VIROLOGY 250, 85–93 (1998)
ARTICLE NO. VY989343

Echovirus 1 Infection Induces both Stress- and Growth-Activated Mitogen-Activated Protein Kinase Pathways and Regulates the Transcription of Cellular Immediate-Early Genes

Pasi Huttunen,*,†[1] Timo Hyypiä,‡ Pia Vihinen,*,§ Liisa Nissinen,*,§ and Jyrki Heino*,§

*MediCity Research Laboratory and †Department of Virology and §Department of Medical Biochemistry, University of Turku, Tykistökatu 6A, FIN-20520 Turku, Finland; and ‡Haartman Institute, Department of Virology, P.O.B. 21, FIN-00014 University of Helsinki, Helsinki, Finland

Received March 20, 1998; returned to author for revision April 17, 1998; accepted July 22, 1998

We have previously shown that echovirus 1 (EV1) infection increases the mRNA levels of cellular immediate-early (IE) genes in host cells. Here we provide further evidence that the induction of *junB,* c-*jun,* and c-*fos* genes is due to active viral macromolecular synthesis rather than to the interaction of EV1 with its receptor, $\alpha_2\beta_1$ integrin. Nuclear run-on transcription assays indicated that differences in mRNA levels in infected and uninfected cells are brought about by regulation at the transcriptional level. EV1 infection induced the phosphorylation of both the stress-related p38 mitogen-activated protein kinase (MAPK) and the growth signal-related ERK1/2 MAPKs. Studies with selective MAPK inhibitors revealed that p38 was the main inducer of *junB* expression, whereas both MAPK pathways were involved in the induction of c-*fos.* Activation of AP-1 genes was also observed to occur during infections with other enteroviruses and with Semliki Forest A7(74) virus, suggesting that the phosphorylation of MAPKs and induction of AP-1 gene expression may be important regulators of host cell behavior during viral infections.

INTRODUCTION

Echoviruses (EVs) are the largest subgroup of the enterovirus genus of *Picornaviridae.* They cause various clinical manifestations, ranging from mild respiratory infections to fatal illnesses of newborns (Grist *et al.,* 1978). Although the infection cycle of some enteroviruses has been studied in detail, cellular consequences of infection are largely unknown. We have recently shown that infections caused by EV1, which uses $\alpha_2\beta_1$ integrin as its cellular receptor (Bergelson *et al.,* 1992), and EV7, which uses decay-accelerating factor (DAF) as its receptor (Bergelson *et al.,* 1994; Ward *et al.,* 1994), are able to induce the cellular immediate-early (IE) genes c-*jun, junB,* and c-*fos* in a human osteogenic sarcoma (HOS) cell line (Huttunen *et al.,* 1997).

In addition to EV1, several collagens and laminin-1 are ligands of $\alpha_2\beta_1$ integrin. We have demonstrated that the mechanism of IE gene induction in EV1 infection differs from that related to type I collagen. The $\alpha_2\beta_1$ integrin-mediated cell adhesion to type I collagen is known to increase the expression of IE genes, whereas viral replication, and not integrin–EV1 interaction alone, is required for full-scale induction (Huttunen *et al.,* 1997).

The expression of IE genes is rapidly but transiently induced by many extracellular stimuli, such as growth factors, cytokines, UV irradiation, and some pharmacological compounds. In addition, certain polypeptides of DNA viruses [e.g., the HBx protein of the hepatitis B virus (Benn *et al.,* 1996)], are able to activate IE genes. The products of *jun* and *fos* IE genes are the major components of the transcription factor AP-1, which is assumed to regulate the transcription of many genes involved in cell growth, survival, and differentiation. Depending on the extracellular stimulus, different mitogen-activated protein kinase (MAPK) subtypes, including extracellular signal-regulated protein kinases (ERKs), Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38, are implicated in IE gene induction. The precise quantitative response of these subtypes and individual IE genes appears to be characteristic for each stimulus, although there is considerable overlap in gene induction and signaling responses (Cooper, 1994).

Very little is known about the cellular effects of positive strand RNA viruses, including picornaviruses, although many host cell factors interact with viral macromolecules and elicit cellular cascades and stress responses, which may have an important influence on the pathogenesis of the infection. Here we show that the AP-1 gene induction observed during EV1 infection is due to activation of specific MAPK pathways. Importantly, EV1 infection seems to regulate both growth signal- and stress-activated pathways.

RESULTS

Type I collagen and EV1 share the same cellular receptor but induce IE genes by different mechanisms

Integrin-matrix interaction is known to mediate integrin clustering and regulate IE genes (Schwartz *et al.,* 1995). EV1

[1] To whom reprint requests should be addressed. Fax: +358–2-3337000. E-mail: pasi.huttunen@iki.fi.

0042-6822/98 $25.00

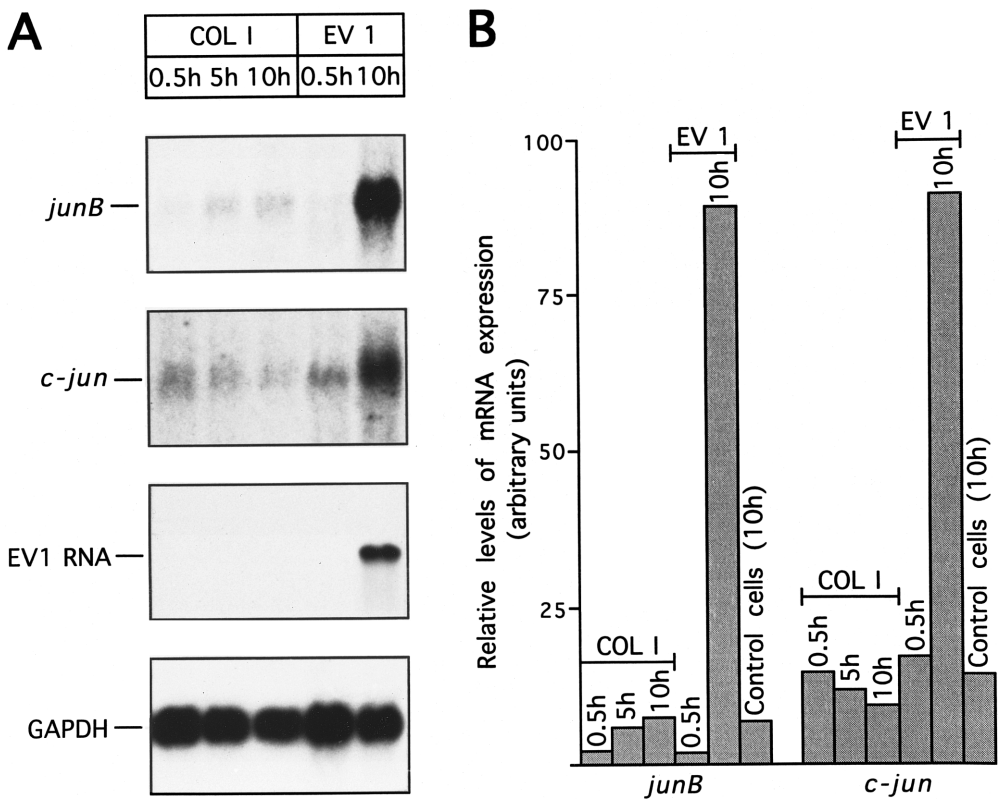

FIG. 1. (A) Expression of *junB* and c-*jun* genes in HOS pα_2AW cells after interaction with type I collagen (COL I) and EV1 infection. Soluble COL I (5μg/cm^2 of cell monolayer) or EV1 (15 pfu/cell) was added to the culture medium, and RNA was isolated 0.5, 5, and 10 h (collagen), or 0.5 and 10 h (EV1) later. *JunB* and c-*jun* mRNA levels were determined by Northern hybridizations. Determination of EV1 RNA levels were used to monitor viral replication, whereas GAPDH mRNA levels were used as controls. (B) Illustration of GAPDH-adjusted relative levels of *junB* and c-*jun* mRNA expression measured from the Northern blot autoradiograms (A).

binds to $\alpha_2\beta_1$ integrin, a major collagen receptor in many cells, and we have shown that infection induces mRNA levels of IE genes and that active viral macromolecular synthesis is needed for the induction (Huttunen *et al.,* 1997). To further study this phenomenon, we analyzed *junB* and c-*jun* gene activation after adding soluble collagen or EV1 to HOS pα_2AW cells. Slight (3.5-fold) induction of *junB* was observed 5–10 h after collagen was added to the cells, whereas the expression of c-*jun* seemed to decrease in the course of time (Fig. 1). In contrast, ~43-fold induction of *junB* and 6-fold induction of c-*jun* were observed 10 h p.i. when HOS pα_2AW cells were infected with EV1 (Fig. 1). This suggests that a small amount (~8%) of the *junB* induction seen during EV1 infection might be mediated by the activation of $\alpha_2\beta_1$ integrin, whereas c-*jun* induction is regulated entirely by virus replication.

To test the cellular effects of virus–receptor interaction, we developed an assay using virus bound to solid phase. We first compared the attachment of α_2 integrin-transfected HOS cells to EV1 and to the major natural ligand of $\alpha_2\beta_1$ integrin, type I collagen (COL I). Within 3 h, HOS pα_2AW cells had attached to COL I and spread (Fig. 2A). Adherence to EV1 showed a nearly linear correlation to the amount of virus (Fig. 2A), although it was slower and the number of attached cells was smaller than those on COL I. Furthermore, the cells did not spread when plated onto EV1 (not shown), which was in contrast to cells plated onto collagen. Thus, despite the fact that the cells use $\alpha_2\beta_1$ integrin in both collagen and EV1 binding (Huttunen *et al.,* 1997), ligand binding can lead to different cellular consequences. To study whether IE genes are induced when the HOS pα_2AW cells are bound to EV1 without internalization of the virus, we carried out an assay in which cell culture dishes were coated with EV1 or COL I, the cells were allowed to attach for 5 h, and then the levels of c-*jun* and *junB* mRNAs were analyzed by Northern blotting. The mRNA levels of c-*jun* seemed to be approximately the same in the cells on the dishes coated with COL I or EV1 (Fig. 2B), but the mRNA levels of *junB* were 3.5-fold higher in EV1-coated dishes than in those coated with COL I. The two ligands thus may have distinct effects on $\alpha_2\beta_1$ integrin function. Another possibility is that other receptors are involved in EV1 or collagen binding. However, the most important conclusion is that in accordance with our previous observations (Huttunen *et al.,* 1997), viral macromolecular synthesis seems to be the main cause for the increased IE gene mRNA levels.

Transcription of AP-1 genes during EV1 infection

To study whether IE gene induction is also seen at the transcriptional level, we carried out a nuclear run-on analysis. The nuclei of EV1-infected HOS pα_2AW cells (8

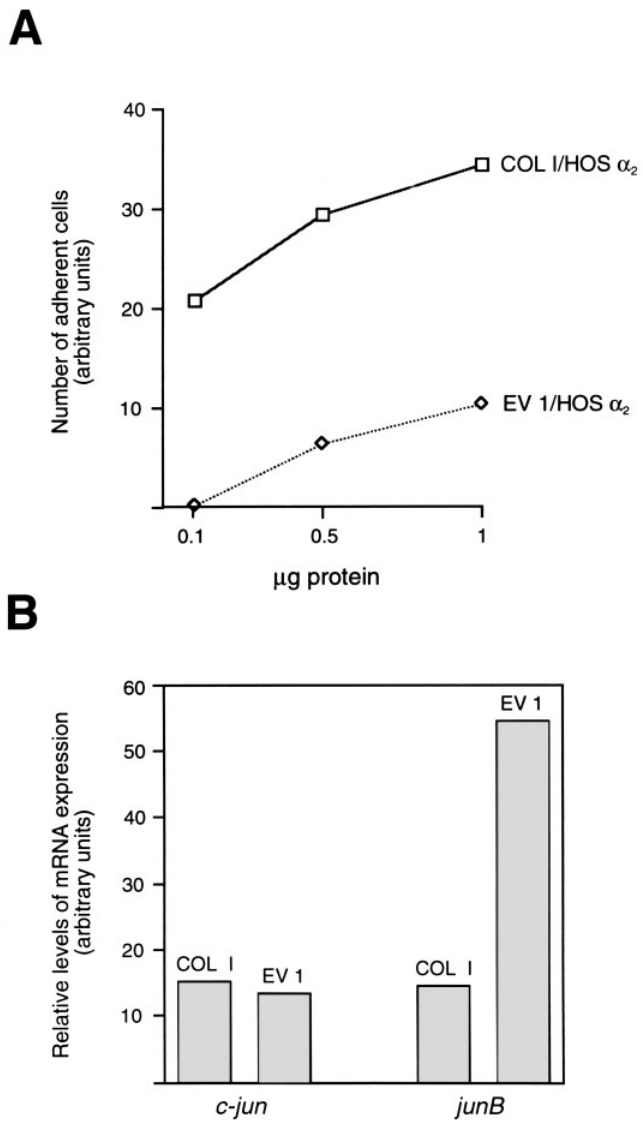

FIG. 2. (A) Adherence of HOS pα_2AW (HOS α_2) cells to COL I and EV1. The cells were detached by trypsin treatment, trypsin activity was inhibited, and the cells were placed on different substrata. After 3 h, the nonadherent cells were washed away, and the adherent ones were fixed and stained with crystal violet. Cell-bound stain was dissolved and measured spectrophotometrically. (B) Illustration of c-*jun* and *junB* gene expression in HOS pα_2AW cells. Cell culture dishes were coated with COL I (5 μg/cm^2) or EV1 (5 μg/cm^2). An appropriate amount of HOS pα_2AW cells was added and allowed to attach for 5 h. Subsequently, RNA was isolated and used for Northern blot analysis. Results are expressed as GAPDH-adjusted optical densities measured from the Northern blot autoradiograms.

h p.i.) were isolated, and the transcription rates of specific genes were analyzed by radiolabeling the newly synthesized mRNA and hybridizing it to nitrocellulose-fixed plasmids containing cDNAs for human *junB,* c-*jun,* and c-*fos* (Fig. 3A). It was found that in the infected cells compared with control cells, the transcription rate of *junB* gene increased 2.5-fold, that of c-*jun* gene increased 2-fold, and that of c-*fos* gene increased 6-fold (Fig. 3B). This suggests that IE gene induction by EV1 infection is, at least partially, due to the increased transcription of these genes. The half-life of the mRNAs was not studied, and we cannot exclude the possibility that it is also elongated by EV1 infection.

EV1 infection induces the phosphorylation of p38 and ERK1/ERK2 MAPKs

Three MAPK subtypes, ERK, JNK/SAPK, and p38, are implicated in IE gene induction. To investigate the activation of two major MAPK subgroups, ERK1/2 and p38, during EV1 infection, we assayed the phosphorylation of these kinases. EV1-infected HOS pα_2AW cells (15 pfu/cell) were collected at 5 and 10 h p.i. and subsequently used in Western blot analysis. Using phospho-specific ERK1/2 antibody, it was found that these kinases are activated as early as 5 h p.i. (Fig. 4A), whereas analysis with phospho-specific p38 antibody revealed that p38 MAPK is activated later (10 h p.i.; Fig. 4B). ERK1/2 represents growth-activated MAPKs, whereas p38 participates in the stress-activated pathway. Our data suggest that EV1 infection activates both of these pathways. A putative inhibitor of the uncoating of EV1, WIN 54954, prevented the phosphorylation of ERK1/2 (Fig. 4C), suggesting that viral replication is necessary for MAPK activation, as it is for the elevation of AP-1 gene mRNA levels (Huttunen *et al.,* 1997).

To obtain further evidence that p38 and ERK MAPK cascades are involved in IE gene induction during EV1 infection, we studied whether the expression of these genes can be inhibited with specific MAPK inhibitors. Two inhibitors were used: SB 203580, a selective p38 inhibitor, and PD 98059, a selective inhibitor of MAPK/ERK kinase 1 (MEK1) activation. MEK1 is an upstream activator of ERK1 and ERK2. It was found that SB 203580 almost completely inhibited the induction of *junB* and significantly inhibited the induction of both c-*fos* (by 75%) and c-*jun* (by 60%; Fig. 5). PD 98059 had a slight effect on c-*jun* induction (inhibited by 35%) and inhibited the induction of both *junB* and c-*fos* by 55% (Fig. 5). SB 203580 and PD 98059 together completely inhibited the induction of c-*fos* and *junB,* whereas the induction of c-*jun* was inhibited by 65% (Fig. 5). As shown in Figure 5, the inhibitors had no effect on viral replication. The data indicate that the expression of *junB* in EV1-infected cells is almost completely dependent on p38 activation, whereas the expression of c-*fos* is regulated by both ERK1/2 and p38 MAPK pathways, and the expression of c-*jun* is regulated by p38 and some other signaling pathway or pathways.

Only partial host cell protein synthesis shut-off occurs in EV1 infection in HOS pα_2AW and LLC-MK$_2$ cell lines

Human enteroviruses, including EVs, are thought to cause specific shut-off of host cell protein synthesis in cultured cells (Rueckert, 1996). This is considered to be a major factor in cellular pathogenesis. To investigate whether EV1 infection induces complete or partial host

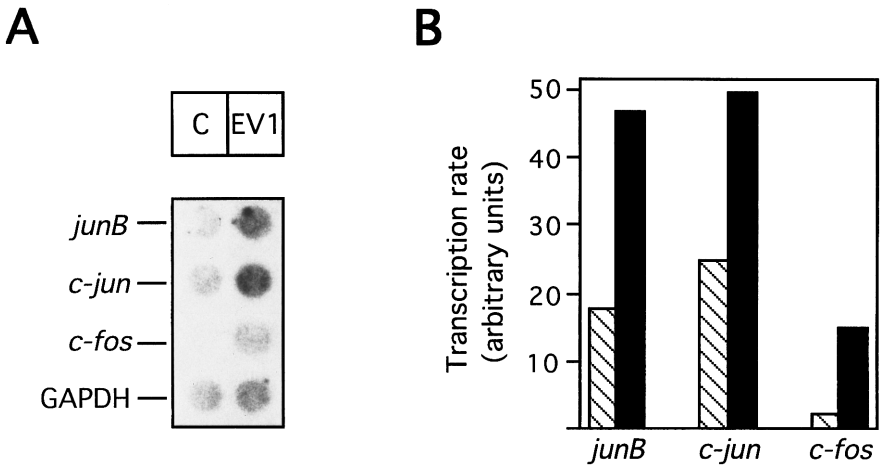

FIG. 3. (A) Nuclear run-on analysis of the IE genes *junB,* c-*jun,* and c-*fos* in EV1-infected HOS pα_2AW cells. The nuclei were isolated 8 h p.i., and newly synthesized mRNA was radiolabeled and hybridized to nitrocellulose-fixed plasmids, including cDNAs for human *junB,* c-*jun,* and c-*fos.* Rat GAPDH cDNA was used as an internal control. (B) Illustration of the GAPDH-adjusted optical densities measured from the nuclear run-on autoradiogram (A). The transcription levels of *junB,* c-*jun,* and c-*fos* genes in EV1-infected cells (solid bars) were compared with those obtained from the control cells (hatched bars).

cell protein synthesis shut-off, we carried out a pulse-chase labeling experiment. Proteins in EV1-infected HOS pα_2AW and LLC-Mk$_2$ cells (monkey kidney cell line generally used for propagation of enteroviruses) were metabolically labeled with [^{35}S]methionine and analyzed by SDS–PAGE. It was found that EV1 infection causes only partial inhibition of host cell protein synthesis in both cell lines (Fig. 6). This suggests that host cell proteins like the IE gene products could be synthesized and processed during EV1 infection. This was also confirmed by a Western blot analysis of JunB protein, indicating a ~2-fold increase in protein levels in EV1-infected cells (10 h p.i.; data not shown).

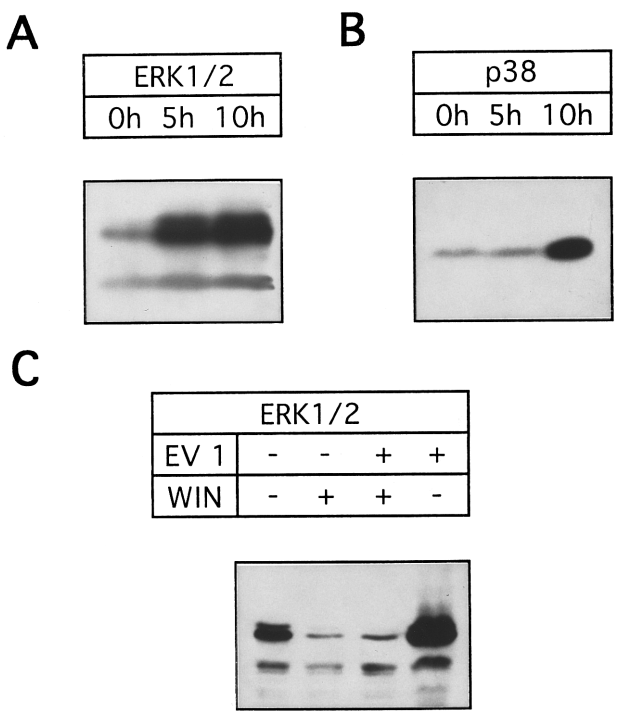

FIG. 4. Western blot analysis of the activation of ERK1/ERK2 (A) and p38 MAPKs (B) in EV1-infected HOS pα_2AW cells. (C) Effect of the antiviral compound WIN 54954 on the activation of ERK1/2 in EV1-infected HOS pα_2AW cells. Samples were collected at 5 and 10 h p.i. (A and B) or 10 h p.i. (C); proteins were subsequently electrophoresed in 10% minigels, transferred to nitrocellulose membrane, and finally probed with phospho-specific ERK1/ERK2 and p38 MAPK antibodies. The results were compared with those obtained from the uninfected (control; 0 h) cells.

Poliovirus 1 and Semliki Forest virus A7(74) infections also activate IE genes but to a different extent and with a different time course

We have previously shown that infection by another EV, EV7, can also activate the expression of IE genes (Huttunen *et al.,* 1997). It was therefore interesting to extent our studies to members of other enterovirus subgroups [e.g., polioviruses (PVs)] and other positive strand RNA viruses [e.g., Semliki Forest virus (SFV), an alphavirus]. PV1 infection (10 pfu/cell) induced the expression of *junB,* c-*jun,* and c-*fos* almost identically to EV1 infection (Fig 7A). SFV A7(74) infection (10 pfu/cell) was able to activate the expression of *junB* and c-*jun* genes but not the expression of *c-fos* (Fig. 7B). Furthermore, the activation occurred at a later time point (8–36 h p.i.). This is probably due to the differences in the replication cycle of SFV and enteroviruses. In conclusion, it seems that enteroviruses and at least one other representative of a positive strand RNA virus [SFV A7(74)] are able to induce the expression of IE genes but that the induction pattern and time course may be characteristic for each virus.

DISCUSSION

The effects of virus infections, particularly those caused by RNA viruses, on cellular functions are still

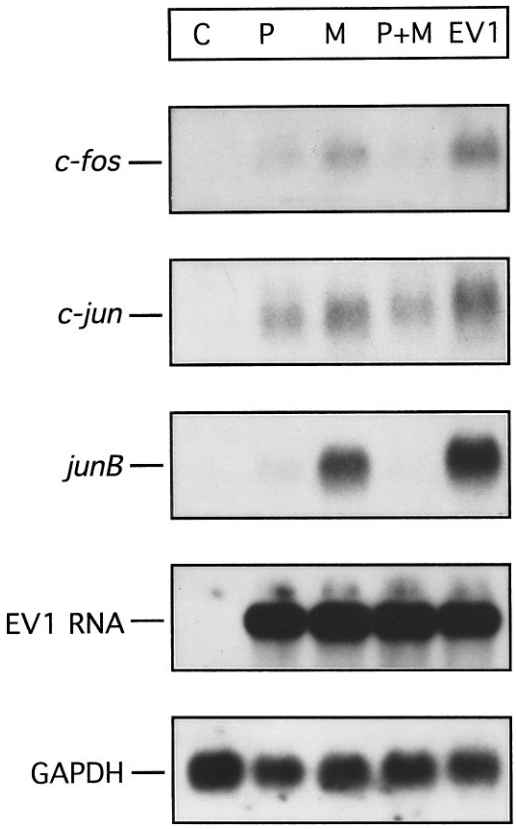

FIG. 5. Effects of two MAPK inhibitors on the expression of c-*fos,* c-*jun,* and *junB* genes in EV1-infected HOS pα_2AW cells. The cells were infected and incubated in the presence or absence of MAPK inhibitors: SB 203580, a selective p38 inhibitor (P) (20 μM); PD 98059, a selective inhibitor of MEK1 (M) (20 μM); or both. (C) Uninfected control cells. (EV1) EV1-infected untreated cells. Detection of viral RNA was used to monitor viral replication. GAPDH mRNA levels were used as a control.

poorly understood. Recently, accumulating knowledge and novel techniques of cell biology have made it possible to investigate the cellular effects of viral infections in detail. It is evident that virus-induced alterations in cell behavior during the infection have important pathogenic consequences.

We previously reported that EV1 infection increases the mRNA levels of cellular IE genes (Huttunen *et al.,* 1997). Here, we provide further evidence that IE gene induction during EV1 infection is mainly due to viral macromolecular synthesis, rather than to activation of the virus receptors. Our initial intention was to investigate whether EV1– $\alpha_2\beta_1$ integrin interaction could activate IE gene-related signal transduction pathways in a manner similar to the natural ligand of $\alpha_2\beta_1$ integrin, COL I. However, it was shown that this could account for only some of the observed IE gene induction (Huttunen *et al.,* 1997). As shown more fully here, virus replication seems to be the main cause for the increased mRNA levels of the IE genes during the infection. Indeed, only ~8% of *junB* induction and virtually none of the c-*jun* induction could be mediated by the activation of $\alpha_2\beta_1$ integrin (Fig. 2). These data, together with observations made using a specific inhibitor of virus replication (Huttunen *et al.,* 1997) and the finding that other enteroviruses and SFV A7(74), which use different receptors for cell attachment, are able to activate IE genes (Huttunen *et al.,* 1997; Fig. 7), suggests that different RNA virus infections, independently of the cell entry process, may have similar cellular effects and pathogenic mechanisms.

MAPK cascades are signal transduction pathways that are used by nearly all cell surface receptors and transduce various extracellular and intracellular signals to coordinate appropriate cellular responses. Three MAPK subtypes, namely ERK, JNK/SAPK, and p38, have been implicated in IE gene induction by diverse physiological, pharmacological, and stress stimuli. JNK/SAPK and p38 MAPKs mediate responses to cellular stress and cytokines, whereas the ERK pathway is stimulated by mitogenic growth signals (Robinson and Cobb, 1997). Our study demonstrated that EV1 infection induces both the growth-related ERK1/ERK2 MAPK pathway and the stress-activated p38 MAPK pathway. The activation of ERK1/ERK2 MAPK was seen earlier (5 h p.i.) than that of p38 MAPK (10 h p.i.; Fig. 4). The balance between these two pathways has been suggested to be critical for certain cellular processes, such as apoptosis (Robinson and Cobb, 1997). Induction of both the growth-related and stress-activated MAPK pathways by EV1 infection is

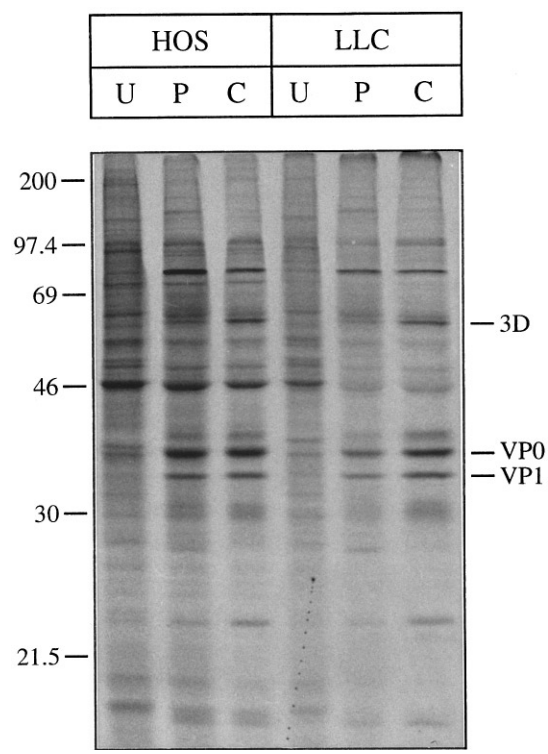

FIG. 6. Protein synthesis in EV1-infected HOS pα_2AW (HOS) and LLC-Mk$_2$ (LLC) cells. For the pulse-chase experiment, the cells were labeled for 10 min (P = pulse) and then chased for 30 min with complete medium (C = chase). The control cells (U = uninfected) were treated with pulse labeling. The pattern of representative viral polypeptides is indicated.

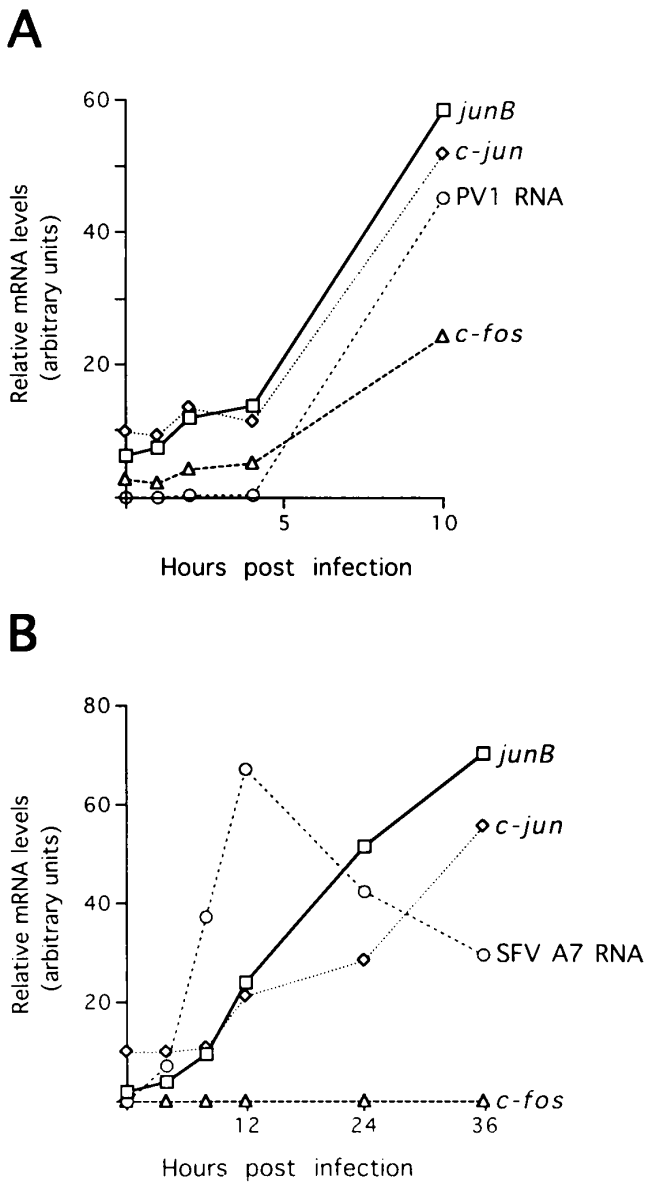

FIG. 7. *junB*, c-*jun*, and c-*fos* gene induction in HOS pα_2AW cells infected with (A) poliovirus 1 (picornavirus) or (B) SFV A7(74). The cells were infected with 10 pfu/cell, and total RNA was extracted at 1, 2, 4, and 10 h p.i. (A) or 4, 8, 12, 24, and 36 h p.i. (B) and used in Northern blot analysis. Determination of viral RNA was used to monitor viral replication. Results are expressed as GAPDH-adjusted optical densities measured from the Northern blot autoradiograms.

an interesting new observation, which may partially explain virus-related changes in host cell behavior.

The mechanism of activation of the MAPK pathways by EV1 infection remains somewhat open. The fact that an antiviral compound, WIN 54954, could prevent the phosphorylation of ERK1/2 suggests that viral replication is essential. Putative inducers of MAPK pathways in RNA virus infections include (1) RNA-dependent RNA synthesis and (2) additional proteases and other viral or virus-induced factors in the replication cycle.

The availability of specific inhibitors of MAPK cascades has further boosted the efforts to verify relationships between MAPKs and IE genes/transcription factors. These inhibitors include SB 203580, which binds and blocks the p38 MAPK subtype, and PD 98059, which blocks the activation of MEK1 and thereby the ERK cascade. In our study, experiments with these inhibitors indicated that in EV1 infection, (1) p38 is the main regulator of *junB* induction, (2) the activation of c-*fos* is regulated by both ERK and p38 MAPK pathways, and (3) the induction of c-*jun* is mainly regulated by some other signaling cascades, but the p38 MAPK pathway also participates in the process (Fig. 8). An obvious candidate as a mediator of EV1 infection-related c-*jun* induction is the stress-activated JNK/SAPK MAPK pathway, which mediates the induction of c-*jun* by diverse stimuli. We have demonstrated the activation of JNK by EV1 infection (P. Huttunen P. Vihinen, T. Hyypiä, and J. Heino, unpublished results). However, with the lack of a specific inhibitor of JNK, its role in the induction of IE genes remains to be shown. It has been recently reported that when the effect of the inhibitor of p38, SB 203580, is studied after the treatment of cells with different inducers of IE genes, it has an essentially identical effect on all genes: the induction of all AP-1 genes is inhibited, their induction is not significantly altered, or the expression of all genes is enhanced (Hazzalin *et al.,* 1997). This is in accordance with the data reported here, since inhibition of p38 with SB 203580 significantly decreased the mRNA levels of *junB,* c-*fos,* and c-*jun* during EV1 infection (Figs. 5 and 8). Despite the fact that p38 participated in the regulation of all three AP-1 genes, it was activated remarkably later than the MEK/ERK pathway. This suggests that the activation of the growth factor-dependent MAPK pathway may be the primary response to virus infection instead of the stress-activated pathway.

Human picornaviruses, including EVs, are thought to cause specific shut-off of host cell protein synthesis (Rueckert, 1996). This is possible because picornavirus mRNA contains an internal ribosomal entry site (IRES) structure and lacks the m^{7}G-cap group, found at the 5′ terminus of most cellular mRNAs, which is needed for initiation of translation. In the case of poliovirus, at least, infection can inactivate cap-binding complex by cleaving

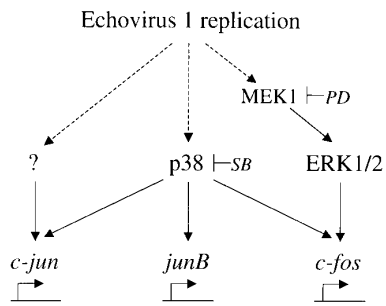

FIG. 8. Our observations suggest that EV1 infection regulates both growth- and stress-activated MAPK pathways. MAPK activation leads to the induction of AP-1 genes. (SB) SB 203580, a selective p38 inhibitor. (PD) PD 98059, a selective inhibitor of MEK1.

the p220 polypeptide, one of the essential components of the complex, by the action of the viral 2A protease (Rueckert, 1996). Interestingly, Zhang and Racaniello (1997) reported that when mouse L cells, which lack receptor for EV1, were made susceptible to the infection by transforming the cells with cDNAs coding for human $\alpha_2\beta_1$ integrin, the infected cells developed a typical cytopathic effect (CPE), but no inhibition of host cell protein synthesis was observed. This suggests that virus-induced inhibition of host cell protein synthesis can vary between closely related viruses and within different cell systems. In our study, complete shut-off was not observed in either EV1-infected HOS pα_2AW or LLC-Mk$_2$ cells (Fig. 6), even though typical CPE was seen. Therefore, it is likely that cellular proteins, including IE gene products, are synthesized during the infection and thus possibly contributing to the cellular pathogenesis.

The exact role of the IE gene induction in EV infections remains unknown. Interestingly, the AP-1 transcription factor is known to be critical for apoptosis in some cell lines (Sawai *et al.,* 1995), whereas in some other cells, apoptosis can take place in the absence of AP-1 components (Roffler-Tralov *et al.,* 1996). Furthermore, the induction of the stress-activated MAPKs has been considered to promote apoptosis, whereas the activation of growth signal-associated MAPKs may protect cells (Xia *et al.,* 1995). Thus, the balance between different MAPK pathways may be critical for cell survival. According to our preliminary results, at time point 12 h p.i., 10% of EV1-infected HOS pα_2AW cells are in apoptosis, whereas 15% of SFV A7(74)-infected HOS pα_2AW cells undergo apoptosis at 24 h p.i. (P. Huttunen, J. Heino, and T. Hyypiä, unpublished results). SFV A7 has also been shown to induce apoptosis in many other cell lines (Glasgow *et al.,* 1997; Scallan *et al.,* 1997). Certain poliovirus mutants are the only enteroviruses that have been reported to induce apoptotic reaction (Tolskaya *et al.,* 1995). It is tempting to speculate that virus-specific differences in the activation of distinct MAPK pathways or AP-1 genes dictate the course of cell survival and death. It is also an interesting possibility that the viruses can activate signals and prolong the survival of their host cells. Our observation that EV1 infection can regulate the putatively antiapoptotic ERK pathway (Xia *et al.,* 1995) suggests that further research should be performed in that area.

MATERIALS AND METHODS

Viruses and cell lines

EV1 (Farouk strain) and PV1 Mahoney strain were obtained from the American Type Culture Collection (ATCC). SFV A7(74) (Bradish *et al.,* 1971) was obtained from Dr. H. E. Webb (Department of Neurology, Rayne Institute, St. Thomas' Hospital, London, UK). EV1 and PV1 were propagated in LLC-Mk$_2$ cells and purified by the method described originally for rhinoviruses by Abraham and Colonno (1984). The purified viruses in PBS containing 0.5 mM $MgCl_2$ were stored at −70°C until used. Human osteogenic sarcoma (HOS) cells also were from the ATCC. HOS pα_2AW cells, constantly overexpressing α_2 integrin cDNA in pAWneo2 vector, were established as described elsewhere (Riikonen *et al.,* 1995). We have shown previously that overexpression of α_2 integrin in HOS cells increases the infectivity of EV1 (Huttunen *et al.,* 1997). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS; Life Technologies, Inc.) and 400 μg/ml G418 (GIBCO). When the cells were infected with the virus, culture medium containing DMEM supplemented with 1% FCS was used. MAPK inhibitor PD 98059 (Alessi *et al.,* 1995) was obtained from Calbiochem, and SB 203580 (Cuenda *et al.,* 1995) was provided by SmithKline Beecham. For MAPK inhibition assays, SB 203580, PD 98059, or both were added to the culture medium (final concentration, 20 μM) before virus inoculation. Antiviral compound WIN 54954 (used at a concentration of 10 μg/ml) was a generous gift from Sanofi Winthrop Inc.

Northern blot analysis

Total cellular RNA was extracted using the thiocyanate–CsCl method (Chirgwin *et al.,* 1979). RNAs were separated in formaldehyde-containing agarose gels, transferred to nylon membranes (Zeta-probe; BioRad). and hybridized with ^{32}P-labeled (Amersham, Inc.) cDNA probes. The following cloned cDNAs in plasmid vectors were used as probes: human c-*jun* (Angel *et al.,* 1988), human *junB* (Schütte *et al.,* 1989), human c-*fos* (genomic fragment from Amersham, Inc.), and full-length EV11 genome (Dahllund *et al.,* 1995). A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Fort *et al.,* 1985) was used to control the proportional quantity of cellular RNA. All autoradiograms were also analyzed by using the Microcomputer Imaging Device version M4 (Imaging Research, Inc.), and the resulting measurements were adjusted to correspond to GAPDH mRNA levels.

Cell adhesion assay

We used 96-well immunoplates (Maxi Sorp; Nunc) in the cell adhesion assay. Wells were coated with 1, 5, or 10 μg/ml collagen (COL I from rat tail; Sigma) or the same amount of purified EV1 in PBS (pH 7.4) overnight at 4°C. Residual protein absorption sites on the wells were blocked with 1% bovine serum albumin in PBS solution containing 0.5 mM $MgCl_2$ for 1 h at room temperature. HOS pα_2AW cells were detached from confluent monolayer cultures with 0.01% trypsin and 0.02% EDTA. To inhibit trypsin activity, the cells were washed with 1 mg/ml soybean trypsin inhibitor (Sigma) and then suspended in minimal essential medium (MEM) (GIBCO). Next, 10,000 cells in 100 μl of MEM were transferred into

each well and incubated for 3 h at 37°C. Nonadherent cells were removed by flushing the wells with the medium. Adherent cells were fixed with 2% paraformaldehyde, stained with 0.5% crystal violet, and washed with distilled water. The plate was allowed to dry, and then the stain associated with the cells was dissolved in 10% acetic acid and measured spectrophotometrically (Multiscan Plus; Labsystems) at a wavelength of 600 nm.

Pulse-chase labeling assay

For labeling with [^{35}S]methionine, the EV1-infected (15 pfu/cell) HOS pα_2AW and LLC-Mk$_2$ cells were incubated at 37°C until most cells showed cytopathic effect (10 h p.i.). Cells were incubated for 30 min in methionine-deficient medium, which was then replaced with medium containing 50 μCi/ml of [^{35}S]methionine (Amersham, Inc.). The cells were harvested after 10 min (pulse) or after subsequent chasing for 30 min with complete medium. The control (uninfected cells) was subjected to pulse labeling. Labeled viral and cellular proteins were analyzed by SDS–PAGE.

Transcriptional nuclear run-on analyses

In vitro nuclear run-on analyses were carried out with an equal number of isolated nuclei (10^7/reaction) in the presence of 100 μCi of [α-^{32}P]UTP (3000 Ci/mmol, New England Nuclear) as described earlier (Banerji *et al.,* 1984). Radiolabeled mRNA was hybridized with 2 μg of nitrocellulose-fixed plasmids, which included cDNAs for human c-*jun* (Angel *et al.,* 1988), human *junB* (Schütte *et al.,* 1989), human c-*fos* (genomic fragment from Amersham, Inc.), and rat GAPDH (Fort *et al.,* 1985). The hybridization and washing conditions were as described previously (Sistonen *et al.,* 1992). Autoradiograms were analyzed with the Microcomputer Imaging Device version M4 (Imaging Research, Inc.), and the resulting measurements were adjusted to correspond to GAPDH mRNA synthesis.

Immunoblotting

For analysis of the phosphorylation of p38 MAPK and ERK1/ERK2 MAPK, equal numbers of cells were lysed in SDS sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue. Proteins were electrophoretically separated in 10% minigels and transferred to nitrocellulose membrane in transfer buffer (25 mM Tris base, 0.2 M glycine, and 20% methanol). The membranes were blocked with buffer containing 1× TBS and 0.1% Tween 20 with 5% nonfat dry milk for 1 h, and then probed with rabbit polyclonal phospho-specific p38 MAPK antibody (New England Biolabs, Inc.) or phospho-specific MAPK antibody detecting ERK1 and ERK2 (New England Biolabs, Inc.). Membranes were washed 3 times for 5 min with TBST (1× TBS and 0.1% Tween 20) after and before incubation with the primary antibody. Anti-rabbit immunoglobulin horseradish peroxidase (DAKO) was used as a secondary antibody, and the detection was carried out with ECL Western blotting reagents (Amersham, Inc.).

ACKNOWLEDGMENTS

The study was financially supported by grants from the Academy of Finland, Sigrid Jusélius Foundation, Paulo Foundation, Finnish Medical Foundation, and Turku Graduate School of Biomedical Sciences. Drs. Ari Hinkkanen and Veli-Matti Kähäri are acknowledged for stimulating discussions, and Dr. Glyn Stanway is acknowledged for checking the manuscript.

REFERENCES

Abraham, G., and Colonno, R. J. (1984). Many rhinovirus serotypes share the same cellular receptor. *J. Virol.* 51, 340–345.

Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J. Biol. Chem.* 270, 27489–27494.

Angel, P., Allegretto, E. A., Okino, S. T., Hattori, K., Boyle, W. J., Hunter, T., and Karin, M. (1988). Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. *Nature* 332, 166–171.

Banerji, S. S., Theodorakis, N. G., and Morimoto, R. I. (1984). Heat shock-induced translational control of HSP70 and globin synthesis in chicken reticulocytes. *Mol. Cell. Biol.* 4, 2437–2448.

Benn, J., Su, F., Doria, M., and Schneider, R. J. (1996). Hepatitis B virus HBx protein induces transcription factor AP-1 by activation of extracellular signal-regulated and c-Jun N-terminal mitogen-activated protein kinases. *J. Virol.* 70, 4978–4985.

Bergelson, J. M., Chan, M., Solomon, K. R., St. John, N. F., Lin, H., and Finberg, R. W. (1994). Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. *Proc. Natl. Acad. Sci. USA* 91, 6245–6248.

Bergelson, J. M., Shepley, M. P., Chan, B. M. C., Hemler, M. E., and Finberg, R. W. (1992). Identification of the integrin VLA-2 as a receptor for echovirus 1. *Science* 225, 1718–1720.

Bradish, C. J., Allner, K., and Maber, H. B. (1971). The virulence of original and derived strains of Semliki Forest virus for mice, guinea-pigs and rabbits. *J. Gen. Virol.* 12, 141–160.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 10, 5294–5299.

Cooper, J. A. (1994). MAP kinase pathways: Straight and narrow or tortuous and intersecting? *Curr. Biol.* 4, 1118–1121.

Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* 364, 229–233.

Dahllund, L., Nissinen, L., Pulli, T., Hyttinen, V.-P., Stanway, G., and Hyypiä, T. (1995). The genome of echovirus 11. *Virus Res.* 35, 215–222.

Fort, P. L., Marty, L., Piechaczyk, M., El Sabrouty, S., Dani, C., Jeanteur, P., and Blanchard, J. M. (1985). Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphatate-dehydrogenase multigenic family. *Nucleic Acids Res.* 13, 1431–1442.

Glasgow, G. M., McGee, M. M., Sheahan, B. J., and Atkins, G. J. (1997). Death mechanisms in cultured cells infected by Semliki Forest virus. *J. Gen. Virol.* 78, 1559–1563.

Grist, N. R., Bell, E. J., and Assaad, F. (1978). Enteroviruses in human disease. *Prog. Med. Virol.* 24, 114–157.

Hazzalin, C. A., Cuenda, A., Cano, E., Cohen, P., and Mahadevan, L. C. (1997). Effects of the inhibition of p38/RK MAP kinase on induction of five *fos* and *jun* genes by diverse stimuli. *Oncogene* 15, 2321–2331.

Huttunen, P., Heino, J., and Hyypiä, T. (1997). Echovirus 1 replication, not

only virus binding to its receptor, VLA-2, is required for the induction of cellular immediate-early genes. *J. Virol.* 71, 4176–4180.

Riikonen, T., Westermarck, J., Koivisto, L., Broberg, A., Kähäri, V.-M., and Heino, J. (1995). Integrin $\alpha_2\beta_1$ is a positive regulator of collagenase (MMP-1) and collagen 1(I) gene expression. *J. Biol. Chem.* 270, 13548–13552.

Robinson, M. J., and Cobb, M. H. (1997). Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* 9, 180–186.

Roffler-Tarlov, S., Brown, J. J., Tarlov, E., Stolarov, J., Chapman, D. L., Alexiou, M., and Papaioannou, V. E. (1996). Programmed cell death in the absence of c-Fos and c-Jun. *Development* 122, 1–9.

Rueckert, R. R. (1996). Picornaviridae: Viruses and their replication. *In* "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), pp. 636–641. Lippincott-Raven, Philadephia.

Sawai, H., Okazaki, T., Yamamoto, H., Okano, H., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Ishikura, H., Umehara, H., and Domae, N. (1995). Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. *J. Biol. Chem.* 270, 27326–27331.

Scallan, M. F., Allsopp, T. E., and Fazakerley, J. K. (1997). *bcl-2* acts early to restrict Semliki Forest virus replication and delays virus-induced programmed cell death. *J. Virol.* 71, 1583–1590.

Schütte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J., and Minna, J. (1989). *Jun-B* inhibits and *c-fos* stimulates the transforming and trans-activating activities of *c-jun. Cell* 89, 987–997.

Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995). Integrins: Emerging paradigms of signal transduction. *Annu. Rev. Cell. Dev. Biol.* 11, 549–599.

Sistonen, L., Sarge, K. D., Phillips, B., Abravaya, K., and Morimoto, R. I. (1992). Activation of heat shock factor 2 during hemi-induced differentiation of human erythroleukemia cells. *Mol. Cell. Biol.* 12, 4104–4111.

Tolskaya, E. A., Romanova, L. I., Kolesnikova, M. S., Ivannikova, T. A., Smirnova, E. A., Raikhlin, N. T., and Agol, V. I. (1995). Apoptosis-inducing and apoptosis-preventing functions of poliovirus. *J. Virol.* 69, 1181–1189.

Ward, T., Pipkin, P. A., Clarkson, N. A., Stone, D. M., Minor, P. d., and Almond, J. W. (1994). Decay-accelerating factor CD55 is identified as the receptor for echovirus 7 using CELICS, a rapid immuno-focal cloning method. *EMBO J.* 13, 5070–5074.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinase on appropriate. *Science* 270, 1326–1331.

Zhang, S., and Racaniello, V. R. (1997). Persistent echovirus infection of mouse cells expressing the viral receptor VLA-2. *Virology* 235, 293–301.