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Review

The 3 facets of regulation of herpes simplex virus gene expression: A critical inquiry

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ABSTRACT

On entry into the body herpes simplex viruses (HSV) replicate in a series of steps that involves derepression of viral DNA activated by VP16, a virion protein, and sequential transcription of viral genes in a cascade fashion. HSV also enters into neurons in which viral DNA maintained as heterochromatin and with few exceptions viral gene expression is silenced. A third face of the interaction of HSV with its host cells takes place at the moment when the silenced viral genome in neurons is abruptly derepressed. The available data do no reveal evidence that HSV encodes different regulatory programs for each facet of its interaction with its host cells. Rather the data point to significant gaps in our knowledge of the mechanisms by which each facet is initiated and the roles of the infected cells at each facet of the interaction of viral gene products with the host cell.

Contents

Introduction

If herpes simplex viruses (HSV) could unveil their moto, it would read “Multiply, Persist and Disseminate”. Indeed for more than a century it has been recognized that HSV infect people by direct contact between tissues of individuals with a herpetic lesion and those of a healthy individual. In the course of multiplication at the portal of entry the virus infects nerve endings and is transported to a dorsal root or sensory neuron where it remains quasi-silent or, in the traditional terminology, latent. In some individuals the virus remains latent for a life time. In others it periodically replicates and is transported anterograde to a site at or near the portal of entry into the body where it replicates and is transmissible by contact (reviewed in Roizman et al., 2013. That HSV is successful in its endeavor to replicate, persist and disseminate is evident from the fact that in many populations the incidence of HSV-1 infections approaches 100%.This extraordinary achievement is due in large part to the exceptional control of viral gene expression during initial replication at the portal of entry, In the course of latent infection and even in the course of reactivation from latent state. In every sense of the word, HSV is a control freak. It is convenient to consider each of the 3 states of HSV sojourn in human bodies separately.

Productive infection at the portal of entry

Viral replication at the portal of entry into the body is a multicycle event that ultimately becomes arrested by the immune

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system. The accepted model of the events that take place in the course of a single replicative cycle is the cell culture in which most if not all cells can be synchronously infected. Current understanding of the events taking place in the infected cell may be summarized as follows:

On infection HSV brings into the cells a capsid containing DNA and approximately 20 proteins packaged in the tegument—a compartment located between the capsid and envelope (Roizman and Furlong, 1974). The capsid is transported to the nuclear pore where it releases the viral DNA into the nucleoplasm. Among tegument proteins 3, i.e. the virion host shutoff (VHS) RNase, VP16 and U_L47 are also translocated to the nucleus (Shu et al., 2013)

On entry of viral DNA into the nucleus several interrelated events take place. Foremost, DNA sensors trigger innate immune responses. A key component of the response is the induction of numerous stress response mRNAs that are degraded by VHS (Esclatine et al., 2004). A second event with significant consequences is the assembly at viral DNA of a dynamic structure designated formally as ND10 or PML bodies and regulated by the promyelocytic leukemia (PML) protein (Ishov and Maul 1996, Maul et al., 1993, 1996). A third key event is insertion of repressive modifications that preclude the expression of viral genes (Bryant et al., 2011, Cliffe et al., 2009; Knipe and Cliffe, 2008; Kristie, 2007; Huang et al., 2006; Liang et al., 2009; Silva et al., 2008; Narayanan et al., 2007).

The order of expression of viral genes. The α genes

The earliest studies focused on the order of viral gene expression in productive infection. Thus studies on the inhibitors of protein synthesis identified 5 genes expressed after infection in the absence of *de novo* protein synthesis. The products of these genes were designated as Infected Cell Proteins (ICPs) 0, 4, 22, 27, and 47 on the basis of their migration on electrophoresis in denaturing gels (Honess and Roizman, 1974, 1975). A second, much larger group of ICPs accumulated in infected cells treated with phosphonocetate, an inhibitor of viral DNA synthesis. Lastly, the early studies identified a larger group of ICPs that accumulated in infected cells in the absence of inhibitors (Honess and Roizman, 1974, 1975). In principle these groups corresponded to immediate-early, early and late nomenclature adapted from bacteriophage. The subdivisions however were more complex. Among the early protein some, (e.g. ICP8) are made very early whereas others (e.g. the thymidine kinase) are made much later. Among the late proteins some are made in small amounts in the absence of viral DNA synthesis whereas others required de novo synthesis of viral DNA in order to accumulate in infected cells. To avoid a complicated nomenclature that described these diverse groups as immediate-early, early-early, early-late, late-early and late-late they were designated as α, β_1, β_2, γ_1,and γ_2, respectively. The complexity of requirements for the sequential synthesis of viral gene products did not rest there. In recent studies it was shown that only ICP0 was made in cells depleted of the histone acetyl transferase CLOCK (Kalamvoki and Roizman 2010). In essence, these studies revealed that ICP0 recruits Bmal1 which in turn recruits its partner CLOCK to the viral transcriptome. This enables the transcription of other α genes.

Finally there is evidence that the expression of some open reading frames (e.g. ORF P and ORF O) is blocked by α proteins as are some miRNAs that accumulate preferentially in cells exposed to inhibitors of protein synthesis at the time of infection (Randall et al., 1997; Du et al., 2015).

The fundamental principle guiding the early studies on productive infections is that on entry of viral DNA into the nucleus the expression of viral genes is sequentially ordered (activated) in a cascade fashion. In quick succession α gene promoters were found to share common sequences (5 G/CTAATGAG/AATTCC/TTTGNGGG3') containing binding sites for Oct1 and VP16, a viral structural protein brought into cells along with viral DNA and a cellular protein designated Host Cell Factor 1 (HCF1). (Mackem and Roizman, 1982, Pellett et al., 1985, Kristie and Roizman, 1987, McKnight et al., 1987, Kristie and Roizman, 1988) Elegant, compelling studies have shown that VP16 enables the assembly of Oct1, HCF1 and lysine specific demethylase 1 (LSD1) along with transcriptional factors to derepress and initiate the transcription of α genes (Liang et al., 2009). LSD1 plays a key role in the derepression of α genes. In uninfected cells the protein is unstable in the absence of its partner (CoREST) (Shi et al., 2005, Yang et al., 2006). The available data suggest that LSD1 is recruited by VP16 from its partner (Zhou et al., 2010). Once ICP0 is made, additional proteins (e.g. CD4, CLOCK, etc) are recruited to enhance genes expression (Kalamvoki and Roizman 2010).

All α gene promoters contain the response elements required for the binding of the VP16/Oct1/HCF1/LSD1 complex(Mackem and Roizman, 1982, Pellett et al., 1985, Kristie and Roizman, 1987, McKnight et al., 1987, Kristie and Roizman, 1988) and hence they are predicted to be derepressed. At the same time the observation that the synthesis of ICP4, ICP22, ICP27, and ICP47 but not that of ICP0 depends on the recruitment of CLOCK (Kalamvoki and Roizman 2010) suggests that the interactions of ICP4, ICP22, IC27 and ICP47 promoters with the VP16/Oct1/HCF1/LSD1 complex are necessary but not sufficient for their expression. The data also predict that there may be some fundamental differences in the response elements present in the promoter of ICP0 as compared to those of other α genes.

Expression of β and γ genes

The promoters of β and γ genes lack the response elements for binding of VP16/Oct1/HCF1/LSD1 complex. Numerous studies, too numerous to cite here, focused on finding group-specific response elements in β and γ genes to no avail (Reviewed in Roizman et al., 2013). The available data again, too numerous to cite here, indicate that derepression of α genes does not ipso facto result in the expression of all viral genes. Numerous studies have pointed to ICP0 as the key element necessary but not sufficient for the expression of all viral genes.

ICP0 is the epitome of a multifunctional protein. It binds numerous proteins and performs multiple functions throughout the replicative cycle of the virus (Reviewed in Roizman et al., 2013). It resides in the nucleus during the first 6 to 7 h after infection and then mysteriously disappears from the nucleus and appears in the cytoplasm. The duration of the sojourn in the nucleus is depended on the amount of DNA introduced into the cells along with the virus (Lopez et al., 2001; Kalamvoki and Roizman, 2008). Relevant to the expression of β and γ genes are two functions. In the order of discovery, ICP0 acts as an E3 ubiquitin ligase. In combination with the UbCH5A ubiquitin conjugating enzyme it degrades PML and SP100. (Boutell et al., 2002, Gu and Roizman, 2003) A consequence of the degradation of PML and SP100 is the dispersal of the constituents of the ND10 bodies). What remains in their place are host and viral proteins recruited by α proteins. They form the nucleus of the replication compartments in which viral DNA is transcribed and replicated (Reviewed in Roizman et al., 2013). The second function of ICP0 is to bind CoREST and displace HDAC1 or 2 from to HDAC/CoREST/LSD1/REST (HCLR) complex (Gu et al., 2005, Gu and Roizman, 2007). The known functions of the HCLR complex are to repress neuronal genes in non-neuronal cells (Ballas and Mandel, 2005). REST is a highly postranslationally modified protein with repressor binding sits at both ends of the protein. It binds a somewhat degenerate response element (RE1). On displacement of HDAC1 by ICP0 the complex falls apart and its components are translocated to the cytoplasm (Gu et al., 2005, Gu and Roizman, 2007)

Both degradation of PML and disassembly of HCLR complex have been linked to the derepression and expression of β and γ genes and their functions are not clearly separable (Lopez et al.,

2002, Du et al., 2010, Gu and Roizman, 2009, Everett et al., 2006). Nevertheless three studies show considerable light on the two cited functions of ICP0.

Thus, overexpression of PML precluded the dispersal of ND10 bodies but had no effect on accumulation of infectious virus or its proteins (Lopez et al., 2002). Next, this laboratory constructed a dominant negative (dn) CoREST in which the binding site for HDAC1 was deleted. A recombinant virus in which ICP0 was replaced by the dnCoREST replicated 10 to 100 fold higher yields than the ICP0 null mutant in a cell type dependent manner (Du et al., 2010). These and other studies (Zhou et al., 2010) implied that displacement of the HCLR complex is a key requirement for the expression of β and γ genes (Du et al., 2010, Zhou et al., 2010). Lastly, another key finding stemmed from comparisons of the replication of wild-type and ΔICP0 mutant virus in murine wild type (PML+/+) and PML−/− cells. Specifically the wild-type and ΔICP0 mutant each replicated equally well in both cell lines. The difference emerged on pretreatment of the cell lines with interferon α or γ. Interferon pretreatment had little or no effect on or the replication of both viruses in PML−/− cells but had a very significant effect on the growth of the two viruses in PLM+/+ cells (Chee et al., 2003). The results suggest that PML acts s regulator of antiviral effects of interferon. In the absence of interferon, the two cell lines were to the extent tested indistinguishable. If degradation of PML were the key requirement for the expression of β and γ genes we could have expected higher yields for ΔICP0 mutant in PML−/− cells.

Conclusions

The fundamental conclusions of the early studies were that α proteins perform multiple functions that result in the recruitment to the viral transcriptome critical components necessary for effective transcription as well as in preemptive degradation of cellular defense mechanisms-functions not covered in this review. The fundamental conclusions of the past decade are that (a) viral genes are repressed on entry of viral DNA into the nucleus, (b) viral genes form clusters that are sequentially derepressed and (c) the identity of the key players has been revealed. We are faced, however with enormous gaps. Although promoter substitutions revealed that the sequences conferring γ_2 specificity resides in the 5' transcribed noncoding domain downstream of the TATA box (Mavromara-Nazos and Roizman, 1989), we have no cohesive pictures for the differential expression of β and γ_2 genes.

We are also confronted with a puzzle. Derepression of viral genes by dnCoREST implies the presence of numerous REST response elements in the viral DNA. We estimated as many as 30 based on a somewhat liberal interpretation of the somewhat degenerate REST binding site (G. Zhou and B. Roizman, unpublished studies). Implicit in such a scheme is that the virus evolved rather then attempted to rid itself of repressive sites. What would be the purpose of a vast layer or repressive sites that could interfere with viral replication?

Expression of viral genes in the latent state

Although latent HSV infections have been established in marmosets, Aotus monkeys, guinea pigs, and rabbits the most common model is the mouse. Mice are commonly inoculated by the skin, corneal or vaginal routes. After 4 weeks all traces of infectious virus disappear. Infectious virus can be recovered by explantation in culture of sensory ganglia innervating the site of inoculation of the virus (Reviewed in Roizman et al., 2013). The mouse model presents numerous advantages for the study of the latent sate. Specifically, while trace amounts of mRNAs of various viral genes have been detected during the latent state, there are no frequent spontaneous reactivation of latent virus to complicate analyses of viral gene expression during latency (Margolis et al., 2007). One confounding issue does exist: in the mouse HSV replicates in the ganglia after inoculation. Following corneal inoculation replicating virus can be detected in ganglia within 24 h after inoculation. The virus reaches peak levels sometime between day 5 and 14 and then rapidly subsides (Du et al., 2011, 2013). The fundamental question raised by these observations is whether the neurons in which the virus replicates differ from those in which the virus remains latent. Is replication an essential prelude for the establishment of latency?

Latently infected ganglia express a 2.0 and a 1.5 Kb latency associated transcripts (LATs) derived by splicing from a much larger transcript (Farrell et al., 1991, Zwaagstra et al., 1990). LATs appear to be unresolved lariats which may explain their stability but not the rapid disappearance of the precursor from which they are derived. In addition to LAT neuronal cells contain a subset of viral miRNAs (Umbach et al., 2008, Jurak et al., 2010, Sun et al., 2012). HSV DNA is in heterochromatin structures that enable both LAT and miRNA precursor RNAs to be synthesized (Wang et al., 2005, Cliffe et al., 2009, Kristie et al., 2008, Kwiatkowski et al., 2009, Neumann et al., 2007).

There is a tacit assumption that the failure to express the α genes is due to the failure of translocation of VP16 and HCF1 to the nucleus of newly infected neuron. That hypothesis does not explain the observation that in many neurons the virus does replicate after corneal inoculation. Another related hypothesis is that transport of VP16 lags behind that of capsids carrying DNA and that in neurons in which the virus does replicate the relatively short distance between the site of inoculation and the neuronal body enables VP16 and associated proteins to reach the neuron within a time frame necessary to enable derepression of α genes (La Boissière et al., 1999).

There are 3 fundamental questions regarding the latent state. First, the question arises as to whether the HCLR complex plays a role in the formation of viral heterochromatin. To answer this question we inserted into the wild-type genome an intact REST, a REST from which the N and C terminal repressor binding sites had been deleted (dnREST), or a set of stop codons to control for the disruption of viral DNA sequence at the site of insertion of wild-type or dnREST genes (Du et al., 2010). The expectations were that if REST plays no role in the silencing of viral DNA in neurons the dnREST would have little or no effect on the silencing of viral DNA. If REST is involved in the establishment of latency, the dnREST would compete with wild-type REST for RE1 sites and block the binding of repressors that constitute the HCLR complex. As a consequence there would be fewer neurons harboring latent virus. The results were unexpected: the dnREST virus was far more lethal than the wild-type parent (Du et al., 2010). The significance of the results stems from the implication that HSV could have evolved into a far more virulent virus but did not. An obvious conclusion stems from the well-established fact that HSV depends on physical contact of uninfected and infected tissues for transmission. Incapacitated patients are unlikely to transmit the virus by physical contact of infected and uninfected tissues. Viruses acquiring mutations that cause them to become virulent are not likely to be disseminated.

The second and equally significant question stems from the necessary conclusion that dnREST was expressed at least in amounts sufficient to compete with wild-type REST. To have this effect, the competition would have to have occurred in neurons on the path to repress HSV gene expression. Do the data imply that a silent latent state can be established even after expression of proteins encoded by the virus?

Finally the ability of the transcriptional machinery of the neuron to discriminate between the sequence encoding the LAT precursor RNA and those encoding the transcripts of silenced genes remains a puzzle. The problem stem from the implied conclusion that the regulation of expression of the LAT precursor mRNA is independent from that of other genes. If this were true, it would be expected that in productively infected cells LAT would be expressed concurrently with α gene products. In fact, LAT accumulates late in infection sug-

gesting the possibility that it may require other viral gene products for its expression.

Activation of silent genome in latently infected neurons

Reactivation of silent genomes in latently infected neurons in vivo involves two steps. In the first, the resident silent DNA initiates the expression of its gene and infectious particles are assembled.

In the second the infectious particles are transported anterograde to cells in which they initiate a productive infection (Reviewed in Roizman et al., 2013). In this step initiation of infection is the result of entry of virion and its components into uninfected cells and therefore the course of the infection would be predicted to be not different from that of virus entering cells at a mucous membrane. To define the events of reactivation it is critical to uncouple the two steps of the reactivation process. The focus of this section is on the first step. It takes place in the absence of either VP16 or ICP0 and the question posed here is how viral genes become derepressed in the absence of these proteins and in what order the genes are transcribed.

In principle two models have been described. The first involves heating the mouse for a precise time interval in a water bath (Sawtell and Thompson, 1992). Our attempts to duplicate this model were unsuccessful. The mortality was very high and among survivors few mice exhibited evidence of reactivation. The model we adapted involved excision of the ganglia and immediate incubation in medium that contained anti-NGF antibody to accelerate reactivation or both NGF and EGF to maintain the virus in latent state. Earlier studies have shown that virus reactivates from neurons harboring latent virus in the absence of NGF (Wilcox and Johnson 1987; Wilcox et al., 1990). The problem is that depletion of NGF in excised ganglia is slow: NGF is made by many different cells. The antibody to NGF accelerates the depletion within a time frame of a single replicative cycle (18–24 h). In experiments in which ganglia were excised and incubated in medium containing antiNGF antibody transcripts of representative α, β, γ_1 and γ_2 began to accumulate as early as 5 h after excision of the ganglia (Du et al., 2011). The conclusion that all viral genes were expressed at the same time independently of each other emerged from the observation that transcripts of representative α, β, γ_1 and γ_2 genes accumulated with the same kinetics in ganglia incubated in medium containing 100 mg of cycloheximide per ml of medium (Du et al., 2011). A necessary conclusion of these studies is that sequential order of gene expression observed during productive infection is at least in part an artifact imposed by sequential derepression of viral genes. The studies on ganglionic organ cultures described above raise numerous questions. Foremost, the basic finding must be confirmed in an intact animal model.

The obvious question that plagues these studies is why in this model all viral genes are expressed at once whereas in productive infections in cell culture and presumably at the portal of entry the genes are expressed sequentially. Expression of all genes at once implies a catastrophic event that cannot be highly productive in terms of virus yield. Sequential synthesis of regulatory protein, enzymes involved in viral DNA synthesis and structural proteins is likely to maximize virus yields whereas simultaneous synthesis of viral DNA and structural protein is likely to reduce yields. One hypothesis that could account for the data is that the portal of entry into the body is doorway to latent infection and also the site of transmission of the virus from one individual to another. Transmission is enhanced by frequent reactivations and appearance of virus at the portal of entry. Thus at the portal of entry the virus requires high yields to insure both seeding of sensory neurons and a critical mass for transmission of virus from one individual to another. To insure a high critical mass, at the portal of entry regulatory and defensive proteins are made first, viral DNA second and finally the structural proteins that package the DNA into virions define the final yield. In contrast the desirable amount of virus in reactivating ganglia should be sufficient for anterograde transmission and infection of cells at the portal of entry but not so high as to break through satellite cells and be transmitted to CNS.

The HCLR complex does not appear to play a role in the reactivation process. Thus wild-type HSV carrying a wild-type REST gene established latency but the virus did not reactivate on incubation in medium containing antibody to NGF. Reactivation as measured by accumulation of viral mRNAs did occur in medium containing cycloheximide. The data support the hypothesis that the HCLR is involved in the silencing of viral DNA during establishment of latency but not during reactivation (Zhou et al., 2013).

Finally, concurrent with accumulation of representative α, β, γ_1 and γ_2 gene transcripts the amounts of LAT and of the viral miRNAs decreased (Du et al., 2011). This decrease did not take place in ganglia treated with cycloheximide suggesting that unlike the accumulation of viral mRNAs the decrease in LAT and miRNAs required prior protein synthesis. Studies involving Act. D chases indicated that miRNAs and LAT were intrinsically stable over many hours and hence the degradation of LAT and of miRNAs is an active process involving an as yet unidentified RNase (Du et al., 2011). The data strengthen the argument that LAT and miRNAs play a role in maintaining the viral genome in silent state.

Subsequent studies have shown that in ganglia maintained in medium containing NGF and EGF viral reactivation could be delayed for at least 36 h. In these ganglia latent HSV was in a dynamic equilibrium defined by the status of HDAC 1 and 4, and STAT3 and p300 and the numerous protein (e.g. PI3K, AKT, mTOR, etc.) that in turn control the functions of these proteins (Du et al., 2013 and unpublished data).

Concluding remarks

The passage of nearly 40 years since the publication of studies that described the order of viral gene expression (Honess and Roizman, 1974, 1975) has not dimed the interest in defining how HSV expresses its genes during its sojourn in human cells. The issues raised in this review may be summarized as follows:

HSV has selected for its sojourn in humans replication mucous membranes as the portal of entry into the body and sensory neurons in which to persist. To express its genes and replicate the virus must use cellular functions. The differences in the viral gene expression in cells at the portal of entry and in sensory neurons underscore the differences in the genetic environment of these two cell populations

The silent state observed during latent infection in sensory neurons cannot be duplicated in cultured cells except by the use of drugs or deletion of key viral genes (Wigdahl et al., 1984a, 1984b, Samaniego et al., 1998). Obviously the repression of viral gene expression seen in cultured cells modeling the initial infection at the portal of entry into the body bears little resemblance to the silent genome in latently infected neurons. The silent genome serves the virus well in that it keeps the viral genome hidden from the immune system until a propitious time for it to become active.

Conversely the gene expression cascade observed in cultured cells requires sequential functions expressed by viral genes. No such requirement has been noted on reactivation of virus from latent state. As noted earlier in the text, such controls serve the virus well in that they maximize virus yields-a necessity to enhance the probability of transmission from person to person. They are effective however at the portal of entry and not on reactivation from latent state.

Another puzzle is the regulation of LAT and its associated miRNAs during productive infection and during the latent state. In productive infection LAT and its miRNAs are expressed late in infection and at least the miRNAs appear to require prior viral gene expression for their synthesis (Du et al., 2015). No such requirement is imposed in latently infected ganglia. Does a neuronal

protein substitute for the viral inducer in the productively infected cells to enable LAT expression?

Finally, numerous observations hint that HSV has evolved elaborate mechanisms to preclude tissue damage to the point where transmission from person to person would be curtailed. For example the enhanced virulence of a mutant expressing a dominant negative REST incapable of binding CoREST suggests that REST response elements is to reduce the potential virulence of the virus. Another relevant observation is that the population of viral miRNAS expressed during the latent state is similar to that of accumulating in productively infected cells and generally different from that emerging during reactivation. Again, one interpretation of the data is that the miRNAs in productively infected cells and in latently infected ganglia perform functions to protect the host and in so doing enhance the long term interaction between the virus and its host.

Clearly, to understand the nuances of the 3 facets of HSV sojourn in the human host we must understand the how the different metabolic and regulatory pathway in the two cell populations interact with and mold the expression of viral genes. This review, written in celebration of the 60th anniversary of Virology merely touches the surface of the many issues that still need to be resolved if we are to fully understand the multiple facets of HSV infection.

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