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Neuronal reactivation of herpes simplex virus may involve interleukin-6

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Interleukin-6 (IL-6) is an inflammatory cytokine produced in many tissues, including the cornea and trigeminal ganglion. IL-6 acts by binding to its specific receptor, stimulating a cascade of signal proteins that induce the transcription factors NF-IL6 and STAT3. These IL-6-induced transcription factors change cellular gene transcription. Neutralization of IL-6 *in vivo* inhibits herpes simplex virus type 1 (HSV-1) ocular reactivation in mice. There are IL-6 response elements, possible binding sites of the IL-6 induced transcription factors, within the HSV-1 genome. These IL-6 response elements are concentrated in the inverted repeat regions of the genome, occurring in a non-random fashion in the promoters of the LAT and ICPO genes. Viral constructs containing deletions of IL-6 response elements in the LAT promoter region reactivate at a lower frequency compared with similar constructs lacking such deletions. HSV-1 may have evolved to exploit the relationship between a major inflammatory cytokine, IL-6, and conditions favorable for neuronal reactivation and subsequent replication in the epithelium. Exploring the role of IL-6, its receptor, and induced transcription factors in HSV-1 reactivation is a promising new avenue of research into the mechanism of HSV reactivation.

Keywords: HSV; latency; reactivation; cytokines; IL-6; STAT3; trigeminal ganglion

Introduction

At least 20% of the US population, over forty million people, experience periodic vesicular herpes simplex virus (HSV) lesions on the lip, face or mouth at some time during their lives (Spruance 1995). HSV-1 normally infects and reactivates in the head and neck region, with lesions most often occurring on the lip. HSV-2 typically infects and reactivates in the anogenital area. Both HSV types share the property of primary infection followed by generally less severe recurrences at the same anatomic site. HSV recurrences are usually self-limited, typically lasting from 7–14 days. However, in some persons HSV recurrences are especially frequent, occurring as often as every 2 or 3 weeks.

Eye infections and recurrences are an important subset of HSV-1 induced disease, afflicting an estimated half a million persons in the USA (Dawson 1995). The incidence of ocular herpes is estimated to be 20 000 cases of primary disease and 28 000 cases of recurrent disease per year in the USA. Five percent of all visits to ophthalmology clinics are due to ocular HSV infections (Hirsch 1995). About 6000 patients per year develop stromal scarring leading to significantly reduced visual acuity. Therefore, herpes simplex keratitis is one of the most common indications for corneal transplantation in developed countries. Unfortunately, recurrences of herpetic eye disease are common in these grafts, and repeat keratoplasties are plagued by a lower long-term success rate (Arffa 1991).

Ultraviolet radiation (UVR) (Laycock *et al*, 1991; Rooney *et al*, 1991; Spruance *et al*, 1991), fever, hyperthermia (Sawtell and Thompson 1992), hypothermia (Varnell *et al*, 1995), dental trauma (Shimizu *et al*, 1989), and surgical manipulation of the trigeminal ganglion (Pazin *et al*, 1978) are stimuli associated with reactivation of HSV-1. Several of these stimuli affect the immune system by inducing the production of inflammatory cytokines. Although the sequence of HSV gene activation during productive infection is well known, the

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mechanism whereby certain environmental stimuli cause latent virus to reactivate is unknown (Hill *et al*, 1997; Roizman and Sears 1996). New findings suggest that interleukin-6 (IL-6) and the related cytokine ciliary neurotrophic factor (CNTF) play a role in the pathogenesis of these outbreaks (Kriesel *et al*, 1994; Kriesel *et al*, 1997). The relationships between neurotrophic cytokines, inflammation, IL-6, and newly identified IL-6 response element sequences within the HSV-1 genome are explored in this report.

Neurotrophic factors and HSV reactivation

Nerve growth factor (NGF) is a protein present in nervous tissues that supports the survival and function of peripheral nerves. Using an *in vitro* model of HSV-1 reactivation from rat ganglion cells, a role for nerve growth factor in HSV reactivation was reported (Wilcox and Johnson 1987, 1988). These studies showed that brief removal of NGF from the medium bathing the latently infected cells stimulated the appearance of HSV antigens and live virus. This process was shown to be mediated by the NGF receptor and could be blocked by anti-NGF antibodies. *In vitro* HSV-1 reactivation was stimulated by drugs that destroy neurons (dopamine), interfere with microtubule assembly (colchicine), or block protein synthesis (cycloheximide) (Wilcox *et al*, 1990). These findings support a role for NGF in the maintenance of HSV latency. Recently, convincing support for a role of NGF in the maintenance of HSV latency has come from animal model studies. Administration of anti-NGF antibodies to latently infected mice (Laycock *et al*, 1994) and rabbits (Hill *et al*, 1997) stimulates reactivation of HSV-1. Anti-NGF antibodies do not affect the establishment of viral latency, the expression of latency-associated transcripts (LAT), or the quantity of HSV DNA in latently infected ganglia. Rather, these studies support a more direct role for NGF in neuronal homeostasis and the prevention of HSV reactivation.

NGF is not the only neurotrophic factor or protein that appears to play a role in HSV-1 reactivation. Levels of the nerve growth associated protein GAP-43 changed during the process of acute ocular HSV-1 infection, establishment of latency, and reactivation in rabbits (Martin *et al*, 1996). The expression of GAP-43 seemed to follow viral replication in this study, suggesting that this protein could be a marker for HSV reactivation.

Ciliary neurotrophic factor (CNTF) promotes the survival and differentiation of a wide range of cell types in the vertebrate nervous system and has been investigated for the treatment of neurodegenerative diseases in humans (Davis *et al*, 1993; Stoop and Poo 1995). During a clinical trial for the treatment of amytrophic lateral sclerosis, patients who received high doses of recombinant human CNTF (Synergen, Inc, Boulder, Colorado) frequently developed herpes labialis (Kriesel *et al*, 1994). The relationship between CNTF administration and HSV reactivation was unexpected, not associated with high fever, and appeared to be dose-dependent. CNTF is a cytokine unique to nervous tissue and was originally identified as a factor responsible for proper innervation of the ciliary ganglion (Richardson 1994; Sendtner *et al*, 1994). CNTF is not actively secreted, but is released following damage to peripheral nerves, possibly to promote repair. Preparations of recombinant human and rat CNTF are commercially available. However, murine CNTF has not been definitively isolated and cloned and hence is not available for mouse experiments. The observation that CNTF is stored in Schwann cells and is not secreted suggests that administration of CNTF to humans followed by HSV reactivation was a purely iatrogenic event. Exogenous CNTF was hypothesized to be mimicking the activity of other cytokines normally induced by reactivation stimuli.

The inflammatory cytokine interleukin-6

The cytokine interleukin-6 (IL-6) is a likely candidate as a mediator of HSV reactivation because it is an important component of the inflammatory response. IL-6 is made by a wide variety of cells including fibroblasts, endothelial cells, keratinocytes, macrophages, and T-cells (Kishimoto *et al*, 1995; VanSnick 1990). Interleukin-1 and tumor necrosis factor can induce the synthesis of IL-6, but the converse is not observed. This supports a role for IL-6 as an immune effector molecule, rather than a cytokine inducer or amplifier. The two best described actions of IL-6 are on hepatocytes where it induces the acute phase response and on B-cells where it acts as a growth factor.

The acute phase response occurs when circulating IL-6 acts on hepatocytes, changing the expression of a variety of genes (Abbas *et al*, 1994; Durum and Muegge 1996). For instance, hepatic synthesis of C-reactive protein (CRP) and hemopexin are increased, while synthesis of transferrin is decreased. CRP functions as a non-specific opsonin, promoting a strong humoral immune response. Hemopexin scavenges toxic heme products, probably to prevent tissue damage. Decreasing levels of transferrin, the major iron transport protein, may prevent microorganisms from obtaining this important element, required for bacterial replication. The acute phase response is common to many diverse organisms, demonstrating its value as a primitive host defense. IL-6 plays a pivotal role in this response by inducing changes in gene expression.

Interleukin-1, tumor necrosis factor, and IL-6 are made within a few hours of tissue damage, leading to inflammation. Increased levels of IL-6 and its message have been observed during fever and after skin exposure to UVR (Dinarello and Wolff 1995; Hirano and Kishimoto 1991; Urbanski *et al*, 1990; VanSnick 1990). Dermal production of IL-6 appears to be important in the subsequent cellular responses

to UVR-induced cellular damage (McKenzie and Sauder 1994). Acute HSV-1 infection of the cornea also leads to corneal production of IL-1 and IL-6, but not TNFα or IFN-γ (Staats and Lausch 1993). The excision of mouse corneas triggers enhanced epithelial cell synthesis of both IL-1 and IL-6 (Lausch *et al*, 1996). IL-6 also accumulates in the medium surrounding explanted mouse trigeminal ganglia within a few hours (author's unpublished observations). Indeed, IL-6 was the most plentiful among the several cytokines studied in the whole-cornea (Staats and Lausch 1993) and trigeminal ganglion explant models (unpublished observations, Dr Daniel Carr). It is reasonable to assume that surgical manipulation of the trigeminal ganglion in humans (as in ablation for trigeminal neuralgia) can elicit a similar inflammatory response, including a local increase in levels of IL-6.

The IL-6 'family' of cytokines (IL-6, IL-11, oncostatin M, leukemia inhibitory factor, and CNTF) act at the plasma membrane by binding to their specific receptors (IL-6R, CNTFR, etc.) and activating the transmembrane signal transducing protein gp130 (Ihle 1995; Ip *et al*, 1992; Kishimoto *et al*, 1995; Taga *et al*, 1992). Cytokine-induced dimerization of gp130 leads to activation of the transcription factors STAT3 and NF-IL-6 (Akira *et al*, 1995). Once inside the nucleus, these transcription factors stimulate synthesis of mRNA's encoding several cytokines and the acute phase proteins. Based on the observations in patients that received CNTF and the relationship of CNTF to IL-6, it was hypothesized that interleukin-6 (IL-6) was acting as a signal between inflamed tissues and neurons, stimulating a cascade of events culminating in HSV reactivation from latency (Figure 1).

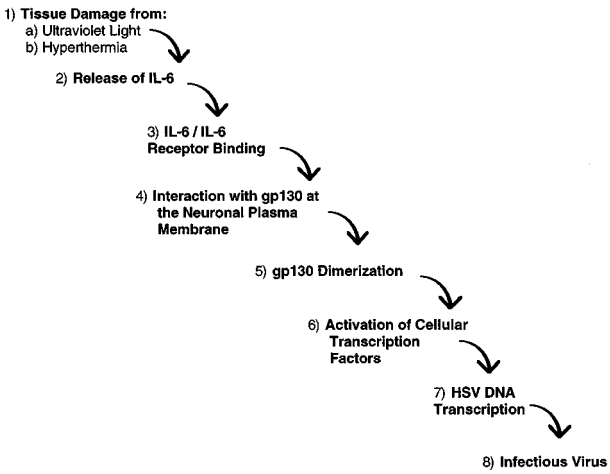

Figure 1 Hypothesis: the role of IL-6 in HSV reactivation.

Anti-IL-6 antibodies inhibit HSV reactivation
Systemic administration of anti-IL-6 antibodies significantly reduced the frequency of viral detection at the corneal surface in two different animal models of induced HSV-1 ocular reactivation (Kriesel *et al*, 1997). Among latently infected mice given hyperthermic stress, a marked reduction in the rate of HSV isolation from the eyes was seen among animals pretreated with a high dose of anti-IL-6 antibodies, compared to those that received a lower dose of anti-IL-6 antibodies or control immunoglobulin. Using the UVR-induced reactivation model, pretreatment with anti-IL-6 antibodies resulted in only 20% reactivation, compared to 75% of control mice. These results were obtained in two independent experiments using the same anti-IL-6 monoclonal antibodies, employing different methods of induced HSV reactivation (hyperthermia versus UVR).

IL-6 response elements within the HSV-1 genome
If IL-6 acts on the latently infected neuron to cause reactivation of HSV-1, there must be some mechanism to induce changes of gene expression within that cell. Interleukin-6 is known to stimulate two kinds of transcription factors within cells: NF-IL-6 and STAT3/APRF (the 'Acute Phase Reaction Factor') (Akira *et al*, 1995; Kishimoto *et al*, 1995). These transcription factors bind to type 1 (NF-IL-6) or type 2 (STAT3) response elements on the genome, thereby altering cellular transcription. NF-IL-6 belongs to the C/EBP family of transcription factors. It induces expression of inflammatory cytokines genes as well as some acute phase genes. In the case of STAT3, binding of the transcription factor to the genome induces the transcription of a variety of genes involved in the acute phase response. The factor IL-6-REBP, related or possibly identical to STAT3, induces expression of hemopexin, an abundant acute-phase protein (Immenschuh *et al*, 1994).

It is our hypothesis that IL-6 acts to induce HSV gene expression by stimulating transcription factors to bind IL-6 response elements within the HSV genome. The consensus recognition sequence for type 1 IL-6 response elements (that bind NF-IL-6) is T(T/G)NNGNAA(T/G), where N is any nucleotide. A consensus recognition sequence for type 2 response elements (that bind STAT3 and/or IL-6-REBP) is C(C/T)GG(A/G)AA (Akira *et al*, 1995; Immenschuh *et al*, 1994). The entire HSV-1 strain 17Syn$^+$ genome was examined for the presence of these IL-6 response element sequences (GenBank database). These IL-6 response element sequences occur commonly within the genome at approximately the expected frequency, but appear to be concentrated in the inverted repeat regions (Figure 2, Table 1).

The inverted repeat long (IR_L) region around the HSV-1 α genes ICP0 and ICP4 is crucial for the initiation of HSV-1 transcription (Roizman and Sears 1996). ICP0, the HSV-1 α0 gene product, is

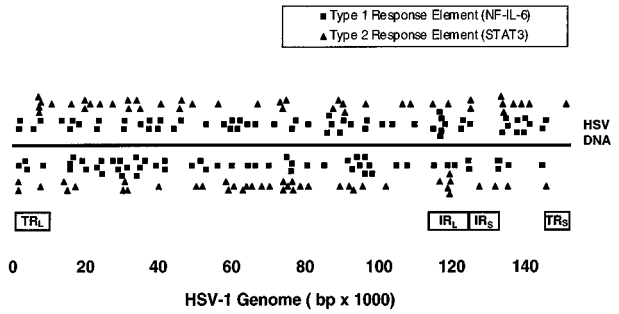

Figure 2 The consensus sequences for type 1 [T(T/G)NNGNAA(T/G) where N is any nucleotide] and type 2 IL-6 response elements [C(C/T)GG(A/G)AA] are mapped to the HSV-1 genome (Akira *et al*, 1995; Immenschuh *et al*, 1994). These consensus sequences are possible binding sites for the transcription factors NF-IL6 and STAT3, respectively. The total number of observed sites is near the expected value for both type 1 and type 2 response elements, but there is an apparent concentration of these sites in the inverted repeat regions (Table 1).

an important trans-activator of HSV gene transcription strongly implicated in viral reactivation from latency; ICP4 is required for optimal activity of ICP0. The latency-associated transcript (LAT), important for the efficiency of reactivation; ICP34.5 is responsible for neurovirulence; and the ORF-P (function unknown) sequence is also located in this region. Multiple type 1 and type 2 predicted IL-6 response elements are located in the inverted repeat regions (Figure 3). Several response elements of each type are located in the LAT and ICP0 promoter regions, situations unlikely to occur by chance alone (Table 1). The area near ICP4 and around the ORI-2 (origin of viral replication) also contain multiple predicted IL-6 response elements. In particular, two type 2 response elements are located 50 bp apart within the ICP0 promoter region and five type 2 response elements are located within the LAT promoter. Closely-spaced type 2 IL-6

Table 1 Predicted binding sites of known IL-6-induced transcription factors

HSV-1 region	*bp*	*IL-6 response elements*	*Factor*	*Observed sites**	*Expected sites***	*Observed/ expected*	P†
Entire genome		type 1	NF-IL6	118	117	1.0	NT
	152 000	type 2	STAT3	85	78	1.1	NT
Inverted repeats		type 1	NF-IL6	19	15	1.3	NS
	20 000	type 2	STAT3	13	10	1.3	NS
LAT promotor		type 1	NF-IL6	2	1.2	1.7	NS
	1500	type 2	STAT3	5	0.8	6.3	0.001
ICP0 promotor		type 1	NF-IL6	3	0.4	7.5	0.007
	500	type 2	STAT3	2	0.3	6.7	0.03
ICP0 promotor		type 1	NF-IL6	0	0	–	–
Tandem sites	50	type 2	STAT3	2	0.03	66.7	<0.001

*The HSV-1 genome was examined for the presence of IL-6 response elements by searching the GenBank dataset. The consensus sequence for type 1 IL-6 response elements (which bind NF-IL-6) is T(T/G)NNGNAA(T/G), where N is any nucleotide (Akira *et al.* 1995). The consensus sequence for type 2 response elements (STAT3 or IL6-REBP) is C(C/T)GG(A/G)AA (Akira *et al.* 1995; Immenschuh *et al.* 1994).

**The expected number of IL-6 response elements within the HSV-1 region was calculated by multiplying the expected frequency of each sequence by the number of nucleotides considered. Both DNA strands were considered by mapping the complementary sequence (e.g. 5′-CTGGGAA-3′ and its complement 5′-TTCCCAG-3′). The G + C content of the HSV-1 genome equals 67% (Roizman and Sears 1996). Therefore, P[G] = P[C] = 1/3; and P[A] = P[T]=1/6. The expected frequency of type 2 IL-6 response elements (potential STAT3 binding sites) is calculated to be: P[C(T or C)GG(G or A)AA] = (1/3)(1/2)(1/3)(1/3)(1/2)(1/6)(1/6) = 1/3888 bp. Therefore, the expected number of type 2 IL-6 response elements within the HSV-1 genome = 152 000/3888 = 39. An equivalent expected number of complementary sites is expected for a total of 78 predicted type 2 response elements. Similar calculations indicate that the expected frequency of type 1 IL-6 response elements (potential NF-IL6 binding sites) is 1/2596 bp.

†Probabilities (P) were derived using the Poisson distribution, applicable due to the relatively low frequency of occurrence for sequences with 6–7 unique nucleotides (Waterman 1995). The formula used was:

$$P(x\ count) = e^{-\lambda}\lambda^{x}/x!,\ where\ \lambda = n \times p$$

Considering type 2 response elements within the approximately 500 bp ICP0 promoter region (row 8 in the above Table):

$n = 500$ bp $\times 2$ (forward and complement strands) = 1000, $P = 1/3888$, $\lambda = 1000/3888 = 0.257$
$P(\geqslant 2$ sites) = 1-P(0 or 1 site)
$P(0$ sites) $= e^{-0.257}(0.257)^{0}/0! = 0.773$; P(1 site) $= e^{-0.257}(0.257)^{1}/1! = 0.199$
$P(\geqslant 2$ sites) $= 1 - [0.773 + 0.199] = 1 - 0.972 = 0.028$

Displayed probabilities are for the observed number of binding sites observed *or more*, as shown in the preceding sample calculation.
NT, not tested, due to the presence of multiple (protein) coding regions in the U_L and U_S regions.
NS, not significant

Probability (T or G) = 0.5 P(N, any nucleotide) = 1.0
P(A) = P(T) = 1/6 P(G) = P(C) = 1/3

response elements can amplify the DNA binding affinity of STAT3 by as much as 80-fold (Brechner *et al*, 1991). These observations suggest that viral evolution has favored the presence of multiple, potentially cooperative, IL-6 responsive elements within these important HSV-1 gene promoter regions.

The presence of predicted IL-6 response elements in these strategic positions could allow IL-6 to directly activate HSV-1 replication. The *in vivo* relevance of these observations needs to be confirmed experimentally, because the secondary and tertiary structures of latent viral DNA could affect the binding affinities of any induced transcription factors. Since IL-6 response elements and their respective transcription factors were discovered to be the effectors of the acute phase response in the liver, these transcription factors have been studied primarily in hepatocytes (Akira *et al*, 1995). The transcription factors produced in ganglionic tissue, the primary site of HSV latency, are currently unknown, but the gp130 signal transducer and STAT3 have been demonstrated in neuronal cells (Ip *et al*, 1992; Wu and Bradshaw 1996).

IL-6 response elements are related to HSV-1 reactivation

Viral deletions within the LAT promoter and intron have been studied to determine regions important for controlling HSV reactivation (Bloom *et al*, 1997; Cook and Hill 1991; Hill *et al*, 1996; Loutch *et al*, 1997; Perng *et al*, 1996; Trousdale *et al*, 1991). These studies showed that deletions from the LAT promoter region display a 'low' (reduced frequency) reactivation phenotype, even in the presence of normal levels of the LAT intron (Figure 4, Table 2). In contrast, deletions within the 2.0 kb LAT intron result in a 'high' (normal frequency) reactivation phenotype. It is possible, but unlikely, that mutations within the LAT intron do not affect the still undiscovered function of this RNA species. Rather, these observations suggest that it is the transcription of the LAT region, rather than the presence of an

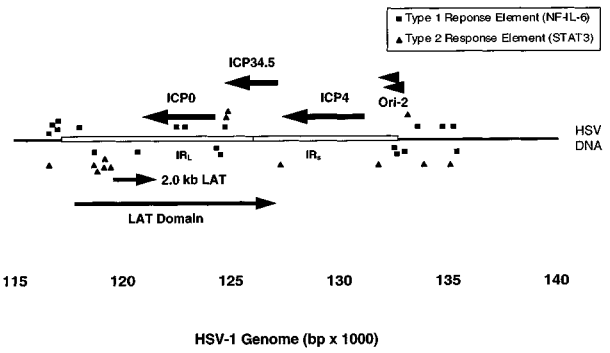

Figure 3 Predicted IL-6 response elements within the inverted repeat regions of the HSV-1 genome are shown. Note the multiple, closely-spaced sites in the LAT domain (promoter region), near ICP0, and around the origin of replication (Ori-2). Concentrations of IL-6 response elements in these regions are likely to be non-random (Table 1).

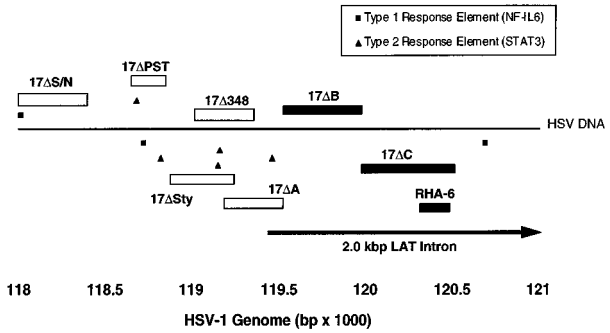

Figure 4 Predicted IL-6 response elements within the LAT promoter and intron regions are shown. The sites of deletions from mutant viruses with high (open bars) and low (solid bars) reactivation phenotypes are displayed. Mutant viruses with high reactivation phenotypes share two properties: (1) their deletions are from the LAT promoter rather than intron region and, (2) they each contain at least one predicted IL-6 response element.

Table 2 The relationship between HSV-1 deletion mutations, the loss of predicted IL-6 response elements, and the subsequent reactivation phenotype. The deletion mutations displayed are all within the LAT promotor or intron regions. (The deletion sites and implicated IL-6 response elements are displayed in Figure 4)

		#Deleted IL-6 response	
HSV-1 deletion mutant	*Deletion size (bp)*	*Elements (P†)*	*Reactivation phenotype*
17ΔA	303	1 (0.15)	low
17Δ348	348	2 (0.01)	low
17ΔS/N	437	1 (0.31)	low
17ΔPst*	202	3 (<0.001)	low
17ΔSty	370	2 (0.01)	low
17ΔB	461	0 (0.74)	high
17ΔC	475	0 (0.74)	high
RHA-6	166	0 (0.90)	high

*The 17ΔPst deletion mutant does not accumulate the LAT intron within latently infected cells. All the other viral deletion mutants produce detectable LAT.

†The probabilities (*P*) that one or more IL-6 response elements would be included in these LAT promotor and intron deletions by chance alone were calculated. The values expressed were calculated for the specific situation observed within each deletion mutation. For instance 17ΔA has a single type 2 IL-6 response element (and no type 1 elements) within its 303 bp sequence:

$$P(x\ count) = e^{-\lambda}\lambda^{x}/x!,\ where\ \lambda = n \times p$$

(a) One type 2 site is observed in 17ΔA: $\lambda = 303$ bp/3888 bp = 0.0779 P(1 or more type 2 sites) = 1 − P(0 type 2 sites) = $1 - e^{-0.0779} = 1 - 0.925 = 0.075$ Doubling to consider the complementary strand: P(1 or more type 2 sites) = 0.015

(b) For type 1 sites, $\lambda = 303$ bp/2596 bp = 0.117, but none are observed in 17ΔA, so P(0 or more type 1 sites) = 1.0

P or more or type 2 sites *and* 0 type 1 sites] = (0.15)(1.0) = 0.15

intact 2 kb intron, that is important for the control of reactivation.

Another possible explanation for the impaired reactivation phenotype among the LAT promoter deletion mutants is the loss of some IL-6 response elements contained within this region (Table 2, Figure 4). The presence of at least one predicted IL-6 type 1 or 2 response element within each of the low-reactivation mutants (17ΔPst, 17Δ348, 17ΔA, and 17ΔS/N) is not improbable (P=0.25, 0.42, 0.37, and 0.52, respectively). However, taken together, the probability of finding at least one IL-6 response element sequence in all these deletion mutants is unlikely (P=0.02). The deletion mutants 17ΔPst and 17Δ348 each contain closely spaced, possibly cooperative, IL-6 response element sequences (P=0.004). Indeed, neurons infected with the mutant containing the most predicted IL-6 response element sequences, 17ΔPst, do not produce detectable LAT. In contrast, the deletion mutants 17ΔB, 17ΔC, and RHA-6 have high-reactivation phenotypes and do not include any predicted IL-6 response elements. The ICP0 promoter deletion mutant ΔTfi recently reported by Davido and Leib (Davido and Leib 1996) included one of three predicted type 1 (NF-IL6) response elements, but did not include the tandem type 2 (STAT3) sites in this region (Figure 3). This mutant virus was less virulent than its wild-type counterpart, but established latency and reactivated normally *in vitro*. In summary, the presence or absence of IL-6 response elements within deletion mutations in the LAT and ICP0 promoter regions appears to predict the reactivation phenotype of the resultant virus.

It is hypothesized that the presence of LAT, a lariat shaped persistent RNA species that does not code for any known protein (Block and Hill 1997), assists IL-6-induced transcription factors in their activation of HSV replication. A positive feedback process is possible whereby IL-6 induced transcription factors favor LAT transcription which in turn enhances transcription factor binding to the HSV inverted repeat regions. This hypothesized amplification process, initiated only in the presence of the inflammatory cytokine IL-6, could result in efficient induction of HSV reactivation. Investigations into transcription factor binding and viral genome activation are necessary to confirm the *in vivo* relevance of IL-6 response elements in the LAT and ICP0 promoter regions.

Abbreviations

HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; UVR, ultraviolet radiation or exposure; CNTF, ciliary neurotrophic factor; IL-6, interleukin-6; NGF, nerve growth factor; GAP-43, 43 kD growth associated protein; CRP, C-reactive protein; STAT3, signal transducer and activator of transcription-3; IL6-REBP, interleukin-6 response element binding protein; LAT, HSV latency-associated transcripts.

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