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Identification of genes involved in the host response to enterovirus 71 infection

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Enterovirus 71 (EV71) infection may be asymptomatic or may cause diarrhea, rashes, and hand, foot, and mouth disease (HFMD). However, EV71 also has the potential to cause severe neurological disease. To date, little is known about the molecular mechanisms of host response to EV71 infection. In this report, we utilized cDNA microarray to profile the kinetics and patterns of host gene expression in EV71-infected human neural SF268 cells. We have identified 157 genes with significant changes in mRNA expression and performed hierarchical clustering to classify these genes into five different groups based on their kinetics of expression. EV71 infection led to increases in the level of mRNAs encoding chemokines, proteins involved in protein degradation, complement proteins, and proapoptotis proteins. cDNA microarray expression comparisons of EV71- and mock-infected cells also revealed the down-regulation of several genes encoding proteins involved in host RNA synthesis. Expression of interferon-regulated proteins was increased early in the infection and then decreased. Expression of proteins involved in cellular development and differentiation, some oncogenes, and transcription and translation regulators were suppressed and then stimulated late in the infection. Our findings illustrate the overall host response to EV71 infection, and will aid in understanding the host response to this virus. *Journal of NeuroVirology* (2004) **10,** 293–304.

Keywords: enterovirus 71; microarray; neuropathogenesis

Introduction

Enterovirus 71 (EV71), a positive-stranded RNA virus in the family *Picornaviridae*, poses a global public health problem. Outbreaks of infection with this virus have occurred around the world (McMinn, 2002). EV71 presents most frequently as a childhood exanthem known as hand, foot, and mouth disease (HFMD). However, acute EV71 infection is also associated with severe neurological disease with significant mortality. Children under 5 years old are particularly susceptible to the most severe forms of EV71-associated neurological complications, including aseptic meningitis, brainstem and/or cerebellar encephalitis, myocarditis, acute flaccid paralysis (AFP), and rapid fatal pulmonary edema and hemorrhage (McMinn, 2002). Such presentations as well as a poliomyelitis-like syndrome have been observed during outbreaks in Taiwan, Malaysia, Singapore, western Australia, Bulgaria, New York, and Europe (Gilbert *et al*, 1988; Alexander *et al*, 1994; Chang *et al*, 1999; Ho *et al*, 1999; Chen *et al*, 2001).

Given the importance of this pathogen to human health, the development of strategies to prevent or possibly treat EV71 infection should be given high priority. Studies directed to a better understanding of the cellular events that follow EV71 infection are

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likely to provide insights that will facilitate the development of such strategies.

The host response to viral infection represents a complex orchestration of divergent pathways designed to eliminate the virus and protect the host. However, many pathways involved in antiviral defense, including the cytotoxic T-cell response, cytokine response, and apoptosis, can result in either dysfunction or death of infected or neighboring uninfected cells. The possibility that a vigorous antiviral response may lead to significant host pathology is especially important in the case of nonrenewable cells such as in the central nervous system (CNS) (Johnston *et al*, 2001). However, the molecular mechanisms underlying the response of human neural cells to EV71 infection still remain unclear.

Viruses impact on many aspects of the host cell's metabolism and function. For example, viral infection can induce the interferon antiviral response, modulate the expression of cell surface molecules, and perturb the host transcription and translation machinery (Cuadras *et al*, 2002). Many of these interactions can be monitored by examining changes in gene expression. However, studies aimed at determining the effect of EV71 infection on cellular mRNA levels have been limited. To date, no studies have been reported describing the global transcriptional response of cells to EV71 infection.

DNA microarray technology makes it possible to assess the expression profiles of thousands of genes (Kudoh *et al*, 2000). This approach is being increasingly used to study the transcriptional responses of cells subjected to a variety of environmental stimuli, including viral infections, such as those due to cytomegalovirus (Zhu *et al*, 1998; Browne and Shenk, 2003), human immunodeficiency virus (HIV) (Geiss *et al*, 2000), influenza virus (Geiss *et al*, 2001, 2002), rotavirus (Cuadras *et al*, 2002), and others (Chang and Laimins, 2000; Morgan *et al*, 2001). With the use of high-density DNA microarrays, it is possible to define changes in gene expression that underlie the host response to viral pathogenesis and to gain specific insights into the molecular mechanism of the host pathways that govern viral pathogenesis.

In this report, we used microarrays containing more than 10,000 human cDNAs to study host response at different time points after EV71 infection. We carried out these studies with the human glioblastoma SF268 cells. We learned that, compared to mock-infected cells, EV71 infection changes the amount of many mRNA transcripts in EV71-infected SF268 cells, with 157 genes changing by at least 0.5-fold at two different time points. These changes represented both up-regulation and down-regulation of specific gene transcripts. Some of these gene products may be necessary for viral replication, others for host defense. Our findings indicate that not only the levels of mRNA encoding certain chemokines, proteins involved in protein degradation, certain complement proteins, and preapoptotic proteins are up-regulated following infection, but also that the time patterns of the up-regulation varies depending on the genes. Knowledge of the host genes that are differently regulated with different kinetics during EV71 infection may be important for predicting cellular response to viral infection.

Results and discussion

Replication of EV71 in SF268 cells

Figure 1 shows the growth curve of EV71 in SF268 cells. Virus yields increased gradually with time and peaked at about 10^6 plaque-forming units (pfu)/ml at 48 h after infection. Cytopathic effect (CPE) was first observed at 32 h post infection and progressed to moderate and severe CPE at 48 and 54 h respectively.

Five clusters of expression profiles

To examine the cellular transcriptional response during EV71 infection, we compared the relative abundance of specific poly(A) mRNAs in infected cells to the same specific poly(A) mRNAs from mock-infected cells at 13 different time points beginning at 2 h and going out to 54 h after infection. Labeled cDNA was prepared from the different RNA samples and then hybridized to cDNA microarrays as described in Materials and Methods. A representative area of a gene filter is displayed in Figure 2 and showing several genes were either increased or decreased in abundance following the EV71 infection.

Normalized data were subjected to cluster analysis by agglomerative nesting and principal-component analyses (Eisen *et al*, 1998). Alterations in the expression of many genes were observed as described in Materials and Methods. The temporal profiles of gene expression were quite diverse. Genes were selected for this analysis if their expression levels in SF268

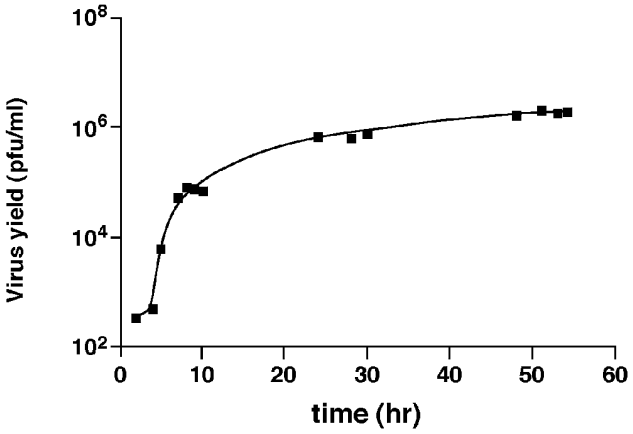

Figure 1 Replication of EV71 in SF268 cells. Confluent cells were infected with EV71 at an m.o.i. of 1 pfu/cell as described in the text and incubated at 37°C. Medium was harvested at serial time points and assayed for infectious virus by plaque formation on Vero cells.

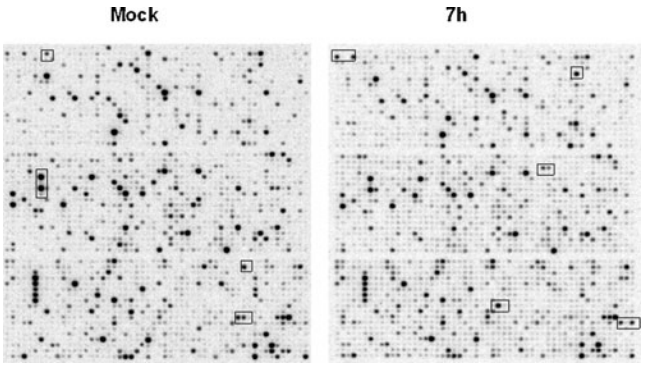

Figure 2 Scanned phosphorimages of cDNA microarrays from mock-infected and EV71-infected SF268 cells. Microarray hybridizations with cDNA derived from SF268 cells 7 h after infection with EV71, or after mock infection, were scanned on a phosphorimager. The images were aligned to show the visually apparent changes in gene expression. Examples of RNAs that are decreased in abundance after infection are boxed in the mock sample (*left*), while RNAs that are increased in abundance after infection are boxed in the 7-h sample (*right*).

cells following EV71 infection were different from those in mock-infected cells by at least a factor of 0.5 at a minimum of two different time points. Using these criteria, 157 genes were included in the cluster, analysis; these fell into five clusters, each with distinct expression kinetics following EV71 infection (Figures 3 and 4). The genes in each cluster are listed in Table 1. Between 1% and 2% of the analyzable genes showed a significant change in expression after EV71 infection. Overall, more genes were up-regulated than down-regulated. In other studies of the transcriptional response to viral infection (Zhu *et al*, 1998; Geiss *et al*, 2000, 2001; Cuadras *et al*, 2002; Chang and Laimins, 2000; Morgan *et al*, 2001), the percentage of genes whose activity was altered varied between 1.3% and 7%. Infection of primary human foreskin fibroblasts with human cytomegalovirus (Zhu *et al*, 1998), and of the CD4+ T-cell line, CEM-CCRF, with HIV-1-LAI (Geiss *et al*, 2000) produced a balanced mix of both up- and down-regulated genes. Similar findings were observed when human keratinocytes were transfected with HPV31 cDNA (Chang and Laimins, 2000). In contrast, influenza virus infection of HeLa cells induced a transcriptional response characterized mainly by down-regulation (Geiss *et al*, 2001).

Genes whose expression was increased following infection (cluster 1)

The expression of the cluster 1 genes, the largest cluster, was increased as early as 2 h post infection and remained up-regulated through out the infection (Figure 3). The majority of these genes fell into functional categories that are known or likely to be important in the host response to viral infections; these include genes encoding chemokine receptors, apoptosis-related genes such as caspase-7 and caspase-3, a p53-responsive gene, and genes that are interferon related or complement related.

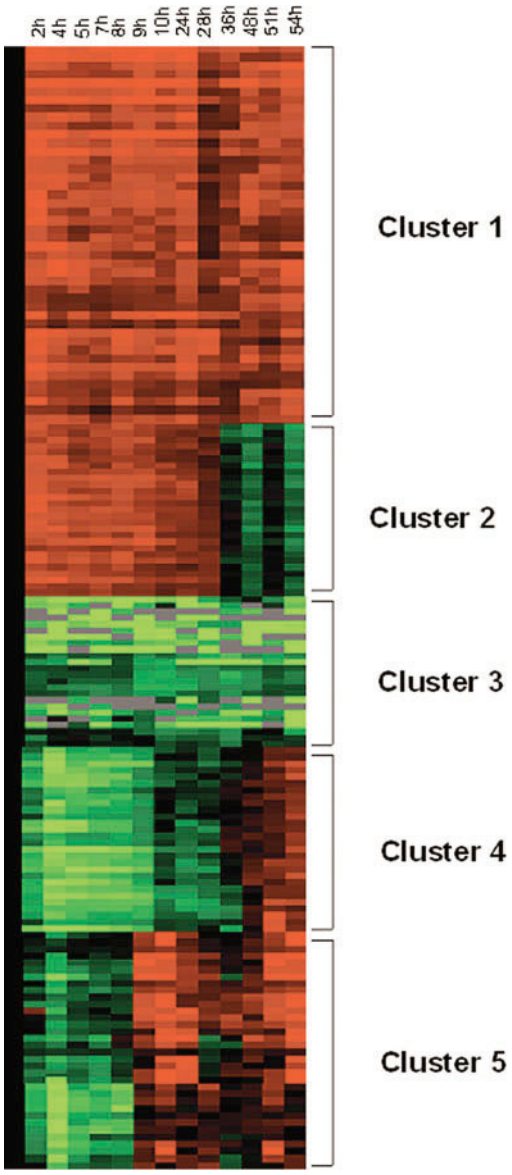

Figure 3 Cluster analysis of genes that were differentially expressed after EV71 infection. Each horizontal row represents a single cDNA, and each vertical column represents a single microarray hybridization. The results are presented in color display; each square represents the ratio of hybridization signal of labeled cDNA prepared from the mRNA of EV71-infected cells relative to mock-infected cells at the same time point. Red squares denote up-regulated genes, green squares denote down-regulation, and black squares denote no significant change in the level of RNA expression.

Complement-related proteins, one class of proteins involved in inflammation, play an important role in neurodegenerative diseases as well as in the innate immune response to microbial invasion (Gasque *et al*, 2000). Hirsch *et al* (1978) reported that treatment of Sindbis virus–infected mice with cobra venom factor to deplete the third component of complement increased the virus titers by 1000-fold in the brain; and Johnston *et al* (2001) found that expression of complement-related proteins was up-regulated in the

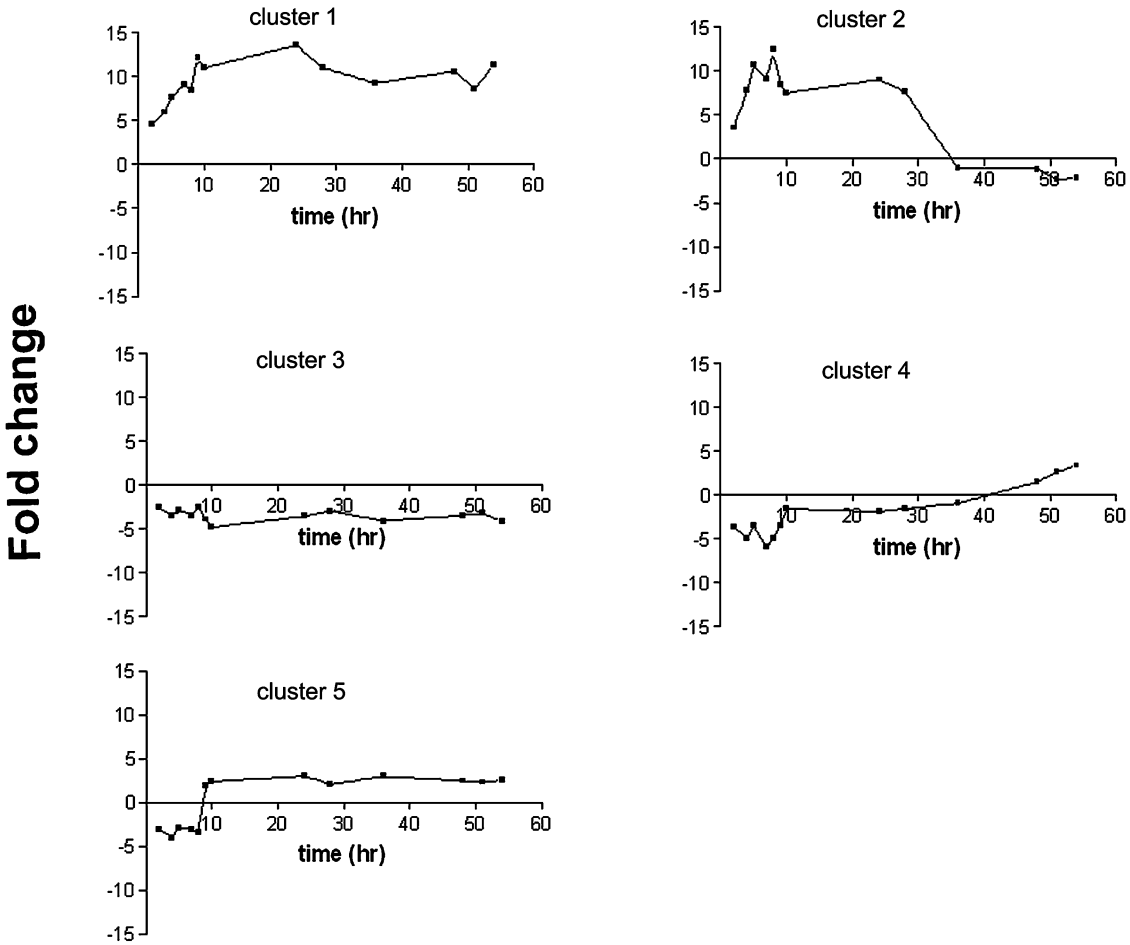

Figure 4 Average expression profiles for each gene cluster. The fold change of mRNA expression of each gene in each cluster was averaged and plotted as a function of time of EV71 infection. Five clusters derived from the analysis show different expression kinetics.

brains of Sindbis virus–infected mice. The increased expression of complement-related genes in EV71-infected neural cells recalls what was observed in the Sindbis virus–infected mouse brains. In addition, the observation raises the possibility that complement may also play a more direct role in antiviral response in the CNS.

Calcium plays a key role in the pathogenesis of many viral infections. It has been shown that the concentration of calcium was increased in HeLa cells during poliovirus and coxsackie B3 virus infection (Irurzun *et al*, 1995; van Kuppeveld *et al*, 1997). The alteration of calcium homeostasis during enterovirus infection has been directly related to the synthesis of specific viral proteins (van Kuppeveld *et al*, 1997; Aldabe *et al*, 1997). Also, this increase in calcium appears to be important in the induction of cell death and the release of viral progeny (van Kuppeveld *et al*, 1997). In this study, we found that one of the components of the voltage-dependent calcium channel, Lα1, was up-regulated. It has been demonstrated in the case of poliovirus infection that the source of the increased intracellular Ca^{2+} was the Ca^{2+} in the culture medium, which enters the cells through voltage-sensitive calcium channels (Irurzun *et al*, 1995). Treatment of cells with verapamil, an inhibitor of L-type voltage-gated calcium channel, reduced the intracellular [Ca^{2+}]. Moreover, poliovirus-infected HeLa cells placed in medium without calcium did not undergo the increase of intracellular [Ca^{2+}]. Thus, it is likely that in the EV71-infected SF268 cells, the increased entry of Ca^{2+} into the infected cells resulted from the opening of calcium channels by virus infection.

We also found that the genes encoding S100A8 and S100A11, two members of a family of low-molecular-weight Ca^{2+}-binding proteins known as S100, were up-regulated early in infection. S100 proteins have been shown to regulate protein phosphorylation, calcium homeostasis, inflammation, and the dynamics of cytoskeleton function in a Ca^{2+}-dependent manner. The three major components of cytoskeleton, microtubules, microfilaments, and intermediate filaments, are targets of the S100 proteins (Donato, 1999). Members of the S100 protein family inhibit tubulin polymerization and cause disassembly of preformed microtubules in the presence of Ca^{2+}. Interestingly, the keratin 6A and laminin B receptors

Table 1 List of genes in each cluster

Accession no.	*Gene name*
Cluster 1	
AA419015	Annexin A4
AA418907	Cytochrome P450, superfamily I polypeptide 1
AA916906	TNF receptor superfamily 1A
AA464771	Complement component 3a receptor 1
AF347015	COX 11 (yeast) homolog
AA416757	Programmed cell death 5
AA034501	
AA086471	S100 calcium-binding protein A8
T50675	Caspase-7
AA451895	Annexin A5
AB023654	Small inducible cytokine A5 (RANTES)
N68271	EST
AA598826	TNF receptor-associated factor 4
H52673	BCL2-antagonist/killer 1
R68555	Programmed cell death 10
AI307134	p53-responsive gene 5
R25377	DEK oncogene (DNA binding)
AA453766	Caspase 8 and FADD-like apoptosis regulator
AA633658	Amyloid beta (A4) precursor protein
AI417775	Chemokine (C-C motif) receptor 5
N66139	Neurochondrin
AI088984	Class I cytokine receptor
AA189050	Signal recognition particle 68 kD
AA437226	Interleukin 10 receptor, alpha
H94466	EST
AI380234	Complement component C1q receptor
N59150	Interferon (alpha, beta, and omega) receptor 1
AA709271	Neural cell adhesion molecule 2
AA644657	Major histocompatibility complex, class I, A
R09561	Decay accelerating factor for complement
AA702361	
AA777034	Brain-specific angiogenesis inhibitor 2
AA458472	Major histocompatibility complex, class II, DQ beta 1
AI675634	BCL2-associated athanogene 5
AA464731	S100 calcium-binding protein A11
AA011446	Caspase-3
W70062	Fas (TNFRSF6)-associated factor 1
AI369284	G-2 and S-phase expressed 1
AI656802	Neuroblastoma protein
AA459292	CDC 28 protein kinase 2
AA102526	Interleukin-8
AI202954	Calcium channel, voltage-dependent, L type, alpha 1
AA455448	CD 47 antigen
N65972	
Cluster 2	
AA191488	Solute carrier family 31, member 1
AA001219	STAT induced STAT inhibitor 3
AA978042	Solute carrier family 2, member 4
H58949	
AA416883	Interferon regulatory factor 2
AA903500	TGF (beta)-induced transcription factor 2
N95249	V-Ki-ras 2 Kirsen rat sarcoma viral oncogene homolog
H59916	CD24 antigen
H48647	
T89391	Caveolin 2
AI653069	KIAA0694 gene product
AA400068	EST
H56929	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
AI337424	KIAA0479 protein
AA282936	M-phase phosphoprotein 1
R56774	Bone morphogenic protein 1
H08231	EST
R99331	EST
H53121	Interleukin 10 receptor, beta
R48796	Integrin, alpha L
AA608557	Damage-specific DNA binding protein 1
AI300241	Myogenic factor 3
W72696	V-akt murine thymoma viral oncogene homolog 1
R11184	EST
H77715	
AA708279	KIAA0426 gene product
AA664406	Complement component 4A
Cluster 3	
AA428959	Cyclin G–associated kinase
AA018906	Transcription factor AP-2 beta
T72628	Splicing factor, arginine/serine-rich 1
T66823	EST
AA676590	Telomeric repeat binding factor 2
R99331	EST
R07870	Baculoviral IAP repeat-containing 3
H97566	Ubiquitin specific protease 12
AA150532	Keratin 6A
AI278206	Homo sapiens clone IMAGE 21721
AA872296	U5 snRNP-specific protein, 116 kD
N91900	EST
AA283744	MADS box transcription enhancer factor 2A
R21416	Oncogene TC21
R09497	EST
W88566	V-raf murine sarcoma viral oncogene homolog B1
AA521469	GTP-binding protein 1
AA099136	Laminin B receptor
AA156821	RAB 18, member RAS oncogene family
AA453015	Mitochondrial ribosomal protein L23
AI362062	Neuro-oncological ventral antigen 1
AA427934	Rho GTPase activation protein 1
W93413	Neuronal Shc
H77636	CD68 antigen
Cluster 4	
T72628	Splicing factor 3a, subunit 1
AA424950	E2F transcription factor 1
AA609976	Mitochondrial capsule selenoprotein
AA069414	Glial fibrillary acidic protein
T51539	Macrophage stimulating 1
NM002424	Matrix metalloproteinase 8
T67474	Anaphase-promoting complex subunit 7
AA600189	Adenosine deaminase, RNA-specific
AA425395	Solute carrier family 16, member 2
AA120779	Zinc finger protein 42
R68805	Integral membrane protein 1
N34028	EST
AA776176	GABA receptor subunit alpha
R45014	
AA479888	Homo sapiens mRNA clone DKFZp434N2412
N55087	Hypothetical protein MGC5178
H93118	
AA644088	Cathepsin C
AA521389	Tumor protein p53-binding protein 1
T59417	N-myc downstream regulated gene 2
MMP-8	
AA405532	Hypothetical protein DKFZp762H1311
AA457025	Laminin B2 chain
AA069770	Potassium voltage-gated channel, Shab-related subfamily, member 1
R35665	Epidermal growth factor receptor
H18068	Protein kinase C–like 1
R11184	EST
AA608550	
Cluster 5	
AI343006	Muscle RAS oncogene homolog
AA664180	Glutathione peroxidase 3
H53869	Zinc finger protein
AI682408	Hypothetical protein MGC2650
AA411619	
AA458991	Nuclear transcription factor Y, gamma
T48639	EST

(Continued on next page)

Table 1 List of genes in each cluster *(Continued)*

Accession no.	*Gene name*	*Accession no.*	*Gene name*
AA676471	Eukaryotic translation initiation factor 3 subunit 9	AA725564	Superoxide dismutase 3
T96132	EST	R06712	RAB5B, member RAS oncogene family
AA478273	APEX nuclease (multifunctional DNA repair enzyme)	AA121387	KIAA0560 gene product
AA947176	Nuclear receptor coactivator 2	H94469	Hypothetical protein FLJ12549
AI341099		N63949	Neurotrophic tyrosine kinase, receptor type 2
AA700604	Sorbitol dehygenase	R94592	EST
H94882	EST	AA034945	Oxidative 3 alpha hydroxysteroid dehydrogenase
AI678208	CGI-60 protein	AA884167	Annexin A13
AI654494	Hsp70B	W72310	Fas-activated serine/threonine kinase
AI018624	Eukaryotic translation initiation factor 2B subunit 5	H79651	EST
AI361616	KIAA0446 gene product	AA989521	Potassium channel, subfamily K, member 3 (TASK-1)
AA425446	Ribosomal protein S6 kinase		
AA676970	Phosphoglycerate mutase 1 (brain)	AA992469	KIAA0599 protein
AA121938		H70143	EST

were both down-regulated in EV71-infected cells. We thus speculate that S100A8 and S100A11 participate in the Ca^{2+}-dependent dissociation of cytoskeleton by down-regulation of keratin 6A and laminin B receptors.

Genes that show increased expression early but are repressed at late times following viral infection (cluster 2)

These genes include V-Ki-ras 2 Kirsen rat sarcoma viral oncogene homolog, and transforming growth factor-β (TGF-β) superfamily such as bone morphogenic protein 1.

Genes that are down-regulated at all times following infection (cluster 3)

Many fewer genes are down regulated throughout the period following infection than are up regulated (cluster 1). These include genes encoding keratin 6A, cyclin G–associated kinase, and transcription, splicing, and translation factors.

Oncogenes play a crucial role in tumorigenesis. Interestingly, some of the oncogenes, for example V-raf, a murine sarcoma viral oncogene homolog, oncogene TC21, and a member of the RAS oncogene family, RAB 18, were suppressed in EV71-infected cells. This suggests that if the cytopathogenicity of EV71 could be suppressed, it could perhaps exert on antitumor effect.

Genes that were repressed early after infection but activated at later times (clusters 4 and 5)

There are two clusters of genes that were down-regulated early in the infection and then up regulated late in the infection. Genes in cluster 4 were repressed early in the infection, less so after 10 h post infection (p.i.), and slightly up-regulated after 36 h p.i. These genes included proteases involved in the degradation of extracellular matrix such as cathepsin C and matrix metalloproteinase 8, transcription and splicing factors, and tumor-related genes.

Cluster 5 represents genes that were slightly down-regulated as early as 2 h after infection, but then up-regulated more rapidly than the genes in cluster 4, i.e., by 9 h p.i. This cluster included genes related to oxidative stress, such as glutathione peroxidase and superoxide dismutase, and other stress response genes. These findings suggest that oxidative stress was increased at later times after viral infection. This cluster also included genes related to membrane transporters, protein kinases, and cell proliferation.

Grouping of genes according to their biological function

To facilitate the analysis of our data, we attempted to group the differently regulated genes according to their known biological functions. This is sometimes difficult because many genes participate in more than one biological process. Nevertheless, for the sake of simplicity we listed the genes in only one single functional category and classified them into a large number of groups such as neuron-specific genes, transcription or translation factors, cell cycle–related genes, signal transduction genes, apoptosis-related genes, and genes related to metabolism. A complete list of these genes is presented in Table 2. Effects on the expression of genes involved in apoptosis (nearly all of these are in cluster 1), mitochondrial function, and transcription and translation were anticipated because previous reports had demonstrated a relationship between EV71 infection and these biological processes (Leong *et al*, 2002).

Some of the host genes whose expression is altered may be involved in the host response to EV71 infection. The majority of these genes can be classified into functional groups that are already known to be involved in the host response to infection with EV71 and other neurotrophic viruses (Johnston *et al*, 2001). These include genes encoding antigen presentation molecules, immune cell activation markers (see under immune response genes for example), chemokines, and interferon (IFN)-inducible gene products. The identification of specific genes in each of these functional groups may help elucidate the molecular details of important host response pathways. For example, we found that the expression

Table 2 Different functional groups of EV71-regulated genes

Function and gene name	*Cluster*
Angiogenesis	
Brain-specific angiogenesis inhibitor 2	1
Annexins	
Annexin A4	1
Annexin A5	1
Annexin A13	5
Apoptosis	
TNF receptor superfamily member 5	1
Programmed cell death 5	1
Caspase-7	1
TNF receptor-associated factor 4	1
Bcl-2 antagonist/killer 1	1
Programmed cell death 10	1
Caspase 8 and FADD-like apoptosis regulator	1
Amyloid beta (A4) precursor protein	1
Bcl-2 associated athanogene 5	1
Caspase 3	1
Fas (TNFRSF6) associated factor 1	1
Baculoviral IAP repeat-containing 3	2
Calcium homeostasis	
S100 calcium-binding protein A11	1
S100 calcium-binding protein A8	1
Cell cycle	
G-2 and S-phase expressed 1	1
CDC 28 protein kinase 2	1
CD 47 antigen	1
CD 24 antigen	2
CD 68 antigen	3
M-phase phosphoprotein 1	2
Cyclin–G associated kinase	3
Anaphase-promoting complex subunit 7	4
Cell migration	
Macrophage stimulating 1	4
Cellular development and differentiation	
N-myc downstream regulated gene 2	4
Epidermal growth factor receptor	4
Chemokines	
RANTES	1
Chemokine (C-C motif) receptor 5	1
Class I cytokine receptor	1
IL-8	1
Complement	
Complement component 3a receptor 1	1
Complement component C1q receptor	1
Decay accelerating factor for component	1
Complement component 4A	2
Cytoskeleton and cell structure	
Keratin 6A	3
Laminin B receptor	3
Laminin B2 chain	4
DNA replication	
APEX nuclease (multifunctional DNA repair enzyme)	5
Enzymes	
Sorbitol dehydrogenase	5
Phosphoglycerate mutase 1 (brain)	5
Oxidative 3 alpha hydroxysteroid dehydrogenase	5
Growth factor inhibtor	
BMP1	2
TGF beta superfamily protein	2
IFN response	
Interferon receptor 1	1
Interferon regulatory factor 2	2
STAT induced STAT inhibitor 3	2
Immune response	
Interleukin 10 receptor, alpha	1
MHC class I A	1
MHC class II DQ beta 1	1
Interleukin 10 receptor, beta	2
Kinases and phosphatases	
Protein kinase C–like	4
Ribosomal protein S6 kinase	5
Fas-activated serine/threonine kinase	5
Neurotrophic tyrosine kinase, receptor type 2	5
Membrane transporters	
Solute carrier family 31 member 1	2
Solute carrier family 2 member 4	2
Solute carrier family 16 member 2	4
Integral membrane protein 1	4
Potassium voltage-gated channel, Shab-related superfamily member 1	4
Calcium channel, voltage-dependent, L type, alpha 1C	1
Potassium channel, superfamily K member 3 (TASK-1)	5
Mitochondrial function	
Cytochrome P450, superfamily I polypeptide 1	1
COX II	1
Mitochondrial ribosomal protein L23	3
Mitochondiral capsule selenoprotein	4
Neuron specific	
Neurochondrin	1
Neural cell adhesion molecule 2	1
Neuroblastoma protein	1
Neuro-oncological ventral antigen 1	3
Neuronal Shc	3
Glial fibrillary acidic protein	4
GABA receptor subunit alpha	4
Oncogene	
DEK oncogene (DNA binding)	1
V-Ki-ras 2 Kirsen rat sarcoma viral oncogene homolog	2
V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	2
V-akt murine thymoma viral oncogene homolog 1	2
V-raf murine sarcoma viral oncogene homolog B1	3
Oncogene TC21	3
RAB 18 member RAS oncogene family	3
Muscle RAS oncogene homolog	5
RAB5B member RAS oncogene family	5
p53 regulated	
p53 responsive gene 5	1
Tumor protein p53-binding protein 1	4
Protein degradation	
Ubiquitin specific protease 12	3
Cathepsin C	4
MMP-8	4
RNA synthesis and modification	
Splicing factor, arginine/serine rich 1	3
U5 snRNP-specific protein	3
Splicing factor 3a, subunit 1	4
Adenosine deaminase, RNA-specific	4
Rho GTPase activation protein 1	3
Signal transduction	
Integrin, alpha L	2
GTP-binding protein 1	3
Stress response	
Glutathione peroxidase 3	5
Hsp 70B	5
Superoxide dismutase 3	5
Transcriptional regulators	
MADS box transcription enhancer factor 2A	3
Damage-specific DNA binding protein 1	2
Transcription factor AP-2 beta	3
Telomeric repeat binding factor 2	3
E2F transcription factor 1	4
Zinc finger protein 42	4
Zinc finger protein	5
Nuclear transcription factor Y, gamma	5
Nuclear receptor coactivator 2	5
Translation machinery	
Signal recognition particle 68 kD	1
EIF3S9	5
EIF2B5	5
Vesicle formation and intracellular transport	
Caveolin 2	1
Myogenic factor 3	2

levels of chemokine mRNAs such as RANTES and interleukin (IL)-8 were increased significantly in EV71-infected neural cells as were their receptors. Thus these two CC chemokines may be involved in EV71-induced neuropathogenesis.

Apoptosis-promoting genes that are induced in EV71-infected neural cells

Although the regulation of genes involved in neuronal function may play a role in determining the pathogenesis of EV71 infection, there is insufficient understanding at present of the molecular details of virus-neuron interactions to postulate which genes these might be. Because it has been demonstrated that apoptosis plays an important role in the pathogenesis of several different CNS viral infections (Labrada *et al*, 2002; Johnston *et al*, 2001; Belov *et al*, 2003), it is not surprising that the expression of apoptosis-related genes is affected by infection with EV71. We therefore compared the expression of all known antiapoptotic bcl-2 family members, all cell death signaling molecules, and all caspases included in the DNA microarrays. There was no difference in the mRNA expression of bcl-2 family members between the EV71-infected cells and the mock infected cells (data not shown). However, expression of baculoviral IAP, an antiapoptotic gene, was found to be down regulated (cluster 3). Among the cell death signaling molecules, TNF and Fas receptors were up-regulated throughout the infection (cluster 1). With respect to the caspase family, at 24 h p.i. the mRNA expression of caspase-3 and caspase-7 was increased 12.6- and 13.4-fold respectively (cluster 1).

To validate the reliability of the microarray results, we used quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) to examine the effect of EV71 infection on selected genes. As shown in Figure 5A, expression of baculoviral IAP-3 was repressed throughout the EV71 infection whereas caspase-3 was up-regulated throughout the infection.

Apoptosis is the major mechanism by which multicellular organisms eliminate redundant, damaged, or unwanted cells. The apoptotic response to viral infections is generally believed to benefit the host. However, the use of apoptosis as a means of removing virus-infected cells can be harmful to the host when the infected cells are nonrenewable, such as is the case with neurons. In this instance, apoptosis is believed to contribute to host pathology. However, absence of apoptosis or the failure of an apoptotic pathway to function can also be a problem for the host. Failure to remove infected neurons may lead to the establishment of persistent infection. Thus, apoptosis is a double-edged sword with respect to virus-infected neurons. Its presence may contribute to pathology while its absence may contribute to viral persistence (Levine, 2002). Making use of deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-based labeling techniques, Lewis *et al* (1996) demonstrated that the age- and strain-dependent neurovirulence of Sindbis virus correlates directly with virus-induced apoptosis. In our study, there were many apoptosis-related genes whose expression was modified in EV71-infected SF268 cells. Many proapoptotic genes such as those encoding caspase-3, -7, and -8, TNF receptor, and Fas-associated factor were up-regulated throughout the infection, whereas IAP, one of the antiapoptotic genes, was down-regulated. This finding is consistent with previous reports by us (Li *et al*, 2002) and by Kuo *et al* (2002) that EV71 can induce apoptosis in human neural (SF268), HeLa, and Vero cells. Taken together, these findings indicate that apoptosis may play a crucial role in the CNS pathogenesis of EV71 infection.

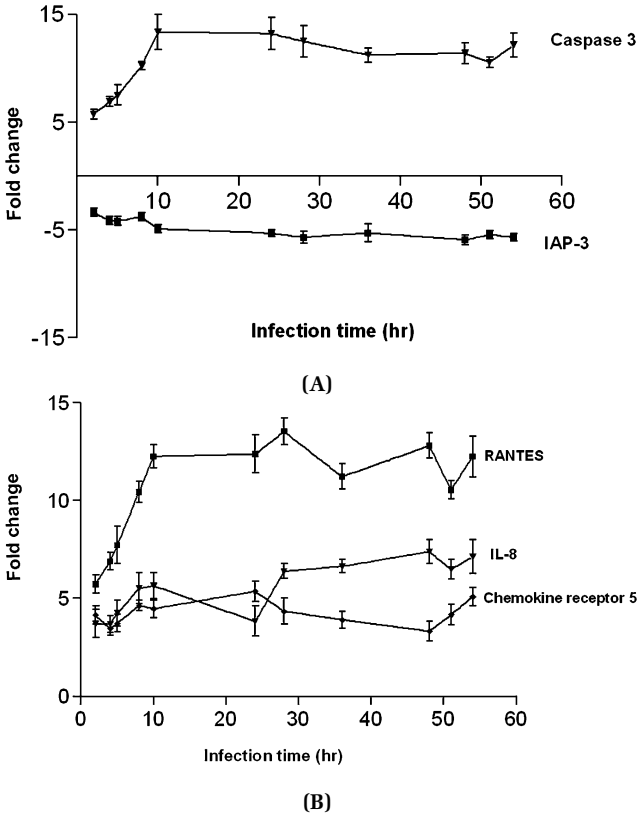

Figure 5 Confirmation of altered gene expression of microarray by real-time RT-PCR. RNA samples from EV71- or mock-infected SF268 cells were extracted at 2 h, 4 h, 5 h, 8 h, 10 h, 24 h, 28 h, 36 h, 48 h, 51 h, and 54 h postinfection. The relative expression of selected apoptotic-related genes (**A**) and of cytokine genes (**B**) was measured by real time RT-PCR. Actin was used as internal control.

Regulation of IFN-related genes

IFNs are a family of related cytokines that mediate a range of diverse functions including antiviral, antiproliferative, antitumor, and immunomodulatory activities (Der *et al*, 1998; Pestka, 2000). IFNs can act directly at various steps of the viral replication cycle through the products of cellular genes induced in the target cells and involved in RNA and protein

metabolism and signaling. Three IFN-regulated pathways are involved in these processes: the double-stranded RNA–dependent protein kinase R (PKR), the 2′-5′A/RNase L system, and the Mx proteins (Espert *et al*, 2003). Previous studies have demonstrated an elevation of IFN-γ in the serum and depletion of lymphocytes in EV71-infected patients with pulmonary edema (Wang *et al*, 2003). Surprisingly, no significant increases of IFN-α, -β, -γ, IFN-induced PKR, or 2′-5′-oligoadenylate synthetase mRNAs were detected in the EV71-infected cells, despite induction of the IFN type 1 receptor, the IFN-signaling molecule STAT 3, and one of the interferon regulatory factors (IRF2). This may reflect the low sensitivity of our microarray analysis compared to previous bioassays used to measure the induction of IFN activity during enterovirus infection (Palmer *et al*, 2000). Alternatively, the elevation of IFN-γ in the serum may be explained by production in cells outside the CNS.

The other possible explanation for the absence of any change in the expression of IFN-α and IFN-β is the existence of a specific viral mechanism designed to eliminate the host IFN response. Various mechanisms of resistance to IFN have been reported for different viruses. For example, Kaposi sarcoma–associated herpesvirus encodes a gene product that has homology to the IRF family of proteins and inhibits the response to IFN (Zimring *et al*, 1998). The influenza A NS1 protein inhibits the activation of IRF3 by sequestering double-stranded RNA, which could potentially activate IRF3 and initiate the induction of an antiviral state (Talon *et al*, 2000). Other viruses such as adenovirus and Epstein-Barr, inhibit the IFN-induced PKR by a virus-encoded RNA that binds to PKR and impairs its activation by double-stranded RNA (Gale and Katze, 1998). Because other viruses have evolved a variety of mechanisms to block the antiviral response of IFNs, we speculate that EV71 may also have developed an IFN-interfering mechanism to overcome the host cell defense. The identification and characterization of such a mechanism will need further investigation.

A subset of chemokines is induced in EV71-infected cells

Chemokines are an important functional subgroup of IFN-inducible cytokines that play a role in both normal CNS development as well as in protective and pathogenic responses during CNS viral infection. Chemokines are currently divided into four distinct subfamilies, CXC, CC, C, and CXXXC, according to the number and spacing of the conserved N-terminal cysteines in their receptors (Asensio and Campbell, 1999). Moreover, in addition to bcl-2 family members, cell death signaling molecules, and caspases, certain chemokines, such as fracalkine, Macrophage derived chemokine CCL22 (MDC), regulated upon activation of normal T cell expressed and secreted (RANTES), and macrophage-inducible protein (MIP)-1β, are involved in regulating susceptibility to neuronal apoptosis (Levine, 2002). Therefore, it is notable that CC chemokine members such as RANTES and CXC chemokine member IL-8 were up-regulated in EV71-infected cells. In addition, genes encoding chemokine receptors were up-regulated coordinately in this analysis. RANTES and monocyte chemoattractant protein (MCP)-1 are up-regulated in several other CNS viral infections (Lane *et al*, 2000; Conant *et al*, 1998; Johnston *et al*, 2001). Therefore, the selective increase in the level of RANTES in EV71-infected cells is of particular importance because RANTES has been directly implicated in the pathogenesis of mouse hepatitis virus-induced demyelination (Lane *et al*, 2000).

Furthermore, we performed real time RT-PCR to confirm the up-regulation of these genes. As shown in Figure 5B, the expression of RANTES, IL-8, and chemokine (CC motif) receptor 5 were all increased throughout the infection. This was consistent with the altered expression of these three genes observed in microarray.

Transcription and translation regulation factors in EV71-infected SF268 cells

Several genes involved in transcription and protein synthesis were down-regulated early after EV71 infection. These include nine transcriptional factors, two translation initiation factors, eIF3S9 and eIF2B5, and one signal recognition particle. No translation elongation factors were found to be modulated in this analysis. Most of the transcription factors, such as E2F and telomeric repeating binding factor, were down-regulated.

Some of the genes involved in host transcription, and translation, such as E2F transcription factor 1 (cluster 4), EIF3S9, and EIF2B5 (cluster 5), were shut off early in the infection and then stimulated hours after infection. This suggests that early EV71 infection mediates a reduction of cellular protein synthesis, favoring translation of viral proteins. The newly synthesized viral proteins may then induce an up-regulation of the protein translation machinery of the host cells to synthesize its own proteins, whereas at the same time host cellular protein synthesis is blocked.

The results of our study provide for the first time a global view of the cellular response to EV71 infection. Although the relationships between cellular mRNA levels and the EV71 replication cycle remain unclear, further characterization of the response of individual genes should provide a better understanding of the interaction between virus and host. In addition, further study should focus on which steps of the virus replication cycle (binding, entry, transcription, translation, assembly, or release) are responsible for the host transcriptional changes observed, which viral genes mediate these changes, and whether the host responses identified in this cell culture model are representative of changes seen in the CNS *in vivo*.

Materials and methods

Cells, viruses, and infection conditions
Human glioblastoma cells (SF268) were grown in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS).

SF268 cells were infected with EV 71 at an multiplicity of infection (m.o.i.) of 1 pfu/cell and maintained after infection at 37°C in RPMI medium with 2.5% FCS. Media from infected cultures were harvested at various times and virus yields were measured by plaque formation on Vero cells (Kuo *et al*, 2002).

Microarrays
The cDNA microarrays were made by the Microarray Facility at the Robert Wood Johnson Medical School–University of Medicine and Dentistry of New Jersey in New Brunswick NJ. They contain probes for 10,692 human expressed sequence tags (ESTs/cDNA elements) corresponding to known genes in the GenBank database, printed on nylon membrane. All the EST clones have been sequence verified.

Isolation of RNA, preparation of labeled cDNA, and hybridization
Human glioblastoma cells SF268 were infected with EV71 as described above. At 2 h, 4 h, 5 h, 7 h, 8 h, 9 h, 10 h, 24 h, 28 h, 36 h, 48 h, 51 h, and 54 h post infection, total RNA was extracted from cells using Trizol reagent according to the manufacturer's protocol (Invitrogen). RNA was also extracted from mock-infected cells at the same times. The quality of the RNA samples was monitored by gel electrophoresis.

cDNA probes were synthesized from total RNA and labeled with [^{33}P]dCTP by oligo dT-primed polymerization using Superscript II reverse transcriptase (Invitrogen). Approximately 5 μg of total RNA was used in each reaction. The nucleotide concentrations in the labeling reaction were 0.5 mM for dGTP, dATP, and dTTP, and 0.2 mM for dCTP. Probes were purified by gel chromatography (BioSpin 6; Bio-Rad, Hercules, CA), ethanol-precipitated, and resuspended in 100 μl of TE buffer; an aliquot was withdrawn for determination of incorporation efficiency. Prior to hybridization, the solution was boiled for 2 min, then allowed to cool to room temperature. An equal number of counts (c.p.m.) from each cDNA sample was used for each hybridization. The hybridization was carried out at 46°C over night. At the end of the hybridization, filters were washed for 20 min in 2× SSC (0.03 μ sodium citrate, 0.3 μ sodium chloride) and 0.2% sodium dodecyl sulfate (SDS) at room temperature, then for 30 min in 0.1× SSC and 0.2% SDS at 60°C, after which the membranes were exposed to a phosphorimage screen for an appropriate time.

Signal detection and data analysis
Array images were scanned on Molecular Dynamics Typhoon Phosphorimager at 100 μm resolution (Molecular Dynamics/Amersham Biosciences, Piscataway, NJ). The scanned images were analyzed using the ImaGene software (BioDiscovery, LA, CA) to generate intensity files which contains mean and median of sample and background. The data was then quantified, corrected for background noise, and normalized using the GeneSight software (BioDiscovery, LA, CA). Genes were selected for this analysis if their expression levels in SF268 cells following EV71 infection were different from that in mock-infected cells. Differences in expression levels of genes in SF268 cells following EV71 infection that increased or decreased by a factor of 0.5 at a minimum of two different time points were considered as significant. For those genes whose expression was changed significantly following virus infection, the patterns of gene expression at different times were log transformed, centered by median, and subjected to cluster analyses by centered correlation and average linkage as the similarity/distance metric, using the hierarchical clustering algorithm in Cluster and TreeView software suite developed by Eisen *et al* (1998). This software clusters genes according to their similarity in the pattern of gene expression and displays the data in dendrogram (TreeView).

Real time RT-PCR
Two-step real-time RT-PCR was performed on selected genes to confirm the differential expression results obtained by microarray experiments. The TaqMan reverse transcription kit (Applied Biosystems Group) was used for the reverse transcription step. For each gene, two specific PCR primers were designed using PrimerExpress software (Applied Biosystems, Foster City, CA). The PCR reaction was performed on Opticon (MJ Research, Waltham, MA) using a SYBR Green PCR Core kit (Applied Biosystems, Foster City, CA). Each RT-PCR experiment was performed three times.

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