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The cellular response to herpes simplex virus type 1 (HSV-1) during latency and reactivation

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In order to learn more about the cellular response to viral gene activity during latency and reactivation of herpes simplex virus type 1 (HSV-1), the authors have employed microarray analysis. On an array of about 1200 cellular genes, approximately 56 genes were found to be differentially regulated in infected trigeminal ganglia of mice, compared to uninfected mice, during latency and reactivation. Of these genes, 10 were examined more closely using quantitative real-time polymerase chain reaction (PCR) to confirm the microarray results. Genes involved in interferon and other signaling pathways appeared to predominate in response to a latent or reactivating HSV infection. Interestingly, some genes found to be differentially regulated in latently infected ganglia are neuronal-specific genes (pro-opiomelanocortinin; zinc finger proteins of the cerebellum 1 and 2). During reactivation, the involvement of several cell signaling molecules that may be important for the initiation of an HSV infection was observed, including various receptors and molecules involved in cell-cell spread. *Journal of NeuroVirology* (2005) **11,** 376–383.

Keywords: HSV-1; latency; microarray analysis; reactivation

Introduction

Herpes simplex virus type 1 (HSV-1) establishes a latent infection primarily in neuronal cells of the peripheral nervous system. When exposed to various types of stress, latent HSV-1 can reactivate and cause recurrent disease in the host. A general understanding of some of the stressors that trigger reactivation, including physical trauma, emotional stress, the effects of exposure to ultraviolet light, and hormonal imbalances, has been gained by study over the years (as reviewed by (Roizman and Knipe, 2001). However, not much is understood regarding the molecular mechanisms involved with reactivation of HSV-1.

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The temporal cascade of viral gene expression during a lytic infection (the immediate early genes, followed by the early, and finally the late genes) was first defined in cell culture in the early 1970s (Honess and Roizman, 1974). More recently, HSV microarrays have been developed where the cascade of viral transcription during a lytic infection has been measured under a variety of experimental conditions (Aguilar *et al*, 2002; Karaca *et al*, 2004; Stingley *et al*, 2000; Sun *et al*, 2004; Yang *et al*, 2002). However, it is thought by some that this same pattern of gene expression may not occur during reactivation because of the different conditions that are present in neuronal cells during reactivation compared to initial infection. *In vitro* studies indicate that peak viral infection in neuronal cells occurs at a later time than in Vero cells (Nichol, 1996). Using *in vivo* studies it was reported that viral gene expression during reactivation does not occur with the typical HSV cascade of immediate early, early, and late genes (Tal-Singer *et al*, 1997). Using the trigeminal ganglia (TG) explant model for reactivation, it was determined that several early gene transcripts, infected protein 6 (ICP6), virion polypeptide 5 (VP5), and thymidine kinase (TK), could be identified by reverse transcriptase–polymerase chain reaction (RT-PCR) by 4 h post explant. The immediate early gene transcripts infected cell polypeptides

0 (ICP0), 4 (ICP4), and 27 (ICP27) were not observed until 24 h post explant using the same experimental conditions (Tal-Singer *et al*, 1997).

Explantation of trigeminal ganglia of mice infected with viruses defective in a functional TK gene product, either by mutation or drug inhibition, results in decreased amounts of immediate early and early gene expression during reactivation (Kosz-Vnenchak *et al*, 1993). Normally, loss of an early gene does not affect the expression of the immediate early gene products, so this suggested a novel regulatory pathway for HSV gene expression in neurons. Other studies suggest that neuronal cells may contain octamer-like proteins that block the ability of virion polypeptide 16 (VP16) to activate immediate early viral gene transcription (Kemp *et al*, 1990; Lillycrop *et al*, 1993). Normally during lytic infection, VP16 interacts with Oct-1, a cellular protein, to allow transactivation of the immediate early genes (reviewed in (Roizman and Sears, 1995; Wysocka and Herr, 2003). A lack of the VP16 protein results in virus that does not form an acute infection but does form a latent infection from which it can reactivate (Steiner *et al*, 1990). Infection with a virus lacking the activation domain of VP16 resulted in decreased levels of HSV transcripts during a lytic infection as measured using an HSV oligonucleotide array (Yang *et al*, 2002). These findings further support the hypothesis that a cellular factor is critical for viral reactivation by replacing the function of VP16 normally observed during an acute HSV infection.

Experiments involving microarray analysis can be especially useful for looking at global cellular gene expression in response to infection. Several of these studies have been done examining different phases and conditions of HSV infection. During lytic infection, experiments have been done showing an overall up-regulation of various transcription factors, stress response genes, cell cycle regulatory genes, and genes involved in apoptotic pathways (Hobbs and DeLuca, 1999; Khodarev *et al*, 1999; Mossman *et al*, 2001; Stingley *et al*, 2000; Taddeo *et al*, 2002). These studies mainly examined up-regulated genes due to complications arising from the nonspecific degradation of cellular mRNAs by the virion host shutoff (vhs) protein encoded by HSV. A few microarray studies have examined mRNAs during the latent and reactivating stages of an HSV infection (Higaki *et al*, 2002; Hill *et al*, 2001; Kramer *et al*, 2003). These studies show a narrower range of genes differentially expressed as compared to those in the lytic studies. Many of them are genes known to be involved in stress or antiviral response pathways, as should be expected during the stressful conditions of reactivation and the presence of HSV.

The above studies were performed with various methods of reactivation, including heat stress and immunosuppressive drug treatment. Another method commonly used is the TG explant model, which results in reactivation of 100% of infected TGs. These other methods of reactivation, including heat stress (Sawtell and Thompson, 1992), exposure to immunosuppressive agents (Openshaw *et al*, 1979), and treatment with cadmium (Fawl and Roizman, 1993), induce reactivation in a smaller percentage of the animals treated. A potential drawback to using the TG explant model system involves the *ex vivo* nature of the stress associated with viral reactivation, and this may not truly represent what occurs during natural HSV reactivation.

Previous work done by Tsavachidou *et al* (2001) used a large scale array representing 18,000 mouse cDNAs to look at regulation of cellular genes during uninfected TG explantation. At 4 h post explant they found 300 mRNAs up-regulated and 500 mRNAs down-regulated (three-fold) from a variety of categories: (i) neuron-specific genes; (ii) transcription and replication factors; (iii) cell cycle–related genes; (iv) signal transduction genes; (v) genes related to metabolism. In the current experiments, we study reactivation using the TG explant model with tissue isolated from mice infected with the F strain of HSV-1. We have examined the events during reactivation because it is believed that the cellular environment plays a role in the establishment of a productive viral infection during reactivation. From the array data gained, several pathways/genes can be described that are differentially regulated during both latency and reactivation.

Results and discussion

Due to the probable involvement of cellular factors during reactivation, the examination of transcription modulation during reactivation should lead to a greater understanding of how HSV-1 takes advantage of its cellular environment to produce effective latent and reactivating infections. Microarray experiments were conducted using trigeminal ganglia from mice infected with the F strain of HSV-1 or uninfected mice. The mice were kept for at least 28 days to ensure that a latent infection had been established. TGs were then extracted and either snap-frozen, to serve as latent samples, or explanted in cell culture conditions for 30 min, to allow for reactivation of the virus from infected samples. These experiments to examine cellular transcription were conducted at a very early time post reactivation (30 min) in order to observe the transcriptional events involved in the switch from latency to reactivation. All RNA samples were then used to hybridize to the Atlas mouse 1.2 cDNA array (Clontech) and analyzed according to genes that were differentially regulated during latency (infected, 0 min post explant versus uninfected, 0 min post explant), reactivation (infected, 30 min post explant versus uninfected, 30 min post explant), and the stress of explantation (uninfected, 30 min post explant versus uninfected, 0 min post explant). Overall, 56 out of approximately 1200

genes on the microarray were differentially regulated by at least 2.5-fold in response to latency and/or reactivation.

Genes involved in immune response and neuronal specific factors altered during latency

During latency we saw 18 genes altered, and of these 5 were found altered only during latency and not during reactivation. These five genes are pro-opiomelanocortinin (POMC), suppressor of cytokine signaling-1 (SOCS-1), protein kinase C beta-II, calmodulin-dependent protein kinase IV, and zinc finger protein of the cerebellum 3 (Table 1). Many of these genes found during latency are neuronal-associated genes such as POMC and the zinc proteins of the cerebellum, while other are involved in various signaling pathways. Some cellular genes were changed during latently infected as compared to uninfected animals and this could be due to the fact that it has been demonstrated that viral TK and ICP4 transcripts can be detected at very low levels during latency (Feldman *et al*, 2002; Kramer and Coen, 1995). It is maintained that whereas basal levels of some gene transcripts may be observed during latency, the stress of reactivation results in a significant overall increase of viral transcription. This minimal level of viral gene transcription could be enough to impact the interferon and immune response pathway genes—such as interferon-inducible protein 1, granzyme A, cathepsin H, STAT1, and interferon regulatory factor 1 and cytokine inducible SH2-containing protein 7 and antiproliferative B-cell translocation gene, respectively. These genes were found to be up-regulated in the array analysis (Table 1). Another group has also noted an abundance of genes involved in immune response to be altered in response to a latent HSV-1 infection using microarray analysis (Kramer *et al*, 2003). We also noted a modulation of a number of brain-specific factors (POMC and the zinc finger proteins of the cerebellum) and these may be involved in the maintenance of viral latency. Previously it has been shown that lytic HSV infection in the brain does result in elevated levels of adrenocorticotropic hormone (ACTH) and corticosterone, which are both downstream elements produced in association with POMC expression (Ben-Hur *et al*, 1995).

Table 1 Genes regulated differentially during latency (In 0 min/Un 0 min)

	Gene name	Genbank number	Fold change	
			Microarray	Q-PCR
R*	Interferon-inducible protein 1	U19119	+6.8	+1.56
L	Pro-opiomelanocortin-alpha (POMC)	K00648	+6.0	+13.8
R	STAT1	U06924	+5.7	+2.09
S	Zinc finger protein of the cerebellum 1	D32167	−5.1	—
R	Granzyme A	M13226	+4.9	+3.43
R	Interferon regulatory factor 1 (IRF1)	M21065	+4.9	—
R	Rho GDP dissociation inhibitor beta	L07918	+4.3	—
R	Delta-like protein precursor (DLK)	L12721	+4.2	—
R	Retinoic acid-inducible E3 protein	U29539	+3.8	—
L	Suppressor of cytokine signaling-1 (SOCS-1)	U88325	+3.7	—
S	CDC 25MM; guanine releasing protein (GNRP)	X59868	−3.5	—
R	Osteopontin precursor	J04806	+3.4	—
R	Cathepsin H	U06119	+3.1	−2.26
L	Protein kinase C beta-II	X53532	−3.1	—
L	Calmodulin-dependent protein kinase IV	X58995	−3.1	—
S	Antiproliferative B-cell translocation gene 2	M64292	+2.6	—
R	P-selectin glycoprotein ligand 1 precursor	X91144	+2.5	—
L	Zinc finger protein of the cerebellum 3	D70849	−2.5	—

*L = only found during latency; R = also found during reactivation; S = also found during the stress of explantation (uninfected).

Elements of the immune response to HSV-1 infection during latency and reactivation

Ten genes found during both latency and reactivation were all up-regulated, a finding that was not surprising given that many belong to the interferon response pathways, including the interferon-inducible protein 1, cathepsin H, STAT1, and interferon regulatory factor 1. Interferon signaling is a common finding in response to a viral infection and studies of this response are fairly comprehensive (Katze *et al*, 2002). Activation of these genes collectively during latency and reactivation indicates that we are observing the elements of a general viral response to HSV infection. Studies of other viruses, including cytomegalovirus (CMV) (Browne *et al*, 2001; Zhu *et al*, 1998), Epstein-Barr virus (EBV) (Cahir-McFarland *et al*, 2004), and adenovirus (Dorn *et al*, 2005), also find an abundance of immune response genes up-regulated in response to viral infection as found by cellular microarray experiments. In addition, much work has been done looking specifically at HSV infection and the various components of interferon response involved. STATs act as signaling molecules in interferon transduction pathways (Levy and Darnell, 2002). In HSV-1–infected TGs, STAT proteins are expressed at the same level during latency and times post reactivation (Kriesel, 2002). This is confirmed in our findings where STAT1 is up-regulated 5.7- and 5.6-fold during latency and reactivation, respectively (Tables 1 and 2). Three potential STAT binding sites within the LAT promoter have been examined and it was found that STAT1 can bind within the 3′ region of the LAT promoter (Kriesel *et al*, 2004). Interferon regulatory factors are activated in response to double-stranded RNAs and other viral specific signals and results in interferon

Table 2 Genes regulated differentially during reactivation (In 30 min/Un 30 min)

	Gene name	Genbank number	Fold change	
			Microarray	Q-PCR
L*	Interferon-inducible protein 1	U19119	+11.0	+2.57
R	Glutathione *S*-transferase A	J03958	+10.2	—
L	Granzyme A	M13226	+7.4	+4.77
R	Gluamate receptor channel subunit gamma	X04648	+6.1	—
R	Cytokine receptor common gamma subunit precursor	L20048	+6.0	—
L	Cathepsin H	U06119	+5.6	+1.04
L	Delta-like protein precursor	L12721	+5.6	—
L	Signal transducer and activator of transcription 1	U06924	+5.6	+2.60
L	P-selectin glycoprotein ligand 1 precursor	X91144	+5.3	—
S	Transthyretin precursor	D89076	−5.2	−7.23
R	Macrophage inflammatory protein 1 beta	M35590	+4.8	—
R	Cell surface glycoprotein MAC-1 alpha subunit precursor	X07640	+4.6	—
R	Gamma interferon-induced monokine precursor	M34815	+4.6	—
R	Laminin receptor 1	J02870	+4.4	—
L	Osteopontin precursor	J04806	+4.3	—
R	CD14 monocyte differentiation antigen precursor	M34510	+4.2	—
R	PLC beta	U43144	−4.2	—
S	von Hipple-Lindau syndrome homolog	U12570	+4.0	−81.6
R	Neuronal kinesin heavy chain	X61435	−4.0	—
R	Inositol 1,4,5-triphosphate receptor type 2	Z71173	+3.5	—
R	Alpha 2 catenin	D25281	−3.4	—
R	Rgs4	AB004315	−3.4	—
S	Bone proteoglycan II precursor (decorin)	X53929	+3.3	—
R	Microsomal glutathione *S*-transferase	J03752	+3.2	—
R	47-kDa heat shock protein precursor	J05609	+3.1	—
R	CD2 antigen	M18934	+3.0	—
L	Rho GDP dissociation inhibitor beta	L07918	+3.0	—
L	Retinoic acid–inducible E3 protein	U29539	+3.0	—
R	cAMP-dependent protein kinase type I-beta regulatory chain	M20473	−3.0	—
R	Calbindin 2	X73985	−3.0	—
R	Defender against cell death 1	U83628	+2.9	—
R	Tubulin beta 4	M28730	−2.9	—
R	Tumor necrosis factor receptor 2 precursor	M59378	+2.8	—
R	Myeloblastin	U43525	+2.8	—
R	Integrin beta 7	M95633	+2.8	—
R	DNA fragmentation factor alpha subunit	AB009376	−2.7	—
R	14-3-3 protein eta	U57311	−2.7	—
R	Intercellular adhesion molecule 1 precursor	X52264	+2.6	—
L	Interferon regulatory factor 1	M21065	+2.6	—
R	Cathepsin W precursor	AF013116	+2.6	—
R	Geminin	AF068780	+2.6	—
R	Granulocyte-macrophage colony stimulating factor receptor	M85078	−2.6	—
S	Insulin-like growth factor receptor II	U04710	−2.6	—
R	Protein phosphatase 2C alpha isoform	D28117	−2.6	—
R	Cytoplasmic beta-actin	M12481	−2.6	—
R	A-myb proto-oncogene	X82327	+2.5	—
R	LIM-homeodomain protein L3	D49658	−2.5	—
R	Neurofilament triplet M protein	X05640	−2.5	—

*R = only found during reactivation; L = also found during latency; S = also found during the stress of explantation (uninfected).

(IFN)-β induction (Katze *et al*, 2002). Interferon regulatory factor (IRF)-1 has been shown previously to be induced during reactivation by differential display and it was suggested that, due to the presence of several potential IRF-1 binding sites in the HSV genome, this factor may be involved in regulating gene expression during reactivation (Tal-Singer *et al*, 1998).

Various other elements of the immune system may play a role in controlling HSV infections. One important defense mechanism, granzyme A, is a serine protease that is found in cytotoxic T cells and natural killer cells that results in cell death via a caspase-independent pathway (Lieberman and Fan, 2003). During a lytic HSV infection, it was found that granzyme A–deficient mice have increased viral loads and subsequently an increased number of infected neurons compared to infected wild-type mice (Pereira *et al*, 2000). This suggests that granzyme A may play a role in controlling the neuronal spread of HSV during a lytic infection. Selectins are adhesion molecules involved in lymphocyte trafficking and are especially important for responses involving the innate immune system (Ley, 2003). P-selectin is up-regulated on the surface of HSV-infected endothelial cells. It requires viral gC expression and results in recruitment of monocytes to the site of infection (Etingin *et al*, 1991). The involvement of these genes during latency and reactivation emphasizes the involvement of the immune system during all phases of HSV infection.

Signaling pathways triggered during HSV reactivation and/or the stress of explantation

A total of 48 genes were differentially regulated during reactivation, with 34 of these genes being specific to reactivation (Table 2). A wide variety of genes representing different cellular, functional categories were observed during this phase of infection. We observed even more elements of the immune system up-regulated, including many

different cytokines, signaling receptors, and cell migration factors that are involved in localizing immune factors. One example is the up-regulation of the MAC-1 cell surface glycoprotein, which is found on macrophages, natural killer cells, some CD8+ T cells, and peripheral mononuclear cells. It has previously been shown to be associated with HSV infected neurons in the trigeminal ganglia (Liu *et al*, 1996).

As reactivation conditions set the stage for initiation of a productive HSV infection in the latent cell, the involvement of various receptors and cell signaling pathways indicate that the virus may be priming the cell for an efficient "infection" in response to the reactivation stimulus. Various receptors, including a member of the TNF family (tumor necrosis factor receptor 2) which has become accepted as being one of the main entry mediators for HSV (Spear, 2004), are shown to be up-regulated in response to HSV reactivation. Other differentially regulated elements found in our experiments are also involved in cell-cell adhesion, including alpha 2 catenin and laminin. They have been studied previously in the context of HSV infection and may play a role in the entry and spread of the virus (Sakisaka *et al*, 2001; Weeks and Friedman, 1997). Stress-related response via inositol 1,4,5-triphosphate (IP_3) receptor signaling has been shown to be important for HSV entry into cells, and up-regulation of this gene in our experiments further suggests that reactivating virus stimulates genes involved in initiating an HSV infection (Cheshenko *et al*, 2003). Several other microarray experiments looked specifically at genes involved in stress response pathways and found several genes differentially regulated in response to HSV infection (Higaki *et al*, 2003; Hill *et al*, 2001). We cannot directly compare the differentially regulated genes with these other microarray studies as they look at times later post reactivation and use different methods of reactivation.

In examining the processes occurring during reactivation, two different stimuli can be shown to affect cellular gene transcription—the virus itself and the stress associated with the explantation process. When looking at uninfected TGs that have been explanted, we see significant changes in expression of 9 out of 16 genes that are found specifically under these mock infection conditions and not during latency or reactivation (Table 3). These genes are mostly involved in various stress response signaling pathways. Another experiment examined differentially regulated cellular genes in response to explanting ganglia from uninfected animals and found several categories of genes to be involved, including neuronal factors, transcription factors, and factors involved in the cell cycle (Tsavachidou *et al*, 2001). We do not find many genes specifically in common with this study but the main categories of stress response and general transcription factors are well represented in both experiments.

Table 3 Genes regulated differentially during explantation (Un 30 min/Un 0 min)

	Gene name	Genbank number	Fold change	
			Microarray	Q-PCR
S*	Early growth response protein	M20157	+11.8	+16.9
L	Antiproliferative B-cell translocation gene 2	M64292	+5.3	—
S	Cytokine inducible SH2-containing protein 3	U88328	+5.0	—
S	TcF AP-1; c-Jun proto-oncogene	J04115	+3.5	—
R	Transthyretin precursor (prealbumin)	D89076	−5.5	−28
L	Zinc finger protein of the cerebellum 1	D32167	−5.1	—
R	Insulin-like growth factor II precursor	M14951	−4.1	—
R	Von Hippel-Lindau syndrome homolog	U12570	−3.4	−2.4
S	Myelin-oligodendrocyte gp precursor (MOG)	U64572	−3.1	—
S	Paired box protein 6	X63963	−3.0	—
L	CDC 25MM; guanine releasing protein (GNRP)	X59868	−2.7	—
S	Mast cell protease (MMCP)-4	M55617	−2.7	—
R	Bone proteoglycan precursor (PG-S2)	X53929	−2.6	—
S	γ-aminobutyric-acid receptor alpha-1 subunit precursor	M86566	−2.5	—
S	Myelin proteolipid protein	M16472	−2.5	—
S	Keratinocyte growth factor FGF-7	Z22703	−2.5	—

*S = only found during the stress of explantation (uninfected); R = also found during reactivation; L = also found during latency.

Real-time PCR confirmation of microarray data

An independent experiment was conducted to confirm the cellular gene transcription levels observed in the microarray experiments. Additional mice were infected with the F strain of HSV-1, or left as uninfected controls, and explanted as described previously. RNA was purified from TGs under the various experimental conditions and used to generate cDNAs using reverse transcriptase meditated PCR with oligo (dT) and random hexamer primers. Cellular gene primers matching the cDNAs found on the cellular array were purchased from Clontech and used in quantitative PCR assays. Although some of the levels are different between these two methods of detection, we still observed the same trend of up- or down-regulation of each gene with only minor exceptions (Figure 1). During latency one gene, cathepsin H, appeared to be down-regulated according to the quantitative (qPCR) assay but it was up-regulated in the microarray experiments. However, the cathepsin H levels matched when looked at during reactivation. The von Hipple-Lindau syndrome homolog gene was reported to be down-regulated by qPCR but up-regulated by microarray analysis during reactivation. Given that these differences occur at different comparisons and not that one gene is always

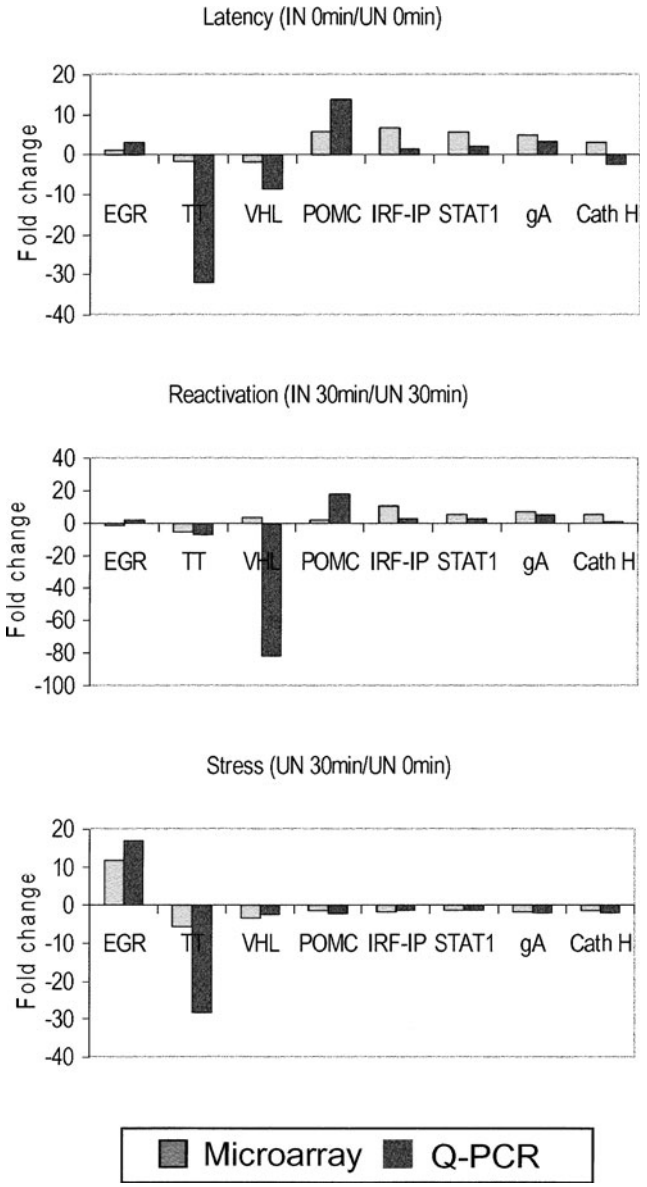

Figure 1 Fold change of cellular gene transcription as measured by microarray or quantitative PCR analysis. Uninfected and infected TGs were harvested for latent or reactivated samples and the RNA was analyzed according to techniques described in the Materials and methods.

misaligned, this suggests that overall the microarray data is accurate.

Conclusions

The changes in gene expression noted here represent changes in many different functional categories of cellular genes. However, the ability of the virus to manipulate any of these cellular genes directly is unclear. The differential regulation of some cellular genes may not play a specific role in reactivation but may be observed as a consequence of the antiviral response of the host cell. It is also possible that the virus may have managed to adapt a function for these normal cellular responses to trigger reactivation.

Materials and methods

HSV-1 infection and explant conditions
Female balb/c mice of 4 to 6 weeks of age were inoculated with 1×10^6 plaque-forming units (pfu) of the F strain of HSV-1 per mouse using a corneal scarification technique, or they were left untreated. The infection proceeded for 30 days and then the mice were sacrificed and the trigeminal ganglia were removed. The ganglia were either removed and frozen immediately in liquid nitrogen, or they were cultured in Dulbecco's modified Eagle medium (DMEM) containing antibiotics and 5% fetal calf serum for 30 min at 37°C with 5% CO_2 to allow viral reactivation to occur. Twelve mice were used for each experimental condition and the TGs were pooled for analysis.

Microarray analysis
Frozen uninfected and infected trigeminal ganglia were submitted to Clontech for RNA isolation, array hybridization, and analysis. The Atlas Mouse 1.2 cDNA microarray from Clontech was used for these experiments. This array contains about 1200 mouse cDNAs that represent many important cellular pathways, including transcriptional regulation, cell signaling, tumor suppression, stress response, apoptosis, and immune responses. Propietary Clontech software (BD AtlasImage) was used to analyze the resulting data. Significantly regulated genes were defined as those above a 2.5-fold difference in expression level.

Quantitative real-time PCR
An ABI7700 machine with Sequence Detection software was used for qPCR analysis (Applied Biosystems). Primer sequences for the cellular genes studied from the microarray are available from Clontech. SYBR green reagents were used to test for double-stranded DNA products resulting from the PCR reactions and dissociation curve analysis tested the specificity of these PCR products. The following PCR cycling conditions were used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 1 min, 68°C for 2 min, and 68°C for 10 min. For dissociation curve analysis the following conditions were used: 95°C for 15 s, 68°C for 20 s, and 95°C for 15 s with the maximum ramping time at this last stage. All data were analyzed using the comparative C_T method of quantitation as described (Applied Biosystems). Each sample was done in triplicate so standard deviations are presented.

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