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Common host genes are activated in mouse brain by Japanese encephalitis and rabies viruses

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This study identified nine genes whose expression is upregulated in the central nervous system (CNS) of mice during Japanese encephalitis virus (JEV) infection. These include: cathepsin S, oligoadenylate synthetase (OAS), GARG49/IRG2, lymphocyte antigen-6A (Ly-6A), macrophage activation gene-2 (Mpa2), early growth response gene1 (Egr1), pyrimidine 5′-nucleotidase (P5N), apolipoprotein D (ApoD) and STAT1. Activation of all nine genes during JEV infection was confirmed by Northern blot analysis. JEV replication was inhibited in the majority of mice immunized with Biken JEV vaccine, and these mice also exhibited reduced expression of JEV-inducible CNS genes. Thus, there is a good correlation between virus load and upregulation of host CNS genes. It was also demonstrated that all the CNS genes activated by JEV are also upregulated during rabies virus infection. In addition, GARG49, STAT1, cathepsin S and ApoD are known to be upregulated in the CNS by Sindbis virus, an alphavirus, and this supports the proposal that common host cell pathways are activated in the CNS by different neurotropic viruses.

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INTRODUCTION

Infection and replication of viruses in vertebrate cells result in the alteration of expression of many cellular genes and these differentially expressed genes can be identified using a variety of techniques such as high-density DNA microarrays, differential display, subtraction hybridization, etc. (Manger & Relman, 2000). Such changes in host gene expression could be a cellular antivirus response, a virus-induced response that is beneficial or even essential for virus survival or a nonspecific response that neither promotes nor prevents virus infection. Global changes in gene expression of virus-infected cells in culture have been reported for several viruses such as human cytomegalovirus (Zhu *et al.*, 1998), herpes simplex virus (Mossman *et al.*, 2001), influenza virus (Geiss *et al.*, 2001a), Kaposi's sarcoma-associated virus (Renne *et al.*, 2001), human papillomavirus (Chang & Laimins, 2000) and human immunodeficiency virus type 1 (Geiss *et al.*, 2001b) etc. However, in the case of neurotropic viruses, which infect the nonrenewable cell populations of the central nervous system (CNS), changes in mRNA expression patterns in normal and virus-infected cells need to be studied in the intact host rather than cells grown in culture. Viruses from several families can infect neurons in the CNS and the study of gene expression changes in the CNS during virus infection can lead to identification of new genes whose function is essential either for the promotion or prevention of virus infection. Such studies have been reported only for a few neurotropic viruses. At least 39 genes are activated in the CNS during rabies virus (RV) infection (Prosniak *et al.*, 2001). In the case of Sindbis virus (SINV), the study of changes in CNS gene expression during virulent and avirulent infection has led to the identification of specific host genes that may play a role in the protective or pathological host response (Johnston *et al.*, 2001).

The family *Flaviviridae* comprises Japanese encephalitis (JE) virus, West Nile virus, yellow fever virus, dengue virus types 1 to 4, tick-borne encephalitis virus and St Louis encephalitis virus (Kuno *et al.*, 1998). Cellular genes such as superoxide dismutase (Liao *et al.*, 2002), mitogen-activated protein kinase and nuclear factor-κB (Su *et al.*, 2002) are activated in JEV-infected cells in culture. However, very little information is available on host gene expression changes induced by flaviviruses in the CNS. In this study, using subtraction hybridization, we have identified nine genes whose expression is upregulated by JEV in mouse brain. We further demonstrate that all these genes are also induced in the CNS by RV. Four of the genes identified in this study are known to be upregulated in the CNS by SINV, an alphavirus. Thus, common host cell pathways are activated during infection of CNS by different neurotropic viruses.

METHODS

Virus infection of mice. JEV strain P20778, an isolate from human brain, was obtained from the National Institute of Virology, Pune, India (Ashok & Rangarajan, 1999). This strain is neurovirulent but not neuroinvasive and is propagated in either the C6/36 *Aedes albopictus* cell line or mouse brain. Intracerebral (i.c.) inoculation of male 6- to 8-week-old outbred Swiss mice with a dose of 10 LD_{50} of the mouse-adapted JEV strain P20778 results in 100 % mortality within 8 to 10 days (Ashok & Rangarajan, 1999). In some experiments, mice were immunized subcutaneously with 100 µl of Biken JEV vaccine (lot no. EJN*173D; Tanabe Seiyaku Co. Ltd, Japan) on day 0 and day 14 and then inoculated (i.c.) with JEV on

day 21 as described (Ashok & Rangarajan, 2000). In the case of RV, i.c. inoculations were done with 80 to 100 LD_{50} of the challenge virus standard (CVS) strain of RV. For peripheral challenge, a virus dose of 1×10^4 i.c. LD_{50} was inoculated into the foot pads of each mouse as described (Biswas *et al.*, 2001). Both peripheral and i.c. inoculation of RV results in 100 % mortality of outbred Swiss mice within 8 to 10 days (Biswas *et al.*, 2001). Mice were sacrificed on days 0 to 6 post-infection (p.i.); their brains were removed, snap-frozen in liquid nitrogen and stored at −80 °C. Uninfected mice or mice inoculated i.c. with saline and sacrificed on day 6 p.i. served as controls.

RNA isolation and analysis. Total RNA was isolated from frozen brain tissues using TRIzol reagent (Invitrogen), and poly(A)$^+$ RNA was isolated using an Oligotex mRNA kit (Qiagen). Northern blot analysis was performed on total brain RNA isolated from normal, saline- or virus-injected mice. RNA (20 μg) was size-fractionated on 1·2 % formaldehyde/agarose gels and transferred to nylon membranes. Radiolabelled cDNA probes were prepared using the Random-primed DNA labelling kit (Amersham Pharmacia). After hybridization and washing, Northern blots were subjected to autoradiography. All blots were first hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to monitor RNA loading and then rehybridized with a probe for the gene encoding the JEV envelope protein (JEVEnv) or RV surface glycoprotein to monitor virus load. JEVEnv gene expression was also analysed by RT-PCR using JEVEnv-specific primers (Ashok & Rangarajan, 1999).

Subtraction hybridization. This was performed using the PCR-Select cDNA Subtraction kit (Clontech) essentially as per the manufacturer's instructions. Poly(A)$^+$ RNA (2 μg) isolated from mice injected i.c. with saline or JEV and sacrificed 6 days later was used as driver and tester respectively. The differentially expressed cDNAs were cloned into pT-Adv vector (Clontech) using the AdvanTAge PCR cloning kit (Clontech). Plasmid DNA was isolated from 100 transformants using the Montage Plasmid Miniprep$_{96}$ kit (Millipore) and the recombinant clones carrying inserts were identified by *Eco*RI digestion of the plasmids. The identity of the genes was established by DNA sequencing and searching the GenBank nucleotide database.

RESULTS

Identification of mouse CNS genes induced during JEV infection

RNA was isolated from the brain tissue of mice at 0, 1, 2, 3, 4, 5, 6 and 7 days after i.c. inoculation of 10 LD_{50} of JEV. JEV gene expression was analysed by Northern blot analysis as well as RT-PCR. The results indicate that the spread of JEV is limited for the first 5 days of infection but rises rapidly on day 6 (Fig. 1B, C). We therefore examined CNS gene expression changes on day 6 p.i. when viral gene expression was maximal. RNA used for subtraction hybridization was isolated from brains of mice injected with JEV or saline and sacrificed 6 days later. The subtracted cDNA was cloned into pT-Adv and transformed into *E. coli*. Restriction enzyme digestion and DNA sequence analysis of plasmids isolated from 100 transformants resulted in the isolation of nine different genes whose identity was established by searching the GenBank nucleotide database (Table 1). Some of these genes have been reported to be upregulated in the CNS by RV (Prosniak *et al.*, 2001) and SINV (Johnston *et al.*, 2001). Upregulation of the host CNS genes during JEV infection

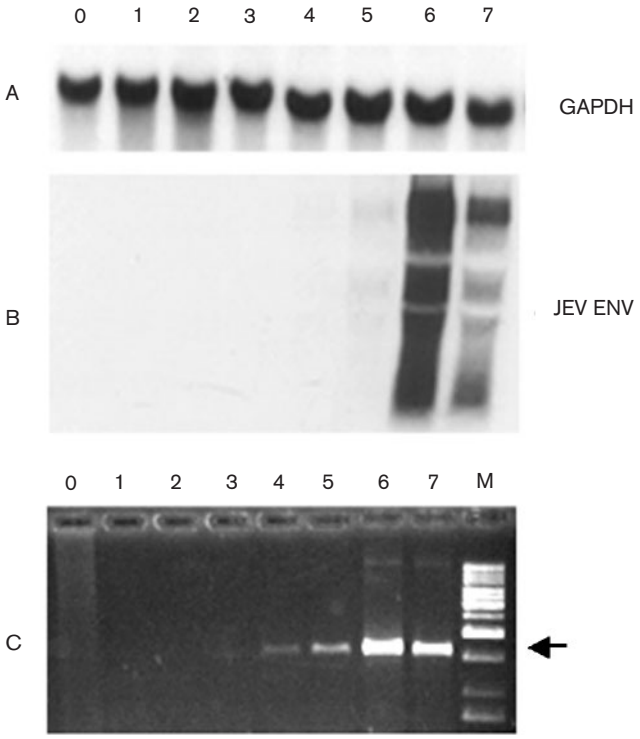

Fig. 1. Analysis of virus load in JEV-infected mouse brain. RNA was isolated on different days after i.c. JEV inoculation and subjected to Northern blot analysis. The blot was probed with either GAPDH (A) or JEVEnv (B) genes. The RNA samples were also analysed by RT-PCR using primers specific for JEVEnv (C). The numbers (0–7) indicate days after i.c. JEV inoculation. Lane M, DNA molecular mass markers.

was further confirmed by Northern blot analysis of RNA isolated from JEV-infected mice at days 3 and 6 p.i. Mice injected with saline and sacrificed 6 days later as well as

Table 1. Identification by subtraction hybridization of mouse CNS genes induced by JEV

Gene	GenBank accession no.
Apolipoprotein D (ApoD)*	L39123
GARG49/IRG2†	U43086
Cathepsin S†	BC011104
STAT1*	U06924
Macrophage activation gene-2 (Mpa2)	BC007143
Early growth response factor-1 (Egr1)	NM_007913.1
Oligoadenylate synthetase (OAS)	NM_011854.1
Lymphocyte antigen-6A (Ly-6A)	NM_010738.1
Pyrimidine 5′-nucleotidase (P5N)	AK011894

*These genes are known to be induced in the CNS by RV (Prosniak *et al.*, 2001) as well as by SINV (Johnston *et al.*, 2001).
†These genes are known to be induced in the CNS by SINV (Johnston *et al.*, 2001).

uninfected mice served as controls. The results presented in Fig. 2 reveal that expression levels of all nine genes are several-fold higher in JEV-infected brains (Fig. 2, lanes 3 and 4) than that of controls (Fig. 2, lanes 1 and 2).

Immunization of mice with Biken JEV vaccine was shown to confer >90 % protection against i.c. JEV challenge (Ashok & Rangarajan, 2000, 2002). We therefore examined host gene expression changes in vaccinated and JEV-infected mice. Comparison of JEV gene expression in vaccinated and unvaccinated mice indicated that JEV RNA was detectable on day 6 p.i. in the three unvaccinated mice (Fig. 3A and C, lanes 6 to 8), but in only one of the three vaccinated mice (Fig. 3B and D, compare lane 6 with lanes 7 and 8). We reasoned that vaccinated mice in which viral gene expression is low or undetectable should also have low levels of virus-induced host genes. Since the expression pattern of JEV-inducible genes in unvaccinated mice has already been studied (Fig. 2), we examined the status of JEV-inducible genes in vaccinated mice. The results indicate that the level of expression of many of the JEV-induced host CNS genes on day 6 p.i. was much lower in mice with low or undetectable JEV RNA than that with high virus load (Fig. 3D and E, compare lanes 7 and 8 with lane 6).

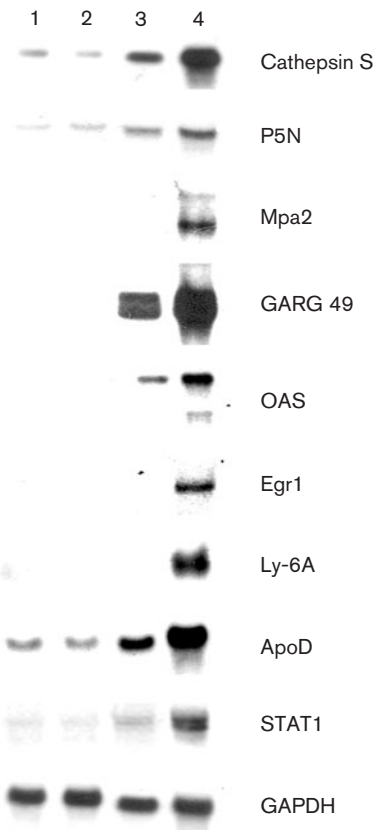

Fig. 2. CNS gene expression changes during JEV infection. Northern blots containing brain RNA isolated from JEV-infected mice at days 3 (lane 3) and 6 (lane 4) p.i. were probed with radiolabelled mouse cDNAs isolated by subtraction hybridization as indicated. RNA isolated from brains of mice injected with saline and sacrificed 6 days later (lane 1) or uninfected mice (lane 2) served as controls. The blots were rehybridized with a probe for GAPDH to monitor RNA loading.

Analysis of CNS gene expression during RV infection

Of the nine genes identified in this study, ApoD and STAT1 are known to be induced in the CNS by both RV (Prosniak *et al.*, 2001) and SINV (Johnston *et al.*, 2001); this alphavirus also induces cathepsin S and GARG49 expression in mouse brain (Johnston *et al.*, 2001). However, there is no report on the activation of Mpa2, Egr1, OAS, Ly-6A and P5N in the CNS by a neurotropic virus. To examine whether these genes are activated by any virus other than JEV, we studied their expression during RV infection of mouse brain. Mice were inoculated with RV either in the foot pads or via the i.c. route as described (Biswas *et al.*, 2001) and sacrificed on days 3 and 6 p.i. Mice inoculated i.c. with saline and sacrificed on day 6 p.i. served as controls. Two mice were sacrificed at every time point and RNA isolated from the brains of these mice was subjected to dot blot analysis (Fig. 4A). RNA from one of these mice was analysed by Northern blotting as well (Fig. 4B). On day 3 p.i., low levels of RV RNA were detectable in the CNS of mice inoculated via the i.c. route but not in those inoculated in foot pads (Fig. 4, compare lanes 2 and 4). A significant increase in the expression of cathepsin S, OAS, GARG49, Ly-6A, Mpa2 and P5N was clearly evident on day 3 p.i. in mice inoculated with RV via the i.c. route but in not those challenged with RV via foot pads (Fig. 4B, compare lanes 2 and 4). On day 6 p.i., when RV genomic RNA became abundant, upregulation of all the CNS genes was observed in mice inoculated with RV via the i.c. route or foot pads. (Fig. 4, lanes 3 and 5). Thus, using two different modes of RV challenge, we clearly demonstrate a direct correlation between virus load in the CNS and upregulation of host CNS genes. Further, all the CNS genes induced by JEV are also induced by RV.

DISCUSSION

Studies on changes in host gene expression during virus infection can be studied using a variety of different techniques and the number of genes isolated depends on the technique used (Manger & Relman, 2000). In this study, we have used subtraction hybridization to identify genes activated in mouse brain by JEV. Cloning of the subtracted cDNA and analysis of the first 100 transformants resulted in the identification of nine genes (Table 1). Northern analysis confirmed that all nine genes are upregulated in JEV-infected mouse brain. We also examined host gene expression changes in mice immunized with Biken JEV vaccine, since vaccination is known to prevent virus replication in brain. The results indicate that on day 6 p.i. only one of the three vaccinated mice developed viraemia in the brain and upregulation of host CNS genes was also observed in the same mouse. Thus, vaccination inhibits virus replication as

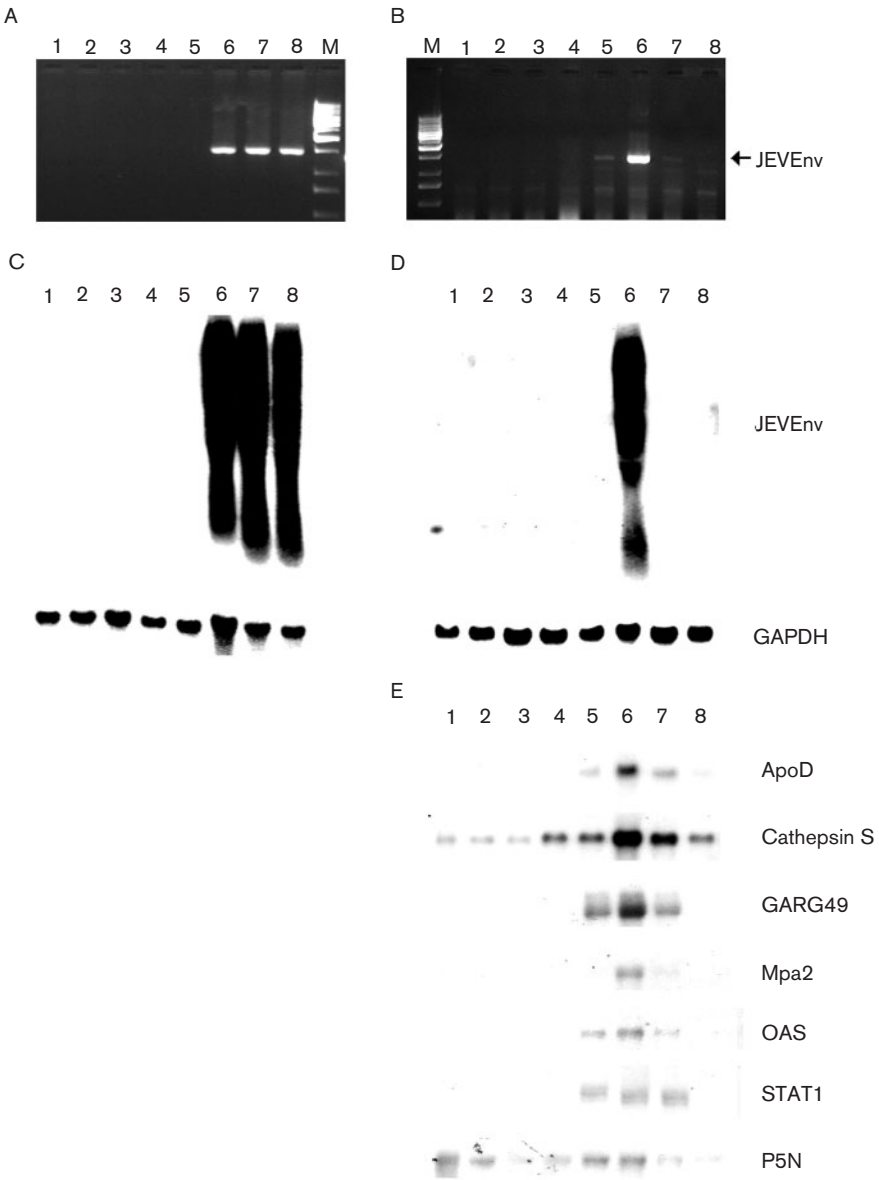

Fig. 3. Effect of immunization on viral and CNS gene expression. RNA was isolated from unvaccinated (A, C) or vaccinated mice (B, D and E) and the virus load was examined at different days p.i. by RT-PCR (A, B) or Northern blot analysis (C, D). Northern blots containing RNA isolated from vaccinated and JEV-infected mice were then probed with different CNS genes as indicated (E). Two mice were injected with saline and sacrificed at day 6 p.i. (lanes 1 and 2), while three mice were injected with JEV and sacrificed at days 3 (lanes 3, 4 and 5) and 6 (lanes 6, 7 and 8) p.i. Lane M, DNA molecular mass markers.

well as virus-induced host gene expression in the CNS. Analysis of CNS gene expression on days 3 and 6 p.i. indicated that the levels of cathepsin S, GARG49, P5N, OAS, ApoD and STAT1 are upregulated even by day 3 p.i. when JEV RNA is barely detectable in the brain and therefore can be designated as early response genes.

Amongst the CNS genes upregulated by JEV, STAT1 is a transcription factor involved in the interferon response pathway and is known to be upregulated during infection by several viruses (Darnell, 1997). Cathepsin S, a lysosomal cysteine protease, is involved in the processing of MHC class II-associated invariant chain in interferon-γ-stimulated microglia (Gresser *et al.*, 2001). Recently, cathepsins were shown to be involved in virus disassembly as well (Ebert *et al.*, 2002). GARG49 is a member of the recently identified glucocorticoid-attenuated response gene (GARG) family, which also includes GARG16 and GARG39 (Smith & Herschman, 1996). GARGs are lipopolysaccharide- and interferon-inducible immediate-early/primary response genes containing multiple tetratricopeptide repeat domains, which are involved in protein–protein interactions in a

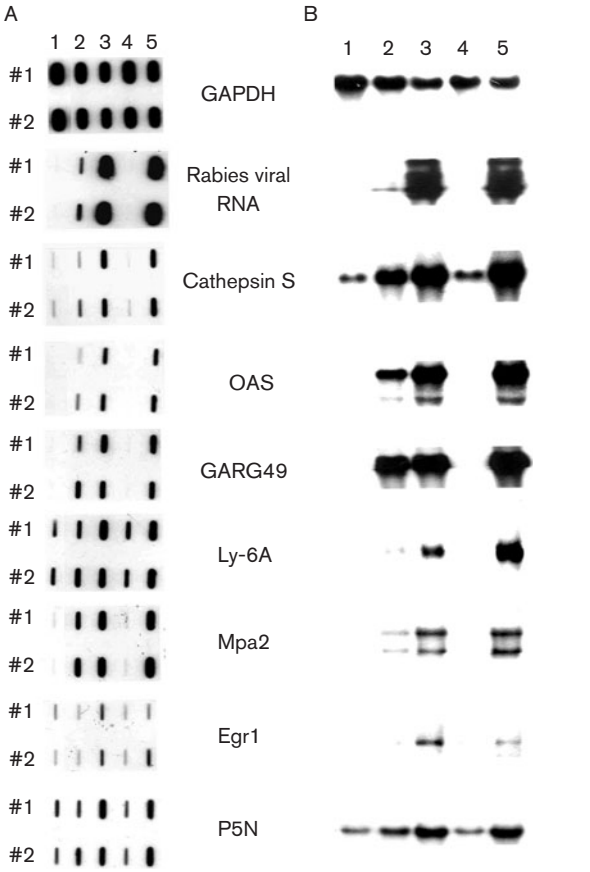

Fig. 4. Upregulation of CNS genes during RV infection. RNA was isolated from brains of uninfected mice (lane 1), mice inoculated with RV via the i.c. route and sacrificed at days 3 (lane 2) and 6 (lane 3) p.i. or mice inoculated with RV in foot pads and sacrificed at days 3 (lane 4) and 6 (lane 5) p.i. Two mice (#1 and #2) were sacrificed at each time point and the brain RNA samples were subjected to slot blot analysis (A). RNA from one of the mice (#1) was subjected to Northern blot analysis (B). Blots were probed with different cDNAs as indicated.

variety of cellular contexts such as mitosis-dependent ubiquitination of cyclin B, transcriptional repression, protein import into mitochondria, etc. (Goebl & Yanagida, 1991; Lamb *et al.*, 1995; Tzamarias & Struhl, 1995). The physiological significance of activation of GARGs in the CNS during virus infection is not clear. The interferon-inducible OAS produces 2′,5′-oligoadenylate, which activates an endogenous ribonuclease involved in the degradation of cellular and viral RNA and leads to reduced protein synthesis (Bonnevie-Nielsen, 1990). While three different isoforms of OAS (OAS1, OAS2 and OAS3) have been identified in humans, the cDNAs so far isolated and characterized in other species, including mouse, rat, pig and chicken, appear to correspond most closely to the OAS1 isoform (Rebouillat & Hovanessian, 1999; Samuel, 2001). In a recent study (Perelygin *et al.*, 2002), mouse strains susceptible to flavivirus infection were shown to produce a truncated form of OAS that is catalytically inactive. Further, a nonsense mutation in the gene encoding the OAS/L1 isoform was shown to be associated with West Nile virus susceptibility in laboratory mice (Mashimo *et al.*, 2002; Samuel, 2002). Thus, the OAS family of genes appears to play an important role in host susceptibility to virus infection and disease. Ly-6A/E is a lymphocyte activation molecule whose expression is inducible by interferon-γ in cultured glial and neuronal cells (Cray *et al.*, 1990). Products of the Ly-6A/E locus encode several low molecular mass cell surface proteins involved in cell signalling and/or cell adhesion processes as well as $CD4^+$ T helper cell proliferation in response to an antigen (Henderson *et al.*, 2002). Thus, upregulation of Ly-6A in the brain during virus infection could be part of the host immune response mounted against the virus. Mpa2 was originally identified as an interferon-γ-inducible gene expressed during the process of macrophage activation (Wynn *et al.*, 1991) and its exact function is not known. Thus, STAT1, OAS, cathepsin S, GARG49, Ly-6A and Mpa2 are interferon inducible-genes upregulated in the CNS during virus infection and may be involved in the host antivirus response.

ApoD is known to be induced in the brain not only during virus infections (Prosniak *et al.*, 2001; Johnston *et al.*, 2001) but also during several neuropathological conditions such as schizophrenia (Thomas *et al.*, 2001a), Alzheimer's disease, (Thomas *et al.*, 2001b) etc. It is considered to be an acute phase protein involved in the removal of lipids during nerve cell degeneration and provision of lipids during the regenerative phase (Reindl *et al.*, 2001). Members of the Egr family of transcription factors are induced by neurotransmitters and neurotrophins and they stimulate the production of many growth factors and cytokines at the site of local tissue injury (Levkovitz & Baraban, 2002). Egr3, another member of the Egr family of proteins was also shown to be induced in the CNS by RV (Prosniak *et al.*, 2001). Thus, the high virus load on day 6 p.i. may result in tissue injury leading to activation of Egr expression. P5N is involved in the hydrolysis of dCMP and UMP and its expression is induced several-fold during erythropoiesis (Hokari *et al.*, 1998). Thus, upregulation of P5N may result in an increase in the cellular mononucleotide pool, which may facilitate virus replication in the brain.

Of the JEV-induced CNS genes identified in this study, Mpa2, Egr1, OAS, Ly-6A and P5N have not been reported to be induced by any other neurotropic virus. Therefore we examined their expression in the CNS during RV infection. The results indicate that all the CNS genes induced by JEV are induced by RV as well. Thus, we have identified seven new RV-inducible CNS genes in this study which have not been reported earlier (Prosniak *et al.*, 2001). As observed in the case of JEV infection, expression of cathepsin S, GARG49, P5N and OAS is induced early during RV infection when the virus load in the brain is very low, further confirming that these are likely to be early response genes. Finally, we conclude that many CNS genes are activated by

Table 2. Host CNS genes induced by at least two neurotropic viruses

JEV-induced genes were identified in this study. RV-induced genes were identified in both this study and by Prosniak *et al.* (2001). SINV-induced genes were identified by Johnston *et al.* (2001). ND, Not determined.

Gene	JEV	RV	SINV
ApoD	+	+	+
STAT1	+	+	+
Cathepsin S	+	+	+
GARG49/IRG2	+	+	+
Mpa2	+	+	ND
Egr1	+	+	ND
OAS	+	+	ND
Ly-6A	+	+	ND
P5N	+	+	ND
IRF7	ND	+	+

more than one virus (Table 2) suggesting that common host cell pathways are activated in the CNS during infection by different neurotropic viruses.

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