lysates were assayed in triplicate on monolayers of Vero or CV-1 cells in 12 well dishes. After 1h, monolayers were covered with DMEM-H containing 2% CS and 0.3% methylcellulose. After 3d incubation at 37^{o}C,

medium was aspirated from the wells and plaques stained with 0.8% crystal violet in 50% ethanol.

Immunofluorescence: Cells were seeded on glass coverslips and allowed to grow until confluent. The monolayers were infected at low multiplicity (MOI of .1 or .01). At the indicated times, cells were washed with PBS, then fixed with 5% paraformaldehyde in PBS for 10 minutes followed by permeabilization with 1% Triton X-100 in PBS. The cells were then incubated in blocking buffer (2.5% normal goat serum, 2.5% normal horse serum and 1% gelatin in PBS) for at least 30 minutes, probed with the indicated primary antibody, and then washed and probed with either Texas red conjugated anti-rabbit or FITC conjugated anti-mouse antibody (Santa Cruz), all in blocking buffer. Primary antibodies were omitted for control cells. Following final washing with PBS, the coverslips were mounted and allowed to set before visualization with an Olympus FV500 confocal laser scanning microscope.

Real time PCR: Cell monolayers were infected at an MOI of 5 as above and then harvested using Promega Wizard SV Genomic DNA Purification System to isolate cellular and viral DNA. Primers against c-Myc (TCAAGAGGTGCCACGTCTCC, TCTTGGCAGCAGGATAGTCCTT) and UL19 (AAACATCGCTGCCTGGAG, GGGGCGCTTAAACTGTACG) were designed by Dirk Dittmer. DNA and primers diluted 1:200 were mixed with SyberGreen and run on an ABI prism 7000 for 40 cycles at 95^{o} denaturing for 30" and 55^{o} annealing for 1'. 7000 systems SDS software was used to collect cycle data. Fold viral DNA copy was calculated based on dC_t of UL19 normalized to the dC_t of c-Myc.

CHAPTER 2

CHARACTERIZATION OF NFκB ACTIVATION FOLLOWING HSV INFECTION

[A portion of the results presented here were published in Gregory et al., 2004 (50)]

At the outset of our research presented here, it had long been known that NFκB was activated by HSV infection, but little was known regarding the cause or reason for NFκB activation. A report by Amici et al had recently demonstrated the activation of the IKK complex following HSV infection by kinase assay of immunoprecipitated IKK complexes (109). To test whether IKK activity was causing NFκB activation following HSV infection, they used PGA1, a prostaglandin, to inhibit IKK activity and assess for NFκB activation by IκBα turnover, NFκB gel shift activity and NFκB reporter activity. In all cases, PGA1 treatment caused loss of IKK activity and failure of NFκB activation. They concluded from this that IKK was behaving normally as part of the canonical NFκB pathway, phosphorylating IκBα and causing its degradation. They also demonstrated, as have others, that loss of NFκB activation greatly reduced virus yield, concluding it to be a necessary component of HSV infection (125).

While these are reasonable conclusions, the Amici et al. study suffers from the standard caveats of using pharmacological inhibitors, namely secondary targets and cytotoxicity. While a previous report by this group did show that other cellular kinases were not as sensitive to PGA1, they did not include such controls in their system for HSV (109).

They did assay for toxicity of PGA1 in their HSV system and claimed no affect. However, their results do show a marked reduction in ICP4 protein levels, which has been shown to contribute to NFκB activation, as well as a general failure of HSV to create a normal [35S] methionine-labeled polypeptide profile (159). Due to these results, the contribution of IKK to NFκB activation and HSV infection remained unclear.

To clarify the role of the IKK complex in NFκB during HSV infection and the involvement of its common upstream effectors, we sought to use a genetic approach. We obtained wild type (wt) mouse embryo fibroblasts (MEFs) or MEFs knocked out for either IKKα or IKKβ. These cell lines were characterized for their ability to facilitate HSV infection and NFκB activation. Our results indicated that IKK is indeed necessary for NFκB activation, with each kinase subunit uniquely contributing to infection. We also sought to determine if PKR, NIK or TAK1, known upstream kinases of IKK, contributed to NFκB activation. MEFs knocked out for functional PKR and cells transiently transfected with dominant negative NIK or TAK1 constructs were characterized for NFκB activation following infection.

Differential roles of IKKα and IKKβ in NFκB activation

Normal, IKK$\alpha^{-/-}$ or IKK$\beta^{-/-}$ MEFs were infected with wt virus and harvested at 8hpi or treated with TNFα for 15 min and harvested. Lysates were fractionated into either cytoplasmic or nuclear partitions. Western blots were performed to assay for cellular components of the NFκB pathway and viral proteins (Fig 2.1). The phenotypes of the cell lines were confirmed; both IKKα and IKKβ were present in lysates from wild type MEFs, while lysates from IKK$\alpha^{-/-}$ and IKK$\beta^{-/-}$ MEFs lacked the respective IKK subunit. Detection of the IKK subunits also served as a loading control for cytoplasmic extracts and a purity

control for nuclear extracts. No IKK proteins were detected in the nuclear fractions (Data not shown (DNS)). Similarly, PARP was used as our nuclear loading control and cytoplasmic purity control and was also not detected in our cytoplasmic fractions (DNS). IκBα is the IKK complex's immediate down stream target and is degraded after IKK dependent phosphorylation. IκBα was lost in wt and IKKα$^{-/-}$ MEFS following both HSV infection and TNFα treatment. IKKβ$^{-/-}$ MEFs however did not lose IκBα. Translocation of p65 from the cytoplasm to the nucleus followed a pattern similar to IκBα loss, as expected. Both wt and IKKα$^{-/-}$ MEF lysates had an increase in nuclear p65 upon HSV infection or TNFα treatment. Both also had increased total p65 following TNFα treatment, possibly due to Sp1 dependent activation of the p65 gene (109). One difference between these two cell lines was the portion of p65 translocated. While wt MEFs translocated the majority of p65 to the nucleus, a significant portion was still detectable in the cytoplasm. This was not the case for IKKα$^{-/-}$, where nearly all the p65 was translocated to the nucleus. This difference may be due to the apparent smaller pool of total endogenous p65 in the IKKα$^{-/-}$ cells. The IKKβ$^{-/-}$ MEFs had some translocation of p65 to the nucleus following HSV infection or TNFα treatment, though much reduced in comparison to the proportions translocated in the other two cell lines, which was consistent with the failure to turnover IκBα in the IKKβ$^{-/-}$ MEFs. These results suggest that IKKβ is the dominant subunit responsible for phosphorylating IκBα and causing NFκB nuclear translocation following HSV infection and may indicate that some upstream signaling components to IKKβ following infection are the same as those involved in TNFα or IL signaling, which also primarily signal through IKKβ.

Viral proteins were next assayed in these lysates. Both IE and E protein levels were similar between all three cell types, as indicated by ICP27 (IE) and ICP8 (E). Both IKKα$^{-/-}$

and IKKβ$^{-/-}$ lines had reduced late protein abundance however. The true late gC was nearly undetectable in both knockout lines, while the leaky late VP16 was reduced by roughly 5 fold in both lines. The failure to produce late proteins suggests either a failure to properly replicate the viral genome or disruption of viral late gene transactivation.

To assess NFκB DNA binding ability, another marker of activation, the nuclear lysates were subjected to electrophoretic mobility shift assay (EMSA). NFκB complexes were detected in all cell lines following either HSV infection or TNFα treatment (Fig 2.2). However, the level of a slower mobility complex in both IKKα$^{-/-}$ and IKKβ$^{-/-}$ lines was greatly reduced, slightly more so in the IKKβ$^{-/-}$ MEFs than the IKKα$^{-/-}$ MEFs. Previously, p65 and p50 were reported to form the dominant NFκB dimer activated by HSV (11, 45, 74, 175). In order to determine if that was the case in our system, we used antibodies to supershift the complexes. Antibody against E2F2 was used as an irrelevant control and did not shift either complex. Antibody against p65 shifted the more abundant slower mobility complex and antibody against p50 shifted both the abundant slow mobility complex and a less abundant fast mobility complex. This pattern suggests that HSV primarily activates p65/p50 heterdimers and to a lesser extent p50 homodimers in these MEFs. Some complexes appeared to not be shifted following incubation with the p65 or p50 antibodies. The make up of these complexes is unclear; possibly they are other NFκB dimers or simply p65/50 or p50/p50 dimers that were not bound by antibody.

As a final assessment of these MEFs, yield assays were performed. Monolayers were infected and harvested at either 16 or 24 hpi. Virus was then titered on Vero monoloayers (Table 2.1). Both knockout lines were deficient in viral yield, though the IKKβ$^{-/-}$ MEFs much more so than the IKKα$^{-/-}$ MEFs. The IKKα$^{-/-}$ MEFs yielded 5.4 and 6.2 fold less virus

at 16 and 24 hpi respectively than wt MEFs. The IKKβ$^{-/-}$ yields were 16.3 and 28.5 fold less than wt. These results are consistent with the Western blot and EMSA data, showing a greater contribution of IKKβ to NFκB activation. However, the large difference in yield is inconsistent with the similar deficiencies in late protein levels in the two cell lines and may indicate further deficiencies of the IKKβ$^{-/-}$ cells.

NFκB activation in PKR$^{-/-}$ MEFs

As active IKK is clearly necessary for NFκB activation and viral replication, we sought to determine what was leading to IKK activation. The double-stranded RNA dependent protein kinase (PKR) is activated by a number of physiological stresses and can cause NFκB activation through Trafs, NIK and IKK (20). PKR is activated following HSV infection, likely due to substantial symmetrical transcription following viral DNA replication (175). One of the primary targets of PKR is eIF-2α, which, once phosphorylated by PKR, inhibits translation. Normally HSV antagonizes PKR via the late protein ICP34.5, which promotes dephosphorylation of eIF-2α. However, because PKR is not itself inhibited, it may still activate NFκB. To determine if this was the case we characterized HSV activation of NFκB in wt MEFs or MEFs lacking functional PKR (PKR$^{+/+}$ and PKR$^{o/o}$ respectively).

To assess NFκB activation following HSV infection of these cell lines, EMSA and Western blotting were performed (Fig 2.3). NFκB DNA binding was induced following HSV infection or TNFα treatment in the wild type cells. The p65/p50 and p50/p50 EMSA complexes were again present, though in contrast to our previous results, HSV appeared to induce p65/p50 and p50/p50 equally. This difference is likely caused by differential transformation states of the wt cell lines. In the PKR$^{o/o}$ lines, there was p50/p50 complex binding in untreated cells, indicating a constitutive activation of NFκB. Both HSV infection

and TNFα treatment induced more NFκB binding, though primarily of the p50/p50 complex. TNFα induction of p65/p50 complex was reduced in comparison to the wild type cell, consistent with previous reports (27). HSV induction of the p65/p50 complex in $PKR^{o/o}$ cells was unaffected, suggesting that PKR is not necessary for HSV activation of NFκB. Western blotting of protein equivalent samples supported this, as IκBα was lost following infection in both cell types. The amount of ICP4 however, was greater in the $PKR^{o/o}$ cell line.

NFκB activation in the presence of dominant negative TAK1 and NIK

TAK1 and NIK are important for NFκB activation from a variety of signals. At the time of this research, it was not know if either was activated following HSV infection, though evidence for TAK1 activation has since be reported (93, 141). Given their prominent roles in a diversity of NFκB activation routes, we wished to ascertain whether they were necessary for HSV NFκB activation. To this end, we transiently transfected HEp-2 cells with dominant negative constructs of either TAK1 or NIK and then infected them with HSV. Cells were harvested at 8 hpi and Western blotting was performed (Fig 2.4). Increased levels of TAK1 or NIK in their respective transected cells confirmed the expression of the dominant negative constructs. The level of IκBα was comparable in the uninfected samples of all three treatments. This result confirmed that the constructs were functionally inactive, as overexpression of wild type TAK1 or NIK results in NFκB activation (3, 109). In all cases, IκBα was also lost following infection, suggesting that HSV either does not utilize either TAK1 or NIK in activating NFκB or uses redundant pathways to activate NFκB. Also, neither dominant negative construct inhibited NFκB DNA binding activity as assayed by EMSA (Gregory and Bachenheimer, unpublished observation).

Significance of Results

HSV activation of NFκB is essential for viral replication (64, 81-83, 150, 151). The results here help further our understanding of how NFκB is activated by HSV. Both IKKα and IKKβ appear to have distinct necessary functions for HSV NFκB activation. PKR does not appear to be necessary for NFκB activation, though it is also activated by HSV infection. Likewise, NIK and TAK1 activity is not necessary for NFκB activation following infection.

The IKK subunits are both necessary for efficient replication though for different reasons. Loss of IκBα and thus translocation of p65 to the nucleus were almost wholly dependent upon IKKβ, while mostly independent of IKKα. There was still some observable p65 translocation and IκBα loss in the absence of IKKβ, likely due to compensating activity of IKKα. Both subunits are critical for NFκB DNA binding, and thus likely for transactivation ability. These roles are the same as those reported in the canonical NFκB pathway following TNFα induction in MEFs. IKKβ was shown to be critical for IκBα phosphorylation and degradation, and NFκB translocation, DNA binding and gene transactivation, while IKKα had a minimal role in IκBα phosphorylation and degradation and NFκB degradation, but was also important for NFκB DNA binding and gene transactivation (149). Studies utilizing siRNA knockdown of the IKK subunits in HeLa cells also demonstrated that IKKβ was the more important of the two subunits, but that the two IKKs had a greater redundancy than the MEF studies indicated (109). Using siRNA knockdown would also benefit our understanding of HSV NFκB activation in more biologically relevant human cell lines.

The IKK mediated NFκB activation in MEFs is clearly relevant to HSV replication, as failure of the activation causes up to 6 and 28 fold reductions in virus yield in IKKα and

IKKβ cells respectively, and appears to be important for late stages of infection. While there was no deficiency in IE and E protein levels, late protein levels were greatly reduced. This result is different from that seen previously following infection of IκBα-SR transfected C33 cells, where there was no observable change in viral proteins, including gC, despite a ~5 fold reduction in yield and inhibition of IκBα loss and NFκB DNA binding (50). However, repetition of the DN-IκBα experiment in HEp-2 cells resulted in a similar decrease in gC, suggesting that the difference in results is due to cell type differences (55). This result, along with the results from the MEFs, indicates NFκB serves an important function for viral DNA replication and/or late gene expression.

While ICP27 is likely the viral activator of the sustained NFκB activation, what other viral or cellular factors mediate this activation has not yet been established fully (147). Our results with the $PKR^{o/o}$ MEFs indicate that PKR is not necessary for HSV NFκB activation, however, this is the opposite of the conclusion reached by Taddeo et al., who reported that NFκB was not activated in this same cell line (136). In their report, they showed IκBα loss was impaired and IKK kinase activity did not increase following infection in $PKR^{o/o}$ cells compared to wild type cells. In contrast the results presented above show no difference in IκBα loss and increase in NFκB DNA binding activity. The difference in results with IκBα might be explained by differences in HSV strains used; KOS1.1 vs F. It should be noted however, that although they did not see an increase in IKK kinase activity following infection in the $PKR^{o/o}$ cells, the basal level in the $PKR^{o/o}$ cells was greater than they observed in the wt cells even after infection, suggesting a constitutive activation of the NFκB pathway in the PKR deficient cells. This is actually consistent with our observation that the $PKR^{o/o}$ cells have p50 homodimer DNA binding activity in the absence of external stimuli.

If the $PKR^{o/o}$ cells have an altered NFκB pathway, any results regarding HSV NFκB activation are suspect. To more clearly determine if PKR is involved in HSV NFκB activation a different approach, such as siRNA or a specific PKR inhibitor, will have to be used.

Our results also indicate that TAK1 and NIK are not necessary for HSV NFκB activation. Expression of dominant negative constructs of TAK1 or NIK did not interfere with NFκB activation by HSV as determined by IκBα loss. TAK1 is essential for NFκB activation initiated through a number of ligands and signaling pathways and operates by directly activating IKKβ leading to IκBα degradation (27). Though there is some evidence that TAK1 can be activated by ICP0, it is unclear whether this actually occurs during HSV infection (87, 93, 141). Even if it is activated, it appears that HSV activates NFκB primarily by other mechanisms. Whether NIK is activated following HSV infection is unknown, though it also appears to play no role in IκBα degradation following infection. NIK normally targets IKKα and is not necessarily critical for IκBα degradation. However, expression of a dominant negative NIK antagonizes NFκB activation by several pathways mediated by Traf2, Traf6, IRAK and other factors (85, 137, 164). Because DN-NIK does not hinder HSV NFκB activation, it is likely that HSV mediates IKK activation through means normally not utilized by the cell in response to cytokines. As such, discovering the immediate upstream effectors of IKK might need to be done through assays of direct interaction following infection, such as IPs.

Figure 2.1

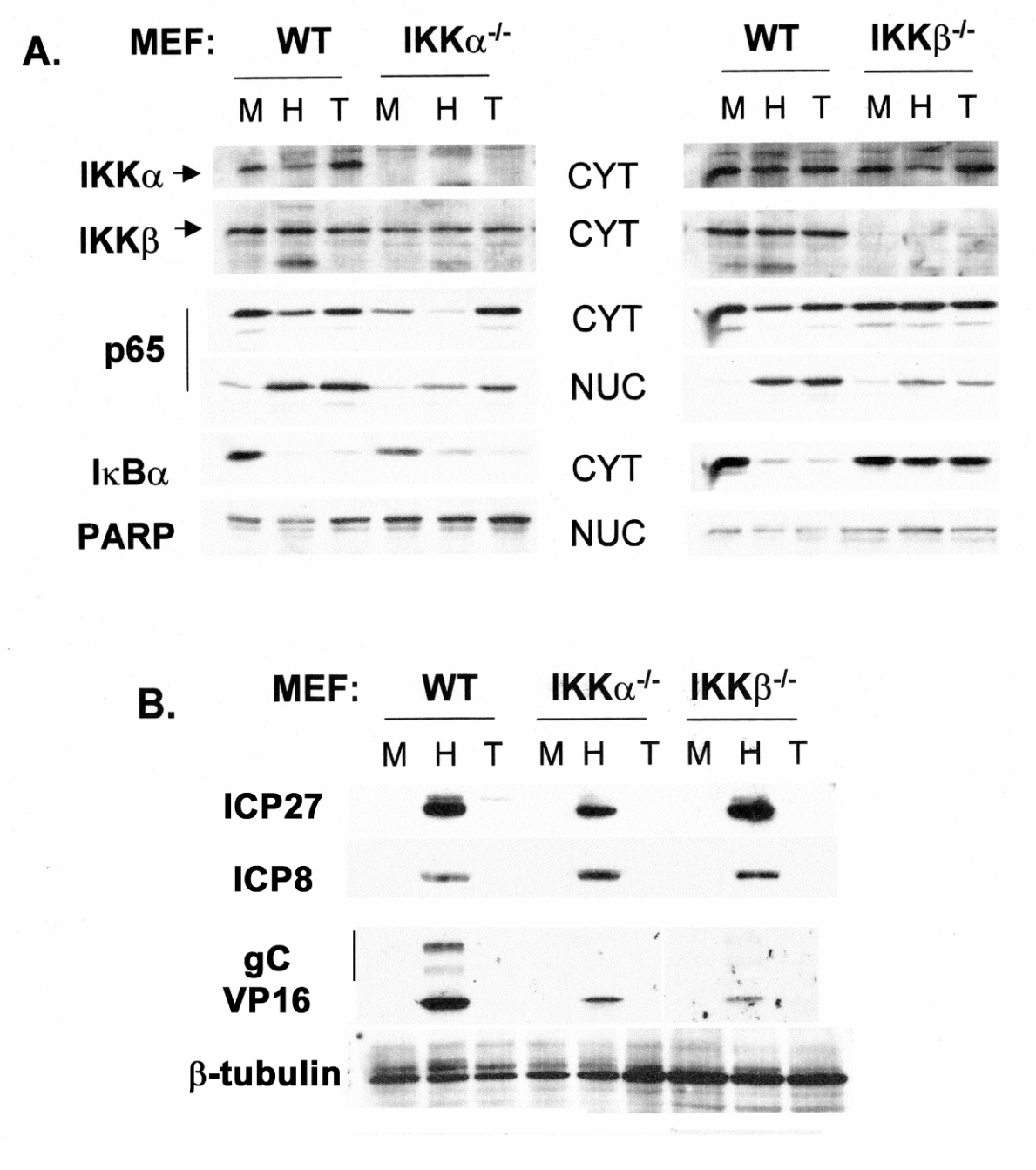

Figure 2.1. IKKβ is the primary kinase for IκBα loss and p65 nuclear translocation. Fractionated lysates from wt, IKKα$^{-/-}$ or IKKβ$^{-/-}$ MEFs harvested at 8 hpi were assayed for cellular (A) or viral (B) proteins by Western blot. M, mock infected. H, infected with wt HSV. T, treated with TNFα for 15'.

Figure 2.2

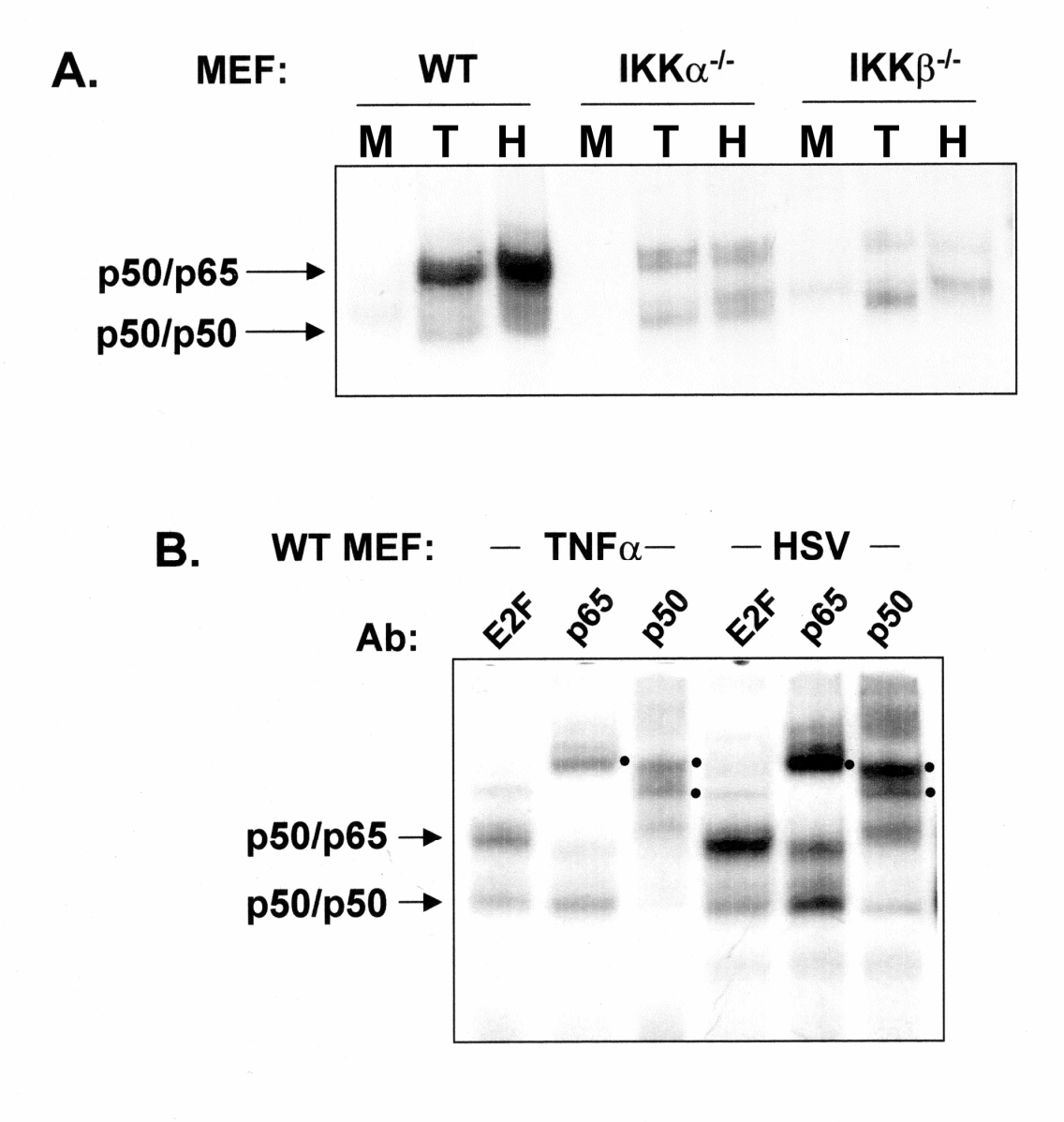

Figure 2.2. IKKα and IKKβ are necessary for NFκB DNA binding activity following infection. A. Nuclear lysates from wt, IKKα$^{-/-}$ or IKKβ$^{-/-}$ MEFs harvested at 8 hpi were assayed for NFκB DNA binding activity by EMSA. B. Nuclear lysates from wt MEFs infected with HSV or treated with TNFα were subjected to EMSA with antibodies against E2F, p65 or p50. M, mock infected. T, treated with TNFα for 15'. I, infected with wt HSV.

Table 2.1

Cell type	Results at 16 hpi		Results at 24 hpi	
	Yield (PFU/culture)	Fold decrease	Yield (PFU/culture)	Fold decrease
Normal	$(3.86 \pm 1.5) \times 10^7$		$(3.74 \pm 0.36) \times 10^7$	
IKK$\alpha^{-/-}$	$(7.09 \pm 3.4) \times 10^6$	5.4	$(6.0 \pm .37) \times 10^6$	6.2
IKK$\beta^{-/-}$	$(2.4 \pm 0.16) \times 10^6$	16.3	$(2.62 \pm 0.33) \times 10^6$	28.5

Table 2.1. Virus yield from wt, IKK$\alpha^{-/-}$ and IKK$\beta^{-/-}$ MEFs at 16 and 24 hpi. As per the Materials and Methods in Chapter 1, cells and media from three independent experiments at each time were collected and virus yields determined. Fold decrease is ratio of the yield from the wt cells to the knockout cells.

Figure 2.3

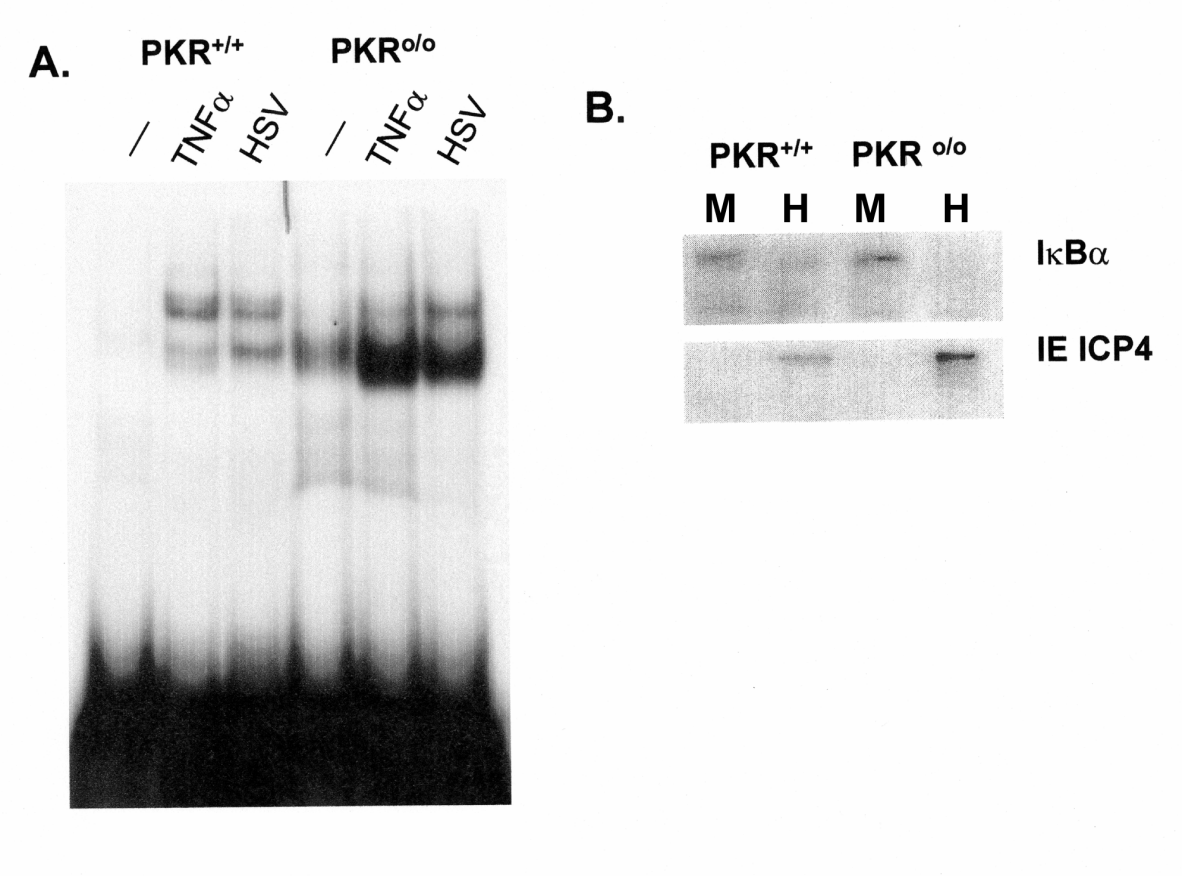

Figure 2.3. PKR is not required for NFκB activation following HSV infection. Cells with or without functional PKR were infected or treated with TNFα. A. Nuclear lysates were subjected to EMSA to detect NFκB DNA binding. B. Whole cell lysates were subjected to Western blotting to detect IκBα or ICP4. M, mock infected. I, infected with wt HSV.

Figure 2.4

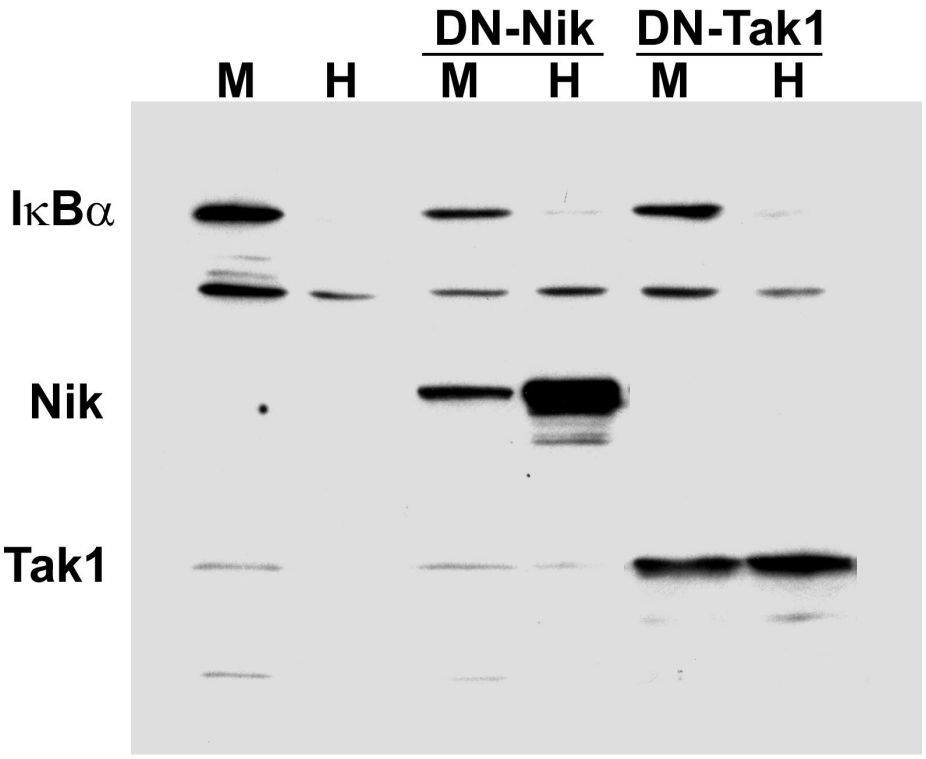

Figure 2.4. NIK and Tak1 are not necessary for NFκB activation. HEp-2 cells were transfected with plasmids expressing either dominant negative NIK or Tak1. After 16h the cells were infected with wt HSV and harvested at 8 hpi. Lysates were assayed for IκBα loss and NIK and Tak1 expression by Western blot. M, mock infected. K, infected with wt HSV.

CHAPTER 3

ACTIVATION OF THE DNA DSB RESPONSE FOLLOWING HSV INFECTION

Recently, many studies have begun to characterize the cellular the DSB response following infection with HSV, the results of which were detailed in Chapter 1. For the following part of my dissertation research I studied the DSB response in two biologically relevant cell lines; hTERT immortalized human keratinocyes (HFKs) and normal human lung fibroblasts (HELs). I initially characterized the status of the machinery most upstream in the DSB response: the MRN complex and ATM. Upon discovering that the DSB response was activated and that Mre11 was lost following infection, I further characterized the requirements for each. The latter is the focus of Chapter 4. Here I will describe my findings regarding the activation of the cellular DSB pathway.

The DSB response and Mre11 Loss Following HSV-1 Infection

Initially I sought to determine the status of the DSB response components following HSV infection. HFKs (Fig 3.1) were infected with wild type HSV-1 at an MOI of 10 and harvested at the indicated times. Lysates were analyzed by Western blot for viral and host DSB response proteins. The IE protein ICP0 was detectable by 1 hour post infection (hpi) and increased until 2 hpi, after which its accumulation remained unchanged. The early (E) protein ICP8 was detectable by 2 hpi and reached a steady level by 4 hours. By 4 hpi, the late (L) proteins VP16 and gC could be detected and increased in levels until at least 8 hpi. This pattern suggests that viral DNA replication began between 2 and 4 hpi.

Between 2 and 4 hpi I also observed activation of the DSB response proteins ATM and Nbs1. Others have reported that such activation resulted from viral DNA replication (85). Activated Chk2, however, was detected at 2 hpi. Chk2 activation preceding detectable activation of other DSB response components following HSV-1 infection was also reported by Lilley et al. and possibly indicates a limited amount of viral DNA replication at this earlier time (85, 137, 164). I also observed loss of DNA-PKcs following infection in HFKs. The loss began as early as 1hpi, though complete loss did not occur until after 2 hpi. Total levels of Rad50 and Nbs1 did not significantly change following infection in HFKs, similar to previous reports of other cell types (85, 137, 164). Essentially identical results were obtained using HEL cells (DNS).

Mre11 levels were also reported to be unchanged following HSV-1 infection (85). However, I observed a sharp decline in Mre11 levels between 2 and 4 hpi in HFKs and HELs. This loss was slightly delayed when compared to DNA-PKcs loss. Due to the temporal resolution of this experiment, I was unable to determine whether Mre11 loss coincided with, or was preceded by, viral DNA replication. However, as Mre11 is necessary for efficient viral DNA replication, the latter explanation is more likely (85, 137, 154, 164).

By immunofluorescence microscopy (IF), all three MRN components have been seen localized at sites of HSV DNA replication (40, 116). To see how Mre11 and Nbs1 behaved in our cell culture system, I infected either HFK or HEL at 0.01 MOI and fixed cells at various times post infection up to 8h, then immuno-stained for either Mre11 or Nbs1, and ICP8 and visualized by confocal microscopy (Fig 3.2A). By using a low MOI, I could observe several early stages of infection as judged by ICP8 localization patterns (diffuse, punctuate, small and large globular). Mre11 and Nbs1 were both localized diffusely

throughout the nucleus with nucleolus exclusion in uninfected cells. Early during infection, prior to detection of ICP8 or when ICP8 was diffuse, Mre11 and Nbs1 localization was indistinguishable from uninfected cells (Fig 3.2 A&B). However, once ICP8 became punctuate or globular, indicating localization to prereplicative sites and then replication compartments, these proteins became enriched co-locally with ICP8, indicating their presence at prereplicative sites and in replication compartments. Mre11 levels did not appear to change during these times.

When I allowed the infection to proceed for 24h or 48h, I detected cells with bright ICP8 staining throughout the nucleus but with little or no Mre11 staining (Fig 3.2C). In contrast, Nbs1 staining persisted at these late times in cells with bright ICP8 staining. Results from quantification of cellular staining patterns are shown in Table 3.1. These data support the Western blot results in Figure 3.1 in suggesting Mre11 is lost following viral DNA replication. The discrepancy in times at which Mre11 is lost is likely due to the differences in MOI.

The requirement of *de novo* gene expression for the DSB response and Mre11 loss

Input HSV genomes are linear, but circularize shortly following infection, possibly due to activity of the DSB response (85, 137, 164). As such, it would be expected that input genomes are able to activate the DSB response. Also, HSV-1 infection inhibits host gene expression, which would be expected to result in loss of unstable proteins as they turn over and are no longer replenished. To test if input viral genomes activate the DSB response or if Mre11 loss was due to instability, I infected HFKs or HELs in the presence or absence of actinomycin D or cycloheximide to inhibit mRNA or protein synthesis respectively. Cells were harvested at 16 hpi and the lysates analyzed by Western blot (Fig 3.3). Treatment with

either drug prevented viral gene expression, as indicated by the lack of detectable ICP0. Some gC was detected following drug treatment, likely from input virions. No Nbs1 activation was observed in the presence of either drug, indicating that input genomes are not sufficient to cause detectable activation of the DSB response. Also, drug treatment did result in roughly 50% reduction of Mre11 and Nbs1 levels, in both infected and uninfected cells. However, HSV-1 reduced Mre11 levels to 10% of the untreated mock control, indicating that loss was due to more than protein instability. These data also indicate that *de novo* viral gene expression is needed for full Mre11 loss, consistent with the observed kinetics in Fig 3.1.

DSB response activation and Mre11 loss are dependent upon a late viral event

Our initial Western blot analysis (Fig. 3.1) suggested that the action of either early or late viral functions were the effectors of DSB response activation and Mre11 loss. In order to differentiate between early and late functions, I used various mutants to halt infection at specific stages of gene expression. Mutants which fail to express either ICP4 (n12) or ICP27 (d27) are deficient in early and late gene expression. Infecting either HFKs or HELs with these viruses did not result in DSB response activation or Mre11 loss (Fig 3.4). The amount of ICP8 was reduced in n12 infection as expected, while ICP8 levels in d27 infection were equivalent to wt. I did detect gC in both infections, though the minimal amounts are likely the contribution of input virions. No ICP4 or ICP27 was detected in the lysates of cells infected with their respective mutants, confirming their genotype. These data indicate that DSB response activation and Mre11 loss require ICP4 and ICP27, as well as the expression of early or late proteins dependent upon ICP4 and ICP27.

To allow E gene expression, but prevent L gene expression, I infected cells with mutants deficient in ICP8 (d301) or viral DNA polymerase (HP66), or with wt virus in the

presence of PAA, an inhibitor of the viral DNA polymerase. These conditions prevent viral DNA replication and thus prevent true-late gene expression. HFKs or HELs infected with the two mutants exhibited gC amounts above that seen after n12 infection, though reduced in comparison to wt infection, while PAA treatment resulted in gC levels equivalent to that seen with n12. IE and E gene expression was largely unaffected as demonstrated by ICP4, ICP0, ICP27 and ICP8 levels. I did not observe Nbs1 activation under conditions which prevented viral DNA replication, which is in contrast to previous reports that observed activation of ATM and Nbs1 following HSV infection in the presence of PAA or acyclovir (another inhibitor of viral DNA replication), though not following HP66 (pol^-) infection (85). I did not observe Mre11 loss under these conditions, suggesting that viral DNA replication and/or L gene expression are necessary for its loss.

Importance of the DSB response for HSV replication

Previous reports on the importance of ATM in HSV infection are contradictory. Lilley et al. reported that fibroblasts from Ataxia Telangiectasia (AT) patients and cells treated with caffeine, which inhibits ATM activity, do not support efficient viral replication (137). Shirata et al. also showed that AT fibroblasts were unable to support efficient viral replication. However, using siRNA to knockdown ATM in 293T cells, they found no defect in viral replication (58). To elucidate ATM's importance to HSV replication and determine if activation of ATM's targets are necessary for Mre11 loss, HFKs were infected and either left untreated or treated with caffeine or KU-55933 (ATMi), a specific inhibitor of ATM (85, 137, 164). Cells were harvested at 8 hpi for Western blotting or at 24 hpi for virus yield or quantification of viral DNA by real time PCR (Fig 3.5 & Table 3.2). Western blot analysis showed that while both caffeine and ATMi inhibited Nbs1 phosphorylation by ATM, ATMi

was much more effective. Also, while caffeine greatly reduced gC levels, ATMi had no effect. Neither prevented Mre11 loss, indicating that the loss was not dependent upon events downstream of ATM in the DSB response. Caffeine treatment also resulted in a ~30% reduction in viral genome copy number and a 10 to 38 fold reduction in virus yield while ATMi treatment had no effect on genome copy number and only caused up to a 4 fold reduction in yield. These results suggest that caffeine's effects on HSV infection are largely due to activities other than ATM inhibition, and that while ATM activation does contribute to HSV replication, it is not essential.

Significance of results

The DSB response is activated following HSV infection. Input virus alone was insufficient to trigger this activation and the activation required viral DNA replication and/or late gene expression. While activation of this pathway did aid virus replication to some degree, it was not necessary for replication. These observations are mostly in keeping with those reported by others (137).

Most evidence suggests that activation of the DSB response is tied closely to viral DNA replication. While input viral genomes are linear, and thus potentially recognizable by the DSB response machinery, there is no evidence that they activate a large scale DSB response. Infection with UV inactivated and wt virus in the presence of gene expression inhibitors failed to cause detectable activation of the DSB response (85). Interference with viral DNA replication does, however, hinder activation of the DSB response. Lilley et al. reported that PAA treatment did inhibit activation of the DSB response following HSV infection at low, but not high MOIs (137). Shirata et al. confirmed this result and also demonstrated that acyclovir was able to interfere with the DSB response (164). Wilkinson

and Weller also saw inhibition of the DSB response following PAA treatment and also saw no activation by HP66 (pol$^-$) infection (154). These reports, and that of Taylor and Knipe (85, 137), also demonstrated that DSB components localize to sites of viral DNA replication, and ATM and Nbs1 at these sites are activated. Taken together with my results, these reports indicate that viral DNA replication triggers activation of the DSB response.

One discrepancy between our results and those of others is the effect of PAA on inhibition of the DNA damage response. I used a relatively high MOI in my experiments, which, according to Lilley et al. and Shirata et al. (164), should have been refractory to PAA's effects on Nbs1 activation. One likely reason that PAA, and also acyclovir, do not always inhibit activation of the DSB response is that, depending upon the exact conditions, these drugs allow some limited DNA replication. There were cell type and virus strain differences between our study and others, which might account for different extents of viral replication following drug treatment. Viral DNA replication was never directly assayed, though I used gC as a surrogate and Wilkinson and Weller used IF to visualize replication compartment formation (85, 137, 154, 164). In the future, a direct assay of viral DNA replication, such as quantitative PCR, will be able to determine if it explains the different results.

While the DSB response is likely activated by viral DNA replication, its importance to viral replication is still not understood. Due to the localization of DSB machinery to replication compartments, it has been proposed that the machinery helps facilitate viral DNA replication (85). This hypothesis is supported by Lilley et al.'s results (137) showing that cells from A-TLD or AT patients, thus deficient in Mre11 or ATM respectively, fail to facilitate efficient viral replication with over a log reduction in yield. Cells treated with

caffeine or expressing adenovirus E1B and E4orf6 proteins, which respectively are inhibited for ATM activation or have the MRN complex degraded, also had over a log reduction in yield. Mre11 deficient cells were also impaired for viral DNA replication. These results are called into question by the report by Shirata et al. (137). They also noted a deficiency in viral replication at the level of viral E protein in cells from AT or NBS (Nbs1 deficient) patients. However, if the DSB response primarily plays a role in aiding viral DNA replication, it should only cause reductions in L protein levels and not IE or E protein levels. They also went on to show that siRNA knockdown of ATM in 293T cells had no effect on virus yield. It is unclear what differences there might be in the cell types to account for the discrepant results. It is possible that SV40 large T antigen is disrupting the normal DSB response in 293T cells, though Shirata et al. demonstrated that it was intact following irradiation (58).

In our cell system, it appears that the DSB response, at least at the level of ATM activation, is not important for viral DNA replication and contributes only moderately to virus replication. Because caffeine has targets other than ATM, I also used the newly developed KU-55933 to specifically inhibit ATM activation (85, 152, 165, 171). While caffeine did drastically reduce both viral yield and DNA replication, ATMi had no effect on viral copy number and only moderately reduced virus yield. Thus, caffeine's inhibitory effects on virus replication are likely due to effects on targets other than ATM. How ATMi affected viral replication is unclear, as there also seemed to be no defect in late gene expression or viral genome copy number. One possibility is that failure to activate ATM somehow interferes with proper assembly or egress of progeny virions. Another explanation is that while viral DNA replication is able to produce equivalent genome copies with or

without ATM activity, its absence results in defective genomes which are unable to contribute to infectious progeny virus.

The results with ATM do not, however, provide insights into what role the MRN complex, which is upstream of ATM, might play in HSV replication. Because recombination is likely used by HSV to replicate its genome, and the MRN complex is normally necessary for such mechanism, it is very likely that the MRN complex is necessary for viral DNA replication, as suggested by the A-TLD and adenovirus protein expressing cells (85). Because the results from the ATM deficient AT cells calls into question the results from A-TLD cell lines and because of the possible multiple effects of adenovirus E1B and E4orf6 that cause MRN degradation, further studies are needed to elucidate the role of MRN in HSV infection.

Another aspect of the DSB response in HSV infection is its implications for the establishment of latency. Lilley et al. suggested that there may be a link between latency and the DSB response, as neurons are deficient in the DSB response (146). They went on to show that *in vitro* differentiated neurons infected with HSV do not activate the DSB response and also do not form replication compartments. They suggested that the inability of neurons to mount a DSB response causes a failure in viral replication leading to establishment of latency. I find this unlikely however; as such defects would also likely equally impair reactivation.

Figure 3.1

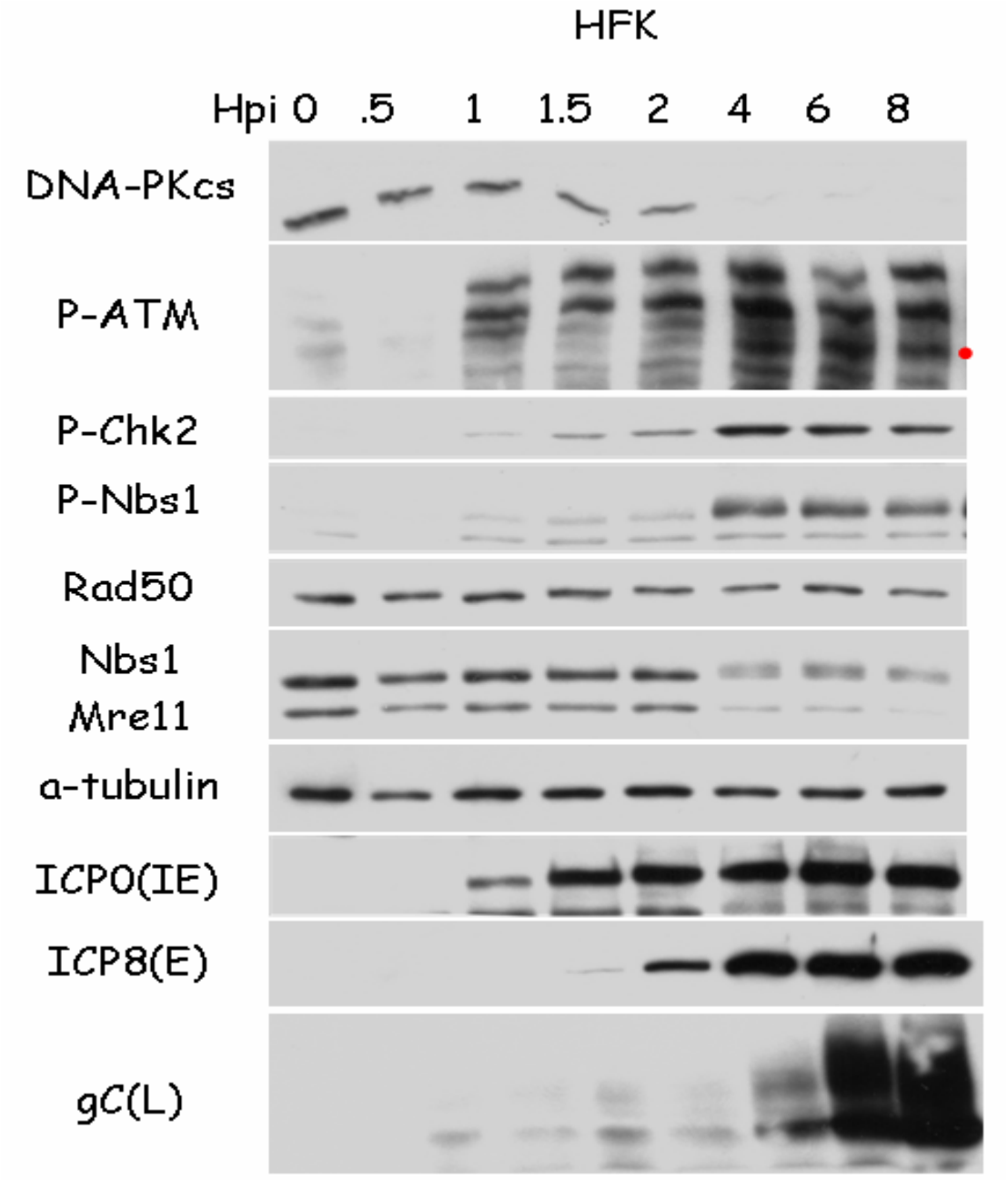

Figure 3.1. The DSB response is activated and Mre11 lost following HSV infection. HFKs were infected and harvested at the indicated times post infection. Whole cell lysates were probed for cellular components of the DNA DSB response and viral proteins by Western blot.

Figure 3.2A

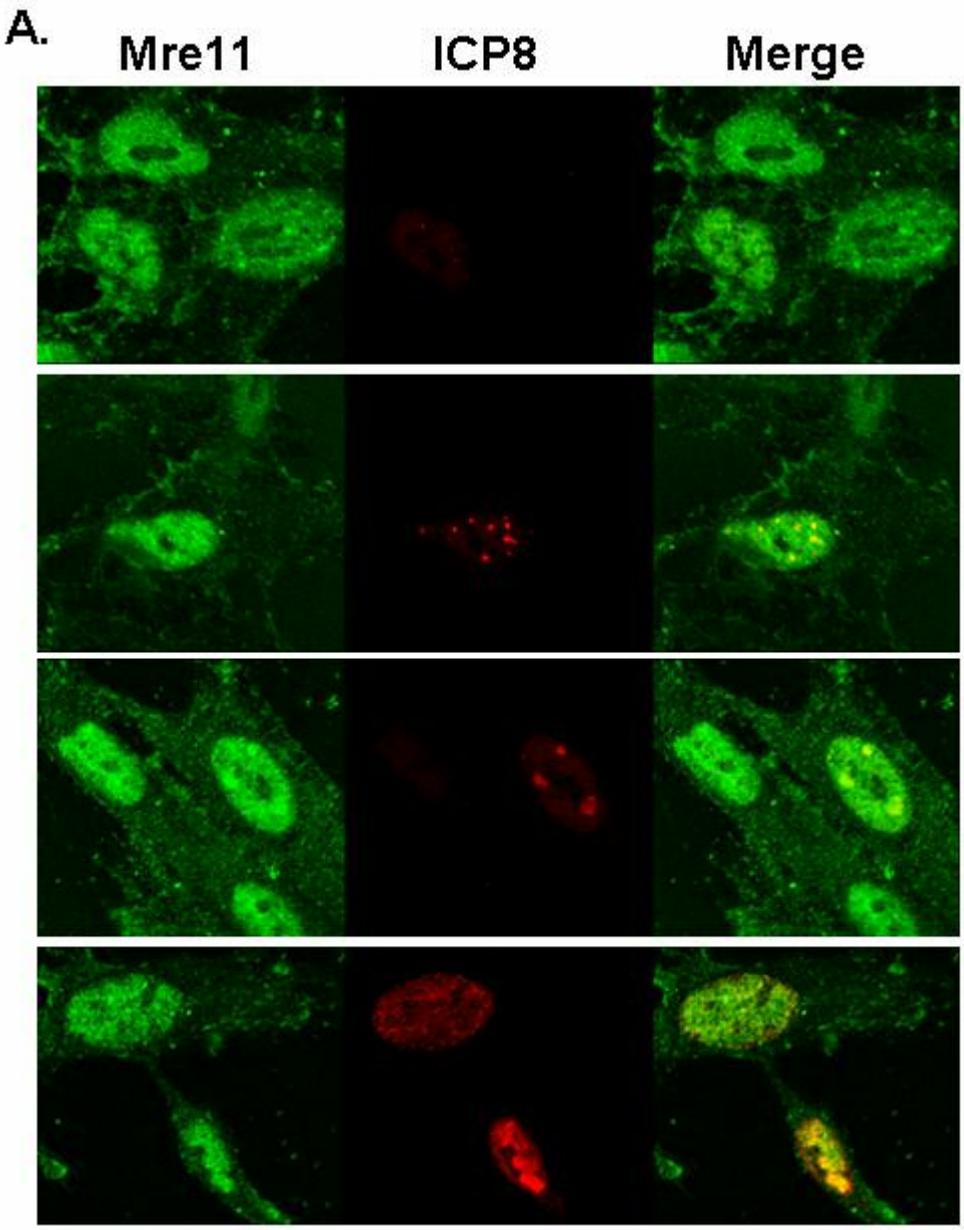

Figure 3.2B

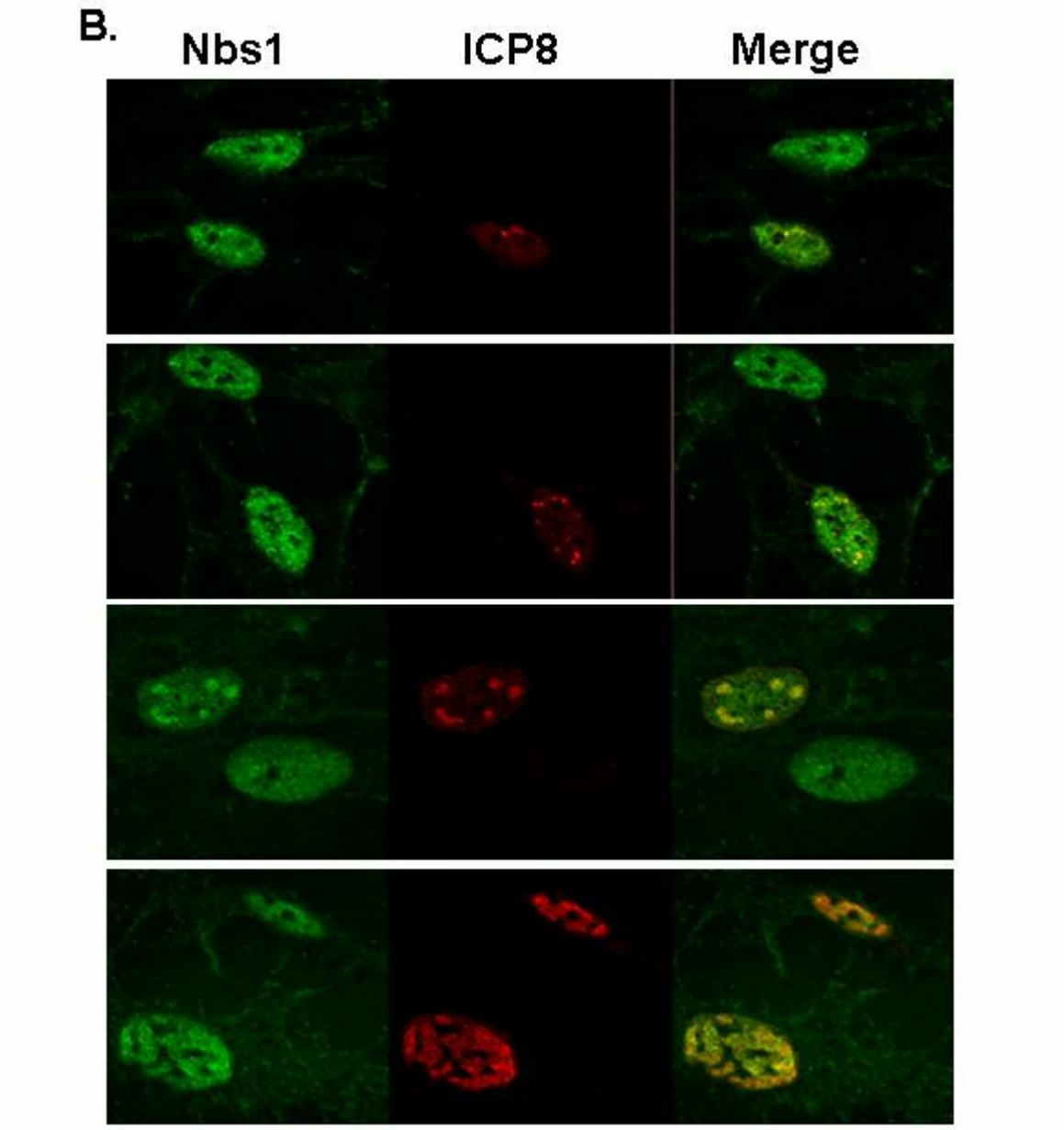

Figure 3.2C

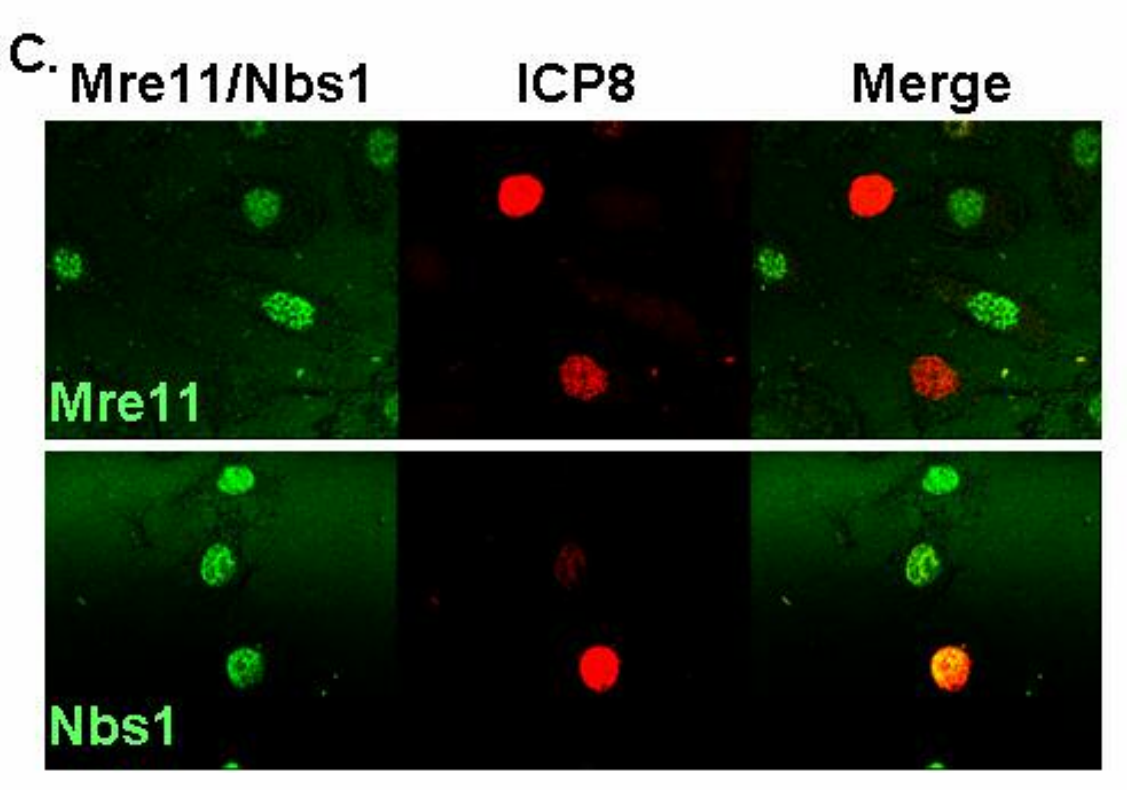

Figure 3.2. Mre11 and Nbs1 localize to sites of viral DNA replication. A & B. HFKs were infected at an MOI of 0.01 and fixed at 2, 4, 6 & 8 hpi. IF was performed according to the Materials and Methods in Chapter 1. Displayed cells are ordered to depict progression of infection and do not represent particular time points. C. HFKs were infected as in A & B, fixed at 24 or 48 hpi and subjected to IF.

Table 3.1

Cells w/o Mre11	24hpi	48hpi
Uninfected	14.7% (n=129)	22.7% (n=79)
Infected	48.1% (n=27)	70.8% (n=24)

Table 3.1. Mre11 is lost at late times following HSV infection. Cells visualized by IF as seen in Figure 3.2C were counted for the presence of Mre11 staining in conjunction with ICP8 staining.

Figure 3.3

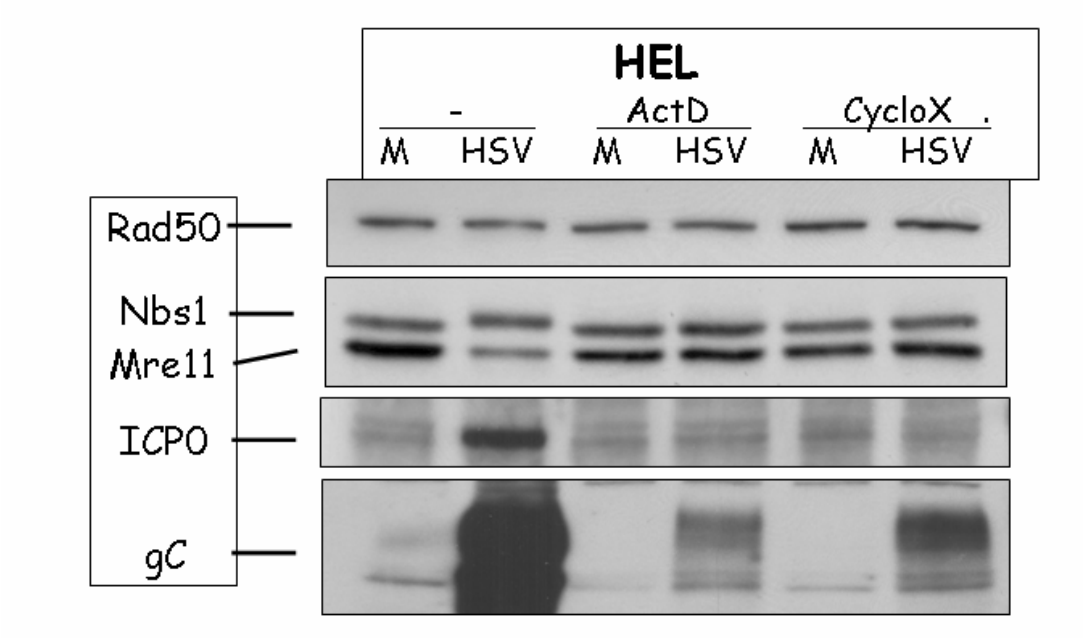

Figure 3.3. Activation of the DSB response and Mre11 loss following infection require *de novo* gene expression. HELs were infected in the presence or absence of Actinomycin D (ActD) or Cycloheximide (CycloX). Whole cell lysates were assayed for components of the MRN complex or viral proteins by Western blot. M, mock infected.

Figure 3.4

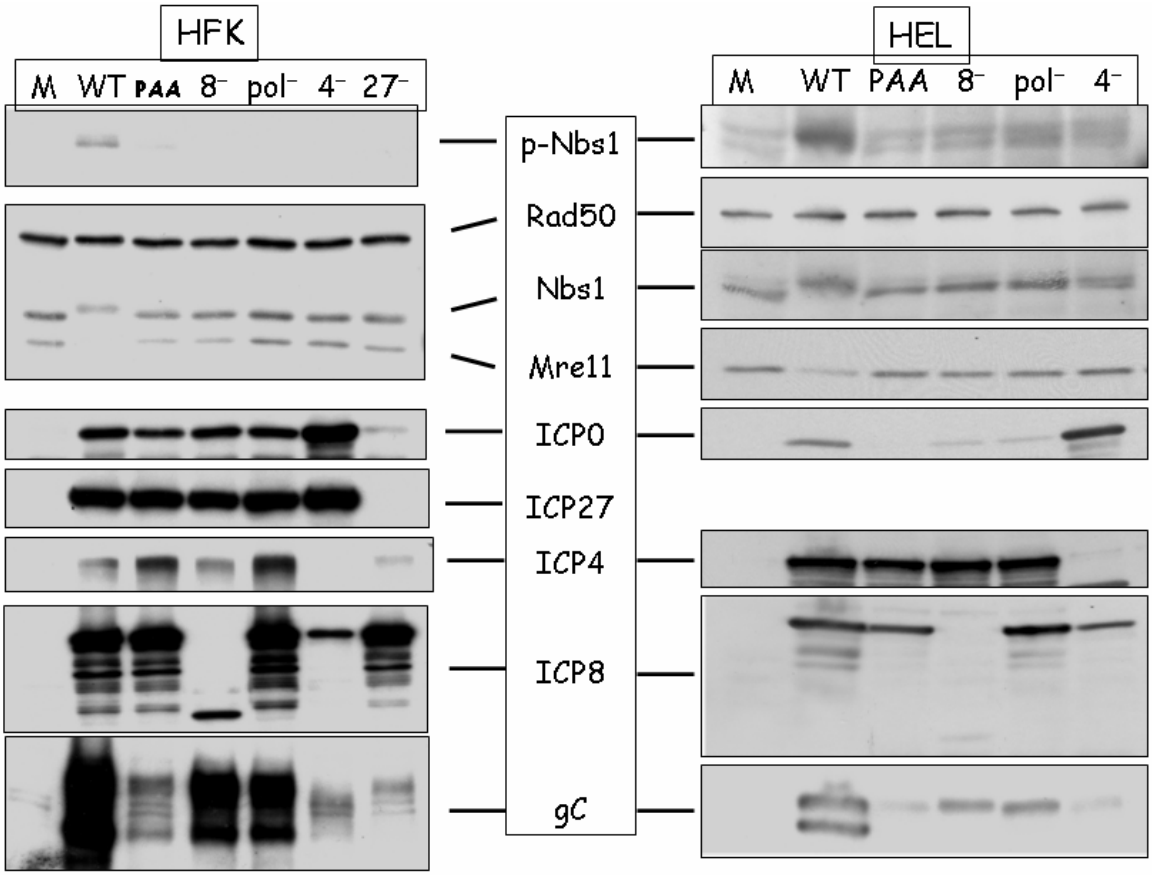

Figure 3.4. The DSB response activation and Mre11 loss require viral DNA replication. HFKs and HELs were infected with wt virus in the presence or absence of PAA or with the mutants deficient in ICP8 (8^-), polymerase (pol^-), ICP4 (4^-) or ICP27 (27^-). Lysates were assayed for cellular or viral proteins by Western blot. M, mock infected.

Figure 3.5

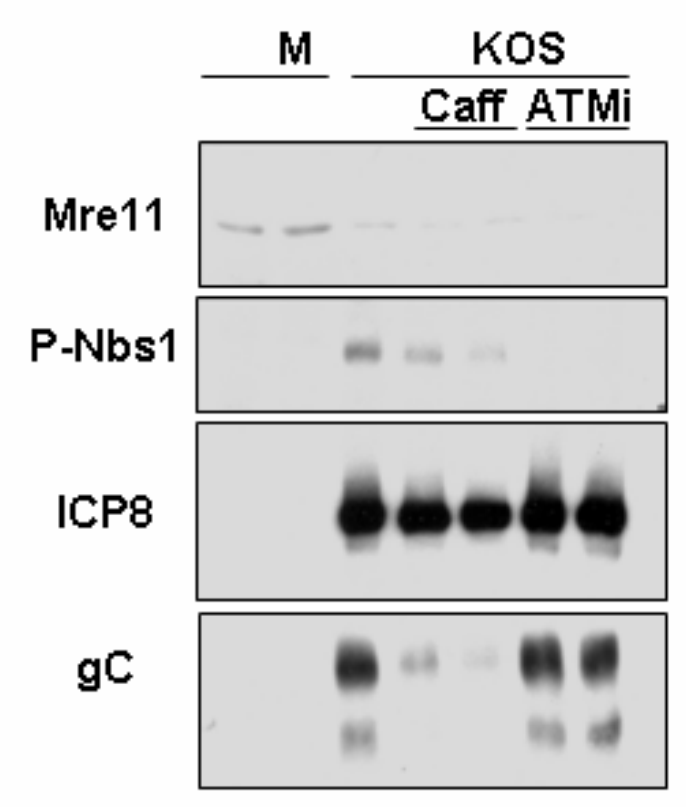

Figure 3.5. ATM activation is not required for Mre11 loss, nor late gene expression. HELs were infected in the presence or absence of caffeine (Caff) or KU-55933 (ATMi) and harvested at 8 hpi. Whole cell lysates were assayed for cellular and viral proteins by Western blot.

Table 3.2

	Yeild	Fold Reduction	DNA Copy
Experiment 1			
KOS	5.67×10^7		1
KOS + Caff	1.50×10^6	37.8	.60
KOS + ATMi	1.93×10^7	2.9	1.1
Experiment 2			
KOS	8.50×10^7		1
KOS + Caff	8.33×10^6	10.2	.74
KOS + ATMi	2.17×10^7	3.9	1.1

Table 3.2. ATM activity is not essential for viral replication. HELs were infected in the presence or absence of Caffeine (Caff) or KU-55933 (ATMi) and harvested at 24 hpi. Parrallel samples were assayed for viral yield or viral DNA copy as described in the Materials and Methods in Chapter 1.

CHAPTER 4

CHARACTERIZATION OF MRE11 LOSS FOLLOWING HSV INFECTION

As presented in Chapter 3, Mre11 is lost following HSV infection of HFKs and HELs. Adenovirus also causes loss of Mre11 in order to protect linear progeny genomes from recognition by the MRN complex and subsequent repair, which would result in their concatermizaration and/or circularization (18, 34, 89, 106). Though HSV DNA replication mechanisms do not result in linear genome accumulation, the replicated DNA must be processed into genome length units in order to be properly packaged into progeny virions. I wondered if HSV might be also be inactivating the MRN complex by degrading Mre11 in order to protect efficient processing and packaging of viral DNA. To test this model, I set out to characterize the mechanism of Mre11 loss following infection. In Chapter 3 I demonstrated that Mre11 loss requires de novo protein synthesis and is not caused by inherit instability of the protein. I have also shown that Mre11 loss requires viral DNA replication and occurs independent of ATM activity.

Mre11 loss is independent of ICP0, the proteasome and lysosome

ICP0 possesses E3 ubiquitin ligase activity and has been demonstrated to cause the proteasomal dependent degradation of many cellular proteins; DNA-PKcs, CENP-A, CENP-C, PMS and Sp100 (33). Though these targets of ICP0 are degraded early during infection, ICP0 is present throughout the course of infection allowing for the possibility that it may target Mre11 for degradation late during infection. To test whether ICP0 was necessary for

Mre11 loss, I infected HFKs with either wild type HSV-1 or the ICP0 mutant 7134 at various MOIs. At 16 hpi, the cells were harvested and lysates analyzed by Western blot (Fig 4.1A).

At low MOI infections, ICP0 mutants are deficient in expression of other viral genes (36). Here I saw no difference in gene expression between wt HSV-1 and 7134, regardless of MOI, indicating that all the MOIs used were above the threshold of this deficiency. As expected, 7134 failed to express ICP0 and thus did not cause DNA-PKcs degradation. However, the absence of ICP0 had no discernable effect upon either activation of Nbs1 or Mre11 loss. I observed similar results with HELs (DNS). These data indicate that ICP0 is not required for Mre11 loss following HSV-1 infection.

The possibility remained that Mre11 was being degraded by the proteasome due to the actions of other viral or cellular proteins. To determine if Mre11 loss was mediated by the proteasome, I infected HFKs with wt HSV-1 in the presence or absence of MG132, an inhibitor of the 26S proteasome. Cells were harvested at 8 hpi and lysates analyzed by Western blot (Fig 4.1B). Treatment with MG132 protected DNA-PKcs from degradation and had only a minor impact on viral protein levels. Activation of Nbs1 and viral protein levels were slightly reduced by treatment with MG132. This was expected, since MG132 was previously reported to hinder the progression of HSV infection (85, 137, 164). Mre11 levels were minimally affected by MG132 treatment. While some Mre11 was retained following MG132 treatment, comparison with DNA-PKcs protection clearly indicated that Mre11 loss was largely due to mechanisms other than proteasomal degradation.

Because Mre11 did not appear to be lost through proteasomal degradation, I decided to test if the lysosome was the cause of loss. HFKs were infected and after 1 hour, to allow for absorption and release of capsids from endosomes, treated with NH_4Cl. The cells were

harvested at 8 hpi and lysates subjected to Western blotting (Fig 4.1C). I observed no change in Mre11 loss with either 10 or 20 mM NH_4Cl, an inhibitor of lysosomal activity. These concentrations were sufficient to prevent productive infection when present at the time of infection (DNS). These data indicate that Mre11 loss is not mediated by the lysosome.

Mre11 loss is cell type specific

Others have reported that Mre11 is not lost following HSV infection of Vero, HeLa or HFF cells (1, 8, 117, 126, 131, 133, 134). To determine if I could observe Mre11 loss in cell types other than HFKs and HELs, I infected HeLa, HEp-2 and CV-1 cells with various HSV strains. HeLa and HEp-2 cells were infected at MOIs of 5 or 10 respectively, with wild type KOS or 17+, and ICP0 mutants 7134, d99 or d22, and harvested at 16 and 20 hpi (Fig 4.2 A & B). CV-1 cells were infected with KOS or 7134 at various MOIs and harvested at 20 hpi (Fig 4.2 C).

Western blotting of lysates from HeLa or HEp-2 cells showed infection with all strains tested resulted in decreased Mre11 levels, while other cellular proteins were largely unaffected. This was not the case with CV-1 cells. Initially I observed no change in Mre11 levels in whole cell lysates from CV-1 cells (DNS). To insure Mre11 was not being inactivated by mislocalization, I prepared fractionated lysates from CV-1 cells infected at various MOIs at 20 hpi. All the MRN components largely localized to the nucleus, with no relocalization or reduction of levels. These results indicate that Mre11 loss is a cell type dependent phenotype, though not a virus strain dependent phenotype.

Analysis of Late mutants for Mre11 loss

Because Mre11 loss did not occur in the absence of viral DNA replication and late gene expression, I decided to assay viruses deficient in expression of various late genes for

their ability to cause Mre11 loss. Because any benefit to HSV replication due to Mre11 loss is likely to be due to preventing detection and subsequent repair of free DNA ends at late times of infection, I felt it likely that Mre11 was being lost in order to protect the efficacy of processing and packaging of progeny genomes, which results in generation of free DNA ends. Thus, I chose to test mutants that have been characterized as deficient in proper processing and packaging of progeny genomes. Mutant viruses individually deleted for UL6 (hr74), UL15 (hr81-1), UL17 (dUL17), UL25 (kUL25), UL28 (GCB) and UL32 (hr64) were tested for Mre11 loss following infection (101). UL6 encodes the portal protein through which DNA enters the capsid (2, 174). UL15 and UL28 gene products are components of the terminase (97, 145). The UL25 gene product is necessary for stabilizing encapsidated DNA and late stages of capsid maturation (75, 153). UL17 and UL32 expression is necessary for correct localization of preformed capsids to sites of DNA packaging (30).

I infected HFKs (Fig. 4.3A) and HELs (DNS) with these mutants and harvested at 8hpi to test for Mre11 loss. I noted that the UL15 and UL17 mutants consistently, and UL6 mutant occasionally, showed less Mre11 loss than wt virus, as seen in Fig. 4.3A. However, further analysis of the lysates revealed reduced levels of selected viral proteins. This defect has been previously seen for these mutants and contributed to a high complementation index when titers were determined on complimenting cell lines (personal communication, S. Weller & J. Baines). These mutants also exhibited reduced activation of Nbs1, indicating inefficient viral DNA replication. As such I could not determine whether the failure to cause Mre11 loss was due to the specific loss of the viral genes or due to the general replication defect.

In order to overcome this defect in general viral fitness for the viral mutants hr81-1, hr74 and dUL17, I repeated infections at an MOI of 200 (Fig. 4.3B). At this MOI, viral

protein levels in the mutant infected cells were equal to or in excess of that of wt virus at an MOI of 20, and Nbs1 activation was also equivalent to wt infected cells. However, under these conditions I also no longer saw any defect in Mre11 loss. This result would indicate that UL15, UL6 and UL17 products are unnecessary for Mre11 loss.

Another late function that could affect Mre11 loss is the viral protease encoded by UL26, which conceivably could directly target Mre11. To determine if UL26 was necessary for Mre11 loss, I infected HFK cells with UL26dZ, a mutant with a deletion of the entire UL26 orf, or UL26H61E, a point mutant disabling the protealytic activity of the viral protease (Fig. 4.3C). At an MOI of 20, these mutants had no defect in Mre11 loss or viral protein accumulation. This result suggests that the viral protease does not contribute to loss of Mre11.

Camptothecin causes Mre11 loss

Though none of the late mutants I tested were defective in Mre11 loss in the end, I did make an interesting observation that Mre11 loss was occasionally observable despite absence of detectable late proteins (DNS). This observation led us to consider the possibility that viral DNA replication, rather than a viral encoded late function, was the trigger of Mre11 loss. As viral DNA replication is the trigger for activation of the DSB response, I considered the possibility that activation of the DSB response could lead to Mre11 degradation, perhaps as part of a negative feedback loop. However, in Chapter 3 I demonstrated that inhibition of ATM and thus the DSB response downstream of ATM did not affect Mre11 loss. Mre11 is upstream of ATM, and thus it would still be possible that engagement of DNA ends by the MRN complex would be sufficient to cause Mre11 loss.

If MRN detection and engagement of DSBs were sufficient to cause Mre11 loss, I would expect this to occur outside the context of HSV infection. To test if this was the case, I treated cells with camptothecin (CPT), which results in the generation of free DNA ends and activation of the DSB response in S-phase cells due to topoisomerase I inhibition. Because CPT primarily functions in S-phase, I serum starved confluent HEL cultures and then released them by passaging in media with serum. After allowing 20 hours for the cells to re-enter the cell cycle, which has previously been shown to result in an enriched S-phase population, cells were treated with CPT and/or ActD (85, 137, 164). ActD was used to control for differences in protein level due to differential cell growth. After 4 hours treatment cells were harvested and lysates subjected to Western blotting (Fig 4.4). Rad50 and tubulin levels were reduced equally in all cells treated with ActD, with or without CPT, in comparison to untreated cells. ActD treatment also resulted in reduced Mre11 levels, though combined ActD and CPT treatment caused further reduction. CPT treatment alone resulted in a further reduction of all protein levels compared to ActD treatment. A possible cause for this might be a greater induction of apoptosis in these cells, though no cytopathology was observable (DNS).

Though the reduction in Mre11 following CPT was insignificant compared to that observed following HSV infection, this result does indicate that activation of the DSB response, or perhaps certain general cellular stresses, can cause Mre11 turnover. I suspect that the loss of Mre11 was less significant due to the fact that CPT treatment most greatly affects cells in S-phase. While my conditions should enrich for an S-phase population, many cells were likely still in G1/G0 or perhaps had progressed beyond S-phase by 20 hours after

release from serum starvation. As such, a portion of the cells were unaffected by CPT treatment, thus reducing the observable loss.

Significance of Results

Mre11 loss following HSV infection would be expected to inhibit the ability of the cellular DSB response machinery to recocognize and respond to DSBs. How this loss might affect HSV replication, if it does at all, is unclear. Here, I have shown that Mre11 loss following HSV infection is a late event that is dependent upon viral DNA replication and perhaps late gene expression. Unlike many other cellular proteins that are lost following HSV infection, Mre11 loss is not caused by ICP0 activity, nor does it depend upon the proteasome or lysosome, though it is possible that NH_4Cl does not fully inhibit the protealytic activity of lysosomes. As these general degradation mechanisms appear not to be involved in Mre11 loss, the actions of specific proteases is left as the most plausible explanation for the loss. Another possibility is that Mre11 is being displaced from the MRN complex, resulting in its instability and degradation. This is unlikely however, as it would still depend upon Mre11 being degraded by the proteasome or lysosome.

Others have not reported that Mre11 is lost following infection, though their systems utilized different cell types and/or virus strains (20, 85, 137, 164). In my hands, I found that Mre11 loss was dependent upon cell type, though not virus strain. The basis for the differences between the cell types is unknown, though notably the one cell type in which I observed no loss of Mre11 at all was a non-human primate derived line, whereas all others tested were of human origin. Further experiments may determine if this is a human specific phenotype.

My results suggest that either viral DNA replication or the function of late genes trigger Mre11 loss following HSV infection. While I have shown a number of late gene functions to be unnecessary for Mre11 loss, it remains possible that there is a functional redundancy amongst those I tested or that a function I have not tested is responsible for Mre11 loss. I expect this not to be the case however, as Mre11 loss is observable in the absence of late protein expression, which leaves viral DNA replication as the likely trigger for loss. To further confirm this, late genes could be expressed from a cosmid library to determine if any are sufficient to cause Mre11 loss.

If viral DNA replication does trigger Mre11 loss, the mechanisms of how this occurs is unclear. It is possible that viral DNA replication allows an IE or E protein to either directly or indirectly target Mre11, leading to Mre11's degradation. However, I have already demonstrated that the most likely candidate, ICP0, is not necessary for Mre11 loss. No other IE or E proteins have characterized functions that would suggest they would be involved in protein loss. Another possible mechanism would involve a cellular protein being triggered by viral DNA replication to cause Mre11 loss. As it is known that viral DNA replication does trigger some cellular stress pathways, such as the DSB response and PKR, this is a reasonable possibility (85).

If activation of such stress pathways does lead to Mre11 loss, I would expect their activation to lead to Mre11 loss out of the context of infection. I found this was indeed the case for the DSB response induced by CPT treatment. This result may indicate that the activation of the MRN complex by viral DNA replication leads to Mre11 turnover. However, as Mre11 loss still occured when ATM activation was inhibited, the loss would not be the result of effectors in the DSB response pathway downstream of ATM. If Mre11 loss

is simply a by-product of DSB response activation, it may be that loss does not contribute to viral replication. Given that Mre11 is likely important for viral DNA replication, its loss may in fact be detrimental (48, 114). Even so, the observed loss of Mre11 following CPT treatment may represent a novel negative regulatory mechanism for the DSB response.

Further experiments are needed to identify the exact mechanism of Mre11 loss and determine if loss following HSV infection and CPT treatment are in fact the same. Use of specific and broad range protease inhibitors might identify the relevant protease and thus also allow the significance, if any, of Mre11 loss to viral infection to be determined. Otherwise, tagging of Mre11 and either pull down experiments or live cell microscopy tracking may provide clues to the fate of Mre11 through identification of interacting proteins or observation of aberrant localization.

Figure 4.1A

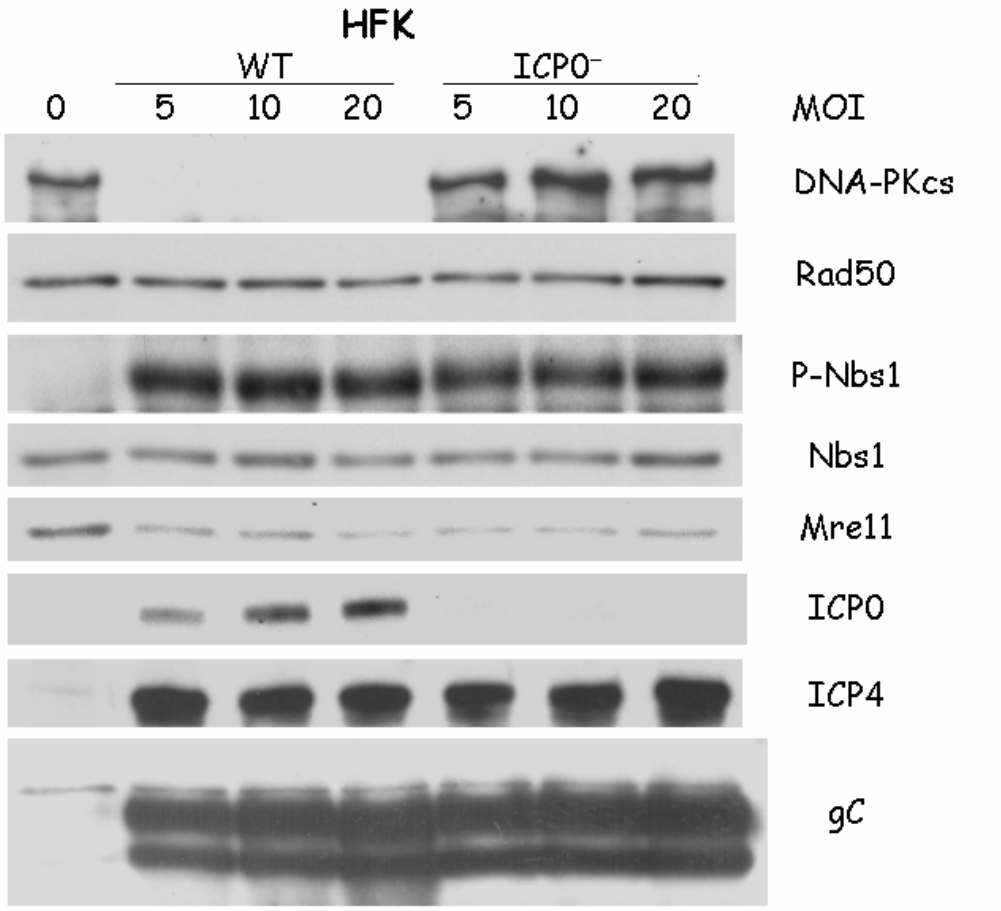

Figure 4.1. Mre11 loss does not require ICP0 or the activities of the proteasome or lysosome. A. HFKs were infected with wt or a ICP0 deficient virus at the indicated MOIs and harvested at 8 hpi. Whole cell lysates were assayed for cellular and viral proteins by Western blot. B & C HFKs were infected with wt virus and were overlayed with media with or without MG132 or NH_4Cl at the indicated concentrations at 1 hpi. Lysates were assayed by Western blot for cellular and viral proteins. M, mock infected. H, infected with wt HSV.

Figure 4.1B

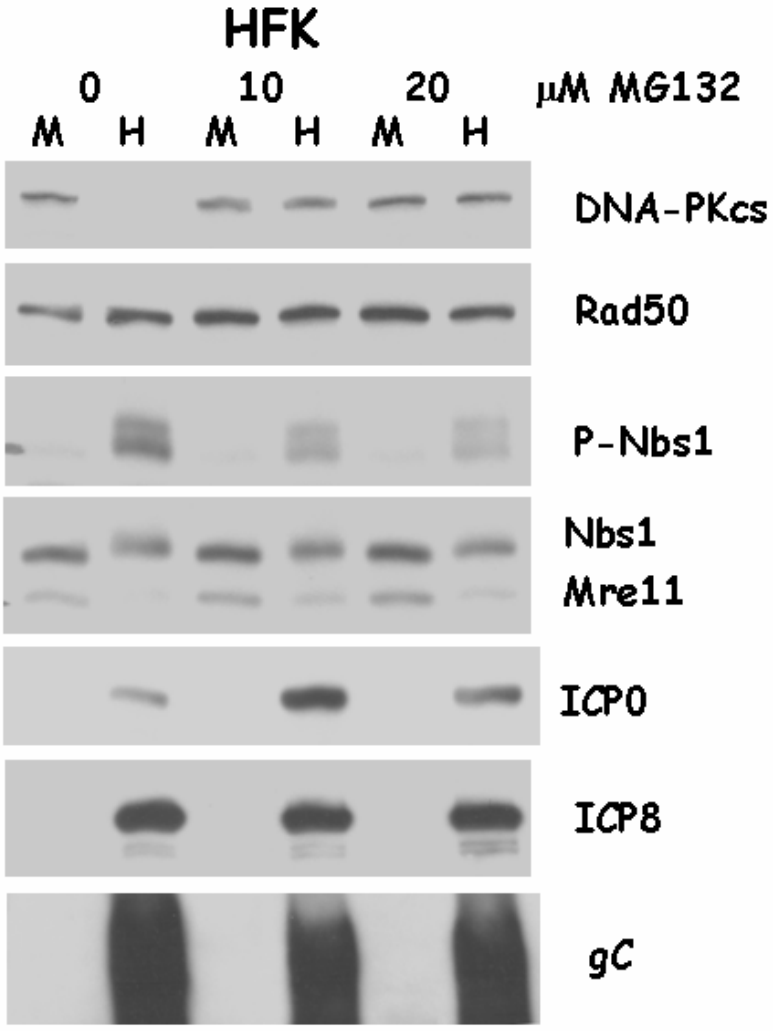

Figure 4.1C

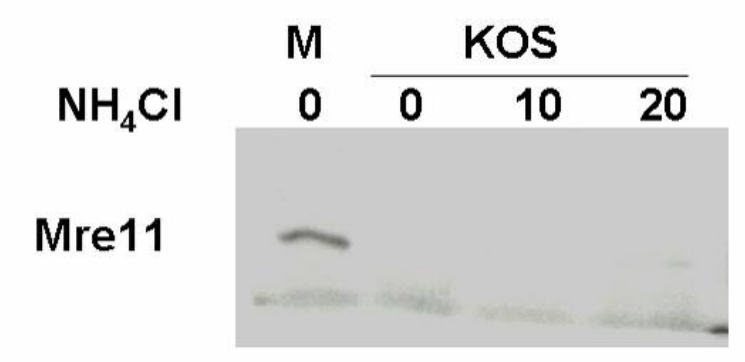

Figure 4.2A

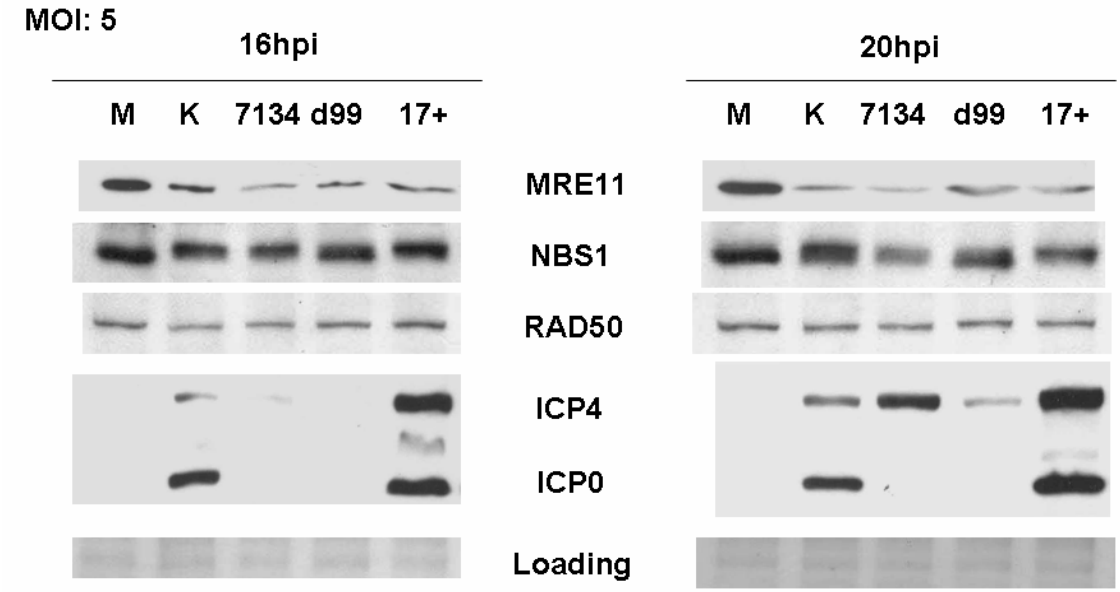

Figure 4.2B

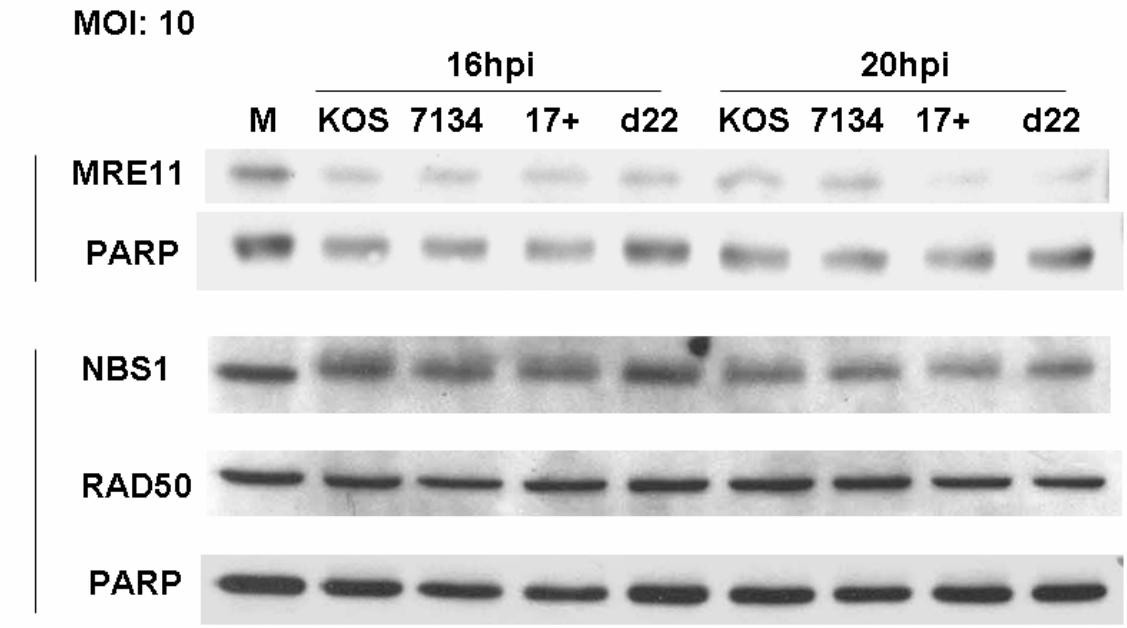

Figure 4.2C

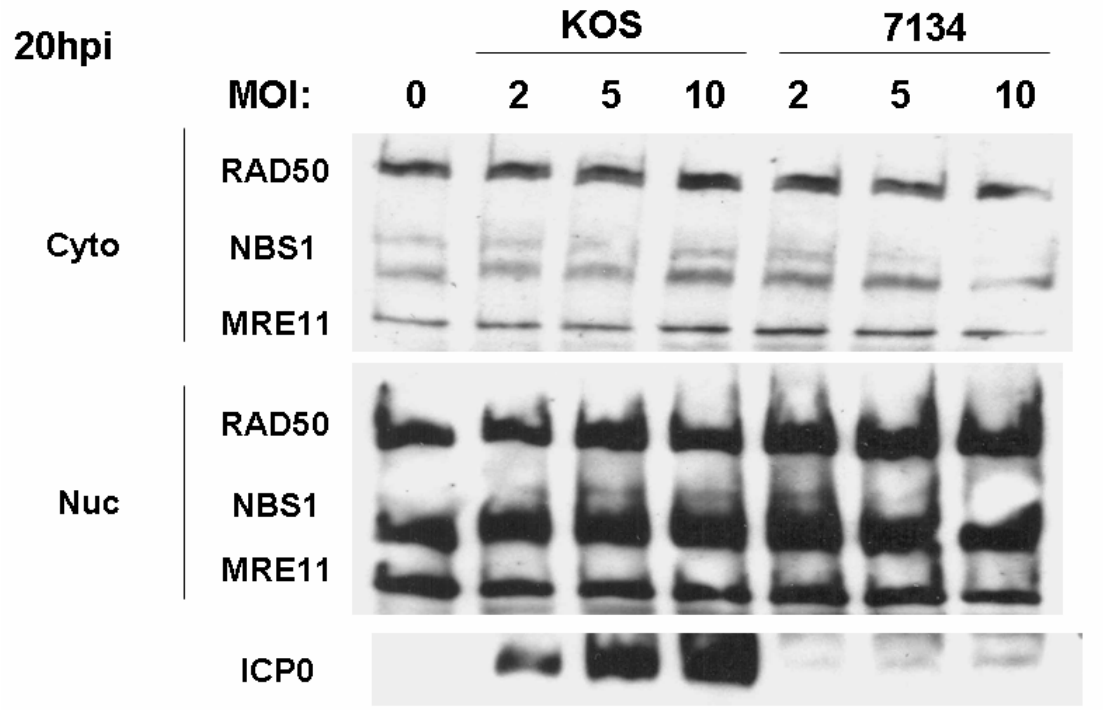

Figure 4.2. Mre11 loss is cell type dependent. HeLa (A), HEp-2 (B) and CV-1 (C) cells were infected with the indicated wt or ICP0 deficient viruses at the indicated MOIs and harvested at the indicated times. A & B. Whole cell lysates were probed for cellular and viral proteins by Western blot. C. Fractionated lysates were likewise probed.

Figure 4.3

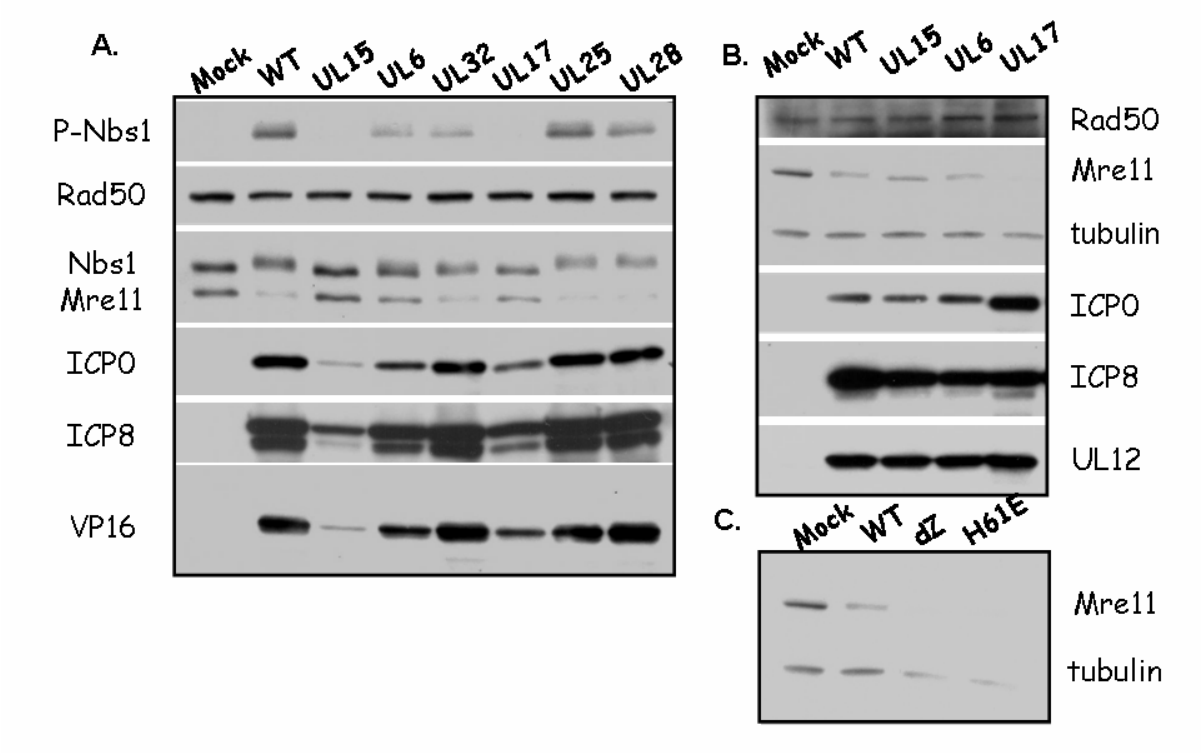

Figure 4.3. Processing and packaging mutants cause Mre11 loss. HFKs were infected with wt or the indicated mutants characterized for defective processing and packaging of viral DNA (A & B) or the viral encoded protease (C). Mutant viruses were used at an MOI of 20 (A) or 200 (B). Lysates were analyzed for cellular and viral proteins by Western blot.

Figure 4.4

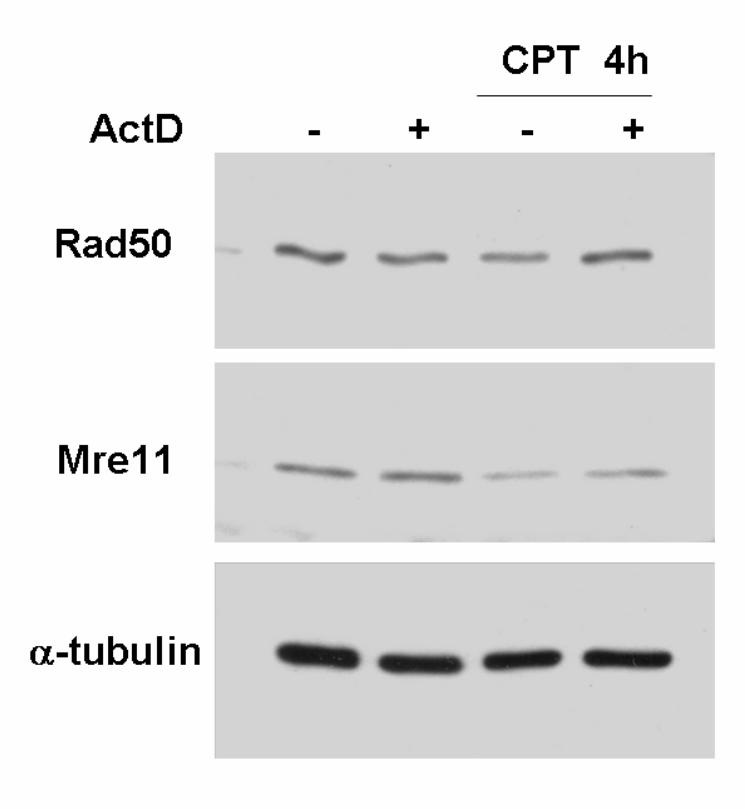

Figure 4.4. Camptothecin treatment causes Mre11 loss. HELs were growth arrested by contact inhibition and serum starvation. 20 hours following release from arrest by replating in fresh media, cells were treated with camptothecin (CPT) or actinomycin D (ActD) for 4 hours. Whole cell lysates were assayed for Rad50, Mre11 and tubulin by Western blot.

CHAPTER 5

CONCLUSIONS

Viruses are efficient pathogens that are absolutely dependent upon a host cell to complete their life cycle. To facilitate viral replication, host cell machinery is co-opted by the virus to carry out necessary tasks in replication. In this dissertation I have characterized two instances in which HSV interacts with host cell signaling machinery to benefit its own replication. First, I have demonstrated the independent importance of the two kinase components to the IKK complex, IKKα and IKKβ, in the activation of the NFκB pathway following HSV infection. Second, I have demonstrated activation of the DSB response and the loss of Mre11, a component of the DSB response, following HSV infection. Here I will discuss the relevance of these findings to our knowledge of HSV life cycle and consider future research in these areas.

NFκB activation

I have shown that the second sustained NFκB activation following HSV infection is dependent upon both IKKα and IKKβ. IKKα was necessary for NFκB DNA binding activity, and thus any transactivation or transrepression activity NFκB may normally have during HSV infection. IKKβ was necessary for IκBa degradation and thus p65/p50 nuclear translocation. It is also possible that IKKβ plays a further role in controlling DNA binding ability or transactivation ability, though it was not possible to assay for these activities due to the upstream defects in the pathway.

IKKα has been shown to be important for NFκB binding to a subset of its responsive promoters, likely due to phosphorylating p65 and or p50 (113). IKKα can also function as a derepressor by targeting transcriptional repressors SMRT and HDAC3 for removal from NFκB target genes and by phosphorylating histone H3 (123). To determine if IKKα has similar functions during HSV infection several experiments need to be performed. If IKKα does directly target p65 or p50 during infection, this could be revealed by comparision of phosphorylated residues detected by Western blot of lysates from cells with or without functional IKKα. Also, cells expressing p65 with alanine substitutions mutations at sites targeted by IKKα could be infected to determine if they replicate the IKKα null phenotype seen in Chapter 2. Conversely, glutamic acid substitutions, which can mimic phosphorylation at these residues, could be used to assay complementation of NFκB activation and HSV replication defects in IKKα$^{-/-}$ cells.

Similarly, Western blotting for phosphorylated sites on repressors, such as SMRT and HDAC3, and H3 from infected cells with or without IKKα could determine if IKKα functions as a derepressor following HSV infection. Also, chromatin immunoprecipitation (ChIP) could be performed to analyze the differential make up of the chromatin structures at HSV dependent NFκB bound sites in cells with or without IKKα. This experiment would also require identifying sites, other than the ICP0 promoter, that are bound by NFκB following infection. The VP16 promoter is the only other site in the HSV genome with identified putative NFκB sites (113). Other sites could be identified by performing ChIP with antibodies against p65, blunt cloning the released DNA fragments and sequencing them. This method would identify any bound DNA not already known or not identifiable by scanning for putative NFκB binding sites in the HSV or cellular genomes.

Determining if IKKβ also has a role in activation of p65/p50 by phosphorylation following infection will be more difficult, as the defect in IκBα turnover and p65 translocation must be bypassed to determine if any such phosphorylations are important for DNA binding or transactivation. Some p65 translocation is seen in IKKβ$^{-/-}$ cells, and thus it may be possible to directly detect phosphorylation sites by Western blot. Sites that are targeted in wt, but not IKKβ$^{-/-}$, cells would determine which residues, if any, are targeted by IKKβ following HSV infection. Alanine replacements could again be tested to determine if any of the IKKβ targeted sites are important. Glutamic acid replacement would be useful if IκBα degradation can be affected without IKKβ activation and activation of other pathways that potentially lead to p65 or p50 phosphorylation.

The observation that IKKβ$^{-/-}$ cells had up to a 28 fold reduction in yield and IKKα$^{-/-}$ cells had only up to a 6 fold reduction while both failed to activate NFκB and allow for substantial late gene expression suggests that IKKβ has additional roles outside of NFκB activation. In addition to the NFκB subunits, IKKβ has been shown to phosphorylate Bcl10, IRS1, Dok1, 14-3-3 and FOXO3a (109). Currently there are no reports of activation of these targets following infection. Assaying for such activations could potentially reveal additional roles for IKKβ in HSV infection.

Though IKKβ is the primary activator of NFκB following infection, the upstream effector is still unknown. I tested two upstream factors known to activate NFκB primarily through IKKβ, PKR and Tak1, as well as Nik, which normally signals through IKKα, for their importance in HSV NFκB activation. PKR was tested using deficient cell lines while dominant negative constructs of Tak1 and NIK were used to test for their contribution. None were necessary for NFκB activation following HSV infection. Whether HSV utilizes

existing cellular pathways that activate IKK or simply encodes its own activating function remains to be determined. Current evidence suggests that IKK activation may involve both of the above.

Both ICP4 and ICP27 contribute to NFκB activation following HSV infection (55). More recent studies by Hargett et al. (54, 55) indicate that ICP27 is necessary and sufficient for NFκB activation. They also demonstrated that p38 and JNK signaling, which ICP27 also triggers, contribute to HSV NFκB activation. That the ICP27 mutant dLeu was capable of activating p38 and JNK, but was deficient in NFκB activation, indicates both that ICP27 activation of p38 and JNK is not sufficient for NFκB activation, and that it either utilizes an as yet undescribed pathway to activate NFκB, or perhaps directly contributes to NFκB activation (4, 49, 50). Further experiments characterizing ICP27 mutants and their physically interacting proteins and other signaling pathways will lead to a more complete understanding of HSV NFκB activation.

The other important aspect of NFκB signaling during HSV infection is its role in virus infection. Currently there is evidence that NFκB activates the ICP0 gene and prevents apoptosis following HSV infection (123). How NFκB affects apoptosis prevention during infection and what other roles it may play are not yet known. Because the promoter of VP16 also contains putative NFκB binding sites, it is likely that NFκB also activates VP16 expression (72). It is also possible that NFκB binds other unidentified sites in the HSV genome and activates other genes. Further studies utilizing ChIP may reveal such sites in relevant cell types, as discussed above.

NFκB anti-apoptotic activity is well characterized following TNFα induction (5, 65). NFκB activates expression of a number of anti-apoptotic genes, such as those encoding IAPs,

Bcl2 family members, TRAF2 & 5 and others. Though it is not known how HSV affects transcription of all of these genes, Bcl-2 and XIAP are upregulated and play a role in preventing apoptosis following infection (85, 137, 164). It is not known whether this upregulation is dependent upon NFκB. Assaying mRNA levels of Bcl-2 and XIAP, as well as other NFκB dependent anti-apoptotic genes, following infection of cells unable to activate NFκB, such as the IKK knockout MEFs or cells transfected with DN-IκBα, will establish whether they are in fact activated by NFκB. Further studies utilizing cells knocked out or down for these upregulated genes will confirm the role for Bcl-2 and XIAP in preventing apoptosis following infection and determine if any other factors play a similar role.

DBS response activation

I have shown that HSV activates the DSB response following infection, dependent upon viral DNA replication, in accordance with reports from others (85). The significance of this observation is not entirely clear. While immunofluorescence and immunoprecipitation data clearly demonstrate interactions between the DSB machinery and viral DNA replication, its role remains unknown. The most likely explanation is that the DSB machinery aids in facilitating later stage recombination dependent viral DNA replication. This might explain my observation that inactivation of ATM did not greatly affect viral genome copy number or virus yield, as ATM is not essential for recombination, though it does contribute to its efficacy.

Mre11 is upstream of ATM and is necessary for recombination. Fibroblasts deficient for Mre11 (A-TLD derived) or cells where Mre11 is degraded (expressing adenovirus EIB and E4Orf6) are defective in facilitating virus replication and, at least in the case of the former, DNA replication (19). WRN is another protein involved in recombination (154).

Cell lines deficient for WRN also have been shown to be defective in supporting viral replication and HSV specific recombination (85). These results strongly suggest that HSV requires DSB and recombination machinery to replicate its DNA. Further studies could be performed to directly assay the resulting replicated DNA structures under conditions where host recombination machinery is perturbed. Knock out cell lines and siRNA mediated knockdown of other components of the DSB response would further our understanding of the exact mechanisms that are used in HSV DNA replication. It may also be possible to use electron microscopy of HSV DNA at various stages of replication to directly visualize the replication mechanisms.

Mre11 loss

Because Mre11 appears to be necessary for efficient viral replication, it is curious that Mre11 is lost following infection . However, because Mre11 loss also only occurs following initiation of DNA replication, it may be that it has served its utility by the time of loss. Another possibility is that Mre11 loss may limit the extent of viral DNA replication. A third non-mutually exclusive possibility, which I originally hypothesized upon observation of Mre11 loss, is that the loss is beneficial to HSV because it prevents interference with processing and packaging of progeny genomes. Packaged genomes are linear and could be acted upon by the DSB response machinery if the free DNA ends were accessible. Though the exact mechanism of packaging is not known, it is thought to be similar to phage packaging and thus packaged genomes are not likely to be accessible to cellular machinery. Nonetheless, packaging would still result in the unpackaged viral DNA having free DNA ends that would be accessible to the host machinery. Processing of these ends by the MRN complex could then be detrimental to further packaging.

To test these models, a method of inhibiting Mre11 loss without interfering with Mre11 activity would have to be developed. While I have done much to advance our understanding of what triggers Mre11 loss following HSV infection, I have not discovered a method of preventing Mre11 loss without also preventing DNA replication where it likely functions to the benefit of the virus. It is clear that unlike the case of most host proteins lost following infection, ICP0 is not responsible for Mre11 loss. Even more curious, neither the proteasome nor the lysosome are effectors of Mre11 loss. While it remains possible that a function encoded by a late gene is responsible for the loss, I have not been able to identify a late protein that is necessary for Mre11 loss. As Mre11 loss was observable in the absence of late gene expression, I hypothesized that activation of the DSB response by DNA replication triggers the loss. Credence for this model is gained from the observation that camptothecin (CPT), which also activates the DSB response, causes Mre11 loss. Inhibition of ATM activation does not prevent Mre11 loss, indicating that the upstream engagement of the MRN complex is sufficient for Mre11 loss absent any signaling downstream of ATM.

Further study is required to confirm that DSB damage can trigger Mre11 turnover. Cell lines expressiong mutant forms of Mre11 or Rad50 which are either unable to bind and process DNA or unable to engage Nbs1 could be used to determine if such activities are necessary for Mre11 loss after HSV infection. Also, direct visualization of live cells with florescence-tagged Mre11 may enable us to track the fate of Mre11 following the induction of DSBs caused by CPT or other damaging agents. Along with confirming the trigger for Mre11 loss, the mechanism of loss must still be determined. As the proteasome and lysosome have been eliminated as mechanisms for Mre11 loss, only specific proteases are left as likely effectors of Mre11 loss.

I have determined that UL26, the HSV encoded protease, is not required for Mre11 loss, leaving either an undiscovered viral protease or a cellular protease as the effecter of Mre11 loss. Because Mre11 loss appears to be a normal cellular response to DSB stimulus, Mre11 is more likely to be targeted by a cellular protease. Broad range and specific protease inhibitors could be used to determine what type of protease is involved. Inhibitory conditions might also allow for the protease to be pulled down with Mre11 by IP, and thus directly identified. Identification of the protease responsible for Mre11 would finally allow determination of whether Mre11 loss is beneficial, neutral or harmful to HSV replication. Effects of inhibition of the protease could be assayed first by yield assay, and then by assaying effects on viral DNA replication, processing and packaging. Because of potential secondary effects of protease inhibition, Mre11 proteins resistant to protease cleavage could be designed to directly assay for the importance of Mre11 loss, provided such changes do not also interfere with MRN activity.

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