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Inhibition of the stress-activated kinase, p38, does not affect the virus transcriptional program of herpes simplex virus type 1

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Abstract

To investigate the impact of stress kinase p38 activation on HSV-1 transcription, we performed a global transcript profile analysis of viral mRNA using an oligonucleotide-based DNA microarray. RNA was isolated from Vero cells infected with the KOS strain of HSV-1 in the presence or absence of SB203580, a pyridinyl imidazole inhibitor of p38. Under conditions that eliminated ATF2 activation but had no effect on c-*Jun*, and reduced virus yield by 85–90%, no effect on accumulation of viral IE, DE, or L transcripts was observed by array analysis or selected Northern blot analysis at 2, 4, and 6 h post infection. Results of array data from cells infected with the ICP27 mutant d27-1 in the presence or absence of SB203580 only reflected the known restricted transcription phenotype of the ICP27 mutant. This result is consistent with a role for p38 activation on virus replication lying downstream of the essential role of ICP27 in DE and perhaps late transcription regulation. No effect of SB203580 on transcription was detected after infection with the ICP0 mutant 7134, at 0.5 or 5.0 PFU/cell, though decreases in the rate of accumulation of all kinetic classes of mRNA could be detected, relative to *wt* virus. These results indicate that inhibiting p38 activity in Vero cells, while significantly reducing *wt* virus yield, demonstrated no obvious impact on the program of viral transcription.

Keywords: HSV-1; p38; JNK; ICP27; ICP0; DNA microarray

Introduction

The well-characterized time-dependent transcriptional program of HSV-1 is regulated by viral and cellular factors (Davido and Leib, 1998; Kim and DeLuca, 2002; Millhouse et al., 1998; Pande et al., 1998; Soares et al., 1996; Weir, 2001; Wysocka and Herr, 2003). The virion-associated factor VP16, in association with two cellular factors Oct1 and HCF, promotes expression of the immediate-early (IE) mRNAs encoding ICP0, 4, 27, 22 and 47. Copies of the *cis*-acting TAATGARAT sequence which mediates VP16/Oct1/HCF binding and IE expression, are present multiple times in each IE gene promoter. Accumulation of ICP4 and ICP27 activates delayed-early gene expression, followed by initiation of DNA replication and late (L) gene expression. The L genes can be further sub-divided into those whose expression is either increased by (γ1 genes), or absolutely dependent (γ2 genes) on DNA replication.

Despite these virus-encoded processes, which can circumvent general cellular transcriptional repression, the replica-

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tion and transcription program of HSV-1 is also strongly subject to the cellular transcriptional environment. The most important biological manifestation of this cell dependence is, of course, latency and stress-induced reactivation in sensory neurons [see (Wagner and Bloom, 1997) for review], (Frazier et al., 1996; Millhouse et al., 1998; Soares et al., 1996). Other evidence for potential dependence of HSV replication on cellular events can be seen in the multiplicity and cell cycle dependence for replication of mutants lacking the immediate-early ICP0 gene (Cai and Schaffer, 1991; Everett et al., 2004; Ralph et al., 1994; Stow and Stow, 1986), and the ability of cellular stress agents to initiate the transcriptional cascade by induction of immediate early gene expression in the absence of αTIF-induced activation [(Yang et al., 2002) and references therein].

Metazoan genomes encode a large family of protein kinases (collectively called the mitogen-activated protein kinases, or MAPKs) that integrate external cues, and propagate intracellular signals that trigger a transcriptional response (Johnson and Lapadat, 2002). Growth promoting, growth inhibiting, differentiating, and death inducing factors, as well as stress-inducers such as ultraviolet light, changes in osmotic pressure, and viral infection can activate one or more of these signal transduction protein kinase pathways. For example, the canonical growth-promoting pathway is triggered by growth factor-receptor interaction at the cell surface, intracellular activation of the small receptor-associated GTP-binding protein RAS, followed by sequential activation of the serine-threonine MAPK kinase kinase (MAPKKK) RAF, the dual-specificity threonine/tyrosine MAPK kinase (MAPKK) MKK1 and the MAPKs ERK1/2. Substrates of ERK include nuclear transcription factors ELK, MYC, and cFos, as well as the cytoplasmic kinases Mnk1/2. While the nature of stress signal receptors and initiating kinases is less well understood, one such pathway involves the MAPKKKs MEKK1 or MEKK4 (also known as mixed-lineage kinases, MLKs), the MAPKK MKK4, and the stress-activated kinase (SAPK) p38 (Gallo and Johnson, 2002). This serine/threonine kinase is reported to directly phosphorylate the transcription factor ATF2, and indirectly CREB and STAT1, the latter two through activation of two downstream kinases, Msk1 and MAPKAPK-2 (Deak et al., 1998; Goh et al., 1999; Li et al., 2004; Ramsauer et al., 2002; Rolli et al., 1999; Wiggin et al., 2002).

Two SAPKs, p38 and Jun N-terminal kinase (JNK), are activated following HSV-1 infection [(McLean and Bachenheimer, 1999; Zachos et al., 1999), and Hargett, McLean and Bachenheimer, manuscript in preparation], the latter mediated by MKK4 (Zachos et al., 1999). Activation of both SAPKs is dependent on immediate-early (IE) viral gene expression (McLean and Bachenheimer, 1999), and in particular on functional ICP27 (Hargett, McLean and Bachenheimer, MS in preparation). Activation of both p38 and JNK contributes to the over-all efficiency of virus replication (McLean and Bachenheimer, 1999; Zachos et al., 2001).

While cognate binding sites for additional cellular transcription factors, including CBP, HiNF-P, NF-κB, AP1, and Sp1 can be identified in lytic viral gene promoters (Deb et al., 1994; Pande et al., 1998; Rong et al., 1992), our understanding of how these sites contribute to the pattern of IE, DE and L gene expression is incomplete. One study of the effect of deleting a portion of the ICP0 promoter containing several cellular transcription factor binding sites indicated that while the level of ICP0 expression and in vivo virulence of the resulting virus were decreased significantly, measures of latency establishment and reactivation were unaffected (Davido and Leib, 1996). Progress has been made in defining promoter modules of delayed-early promoters (Imbalzano and DeLuca, 1992; Imbalzano et al., 1991; Pande et al., 1998). In each case studied in detail, an essential downstream module, containing at a minimum the TATA box, and an essential or augmenting upstream module, consisting of one or more Sp1 sites, participate in activated expression of the gene. In the case of the TK gene, regulation of both temporal and activated expression may be governed by the phosphorylation state of Sp1 (Kim and DeLuca, 2002).

Transcription factor AP-1 activity increases following HSV infection (Jang et al., 1991). Consisting most commonly as homodimers of Jun family members, or heterodimers of Jun and Fos family members, AP-1 binds the TPA-responsive element (TRE) TGACTCA. An activation target of JNK, c-*Jun*, can also heterodimerize with CREB family members such as ATF2, and these complexes bind the related cyclic AMP-responsive element (CRE) TGACGTCA. Reporter genes regulated by either AP1 or CRE sites are activated following HSV infection (McLean and Bachenheimer, 1999). The promoter of the UL9 gene contains a CRE site and was reported to be responsive to addition of dibutyryl cyclic AMP in a transient reporter assay (Deb et al., 1994).

One model explaining the contribution of SAPK activation to HSV-1 replication predicts that p38 and JNK affect directly or indirectly the viral transcriptional program because of their ability to activate cellular transcription factors ATF-2 and c-*Jun*, respectively. These considerations prompted us to initially ask whether inhibition of p38 function affected the transcriptional program of HSV. To test this hypothesis, we compared the kinetics of accumulation of viral IE, DE, and L transcripts in the presence or absence of SB203580, a pyridinyl imidazole drug that specifically inhibits p38 activity (Cuenda et al., 1995; Young et al., 1997).

Results

SB203580 selectively inhibits activation of the p38

The pyridinyl imidazole compound SB203580 (SB) selectively inhibits the ability of p38 to phosphorylate,

and thus activate one of its substrates, ATF2. It is reported to be a selective inhibitor of p38 function by competitively inhibiting ATP binding in the catalytic pocket (Young et al., 1997). Activation of the transcription factor ATF2 can occur through phosphorylation by either p38 or JNK (Gupta et al., 1995; Livingstone et al., 1995; van Dam et al., 1995). Phosphorylation of p38 and JNK by their upstream MAPKKs, as well as activation of ATF2 and c-*Jun* can be monitored with phospho-specific antibodies. Because we wished to use SB to selectively inhibit p38 function and thus prevent activation of ATF2, we compared the phosphorylation status of ATF2, a p38 substrate, with phosphorylation of the JNK substrate, c-*Jun*. Lysates prepared from cells treated either with SB, or the diluent DMSO, and then mock infected, or infected with *wt* KOS or the ICP27 mutant d27-1 were fractionated by SDS-PAGE, Western blotted and probed for total and phosphorylated ATF2 and c-*Jun* (Fig. 1). Relative changes in the amounts of proteins were quantified by ImageJ analysis of autoradiograms as described in Materials and methods. Under our standard conditions of mock infection, low levels of activated p38 (pp38, lane 1) and JNK (pJNK, lane 7) were detectable in Vero cells. SB treatment resulted in a 43% increase in the level of phosphorylated p38 (pp38) when normalized to total p38, but as expected the amount of activated ATF2 (pATF2) was reduced more than 88% in the presence of the p38 inhibitor (pATF2, lanes 1 and 2). The drug had no effect on levels of phosphorylated JNK (pJNK) or activated c-*Jun* (pc-Jun, lanes 7 and 8) in mock-infected cells. Following infection with *wt* HSV, activated p38 and JNK, when normalized to total p38 and JNK, increased both in the absence (150% and 118%, respectively) and presence (59% and 56%, respectively) of SB (lanes 3, 4, 9, and 10), relative to the mock-infected control samples. We detected a 100% increase in activated ATF2 after wt HSV infection compared to mock infection (lanes 1 and 3) including at least one form with reduced mobility relative to the majority of total ATF2. Importantly, when normalized to total ATF2, the amount of activated ATF2 was substantially reduced when *wt* infection was performed in the presence of SB (49%, lanes 3 and 4). In contrast the drug had only a slight effect on the level of activated c-*Jun* (5%, lanes 9 and 10), when normalized to total c-*Jun*. We have determined that expression of the IE viral protein ICP27 is necessary for activation of both p38 and JNK (Hargett and Bachenheimer, manuscript in preparation). As expected, infection with the ICP27 mutant d27-1 failed to activate either ATF2 (lanes 5 and 6) or c-*Jun* (lanes 11 and 12) over levels seen in mock-infected control samples. These results indicate that treatment of cells with SB203580 resulted in the expected inhibition of ATF2 activation, with little detectable effect on JNK function as indicated by c-*Jun* activation.

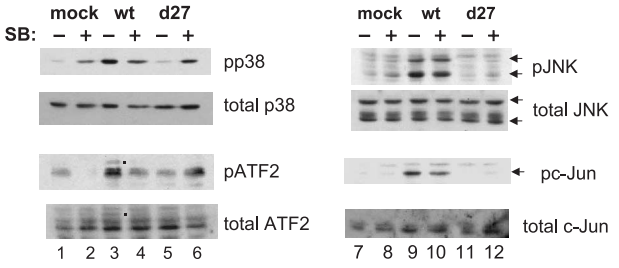

Fig. 1. Effect of p38 inhibitor SB203580 on transcription factor targets of p38 and JNK. Sets of replicate monolayers of Vero cells were pretreated for 30 min with either SB203580 (10 μM) in DMSO or with an equivalent volume of DMSO, and then either mock infected, or infected with *wt* KOS or d27-1. Incubation with drug or DMSO continued until 8 h pi, when whole cell lysates were prepared, fractionated by SDS-PAGE, transferred to membranes and probed for the indicated proteins by Western blot, as described in Materials and methods. Short arrows point to phosphorylated isoforms of JNK, the corresponding total JNK bands, and phosphorylated c-*Jun* in lanes 9–10; dots indicate phosphorylated ATF2 and the corresponding band in total ATF2 in lane 3. *pp38*: phosphorylated p38; *pATF2*: phosphorylated ATF2; *pJNK*: phosphorylated JNK; *pc-Jun*: phosphorylated c-*Jun*.

Effects of MAPK inhibitors on virus yield in Vero cells

We previously demonstrated a role for JNK activation in promoting efficient virus yield. Over-expression of the JNK scaffold protein JIP-1 forced cytoplasmic retention of JNK, failure to phosphorylate and thus activate nuclear c-*Jun*, and decreased virus yield by 70% (McLean and Bachenheimer, 1999). In three independent experiments, we compared the effect of the p38 inhibitor SB203580 with the vehicle control DMSO (Table 1). In one of the experiments, we also compared the effect of SB203580 with the JNK inhibitor SP100625, and the ERK inhibitor UO126 to determine the impact of activation of additional MAPKs on virus yield in Vero cells. Freeze–thaw lysates were prepared at several times post infection (pi), and virus yield determined by standard plaque assay as described in Materials and methods. The results indicated that both the p38 and JNK inhibitors, but not the ERK inhibitor significantly impacted virus yield. Specifically, virus yield at 24 h was reduced 60% to 86% following SB203580 treatment and 90% following SP100625 treatment. The impact of 10 μM SB203580 on virus yield in Vero cells was greater than that reported for the drug used at 30 μM in BHK cells and HeLa cells (Zachos et al., 2001), suggesting cell type-specific effects of p38 inhibition on virus replication. The presence of the ERK inhibitor, UO126 resulted in significant increases in virus yield compared to the DMSO control at both 8 and 16 h, pi. The decreased yield at 24 h may reflect a decrease in the activity of the drug by this time post addition. At earlier times, the ability of UO126 to affect virus yield was consistent with results [(McLean and Bachenheimer, 1999), Hargett, McLean and Bachenheimer, unpublished] indicating that the MAPK pathway leading to ERK activation is inhibited following HSV-1 infection. Together, these results suggest that inhibition of the ERK pathway is important for efficient virus replication, in contrast to our results with the JNK and p38 pathways, indicating that their activation contributes to efficient virus replication.

Table 1
Virus yield following treatment with MAPK inhibitors[a]

Experiment #1[b] MOI = 5				Experiment #2[b] MOI = 5	
SB203580	h, pi	pfu/culture	% reduction	pfu/culture	% reduction
–	8	$1.22 \times 10^5 \pm 0.39$	–	$6.22 \times 10^6 \pm 0.54$	–
+	8	$1.02 \times 10^5 \pm 0.58$	16	$3.63 \times 10^6 \pm 1.2$	41
–	16	$2.13 \times 10^6 \pm 0.64$	–	$7.70 \times 10^7 \pm 1.3$	–
+	16	$1.72 \times 10^6 \pm 0.56$	19	$3.00 \times 10^7 \pm 1.0$	61
–	24	$5.87 \times 10^6 \pm 0.54$	–	$6.95 \times 10^7 \pm 0.56$	–
+	24	$2.15 \times 10^6 \pm 0.82$	63	$2.78 \times 10^7 \pm 0.67$	60

Experiment #3[c]		MOI = 5	
SB203580	h, pi	pfu/culture	% reduction/(increase)
–[d]	4	$4.22 \times 10^5 \pm 0.47$	–
+	4	$3.8 \times 10^5 \pm 1.2$	10
–	8	$1.10 \times 10^7 \pm 0.16$	–
+	8	$3.93 \times 10^6 \pm 1.9$	64
–	16	$3.58 \times 10^8 \pm 0.24$	–
+	16	$4.96 \times 10^8 \pm 1.4$	(27)
–	24	$9.00 \times 10^8 \pm 0.03$	–
+	24	$1.22 \times 10^8 \pm 0.21$	86
UO126			
+	4	$3.87 \times 10^5 \pm 0.39$	8
+	8	$3.19 \times 10^7 \pm 1.5$	(190)
+	16	$1.3 \times 10^9 \pm {}^{0}.16$	(263)
+	24	$5.93 \times 10^8 \pm 1.1$	34
SP100625			
+	4	$2.77 \times 10^5 \pm 1.4$	34
+	8	$2.02 \times 10^6 \pm 0.42$	81
+	16	$1.54 \times 10^8 \pm 0.16$	56
+	24	$7.82 \times 10^7 \pm 3.0$	91

[a] Replicate cultures of Vero cells were pretreated with DMSO or MAPK inhibitors SB203580, SP600125, or UO126 for 30' before infection with HSV-1 KOS. Incubation in DMSO or drug was continued for the indicated times when medium and cells were harvested and lysates prepared. Titer of infectious virus was determined on Vero cells. The % increase or decrease was calculated by dividing the difference between control and drug treated values by the control value ×100. Plaque assays were performed as described in Materials and methods.
[b] Values derived from the average of duplicate cultures.
[c] Values derived from the average of triplicate cultures.
[d] Virus yields at various times post infection in the presence of DMSO were used to calculate % reduction or increase in virus yield for all three drugs.

Effect of p38 inhibitor SB203580 on wt HSV-1 transcription

To assess the impact of inhibition of p38 SAPK on the transcriptional program of HSV-1, we performed a global analysis of viral mRNA with an oligonucleotide-based DNA microarray (Stingley et al., 2000; Wagner et al., 2002; Yang et al., 2002). To succinctly present the large quantity of data generated by such analyses, we have presented it graphically showing only those probes that are diagnostic for a single transcript or family of transcripts that are kinetically the same. A tabular compilation of each group of experimental data is available at the following URL: http://mendel.gti.ed.ac.uk:8080/GPX/cgi-bin/gpx.cgi. We used these values to carry out statistical analyses (t test). This procedure has been described in earlier papers (Stingley et al., 2000; Wagner et al., 2002; Yang et al., 2002).

To verify adequate sensitivity for detection of changes in transcript abundance, array analyses were performed with RNA prepared at 2 and 6 h pi after low (0.5 pfu/cell) and high (5 pfu/cell) multiplicity infections with the ICP0-null mutant, 7134; these data are shown in Fig. 2. At 2 h pi following high MOI infection (panel A) only IE transcripts for ICP27, ICP4, ICP22, and ICP47 accumulated to high levels; this differs from a *wt* infection such as shown in Fig. 3 notably in the decreased accumulation of DE and L transcripts relative to levels of IE transcripts. Indeed, at this time following infection, such a delayed onset of DE and L transcripts is reminiscent of that seen in infections with mutants lacking the VP16 activation domain at 2 h after infection (Yang et al., 2002). While protein lysates prepared from 7134-infected cells did not contain any ICP0 based on Western blot (data not shown), we did see a low hybridization signal with the ICP0 probe. The 7134 mutant contains the lacZ gene in place of the ICP0 coding sequences, but retains the sequences of both 5′ and 3′ UTRs (Cai and Schaffer, 1989). The ICP0 oligos

in the array detect 3′UTR sequences (Stingley et al., 2000). Relative accumulation of IE, DE and L transcripts at 6 h pi (Fig. 2, panel B) more closely resembled the pattern seen at 4 h rather than 6 h pi with *wt* virus (Figs. 3C, D). This is consistent with a delay in the kinetics of the viral transcription program with this mutant even at MOI = 5. Analysis of 2 h and 6 h mRNA from cells infected with ICP0 mutant 7134 at a MOI = 0.5 (Figs. 2C, D), indicated a severely reduced accumulation of all transcript classes even at 6 h.

As shown in Fig. 3, and in contrast to these results, viral transcript accumulation at 2, 4, and 6 h pi following infection with *wt* KOS demonstrated nearly identical patterns of accumulation of IE, DE, and L transcripts whether infections were performed in the presence (panels A, C, E) or absence (panels B, D, F) of SB203580. Numerical data for selected diagnostic transcripts are tabulated both as normalized abundance levels and as relative proportions of total transcript signal at the times under study, and are shown in Table 2. Under these conditions of infection, significant accumulation of most DE and a few selected L transcripts was observed even at 2 h, and the abundance of transcripts from all classes increased by 4 h. At 6 h pi, while the relative abundance of IE and most DE transcripts declined, there were marginal increases in the relative proportion of selected L transcripts,

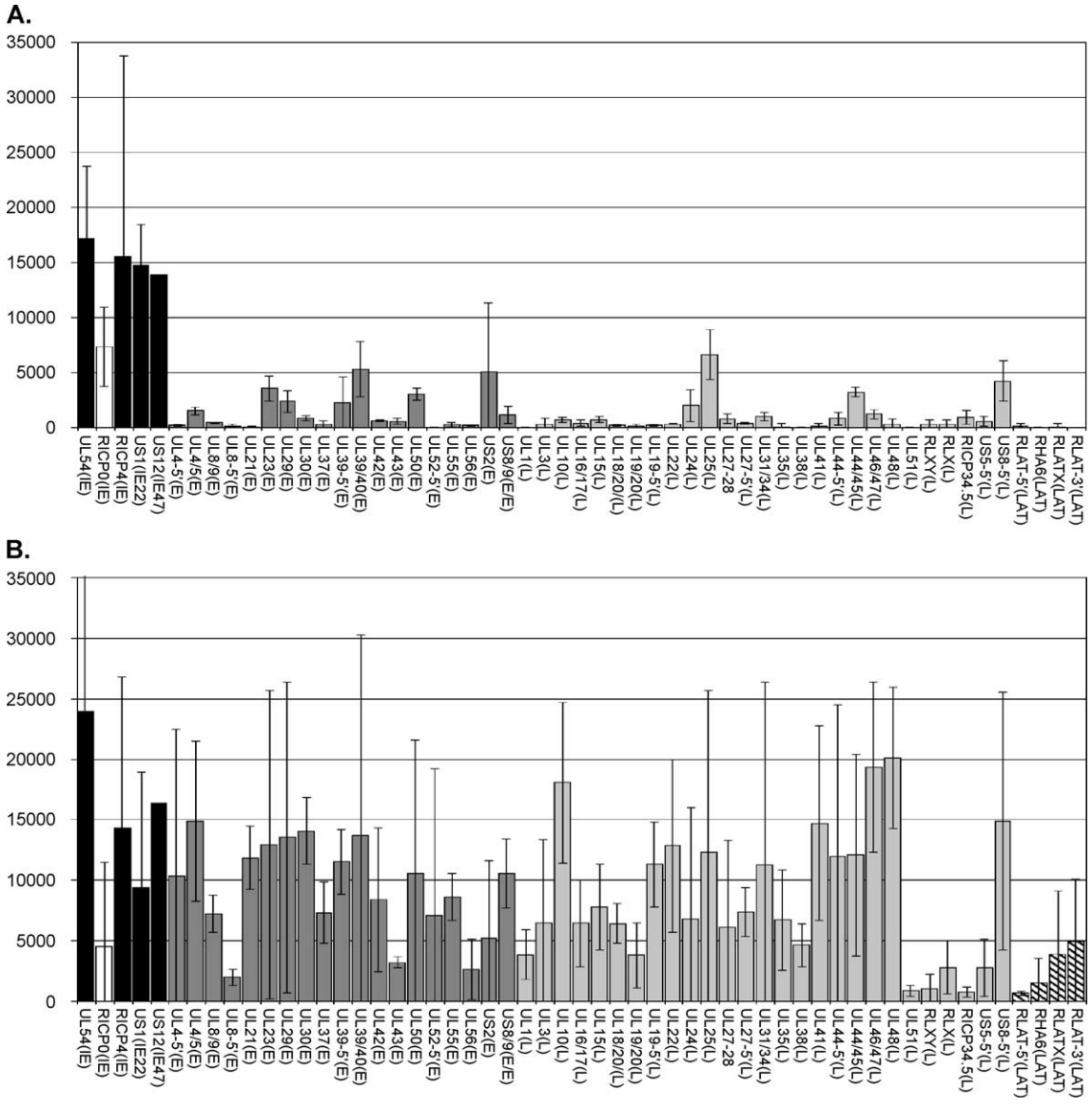

Fig. 2. Transcript accumulation in ICP0 mutant 7134 infected cells. Vero cells were infected with 0.5 or 5 pfu per cell with the ICP0 mutant 7134. RNA was isolated at 2 or 6 h pi, and oligo-dT primed cDNA was prepared and hybridized to an oligonucleotide-based DNA microarray as described in the text and Stingley et al., 2000). *X*-axis: oligonucleotides representing HSV transcripts arranged by kinetic class (black—IE; dark gray—DE; light gray—L; hatched—LAT region of the RL). The bar representing signal from the ICP0 (RICP0) oligo is unfilled. *Y*-axis: signal intensity in arbitrary units. (A and B): infection with 5 pfu/cell 7134; (C and D): infection with 0.5 pfu/cell 7134. Panels A and C: RNA isolated at 2 h pi; panels B and D: RNA isolated at 6 h pi.

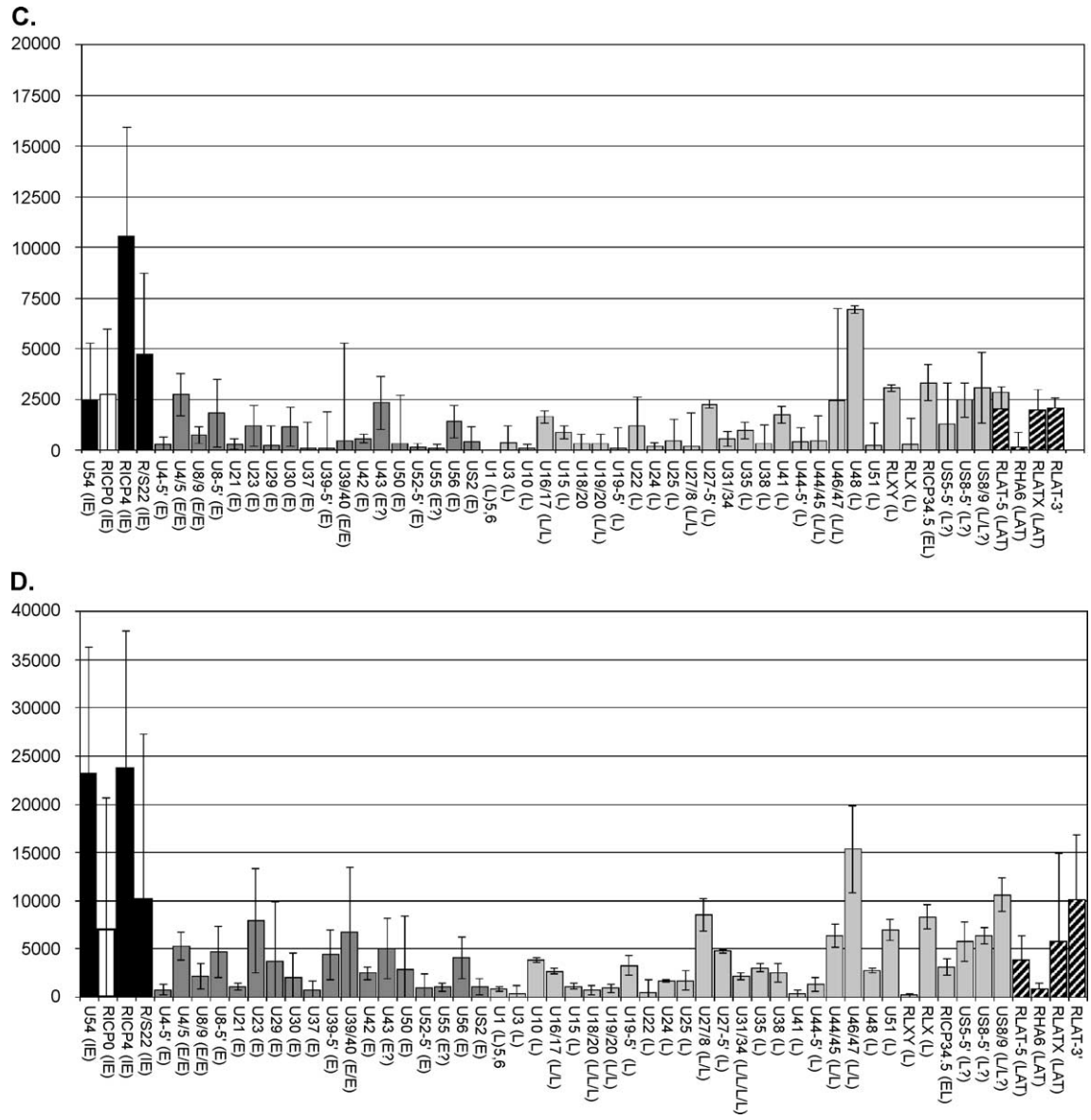

Fig. 2 (*continued*).

for example, UL6 (portal), UL22 (gH), UL19 (VP5), UL35 (hexon tips), UL44 (gC), and UL48 (VP16)—such changes are entirely consistent with our global analyses of the HSV transcription program (for example, see http://darwin.bio.uci.edu/~faculty/wagner/hsv9fnew.html).

At both 2 and 4 h after infection with *wt* KOS, none of the individual transcript groups showed any significant difference ($P < 0.05$) when the drug and non-drug treatments were compared, and there was no significant difference in the total signals. At 6 h, the relatively small difference between the sum of the median-relative signals for all viral transcripts from drug and non-drug treatment samples (460 k vs. 414 k) was measurably significant by ANOVA ($P = 0.025$), but this was not manifest in significant differences in any major hybridization signal. Three probes specific for regions expressed in the LAT primary transcript (LAT-5′, RHA6, and orfOP) showed essentially 2-fold higher total hybridization signals in the drug treated vs. untreated controls, which was statistically significant, but there was no corresponding difference seen with the LATA and LATX probes which measure the transcript level near the 3′ end.

The lack of effect of drug treatment on transcript accumulation was verified by Northern blot analysis for selected IE and DE viral transcripts (Fig. 4). Consistent with the array results in Fig. 3, and the kinetic and metabolic properties of IE and DE mRNAs, ICP0 and ICP27 transcripts were detectable at 2 h pi, while transcripts for the DE essential replication proteins ICP8, UL5, UL42, and UL30 were detectable initially at 4 h pi, and their relative abundance was insensitive to the presence of either the p38 inhibitor SB203580, or the viral polymerase inhibitor PAA.

Effect of SB203580 on ICP27 mutant d27-1 transcription

To confirm that p38 had no direct role in the transcriptional program of HSV, we analyzed mRNA accumulation at 6 h pi with the ICP27-null mutant d27-1 (Fig. 5). As expected, global transcript analysis revealed significant amounts of IE transcripts, with the exception of UL54, the ICP27 gene, and the DE transcripts from UL1 (gL), UL23 (TK), UL27 (gB), UL29 (ICP8), UL39/40 (RR1/RR2), UL50 (dUTPase), US2, and US8/9 genes (panel B). This was in accord with a previous analysis of transcript accumulation in the absence of ICP27 (Stingley et al., 2000). When infection was performed in the presence of the p38 inhibitor SB203580, we observed no overall change in the transcript profile, except for apparent increases in transcripts encoding IE ICP0 (RICP0) and DE ribonucleotide reductase large subunit (RR1/ICP6, UL39) (panel A). The basis for this is currently unknown, but subsequent Western blot analysis for protein accumulation under identical conditions of infection, revealed no change in the level of ICP0 in the presence of SB203580, compared to infection in the presence of DMSO (data not shown).

Accumulation of transcripts for the γ1UL46/γ2UL47 (VP11/12 and VP13/14 VP16-associated proteins) L genes may be explained by the fact that UL46 is a relatively abundant transcript, which is clearly present by 2 h after

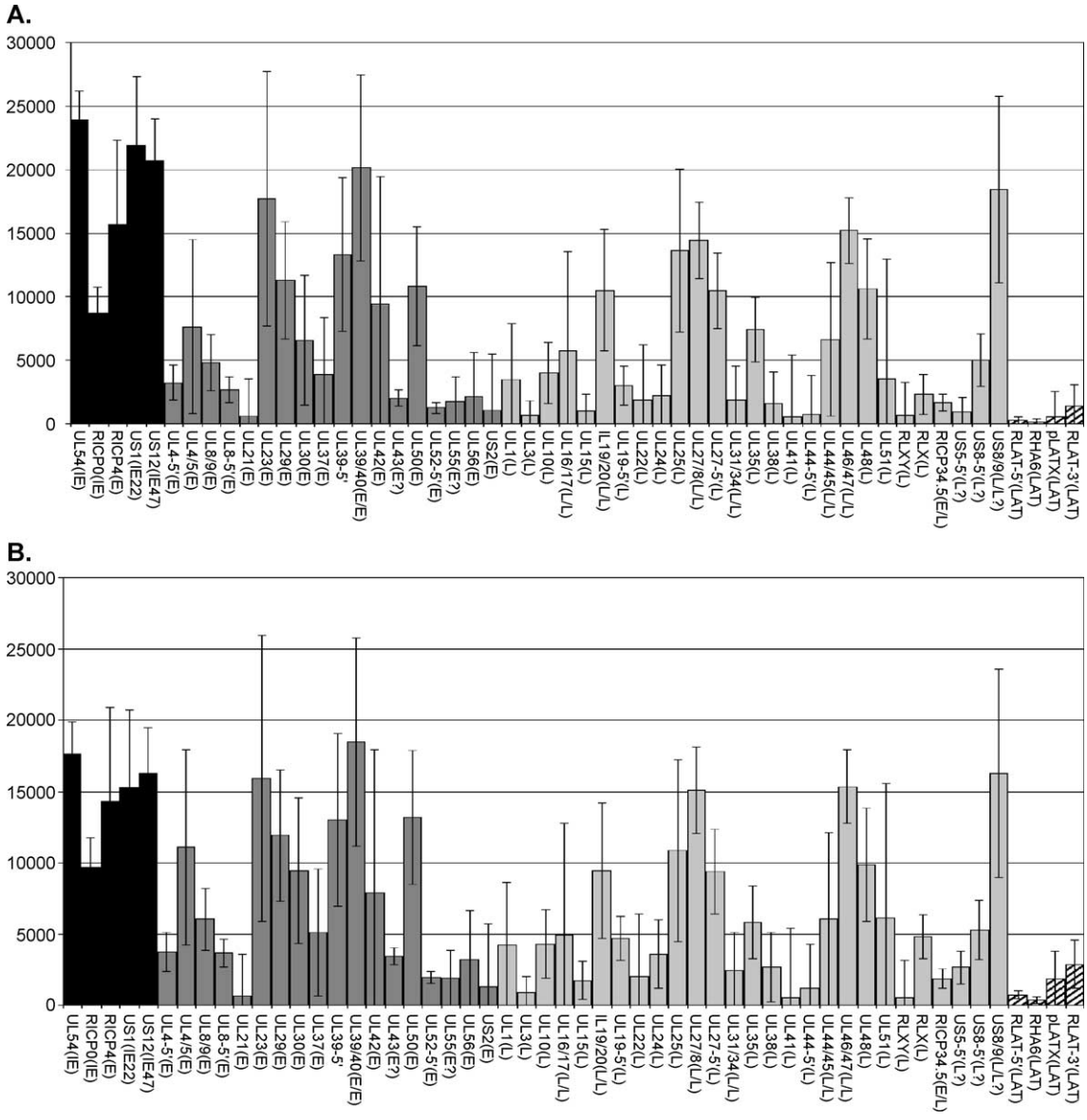

Fig. 3. Effect of p38 inhibitor SB203580 on *wt* HSV-1 transcription. Vero cells were infected in the presence or absence of the p38 inhibitor SB203580 with *wt* HSV-1. RNA was isolated and oligo-dT primed cDNA was prepared and hybridized to an oligonucleotide-based DNA microarray as described in the text and Stingley et al., 2000). *X*-axis: oligonucleotides representing HSV transcripts arranged by kinetic class (black—IE; dark gray—DE; light gray—L; hatched—LAT region of the RL). *Y*-axis: signal intensity in arbitrary units. Panels A, C, and E: infections in the presence of SB203580; panels B, D, and F, infections in the presence of DMSO. (A and B): 2 h infection; (C and D) 4 h infection; (E and F): 6 h infection.

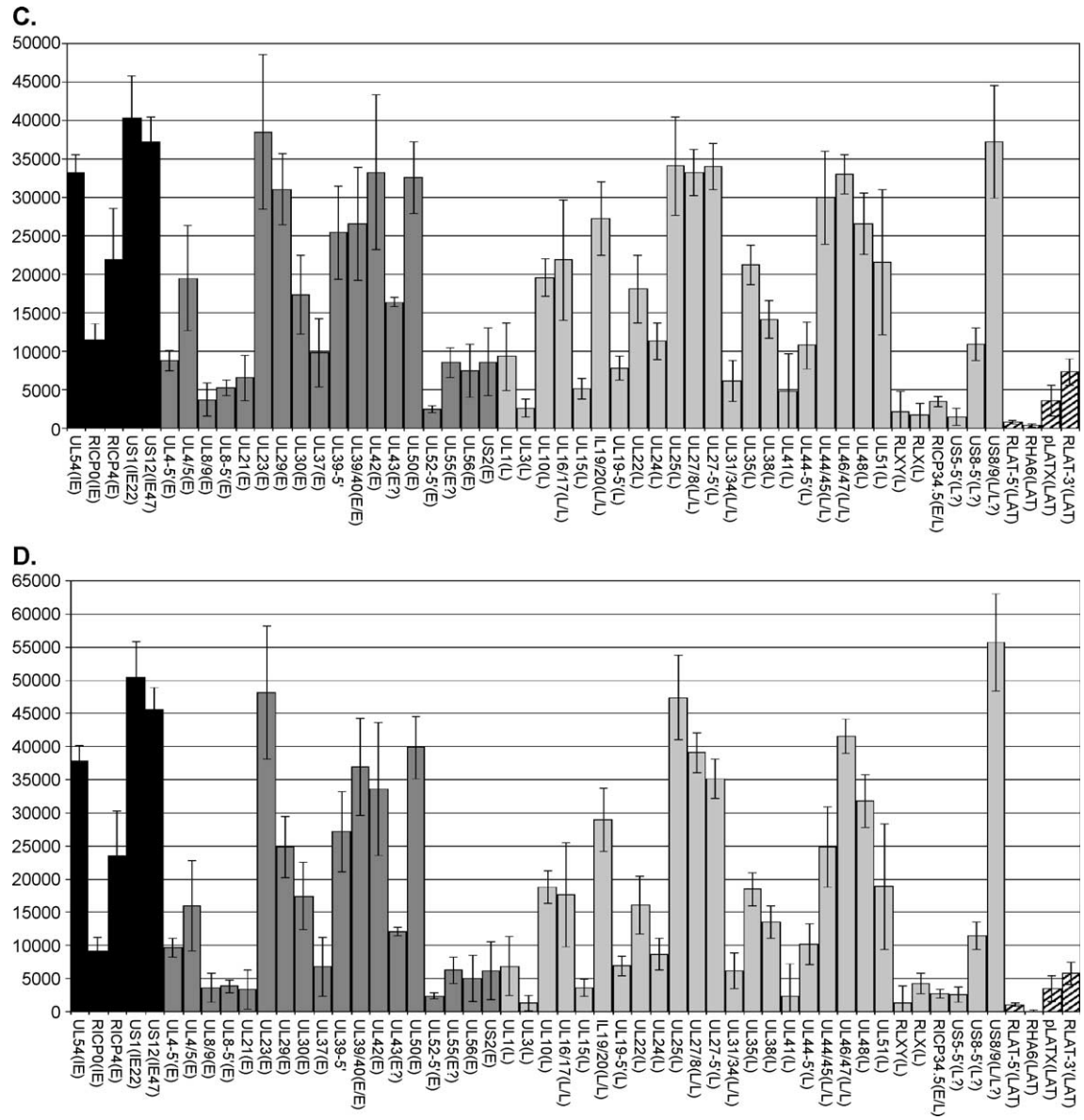

Fig. 3 (*continued*).

infection, and that leaky late gene expression, albeit at a low level, occurs in the absence of ICP27. The remaining pattern of transcript accumulation was otherwise unchanged from the DMSO control pattern.

Discussion

The finding that HSV-1 infection activates the p38 and JNK signal transduction pathways, prompted us to investigate the role of these SAPKs in transcription regulation. These kinases are reported to phosphorylate, and thus activate several transcription factors, most prominently ATF2, CREB by p38, and c-*Jun* by JNK. In this report, we focused on p38 and the effect of the p38 inhibitor, SB203580 to affect the transcriptional program of HSV-1. Using a global gene array approach, we have demonstrated that inhibition of p38 function by this inhibitor did not quantitatively or kinetically affect the IE, DE, and L viral transcript accumulation.

In a study directed at the role of p38 activation in the evasion of the apoptotic response to HSV infection (Zachos et al., 2001), the impact of SB203580 on the accumulation of several viral proteins was analyzed by Western blot. Comparison of drug-treated and control samples harvested at 6, 9, and 12 h pi, indicated a consistent 30 $\pm$ 10% decrease in ICP0 protein in the presence of the p38 inhibitor, but no difference in the levels of ICP4. At 9 h pi, a decrease in ICP27, but not the DE protein UL42 was observed, and decreases in DE UL40 (ribonucleotide reductase small subunit/RR2) and L UL44 (gC), but not in IE/E UL39 (ribonucleotide reductase large subunit/RR1) were observed, though the time point in these latter cases was not specified.

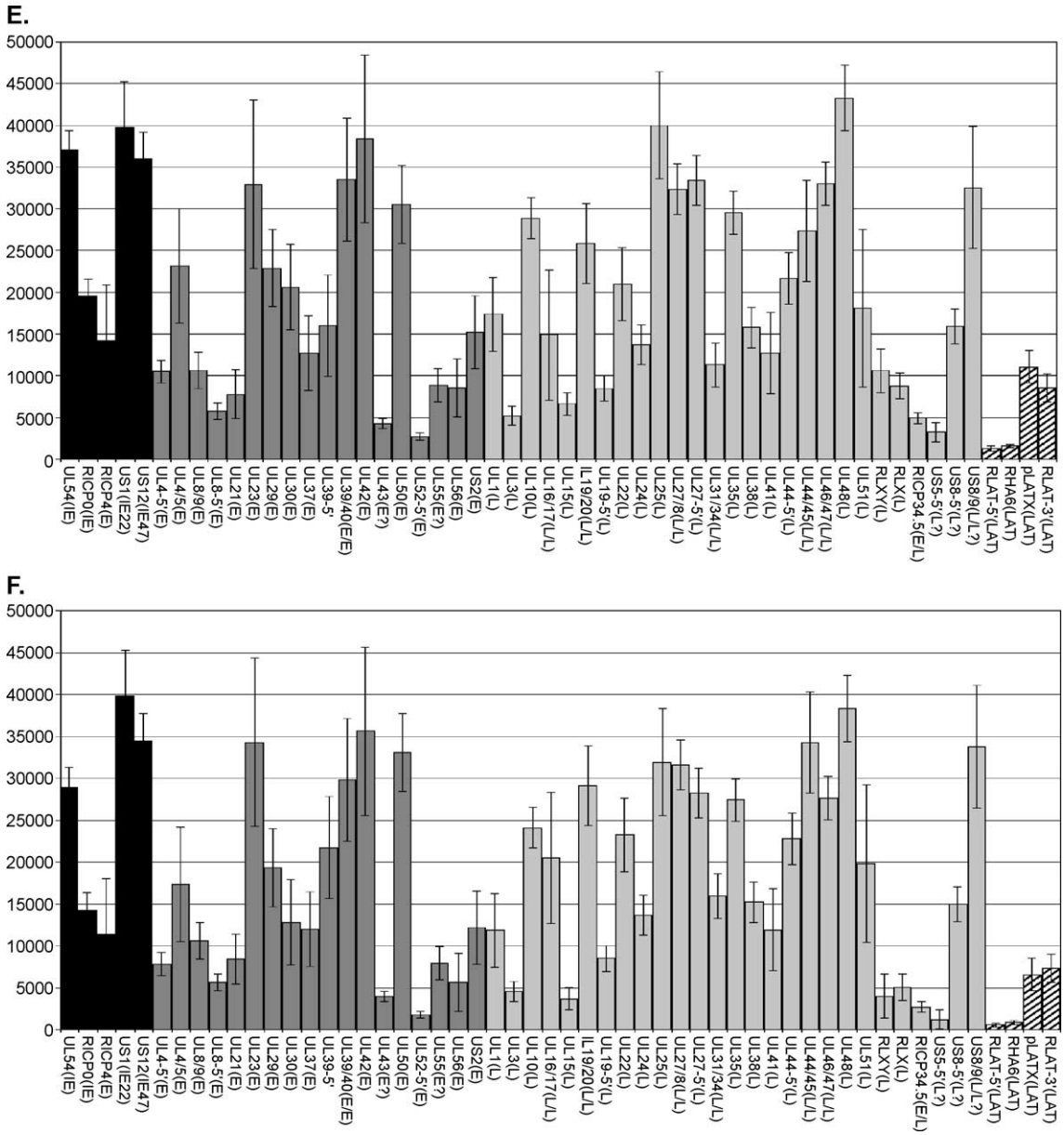

Fig. 3 (*continued*).

Inspection of these genes in our array data [URL:http://mendel.gti.ed.ac.uk:8080/GPX/cgi-bin/gpx.cgi] indicate no statistical difference between drug treated and control RNA samples at 2, 4, and 6 h pi. Obvious differences between these two analyses, besides assays employed for gene expression, are the cell types (Vero vs. HeLa and BHK) and drug doses (10 vs. 30 μg/ml).

While the present experiments were being performed we determined that expression of functional ICP27 in the context of virus infection was sufficient for p38 activation (Hargett, McLean and Bachenheimer, manuscript in preparation). Comparison of transcript accumulation during *wt* KOS virus infection in the presence of SB203580, with accumulation during d27 mutant virus infection indicated that requirements for ICP27 in transcription regulation must lay upstream of any effect of the drug on the viral replication cycle: while the transcription program of *wt* virus was unaffected, accumulation of viral transcripts in the absence of ICP27 was restricted to the IE and DE classes. Early roles of ICP27 have been noted for polyadenylation site selection (Ellison et al., 2000; Hann et al., 1998; McGregor et al., 1996; Sandri-Goldin and Mendoza, 1992), and transcription of genes encoding viral DNA replication proteins (Uprichard and Knipe, 1996). Late functions of ICP27 include reorganization of splicing factors (Lindberg and Kreivi, 2002; Sandri-Goldin et al., 1995), nuclear-cytoplasmic shuttling

Table 2
Normalized and relative abundance of selected HSV-1 transcripts at 2, 4, and 6 h after infection in the presence or absence of the p38 inhibitor SB203580

Probe	Control[a]									SB203520[b]								
	2 h			4 h			6 h			2 h			4 h			6 h		
	med[c]	SD	rel[d]	med[c]	SD	rel[d]	med[c]	SD	rel[d]	med[c]	SD	rel[d]	med[c]	SD	rel[d]	med[c]	SD	rel[d]
UL54 (IE)	17,600[e]	13,400	0.038	37,800	15,600	0.033	29,000	2300	0.026	23,900	7600	0.058	33,200	20,200	0.031	37,100	8600	0.032
RICP0 (IE)	9700	2900	0.021	9100	2800	0.008	14,300	2100	0.013	8700	4200	0.021	11,500	3200	0.011	19,500	4800	0.017
RICP4 (IE)	14,300	8800	0.031	23,600	11,000	0.021	11,400	6600	0.010	15,700	5200	0.038	21,900	4900	0.020	14,200	4500	0.012
US1 (IE22)	15,300	7800	0.033	50,400	10,800	0.044	39,800	5400	0.036	21,900	8300	0.053	40,300	17,700	0.037	39,800	6600	0.034
US12 (IE47)	16,300	8600	0.035	45,600	6800	0.040	34,500	3200	0.031	20,700	7000	0.050	37,200	8300	0.034	36,000	9300	0.031
UL23 (E)	15,900	6200	0.035	48,100	14,100	0.042	34,300	10,000	0.031	17,700	7200	0.043	38,500	20,100	0.036	32,900	5600	0.028
UL29 (E)	11,900	1800	0.026	24,900	25,900	0.022	19,300	4600	0.018	11,300	2600	0.027	31,000	8000	0.029	22,900	5100	0.020
UL30 (E)	9400	1700	0.021	17,400	2200	0.015	12,800	5100	0.012	6600	3000	0.016	17,400	4500	0.016	20,600	6100	0.018
UL39-5'	13,000	9200	0.028	27,100	9100	0.024	21,700	6100	0.020	13,300	5700	0.032	25,400	4900	0.024	16,000	3000	0.014
UL43 (E)	3400	1400	0.008	12,100	18,500	0.011	3900	600	0.004	2000	1400	0.005	16,300	20,600	0.015	4300	1000	0.004
UL50 (E)	13,200	1000	0.029	39,900	10,500	0.035	33,100	4700	0.030	10,800	2100	0.026	32,600	10,600	0.030	30,500	2700	0.026
UL1 (L)	4200	2800	0.009	6900	7800	0.006	11,900	4400	0.011	3500	2100	0.008	9300	3300	0.009	17,400	4400	0.015
UL3 (L)	900	700	0.002	1300	1200	0.001	4600	1200	0.004	700	500	0.002	2600	2400	0.002	5200	1200	0.004
UL6 (L)	4000	2100	0.009	6300	4400	0.006	10,900	2500	0.010	3100	1900	0.007	6500	4700	0.006	11,900	2400	0.010
UL19/20 (L/L)	9400	3200	0.021	29,000	11,300	0.025	29,100	4800	0.027	10,500	2200	0.025	27,200	4600	0.025	25,900	3000	0.022
UL35 (L)	5800	2500	0.013	18,500	8900	0.016	27,400	2600	0.025	7400	2300	0.018	21,200	4000	0.020	29,500	3000	0.025
UL38 (L)	2700	1100	0.006	13,500	7100	0.012	15,200	2400	0.014	1600	1100	0.004	14,100	9800	0.013	15,800	5500	0.013
UL41 (L)	500	1100	0.001	2300	2700	0.002	11,900	4900	0.011	500	500	0.001	4700	2400	0.004	12,700	3100	0.011
UL48(L)	9900	3000	0.021	31,800	30,000	0.028	38,300	4000	0.035	10,600	1100	0.026	26,600	7100	0.025	43,300	7800	0.037
pLATX(LAT)	1800	1100	0.004	3400	1200	0.003	6600	2000	0.006	600	500	0.001	3600	1500	0.003	11,100	3500	0.009
Total signal[f]	459,500			1,137,000			1,096,600			413,600			1,078,700			1,172,000		

[a] Infections were carried out in 0.5 μL/ml DMSO.
[b] See Materials and methods for details.
[c] Median and standard deviation of values of four independent normalized experiments.
[d] Relative level of transcript determined by the ratio of transcript signal to total signal.
[e] Abundance measured in arbitrary florescent units.
[f] Total of signals for all probes.

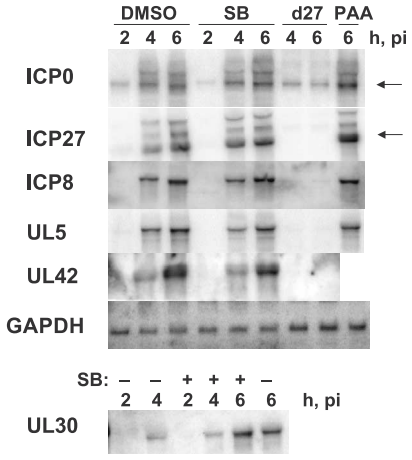

Fig. 4. Northern blot analysis of viral transcripts Vero cells were infected with *wt* HSV-1 KOS in the presence or absence of p38 inhibitor SB203580 or with the viral DNA polymerase inhibitor PAA (400 μg/ml), or infected with the ICP27 mutant d27-1. Cells were harvested at the indicated times and RNA was isolated for Northern blot analysis as described in the text.

and expression of late viral mRNAs (Bryant et al., 2001; Chen et al., 2002; Koffa et al., 2001; Phelan and Clements, 1997; Soliman et al., 1997), and suppression of apoptosis (Aubert and Blaho, 1999; Goodkin et al., 2003). Thus, it seems likely that the role for ICP27-dependent p38 activation is not an adjunct to the role of ICP27 in transcription or post-transcriptional processing. We cannot at this time exclude roles for ICP27-dependent p38 activation in the export or translation of viral mRNA.

The mechanism of two linked aspects of viral gene expression, temporal regulation, and activated expression still lack adequate explanation. An underlying assumption in models of regulation is that cellular transcription factors play an important role in activated expression, given the presence of their cognate binding sites in viral promoters. In the context of virus infection the UL37 promoter contains a downstream HiNF-P site and the UL50 (dUTPase) promoter contains an upstream Sp1 site, both of which are essential for activated expression. In contrast, the upstream Sp1 sites of the DE UL23 (TK) and UL37 promoters only have augmenting roles (Imbalzano and DeLuca, 1992; Imbalzano et al., 1991; Pande et al., 1998). CRE sites, which bind Jun/ATF heterodimers, occur in the intergenic regions upstream of the UL9 and UL54 (ICP27) genes, upstream of the UL4 ORF, and in the middle of the UL15 ORF. None of these sites have been shown to be critical for gene expression in the context of virus infection.

The present study reinforces the notion that activation of a transcription factor following virus infection does not necessarily point to its critical role in activated expression of viral genes. Four possible explanations for the failure to detect a role for p38-dependent transcription factor activation in the context of virus infection can be considered. First, perhaps alternative targets of p38, such as Msk1, MapKap2, and Mnk1/2 kinases (Raught and Gingras, 1999; Roux and Blenis, 2004) and their downstream targets, are relevant for virus replication. Indeed, eIF4e activation in HSV infected cells has been observed [(Walsh and Mohr, 2004), Hargett, McLean and Bachenheimer, unpublished observations]. Second, the importance of activated ATF2 and AP-1 are masked in the tissue cultures models normally used to study virus replication. Third, signal regulated networks have the potential for cross-talk (Gupta et al., 1995; Livingstone et al., 1995; van Dam et al., 1995), and coupled with combinatorial redundancy of regulatory motifs in viral promoters may well exhibit a high degree of robustness even in the presence of a specific SAPK kinase inhibitor. Fourth, the impact of activated p38 targets may be masked by viral encoded factors such as VP16 and ICP0. With regard to this latter possibility, the mutant *in*1820K encoding a mutated VP16, the temperature sensitive allele of ICP4 derived from the tsK mutant, and a substitution mutation in the ICP0 promoter failed to express IE genes. However when infected cells, incubated at 38.5 °C were treated with protein synthesis inhibitors, both ICP0 and ICP27 promoters were activated (Preston et al., 1998). These are conditions that induce JNK and p38 (McLean and Bachenheimer, 1999). Future studies will be directed at addressing the role of stress kinase activation for HSV replication under conditions that take into account these four explanations.

Materials and methods

Cells and virus

Vero cells were propagated in Dulbecco's modified Eagle medium (DMEM-H), supplemented with 5% calf serum, 100 U/ml penicillin, 1% streptomycin, and 1% L-glutamine (all from Gibco).

Virus yield assay

Sub-confluent cultures were pretreated with the p38 inhibitor SB203580 (10 μM), the JNK inhibitor SP100625 (20 μM), the ERK inhibitor UO126 (10 μM), or the vehicle control DMSO (0.5 μl/ml). Drug treatment was continued during infection with HSV-1 strain KOS at MOI = 5, until the time of harvest between 4 and 24 h post-infection (pi). Following three cycles of freezing and thawing, monolayers of Vero cells in 12 well dishes were inoculated in triplicate with serial 10-fold dilutions of the lysates. After 1 h, monolayers were covered with DMEM-H containing 2% CS and 0.3% methylcellulose. After 3 days incubation at 37 °C, medium was aspirated from the wells and plaques stained with 0.8% crystal violet in 50% ethanol.

Global analysis of viral RNA

Replicate monolayers of Vero cells in 150 mm dishes were infected with *wt* KOS at MOI = 5, in the presence or

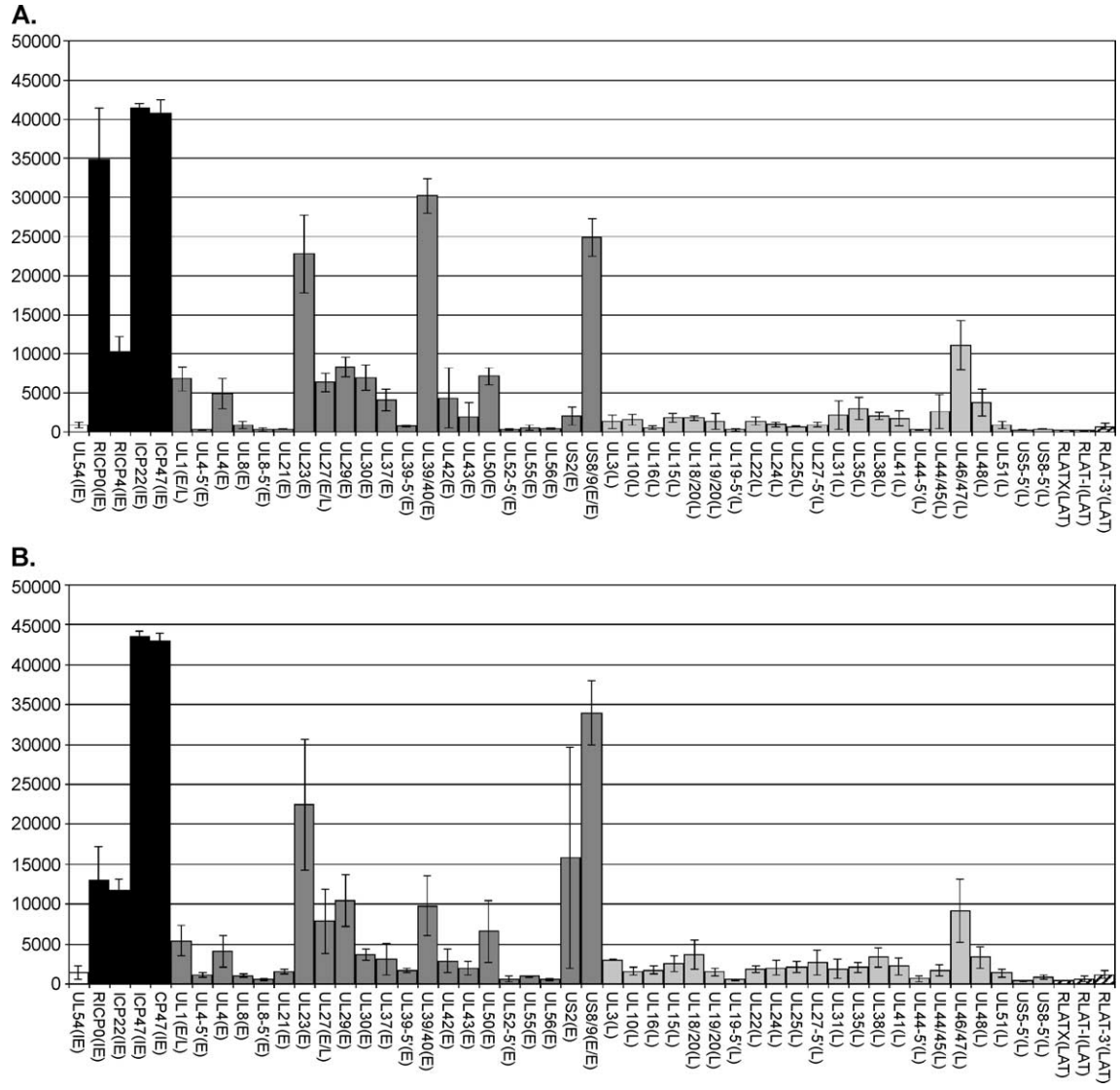

Fig. 5. Transcript profile in ICP27 mutant d27 infected Vero cells. Vero cells were infected in the presence or absence of SB203580 with the ICP27 mutant d27-1. RNA was isolated at 6 h pi, and oligo-dT primed cDNA was prepared and hybridized to an oligonucleotide-based DNA microarray as described in the text and Stingley et al., 2000). *X*-axis: oligonucleotides representing HSV transcripts arranged by kinetic class (black—IE; dark gray—DE; light gray—L; black—LAT region of the R_L). Bar representing signal from the ICP27 (UL54) oligo is unfilled. *Y*-axis: signal intensity in arbitrary units. (A) d27-1 infection in the presence of SB203580; (B) d27-1 infection in the presence of DMSO.

absence of SB203580 (10 μM). RNA was prepared from cells harvested at 2, 4, and 6 h pi under these conditions from three independent (separated in time) sets of infections using Trizol. Isolated RNA was further purified by pelleting through a 5.7 M CsCl cushion by centrifugation for 16 h at 36,000 rpm in a Beckman SW41 rotor.

Fluorescence-labeled cDNA was prepared from 1 μg aliquots of purified poly(A)-containing RNA by random hexamer-primed polymerization using Superscript II reverse transcriptase (Gibco-BRL). The pool of nucleotides in the labeling reaction consisted of 0.5 mM dGTP, dATP, and dTTP and 0.3 mM dCTP and fluorescent nucleotides (Cy3dCTP and Cy5dCTP; Amersham) at 0.1 mM. Fluorescence-labeled DNA was purified by chromatography through Microcon YM-20 columns (Amicon), then heat denatured for 2 min at 100 °C, and incubated 20 to 30 min at 37 °C before use.

Characteristics of the HSV-1 specific oligonucleotide-based DNA chip have described (Wagner et al., 2002). Microarrays were hybridized for 16 h in 5× SSC–0.2% SDS at 68 °C under coverslips with combined Cy5dCTP and Cy3dCTP-labeled cDNA. The entire assembly was enclosed in a commercial hybridization chamber (GeneMachines, San Carlos, CA). After hybridization, the microarray slide assembly was washed for 5 min in 1× SSC–0.2% SDS at room temperature for 5 min, 5 min in 0.1× SSC–0.2% SDS at room temperature, and 1 min in 0.1× SSC, and spun dry in a low-speed centrifuge. Microarrays were scanned using a confocal laser ScanArray 4000 system (General Scanning, Inc.). Data were collected at a maximum resolution of 10 μm/

pixel with 16 bits of depth by using Quantarray software (General Scanning, Inc.).

The scanning process sampled the fluorescence (expressed in arbitrary units) derived from each spotted oligonucleotide. These values were adjusted by subtracting the background fluorescence of an equivalent area within a concentric ring just outside the spotted sample. The data from individual spots were then expressed in a Microsoft Excel spreadsheet, and net values were determined by subtracting the average of values obtained from measuring the fluorescence of a large number (ca. 100) of regions spotted with SSC alone. Typically, and depending on the exact laser power of the scan, this SSC background ranged from 50 to 500 arbitrary units.

Weak fluorescent signals are inherently less reliable than strong ones. Moreover, the ratio of fluorescent signal to actual sample value is linear only to net (−SSC) values of 40,000 or so. To accommodate these limitations, laser power for scanning was adjusted for optimum signal sensitivity to a laser power between 75 and 90 with the photomultiplier set 5 units less than laser power. Standardization studies have shown that increasing laser power by 5 units in the range used increases those signals in the reciprocity range approximately by a factor of 2. Multiple scans at varying laser power settings were used to optimize all signals of interest.

For comparison of data from the various experimental conditions, the net (−SSC) hybridization values were compiled in several steps. First, in each separate experiment (hybridization) and for each transcript set, a median value was calculated from the three replicate probe spots on a given chip. Two completely separate experiments were carried out for each condition used, and each RNA preparation was tagged with c3 and c5 in separate experiments, and these were hybridized individually. Thus, each data point was based on the median for the four separate hybridization values. To compare data from replicate experiments the 75th percentile rank for the total viral hybridization was calculated. One experiment was arbitrarily chosen as the reference and the 75th percentile values of all other determinations were adjusted to this value by appropriate factoring. In this way, chips belonging to each experimental group are scaled accordingly. The relative abundance of each transcript was calculated as the quotient of the final median value for that transcript divided by the sum of the median values for all viral transcripts. These relative abundance values are displayed as percentages. To compare expression levels under different conditions, the sets of median values (or, in some cases, the sets of relative abundance values) from all replicates of the conditions being considered were evaluated using Student's two-tailed *t* test, assuming unequal variance and with the null hypothesis being that the true values under those two conditions are identical. Tabular data is available at http://mendel.gti.ed.ac.uk:8080/GPX/cgi-bin/gpx.cgi.

Northern blot analysis

RNA was isolated from mock and virus infected cells using the RNeasy manufacturer's protocol (Qiagen). RNA (5 μg aliquots) was fractionated on 1.4% formaldehyde agarose gels, transferred to Biotrans nylon membranes (ICN) with 20× SSC and subsequently covalently linked to membranes by UV cross-linking. Plasmids containing coding sequences for UL5 (pCMUL5), UL29/ICP8 (pSV8.3), UL30/pol (pCMUL30), UL42 (pCMUL42), and UL54/ICP27 (pCMUL54) (Uprichard and Knipe, 1996) were linearized with *Hin*dIII, and using specific primer sets, probe sequences were amplified by PCR and then purified using the QIAquick PCR purification manufacturer's protocol (Qiagen). Probe sequences for ICP0 and ICP4 were PCR amplified from *Eco*RI linearized pSG1 (Quinlan and Knipe, 1985), and probe sequences for ICP27 were PCR amplified from a *Bam*/*Sal* fragment derived from pRB112 (Post et al., 1980). Forward (F) and reverse (R) primer sets (5′ to 3′) (Sciortino et al., 2001) were as follows:

ICP0 (F) TGTTTCCCTGCGACCGAGACCT, (R) CACTATCAGGTACACCAGCTTG;
ICP4 (F) GGCGGGAAGTTGTGGACTGG, (R) CAGGTTGTTGCCGTTTATTGCG;
ICP27 (F) TAATTGACCTCGGCCTGGACCT, (R) TGGGCTTTGGTCGGTGGGGGTT;
UL5 (F) TAATGACGTCATCGCGCTCGTG, (R) ACGGGTGTTTGCCTTTGCGGAC;
ICP8 (F) TAGGTACTCGTCCTCCAGGAGC, (R) ATGGTCGTGTTGGGGTTGAGCA;
UL30 (F) GGTGTATTACAAGCTCATGGCC, (R) ACTCTCGGTGATCTTGGCGTTA; and
UL42 (F) GAACCAGCACCACAAAGCTCGT, (R) GGGCGCCGAACTTAATGGAATC.

Labeled probe was generated by subjecting the PCR products to a random priming reaction (Life Technologies) containing α^{32}PdCTP, and separated from unincorporated nucleotides using ProbeQuant G-50 columns (Amersham Pharmacia Biotech). Following prehybridization of filters at 42 °C for 30′ in ULTRAhyb solution (Ambion) containing 20 mg/ml denatured salmon sperm DNA, labeled probe was added, and hybridization continued for 18 h. Blots were then washed in 2× SSC, 0.1% SDS for 10′, and 1×SSC, 0.1% SDS for 5′. Filters were dried and exposed to XAR-5 film (Kodak) at −80 °C with intensifying screens.

Preparation of whole cell lysates and Western blot analysis

At the time of harvest, Vero cells were placed on ice to prevent artifactual induction of stress responses. The medium was removed and the monolayers rinsed with ice-cold Dulbecco's PBS. Cells were scraped directly into 1× SDS sample buffer [3.85 mM Tris-base (pH 6.8), 9.1% β-mercaptoethanol, 1.82% SDS, 4.6% glycerol, and 0.023%

bromophenol blue (in 100% EtOH)] and denatured by boiling. Cell-equivalent amounts of lysate were separated by electrophoresis on 12% SDS-polyacrylamide gels (SDS-PAGE). Proteins were transferred to PolyScreen® PVDF membranes (Perkin-Elmer Life Sciences) followed by blocking in TBST (150 mM NaCl, 20 mM Tris [pH 7.6], 0.05% Tween 20) with 5% milk. All probing and washing of membranes was done in TBST. Antibodies for p38 (9212), phospho-p38 (9211), JNK (9252), phospho-JNK (9251), c-*Jun* (9162), phospho c-*Jun* (9261), ATF-2 (9222), and phospho ATF-2 (9225) were purchased from Cell Signaling Technology and used at a 1:1000 dilution overnight at 4 °C per manufacturer's instructions. Monoclonal antibody for α-tubulin (B-5-1-2, Sigma) was used at a 1:20,000 dilution. Goat anti-rabbit and anti-mouse secondary antibodies were purchased from Amersham Biosciences. The secondary antibody was detected with SuperSignal West Pico Chemiluminescent substrate agent (Pierce). Films were scanned and images stored as 8-bit grayscale JPEG files. The density of each band was determined using Image J (NIH). Relative density values were corrected for average background by subtracting out the density of a blank portion of the film. The corrected values were then used to calculate percent change from mock-infected cell or control cell levels.

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