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Current Status Review: Molecular biology of herpes simplex virus latency

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The mucocutaneous lesions produced by herpes simplex viruses (HSV) types 1 and 2 represent sites of lytic infection with the virus in which viral proteins are synthesized, the viral DNA is replicated and the cells are eventually lysed (for review see Spear & Roizman 1980). Such lytic infections, which can be reproduced by infection of susceptible cells *in vitro*, are readily treatable using the drug acyclovir which inhibits viral DNA synthesis and hence prevents the lytic cycle from proceeding. Following successful treatment of the initial infection however, the lesions can recur repeatedly throughout the lifetime of the affected individual producing considerable pain and discomfort. This pattern of recurrence is dependent on the establishment, at the time of the initial infection, of lifelong latent infections in sensory neurons of the trigeminal or dorsal root ganglia which innervate the site of the peripheral lesions. These latent infections, which are entirely asymptomatic, can be reactivated in response to stimuli such as stress or fever, the subsequent migration of virus to the periphery resulting in the recurrence of the infection in susceptible epithelial cells.

The successful treatment of herpetic disease will therefore require an understanding of the processes which occur in latent infection in order that the recurrent pattern of lesions can be controlled. Such infections also represent a considerable intellectual problem in elucidating how a virulent virus which can kill most cell types is able to establish a long-term relationship with sensory neurons.

Although the involvement of the nervous system in HSV infection has been known since the nineteenth century (for review of the early work see Stevens 1975) the understanding of latent infection has been hampered by the small number and inaccessibility of latently infected cells *in vivo* and the inability to produce such infections successfully *in vitro*. The development of animal models of latent infection (for review see Roizman and Sears 1987) which are experimentally manipulable has yielded much information, but as with human latency, only a small number of cells are latently infected, rendering a molecular analysis of this phenomenon difficult.

However, there has been considerable recent advance in the field of HSV latency using new molecular techniques. This review will consider both the progress that has been made in molecular studies of HSV latency *in vivo*, and the attempts to develop models of latent infection *in vitro* that are more amenable to molecular analysis and which may therefore eventually provide insights applicable to the study of in-vivo latent infections.

HSV DNA in latently infected neurons

HSV has a linear DNA genome which is approximately 152 kilo-base pairs in length and consists of two unique sequences (U_L and U_S) bracketed by inverted repeat sequences (Roizman 1979) (Fig. 1). The ready production of lytic infections *in vitro* has allowed detailed study of viral DNA replication in such infections. Following infection, the genome circularizes and new viral DNA is

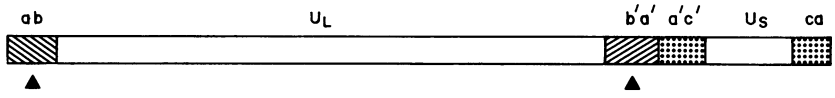

Fig. 1. Schematic representation of the HSV genome. The two unique regions U_L and U_S are shown unshaded whilst the inverted repeats are shaded. The arrows indicate the positions of the two copies of the gene encoding the latency associated transcripts (see Fig. 3).

produced by a rolling circle mechanism in which linear concatameric viral DNA is produced and cleaved into genome-length units for packaging into virions (Poffenberger & Roizman 1985).

Because of the limited material available from latently infected ganglia, studies on the state of latent viral DNA have concentrated on measuring the levels of specific fragments of the viral genome and in particular on determining the amount of the genome termini detectable compared to that of internal fragments. The majority of such studies have been carried out by Fraser and colleagues using latent infections in the mouse brain stem (pons-medulla) as a model system (Rock & Fraser 1983, 1985; Mellerick & Fraser 1987). Although such latent infections of the central nervous system differ from those of peripheral ganglia in being very difficult to reactivate (Fraser *et al.* 1984), in general, insights into the structure of viral DNA obtained in this system have been confirmed in studies of ganglionic DNA in both animal and human systems (Efstathiou *et al.* 1986).

In studies of this type, the termini of the viral DNA could not be detected in the latently infected brain stem (Rock & Fraser 1983) or in similarly infected human ganglia (Efstathiou *et al.* 1986). These studies indicate therefore that unlike the DNA of the infecting virion, the DNA in latently infected cells is not in a simple linear unintegrated form. Subsequent studies (Rock & Fraser 1985) indicated that the termini of the latent genome are joined together to create an internal head-to-tail joint fragment and that junctions of viral and cellular DNA cannot be detected. Such studies eliminate models in which the lack of terminal fragments is due to the integration into cellular DNA of a single linear molecule, since this would lead to junctions of viral and cellular DNA which would be readily detectable. They do not, however, eliminate a model in which the viral DNA integrates as a long concatamer of genome-length molecules joined head to tail resulting in the common occurrence of head-to-tail junction fragments, thus making rare viral–cellular junctions which differ in different integration events undetectable. Alternatively, the data is equally compatible with latent DNA existing as an unintegrated circular episomal molecule similar to that which occurs in latent infections with another herpes virus, Epstein-Barr virus (Lindahl *et al.* 1976) and in which free termini would also be undetectable.

In an attempt to distinguish these two possibilities Mellerick and Fraser (1987) banded the DNA from latently infected cells in caesium chloride density gradients. Because of the higher GC content of viral compared to cellular DNA, unintegrated viral DNA would be expected to band at a different density from viral DNA integrated into cellular DNA. In agreement with a circular unintegrated structure, most of the DNA homologous to viral DNA in latently infected mouse brain banded at the density of free viral DNA. Some hybridization to viral DNA probes was found within the cellular DNA, as would be expected if integration had occurred. However, the interpretation of this phenomenon is rendered difficult by the known ability of some cellular DNA to cross-hybridize with viral DNA probes (Peden *et al.* 1982; Puga *et al.* 1982) which might result in the low level of apparent viral DNA in the cellular fraction.

Such cross-hybridizing sequences also led

to the failure of attempts to study the state of viral DNA by preparing a genomic DNA library from latently infected ganglia and isolating clones containing viral DNA (Puga *et al.* 1982). The clones isolated in such studies consisted entirely of cellular DNA capable of cross-hybridizing with viral DNA and no bona fide viral DNA clones were isolated.

Hence, although the circular episomal structure is the most probable one for viral DNA in the latent state the existence of some integrated concatamer DNA cannot be ruled out. It is probable that the application of the polymerase chain reaction (which is already being used for the detection of HSV DNA and RNA (Lynas *et al.* 1989)) to latently infected ganglia will resolve this question providing appropriate primers for the amplification of the ends of viral DNA that lack homology to cellular sequences can be obtained.

Whatever its precise state, there is evidence (Deshmane & Fraser 1989) that the viral DNA in latently infected cells is packaged with histones into the nucleosomal structure characteristic of both active and inactive cellular genes. Interestingly, in the brain stem model at least it seems that despite the transcriptional inactivity of most of the viral genome (see below) it is not extensively methylated (Dressler *et al.* 1987) in the manner seen for many cellular genes which are inactive in a particular cell type (for review see Cedar 1988). This suggests that the viral genome may be directly regulated by specific cellular transcription factors present in the neuron rather than by blanket long-term repression which shuts down the entire genome. It should be noted, however, that treatment with the demethylating agent 5-azacytidine has been shown to reactivate latent infections of mouse and guinea-pig ganglia (Stephanopoulos *et al.* 1988) indicating either that the brain stem model is unique in the non-methylation of viral DNA or that 5-aza-cytidine is inducing reactivation by a means distinct from demethylation.

HSV RNA in latently infected neurons

In lytic infection the viral genome is expressed in three stages, resulting in the sequential transcription of the immediate-early, early, and late genes (Spear & Roizman 1980) (Fig. 2). The products of the first phase of this process, the five immediate-early proteins, play a crucial role in the lytic cycle, one of these proteins, ICP4, being absolutely required for the subsequent stages of viral gene expression (Preston 1979). Viruses

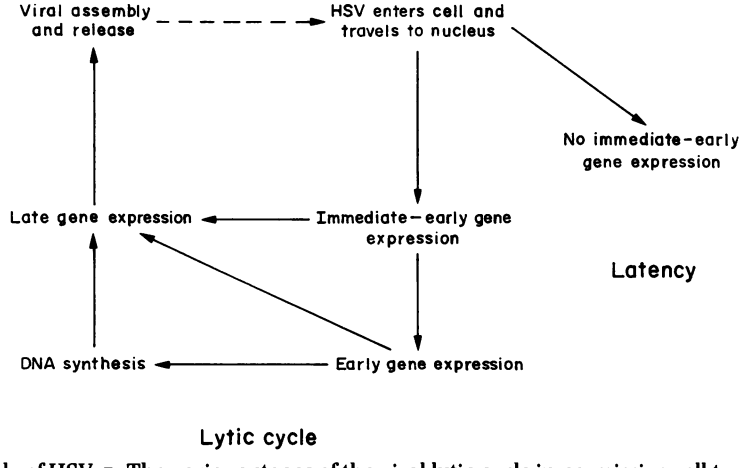

Fig. 2. Life cycle of HSV-1. The various stages of the viral lytic cycle in permissive cell types are indicated together with the point at which this is aborted in latently infected cells.

lacking the gene for this protein (Preston 1979) or that encoding another immediate-early protein ICP27 (Sacks *et al.* 1985) are unable to grow in any permissive cell type whilst those lacking the genes encoding other proteins of this type ICP0 or ICP22 are impaired in lytic growth to a greater or lesser extent depending on the cell type involved (Stow & Stow 1986; Sears *et al.* 1985).

A crucial question therefore is the point at which the lytic cycle is aborted in latently infected cells and in particular whether the immediate-early proteins are synthesized in such cells. Early studies of this problem were complicated by the reactivation of the virus during the experiment resulting in the detection of numerous viral transcripts (see for example Galloway *et al.* 1982). It is now clear, however, from numerous studies using in-situ hybridization that RNAs encoding the immediate-early proteins are not detectable in latently infected trigeminal or spinal ganglia of humans (Coen *et al.* 1987) and mice (Deatly *et al.* 1987; Stevens *et al.* 1987) and in the latently infected mouse brain stem (Deatly *et al.* 1988). Hence the failure of the lytic cycle in latently infected cells is due to the absence of the immediate-early mRNAs and thus of their corresponding proteins.

Interestingly, in all the studies noted above, RNA corresponding to one region of the viral DNA was observed in the latently infected cells. This latency associated transcript (LAT) is derived from a region of the genome overlapping that encoding the immediate-early protein, ICP0, but is transcribed in the opposite direction to the ICP0 mRNA, being produced from the complementary strand of DNA to that encoding ICP0 (Fig. 3). More precise study of the LAT RNA has identified at least three different LAT transcripts 2.0, 1.5 and 1.4kb in size, which are partly co-linear and all of which overlap the ICP0 mRNA (Spivack & Fraser 1988; Wagner *et al.* 1988). These RNAs are made at very low levels in normal lytic infection but are present at high abundance in latently infected cells (Spivack & Fraser 1988) allowing their ready detection by in-situ hybridization (Fig. 4).

Following the discovery of the LATs, much speculation has centred on their functional role (if any) in the establishment and maintenance of latent infection. The fact that the transcripts are predominantly nuclear suggests that they do not encode a protein, a conclusion supported by the fact that antibodies raised against synthetic peptides predicted from the DNA sequence of the LAT gene do not detect a protein in latently infected ganglia (Wechsler *et al.* 1989).

It seems likely rather that these transcripts have a regulatory role. That this might involve an inhibition of ICP0 expression in latently infected ganglia is suggested by the widespread use of so-called antisense RNA derived from the opposite strand to the mRNA in order to inhibit the expression of a particular gene both naturally in bacteria (Simons & Kleckner 1983) and artificially by using appropriate recombinant DNA constructs in eukaryotes (Izant & Weintraub 1984). In this model, the LAT RNA would bind to the ICP0 mRNA by virtue of its

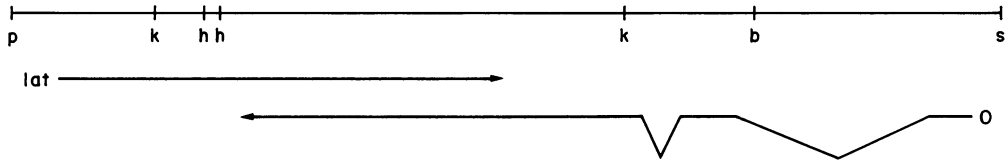

Fig. 3. Region of the HSV-1 genome encoding the major latency associated transcript (LAT) and the spliced IE1 or ICP0 transcript (0). Restriction sites: b, Bam HI; h, HpaI; k, KpnI; p, PstI; s, SstI. Modified from Wagner *et al.* (1988).

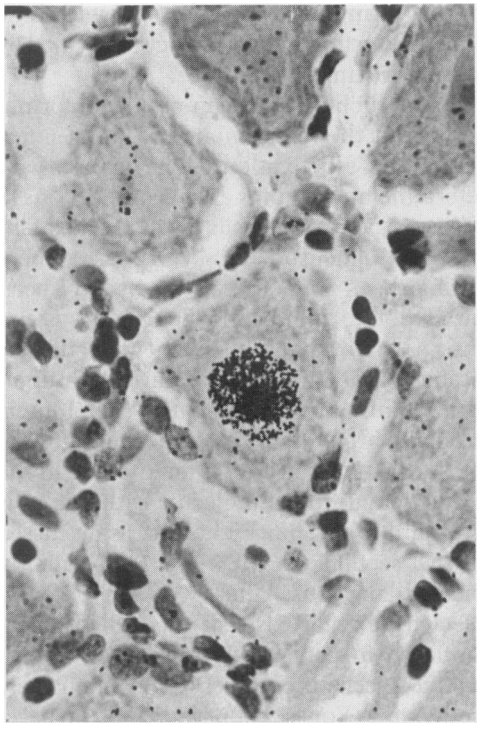

Fig. 4. Localization of the LAT transcripts in the nucleus of a latently infected cell by in-situ hybridization with a ^{3}H-labelled probe specific for these transcripts (courtesy of Dr S. Wechsler).

sequence complementarity (Fig. 3) and would block the production of ICP0 protein either directly by inhibiting translation of the mRNA or indirectly by blocking its transport from the nucleus. Such a model is particularly attractive because of the apparent role played by ICP0 in reactivation of latent virus. Thus viruses mutant in the gene encoding this protein although able to grow lytically to some degree (Stow & Stow 1986), fail to reactivate following latent infection in two different animal systems (Leib *et al.* 1989). Hence in this model LAT RNAs would function to damp down any bursts of ICP0 mRNA synthesis in the latently infected ganglia thus inhibiting the production of ICP0 protein and thereby preventing reactivation.

Unfortunately this model is not consistent with a recent study (Steiner *et al.* 1989) involving an HSV-1 variant which does not synthesize the LAT RNAs. This virus was found to be unimpaired in the establishment or maintenance of latent infection but paradoxically showed a much delayed time course of reactivation leading to the suggestion that the LAT transcripts may accumulate during latent infection in order to fulfil some role in promoting reactivation rather than in its inhibition.

Latency *in vitro*

The difficulties in assigning a function to the LAT transcripts emphasize the problems in studying a phenomenon such as latent infection which can be produced only *in vivo*. Similarly, although in-vivo studies have shown that the immediate-early mRNAs do not accumulate in latently infected ganglia, whereas the LAT RNAs are produced at levels far greater than those observed in lytic infection, the number of cells available *in vivo* have not permitted an analysis of the processes regulating the corresponding genes. Thus it is not clear, for example, whether the absence of immediate-early mRNA in latently infected ganglia is caused by a lack of transcription of the corresponding genes or by some post-transcriptional process such as rapid degradation of newly synthesized viral RNA. For these reasons many workers have attempted to establish in-vitro systems to study the process occurring during the onset and maintenance of latent infections and these will now be discussed.

Three basic types of in-vitro systems for studying latency have been described.

Primary cultures of neurons

Attempts have been made to produce latent infections in primary cultures of sympathetic neurons. In the studies of Wilcox and Johnson (1987, 1988) such cells were infected with very low levels of virus or in the presence of acyclovir to prevent destruction

of the cells by lytic infection. Under these conditions it was possible to establish cultures lacking replicating virus and not expressing viral antigens. Virus could be reactivated from these cultures by various treatments including removal of nerve growth factor or treatment with phorbol myristate acetate (PMA), indicating that a latent, potentially reactivatable infection has been established. This system thus offers a means of readily testing putative agents of reactivation in a situation much simpler and more accessible than testing *in vivo*. Indeed, the fact that removal of nerve growth factor from the primary cultures causes reactivation explains early observations that cutting the axonal process of a latently infected neuron *in vivo* results in reactivation (Carton & Kilbourne 1952) since such treatment would abolish the retrograde transport to the neuronal cell body of nerve growth factor synthesized by the innervated tissue.

Unfortunately a more detailed analysis of how these agents induce reactivation and of the processes which produce the initial latent infection is rendered difficult by the small numbers of cells obtainable in non-dividing primary cultures exactly as with latent infection *in vivo*.

Artificial inhibition of latent infection in permissive cell lines

The problem of cell numbers, inherent in the primary culture system, could be overcome if latent infections were established in cell lines capable of indefinite growth in culture. Most such cell lines are highly permissive for HSV lytic infection and hence attempts to establish latent infections have routinely included the use of various inhibitors of viral growth (see for example, Shiraki & Rapp 1986) or of supra-optimal temperatures (Russell & Preston 1986) to prevent the lytic cycle. Although such systems obviously cannot provide information about how the neuron resists the completion of a full lytic cycle following infection allowing the establishment of latent infection, they have resulted in long-term persistent infections with little virus production which can be reactivated by various treatments.

Before such systems can provide information about the maintenance of latent infection or its reactivation, it is necessary to determine how similar are such infections of normally permissive cells to bona fide latent infections of neurons. In several cases where this has been done, these systems have been found not to mimic the neuronal system. Thus, when apparent latent infection is established in normally permissive human embryo lung fibroblasts by the use of interferon and bromodeoxyuridine to inhibit viral infection, the viral DNA is maintained in the cell as a single linear molecule with free ends (Wigdahl *et al.* 1984) whereas, as discussed in the section on HSV DNA in latently infected neurons, the DNA in latently infected ganglia exists in an endless form. Similarly, in persistently infected semi-permissive T cells the viral DNA is extensively methylated (Youssoufian *et al.* 1982) whereas this does not seem to be the case in neuronal cells, at least in the brain stem.

Until a system of this type is established which more precisely mimics the properties of the neuronal situation, the relevance of such systems as a source of information on latency is doubtful and it remains possible that bona fide latent infection can be established only in neuronal cells.

Immortal cells of neuronal origin

In an attempt to combine relevance to the neuronal situation with the convenience of a cell line, numerous studies have examined the effect of HSV infection on proliferating cell lines, such as neuroblastomas, of neuronal origin. Several such lines have been shown to be relatively non-permissive for the HSV lytic cycle (Adler *et al.* 1978; Levine *et al.* 1980; Vahlne & Lycke 1978) and in at least one case, that of the C1300 mouse neuroblastoma, this is due to a failure of immediate-early protein synthesis following infection (Ash 1986) exactly paralleling the

absence of such proteins during latent infections *in vivo*. Hence these cells offer an attractive system for investigating the reasons for the failure of lytic infection in neuronal cells which represents an essential step in the establishment of latent infections. Indeed the fact that these cells can be grown in sufficient amounts to allow the direct measurement of gene transcription has allowed us to show that they fail to transcribe the viral immediate-early genes following infection (Kemp & Latchman 1989). This is the first time that such a transcriptional block has been demonstrated in cells of neuronal origin and suggests that the lack of immediate-early mRNA in latently infected neurons *in vivo* is due to a failure of immediate-early gene transcription.

The similarity of the processes occurring following infection of C1300 cells to those occurring in the latently infected neuron indicates that these cells might also serve as a suitable vehicle for the establishment of latent infections, allowing a study of the processes involved in the maintenance and reactivation of such infections. Indeed, Nilheden *et al.* (1985) were able to establish such infections with HSV-1 in a hyperresistant clone of C1300 cells and show that such infections could be reactivated by superinfection with the related virus HSV-2. Unfortunately, the amount of virus which could be reactivated declined as the latently infected cells were passaged, becoming undetectable after three passages. This loss of reactivatable virus was due either to overgrowth of the latently infected cells by non-infected cells or more probably to the fact that latent viral DNA was not replicated as the cells divided.

Whatever its cause, the failure of these experiments to result in long-term latent infections illustrates perfectly the problems of studying latency *in vitro*. Thus whilst proliferating cell lines provide sufficient material for study, it is possible that long-term latent infections can be established only in non-proliferating cells. One way to overcome this problem would be to grow cells on a large scale in culture and then switch them to a non-proliferating state in which latent infection could be established. A system of this type has been obtained by fusing C1300 cells with primary cultures of rat sensory neurons. The cell lines obtained in this way (J. N. Wood, personal communication) proliferate indefinitely in culture. In response to treatment with cyclic AMP, however, the cells cease to proliferate and assume the appearance and electrophysiological characteristics of sensory neurons. Such cells appear to be excellent candidates for the establishment of latent infection especially since, like C1300 cells, they are highly non-permissive for HSV and fail to transcribe the viral immediate-early genes following infection (S. C. W. Wheatley & D. S. Latchman, unpublished). Moreover, these cells do transcribe the LAT gene following infection, a phenomenon which is not observed following infection of C1300 cells (Kemp & Latchman 1989). Attempts are therefore currently under way to establish latent infections in these cells.

Conclusions

It is clear that the small numbers and inaccessibility of latently infected cells *in vivo* have considerably hampered the accumulation of information on latent infection with HSV. Nonetheless, the application of molecular biological techniques to these infections both in humans and in animal models has yielded much information and it is likely that the application of new techniques such as the polymerase chain reaction will extend our knowledge considerably. Further understanding of the mechanisms regulating the processes observed *in vivo* is likely to require the development of in-vitro systems to generate sufficient cell numbers for study. Ultimately the application of insights obtained in such systems to the study of in-vivo latency, will allow the understanding and possibly the treatment of the clinically and intellectually fascinating problem of HSV latency.

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