Gene regulation in Herpesvirus saimiri and its implications for the development of a novel gene therapy vector

Review Article

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Summary

We have investigated the potential of HVS as a human gene therapy vector and found that it is capable of infecting an extremely broad spectrum of human cell lines and primary cultures with efficiencies that are at least as good as (and in many cases better than) currently available vector systems. Like others we found that the virus was capable of stably transferring a functional heterologous gene by virtue of episomal maintenance. Although transduced clones can be established in all cases, we have also been able to demonstrate low levels of virus production from these cells. This finding necessitates the development of disabled mutants for potential future clinical applications.

Fundamental research carried out in this laboratory has identified the interactions between the two known transcriptional regulatory genes encoded by HVS. Overall, these results suggest that ORF50 and ORF57 are ideal essential candidate genes to delete in order to produce a replication-disabled HVS. This will provide the basis for a novel gene therapy vector which is theoretically capable of addressing the problems faced by current vector systems.

I. Herpesviruses as gene therapy vectors

Herpesviruses are classified as large DNA viruses having genomes of between 100 and 250 kb. They are divided into alpha, beta and gamma sub-groups on the basis of their biological and genetic properties (Roizman et al., 1981). As a family their advantages as gene therapy vectors relate to an ability to package large DNA insertions and establish lifelong latent infections in which the genomic material exists as a stable episome. Nearly all of the research in this field has focused on the use of Herpes simplex virus (HSV) vectors for gene transfer to the nervous system (Coffin and Latchman, 1996). HSV encodes several proteins which modulate viral and cellular gene expression via a temporal cascade of immediate-early (IE), early and late genes (Honess and Roizman, 1974; DeLuca and Schaffer, 1985). These first generation HSV based vectors were disabled by the deletion of one or more of the immediate early genes. These viruses could only replicate in 'helper' cell lines which provided the IE gene product *in trans* (DeLuca et al., 1985; DeLuca and Schaffer, 1987). The vectors were engineered to carry heterologous genes in the deleted portions of their genomes, but were often toxic to the cells which they infected (Glorioso et al., 1985; Johnson et al., 1992; Sabel et al., 1995).

A second broad category of HSV based vectors are amplicons. These are plasmids containing an HSV lytic replication origin and terminal packaging signals. They can be amplified and packaged into infectious HSV-1 particles in the presence of helper-virus (Spaete and Frenkel, 1982; Kwong and Frenkel, 1984; Geller and Breakefield, 1988; Geller and Freese, 1990). As such they constitute a cloning vehicle which can efficiently carry genetic information between prokaryotic and eukaryotic cells. Amplicons retain many of the characteristics of

standard HSV vectors but viral stocks tend to have lower titres, making them less useful for gene therapy applications.

A problem shared by all HSV based vector systems is the fact that the genome has no 'latent origin' of DNA replication, meaning that a state of episomal maintenance cannot be established in dividing cells. However, Epstein Barr virus (EBV), a member of the gamma-herpesviruses, is capable of establishing a latent state in dividing cells where the viral episome replicates co-ordinately with cell division and is inherited by all progeny cells. Such a vector derived from EBV may be suitable for stem cell gene therapy. However, EBV is associated with a number of human malignancies and lymphoproliferative disorders, necessitating extensive modification of the virus genome to eliminate those genes involved in transformation.

One example of an alternative strategy, which has been employed with some success in the laboratory, is the construction of HSV amplicons containing EBV sequences that maintain the plasmid as an episome in the infected cell nucleus (Wang and Vos, 1996; Wang et al., 1997). Such developments are bound to lead to improvements in Herpes Simplex virus based vectors and the eventual creation of 'niche' gene therapy applications. However, all of the current options suffer from inherent problems and limitations which are far from trivial. This review highlights a potential alternative, Herpesvirus saimiri (HVS), and illustrates the importance of basic research in the quest for better vector systems.

II. Herpesvirus saimiri

Herpesvirus saimiri is a lymphotrophic rhadinovirus (-2 herpesvirus) of squirrel monkeys (*Saimiri sciureus*), which persistently infects its natural host without causing any obvious disease. However, HVS infection of other species of New World primates results in fulminant polyclonal T-cell lymphomas and lymphoproliferative diseases (Fleckenstein and Desrosiers, 1982). Certain strains of HVS are also capable of transforming human T lymphocytes to continuous growth *in vitro* (Beisinger et al., 1992). The genome of HVS (strain A11) consists of a unique internal low G+C content DNA segment (L-DNA) of approximately 110 kbp which is flanked by a variable number of 1444 bp high G+C content tandem repetitions (H-DNA) (Albrecht et al., 1992). Analysis indicates it shares significant homology with other herpesviruses: EBV, bovine herpesvirus 4, Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8) and murine gammaherpesvirus 68 (MHV68) (Albrect and Fleckenstein, 1990; Bublot et al., 1992; Gompels et al., 1988a;b; Neipel et al., 1997; Russo et al., 1996; Virgin et al., 1997). The genomes of EBV, KSHV, MHV68 and HVS have been shown to be generally colinear, in that homologous sequences are found in approximately equivalent locations and in the same relative orientation. However, conserved gene blocks are separated by unique genes with respect to each virus (Albrect and Fleckenstein, 1990; Nicholas et al., 1992; Russo et al., 1996; Virgin et al., 1997).

III. Potential of HVS as a gene therapy vector

All herpesvirus vector systems which have previously been assessed were based on human herpesviruses and are inevitably likely to be ineffective in the majority of individuals due to the inherent immune response induced by the wild type virus. A herpesvirus of non-human origin, capable of infecting human cells without a cytopathic effect therefore represents an attractive candidate as a gene therapy vector, as there should be no immediate inate immune response in the recipient. Earlier publications have demonstrated that a selectable HVS has the ability to persist in a variety of human cell lines for long periods of time apparently without the production of infectious progeny (Grassmann and Fleckenstein 1989; Simmer et al., 1991).

In order to assess the potential of HVS as a possible gene therapy vector, we have generated a HVS recombinant virus (based on a non-transforming strain, A-11) which expresses EGFP (Cormack et al., 1996), and the neomycin resistance gene under the control of distinct promoters. These heterologous genes have been cloned into the repeat regions of the HVS genome, theoretically preventing alteration of the wild type virus phenotype (**Figure 1**). Analysis using this virus, which can be grown to a high titre, has demonstrated infection of a wide variety of human cell lines at approaching 100% efficiency including, A549 (lung carcinoma), HT-29 (colonic adenocarcinoma), MIA-PACA (pancreatic carcinoma), K562 (chronic myelogenous leukaemia), Jurkat (T-cell lymphoma), Molt-4 (T-cell leukaemia) and Raji (Burkitt's lymphoma) cells (**Figure 2**) (Stevenson et al., 1999). In contrast to previously published work we have detected low levels of virus replication in all of these cell lines at early stages post infection, even in the absence of apparent cytopathic effects. However, the virus DNA is clearly able to establish a latent episomal state within the cell which segregates to the progeny upon division. **Figure 3** shows the development of a clone of A549 cells resulting from a single infected cell. The period of this experiment was four weeks, but the clone is still growing (and remains bright green) six months later. This result and similar data generated in other cell lines are extremely encouraging and we believe this system offers enormous potential for the delivery of therapeutic genes to cancerous cells, as well as to bone marrow and stem cells.
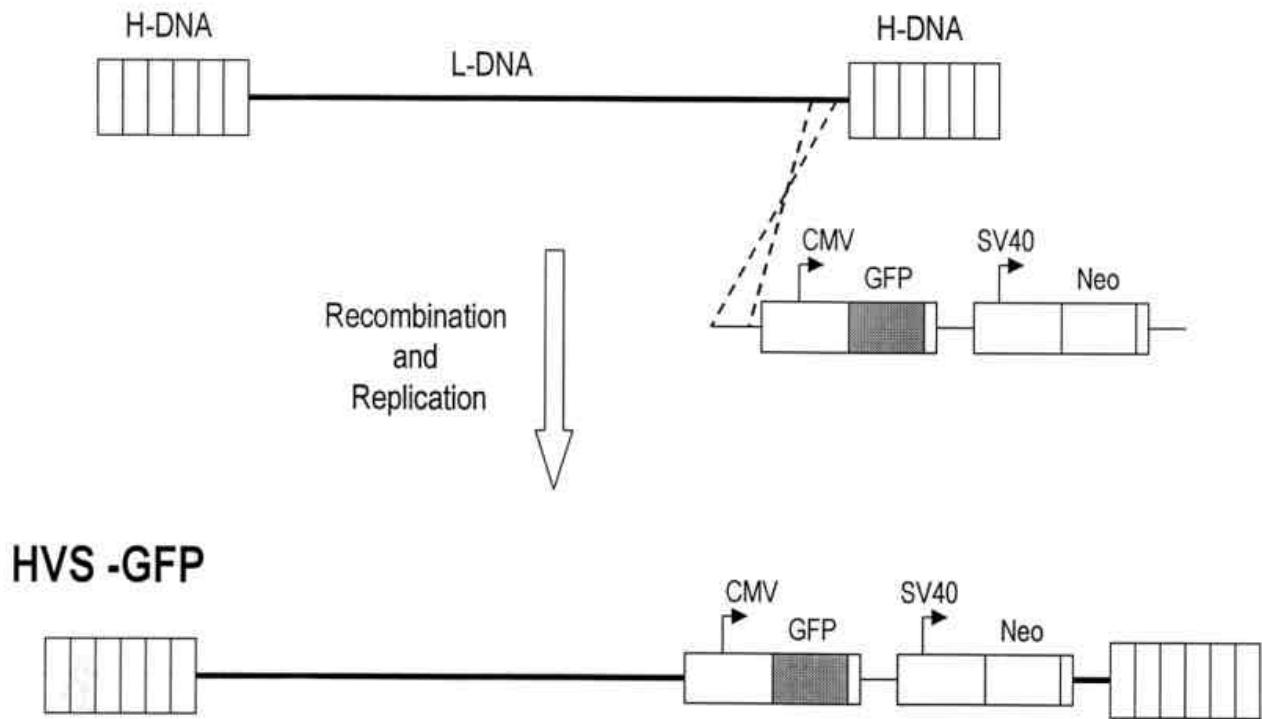
Figure 1. Construction of GFP/Neo virus. A recombinant virus was generated by transfection of OMK cells with the recombination vector followed by super-infection with wild type virus. Recombinants were initially selected for by the addition of Geneticin to the culture medium followed by two rounds of plaque purification.
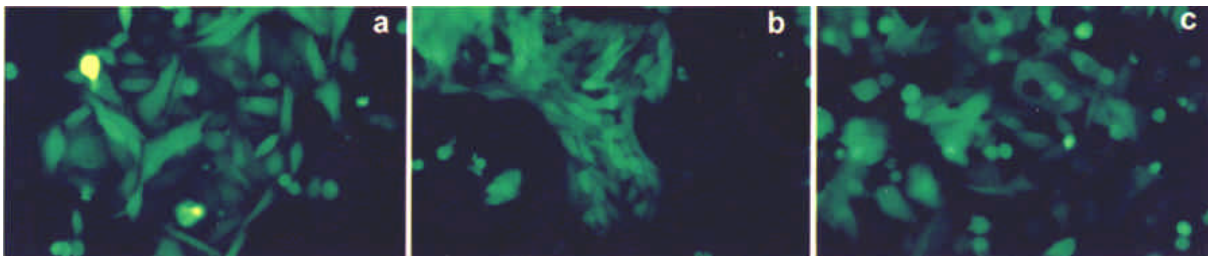

Figure 2. Examples of human cancer cell lines infected with GFP/Neo virus. (a) SW480 (colonic carcinoma) (b) HT-29 (colonic carcinoma) (c) Miapaca (pancreatic carcinoma).

Figure 3. Segregation of GFP amongst dividing human lung carcinoma cells (A549). A549 cells were infected with GFP/Neo virus and selected in the presence of Geneticin. The figure shows the development of an individual clone over the period of four weeks.
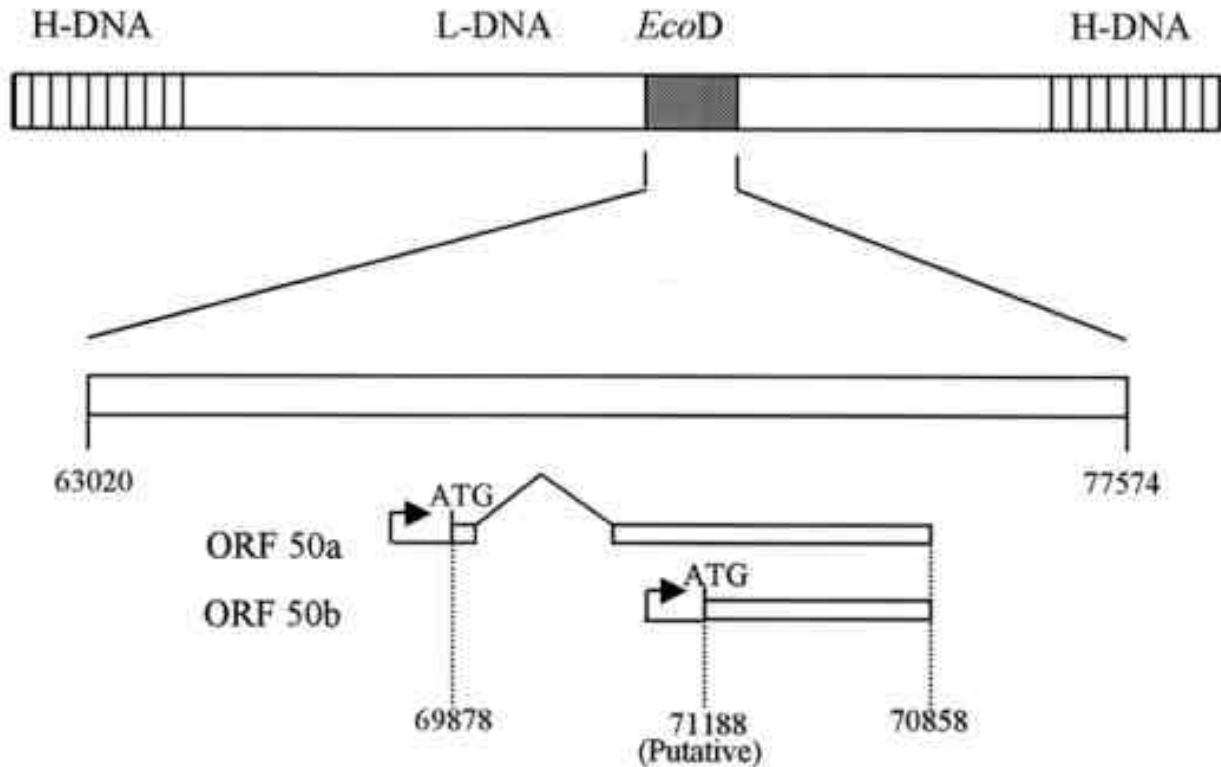
Figure 4. Diagrammatic representation of the ORF 50 transcripts. ORF 50 produces two transcripts, the first is spliced containing a single intron and is detected at early times during the productive cycle, whereas the second is expressed later and is produced from a promoter within the second exon.

To develop this virus further as a gene therapy vector and to minimise the risk of pathogenicity, disabled HVS vectors are required. In order to generate a replication-disabled vector, genes essential for the replication of the virus must be deleted. Ideal candidate genes to disable viruses are those expressed early in the viral replication cycle and which are involved in the regulation of viral gene expression. The following section discusses these genes and the role they play in HVS replication.

IV. Gene regulation in HVS
A. The ORF 50 gene products

We have recently identified the two major transcriptional regulating genes encoded by HVS. The first transcriptional activator is homologous to the EBV BRLF1 gene product, R (Nicholas et al., 1991; Albrecht et al., 1992), a sequence-specific transactivator (Gruffat et al., 1990). The HVS R gene or ORF 50 produces two transcripts. The first is spliced containing a single intron and is detected at early times during the productive cycle, whereas the second is expressed later and is produced from a promoter within the second exon (**Figure 4**). The spliced transcript is 5-fold more potent in activating the delayed-early ORF 6 promoter. The function of the non-spliced transcript is unclear (Nicholas et al., 1992; Whitehouse et al., 1997a). Further analysis of ORF 50 indicates it responds to particular DNA sequences specifically contained within the promoters of the genes it transactivates. Deletion and gel retardation analysis have identified a consensus ORF50-recognition sequence, CCN_9GG, required for transactivation by both ORF 50 transcripts (Whitehouse et al., 1997b). The response elements have significant homology to the EBV.R response element consensus sequences, $GNCCN_9GGNG$. It

has been shown by guanine methylation studies that the CCN_9GG motif is essential for EBV.R binding, suggesting that the R binds to adjacent major grooves of the DNA (Gruffat et al., 1990; 1992; Gruffat & Sergeant, 1994). The ORF 50 response elements map to within 32 bp which contain a CCN_9GG motif. However, the flanking sequences are significantly different to the EBV.R response elements, suggesting that the ORF 50 gene products have different sequences required for recognition and fixation of the proteins to their target. At present we are unable to determine using gel retardation analysis if the ORF 50 gene products bind directly to the response elements, or whether the retarded complex identified is due to the recruitment of host cell proteins by ORF 50.

We believe that ORF 50 probably binds to the response sequences because of its homology with EBV.R protein which has been purified and shown to specifically recognise its response elements (Gruffat & Sergeant, 1994). However, further analysis of the ORF 50 response element by mutagenesis is required as is the production of purified ORF 50 gene products to investigate these hypotheses. The EBV.R protein has been shown to transactivate three promoters. We believe that ORF 50s gene products also transactivate multiple promoters. We have searched the HVS genome for additional ORF 50 response elements using the motif, CCN_9GG and have identified 69 putative response elements. Further characterisation of these putative elements to localise in a promoter region of a defined ORF expressed delayed-early or late in the virus replication cycle, has identified 10 possible promoter regions which may contain ORF 50 responsive elements (Whitehouse et al., 1997b). We are currently examining these genes for possible transactivation by either of the ORF 50 gene products and investigating whether late genes are transactivated by the later ORF 50b transcript. Alternatively, the ORF 50b gene product, which has been shown to transactivate ORF 6 to a lesser extent, may compete with ORF 50a for binding to the response elements, thus acting as a negative regulator of transcription.

B. The ORF 57 gene product

The second transactivator encoded by ORF 57 is homologous to genes identified in all classes of herpesviruses. These include the EBV transactivator encoded by BMLFI, ICP27 of HSV, BICP27 in bovine herpesvirus 1, ORF 4 encoded by varicella-zoster virus, UL69 in human cytomegalovirus, and ICP27 in equine herpesvirus 1 (Davidson and Scott, 1986; Kenney et al., 1989; Nicholas et al., 1988; Perera et al., 1994; Singh et al., 1996; Winkler et al., 1994; Zhao et al., 1995). The ORF 57 gene product has transregulatory functions which, unlike ORF 50's gene products, are independent of the

target gene promoter sequences and appear to be mediated at the post-transcriptional level. At present we are unable to determine the actual effect of ORF 57 i.e. whether it affects the processing, transport or translational efficiency of mRNA. The more widely studied ORF 57 homologue, ICP27, appears to act post-transcriptionally by affecting mRNA processing suggesting ICP27 regulates usage of polyadenylation sites as a means of controlling gene expression (McGregor et al., 1996; McLauchlan et al., 1992). It has also been demonstrated that a bacterially expressed ICP27 fusion protein specifically binds to the 3' ends of RNA leading to accumulation and increased half life of the mRNAs (Brown et al., 1995). The RNA binding motif (residues 138 and 152), is similar to an RGG box motif and this is believed to be an RNA binding determinant (Mears and Rice, 1996). Furthermore, it has recently been shown that ICP27 shuttles between the nucleus and cytoplasm. Shuttling occurs only at late stages during infection and is dependent on the co-expression of HSV late mRNAs, suggesting that ICP27 facilitates the export of late mRNAs (Soliman et al., 1997; Phelan et al., 1997). However, not all ICP27 homologues, including ORF 57, contain an homologous RGG box motif. Nevertheless, ORF 57 does encode an arginine-rich amino terminus, which may contain alternative RNA binding determinants. Deletion and mutational analysis of the N-terminal region of ORF 57 may help to clarify its role, if any, in RNA binding.

In addition to ORF 57's transactivating capabilities we have demonstrated that it can downregulate gene expression, specifically on intron-containing genes (Whitehouse et al., 1998a;b). In addition, the more widely studied homologue, ICP27, has been shown to be involved in the switch from early to late gene expression (McGregor et al., 1996; McLauchlan et al., 1989; 1992; Rice et al., 1993; Sandri-Goldin et al., 1995) and in the downregulation of viral IE and early genes. It is also required for the expression of late genes (McLauchlan et al., 1989; Rice et al., 1993; Sandri-Goldin et al., 1995; Sandri-Goldin and Mendoza, 1992). Furthermore, ICP27 contributes to the shut off of host cell protein synthesis and contributes to a decrease in cellular mRNA levels during infection, as deletion mutant infections result in increased levels of cellular protein synthesis and mRNA than do wild type infections (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Hibbard and Sandri-Goldin, 1995; Schroder et al., 1989).

HVS contains 76 major open reading frames, of which only 4 contain introns. This suggests that this virus makes limited use of the host cellular splicing machinery. Preliminary experiments have shown that during HVS infection, antigens associated with the small nuclear ribonucleoproteins (snRNPs), which are subunits of splicing complexes (reviewed in Kramer, 1995), are

redistributed in the nucleus and become concentrated in specific intranuclear structures (Cooper et al., 1999). This redistribution has also been observed during herpes simplex virus infection (Phelan et al., 1993; Sandri-Goldin et al., 1995). Sequence analysis has shown that ORF 57 is more highly conserved with respect to other members of the ICP27 family at the 3'-terminal region of the gene. We believe that the ORF 57 gene product contains a functional domain within the C-terminus which is required for the repressor function of this protein. It has been demonstrated that the C-terminal domain of ICP27 must remain intact for its inhibitory effect (McMahon and Schaffer, 1990; Sandri-Goldin et al., 1995). This region contains a cysteine-histidine rich region which resembles a single "zinc finger-like" motif or "zinc knuckle" which is conserved in all ICP27 homologs including ORF 57 (histidine residue 383 and cysteine residues 387 and 392 in ORF 57). Similar motifs occur in a number of splicing factors (Sandri-Goldin and Hibbard, 1996). Further studies are been undertaken to determine if this domain is essential for the repressor activity of ORF 57 and to determine which cellular genes interact with ORF 57.

C. A novel feedback mechanism which regulates HVS gene expression

More recently, we have demonstrated that these two major transcriptional control genes interact to regulate HVS gene expression via a novel feedback mechanism summarised in **Figure 5**. (Whitehouse et al., 1998b). The ORF 57 gene is produced at low levels early in the replication cycle until transactivated by the early ORF 50a gene product. Sequences within the ORF 57 promoter contain an ORF 50 response element which are essential for transactivation by the ORF 50a gene product and which result in an increase in RNA levels of the ORF 57 transcript. In addition, ORF 50a transactivates other genes which contain ORF 50 response elements within their promoters, for example the major DNA binding protein (Nicholas et al., 1992; Whitehouse et al., 1997a;b). Once transactivated by ORF 50a the ORF 57 gene product has several functions. As discussed previously, it has been shown to transactivate a range of HVS genes through post-transcriptional modification. Second, it downregulates ORF 50a, due to the presence of the intron within its coding region (Whitehouse et al., 1998a). Therefore we believe a feedback mechanism is in operation involving ORF 50a and ORF 57, which regulates gene expression in HVS, whereby a gene is downregulated by the product of the gene is has previously transactivated. Third, we believe the intron containing ORF 57 gene is responsible for its own downregulation by the same mechanism as that with which it represses ORF 50a, as both genes are

downregulated at a similar time during the replication cycle.

This series of events regulating gene expression in HVS differs from other herpesviruses. IE genes in all herpesviruses are defined as those which can be transcribed efficiently in the absence of *de novo* protein synthesis. Therefore, they mostly encode transcriptional regulators which are required for viral gene expression. However, despite their obvious role in virus replication the major IE genes are not conserved amongst herpesviruses. For example, during HSV replication five IE genes; ICP0, ICP4, ICP22, ICP27, ICP47 are expressed in the absence of viral protein synthesis. The fact that only one of these genes is conserved in HVS (ORF 57 is homologous to ICP27), may be unsurprising as HSV and HVS belong to different subfamilies of the herpesvirus genera. However, EBV, a member of the same subfamily as HVS, also differs from HVS in the IE genes it encodes. Upon reactivation, two major IE genes are expressed which are the key transactivating genes in EBV. The first, the IE BZLF1 gene product, Z, is sufficient to trigger reactivation, when overexpressed in latently infected cells (Buisson et al., 1989; Furnari et al., 1994; Rooney et al., 1989). Z is able to transactivate several promoters containing Z responsive elements, as well as to regulate its own promoter (Furnari et al., 1994; Liebermann et al., 1989; Packman et al., 1990; Rooney et al., 1989). The second IE protein, the BRLF1 gene product, R, is also a sequence specific transactivator. HVS does not encode a Z homologue. However, ORF 50 is homologous to the EBV R protein. Overall, this suggests that the two genes encoded by HVS which are homologous to genes found in other herpesviruses play a critical role in the HVS replication cycle.

Acknowledgements

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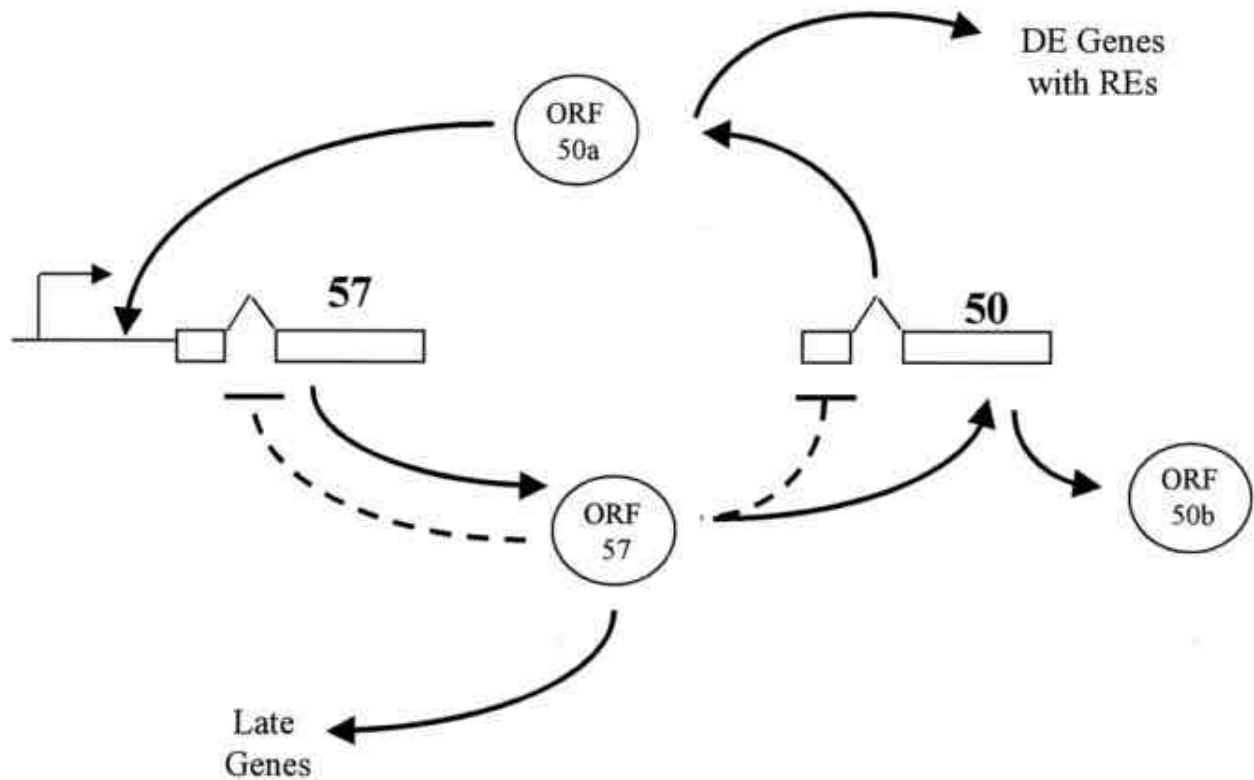
Figure 5. Schematic representation of the role and interactions of the ORF 57 and ORF 50 genes which regulate gene expression in the HVS replication cycle. (Abbreviations, DE - Delayed Early; RE - Response Elements).

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