Journal of NeuroVirology (1999) 5, 161–171
© 1999 Journal of NeuroVirology, Inc.
http://www.jneurovirol.com

A mechanism for selective induction of 2′-5′ oligoadenylate synthetase, anti-viral state, but not MHC Class I genes by interferon-beta in neurons

Paul T Massa[1,2,3], Laurie W Whitney[1,4], Charlene Wu[2], Stacie L Ropka[2,3] and Keith W Jarosinski[3]

[1]Neuroscience program; [2]Department of Neurology and [3]Department of Microbiology and Immunology, State University of New York Health Science Center, Syracuse, New York, USA

Interferon-inducible expression of major histocompatibility class I genes has previously been found to be quantitatively and functionally deficient in neurons compared to other somatic cells or other neural cell types including astrocytes. This deficiency is a key component of neuronal immunoprivilege during viral infections of the CNS. To the contrary, in the present study, induction of functional antiviral state by IFN-β in neurons compared to astrocytes was found to be highly efficient with respect to both viral replication and protection from cytopathic effects. A candidate antiviral state gene found to be efficiently induced in neurons by IFN-β was the 2′-5′-oligoadenylate synthetase (OAS) gene. Unlike MHC class I genes, induction of OAS was comparable in neurons and astrocytes indicating differential expression in these neural cell types. Analysis of OAS gene promoter activity indicated that induction of the OAS gene by IFN-β was dependent on a region containing the interferon stimulated responsive element (ISRE). In contrast, a construct containing the MHC class I-ISRE responsible for induction by IFN-β in astrocytes was not responsive to IFN-β in neurons. Therefore, transcription factor binding to the OAS- and MHC-ISREs was analyzed. While the OAS and MHC Class I site bound equal amounts of the transcriptional repressor IRF-2, the OAS-ISRE preferentially interacted with the transcriptional activator ISGF3 in response to IFN-β. Further, unlike neurons, upregulation of MHC class I genes in astrocytes was related to binding of IRF-1 instead of IRF-2 to the MHC-ISRE. It is proposed that selective activation of anti-viral state genes compared to MHC class I genes by IFN-β in neurons is mediated by preferential induction and binding of ISGF3 to anti-viral state gene ISREs but not the MHC-ISRE.

Keywords: central nervous system infections; virus replication; enhancer elements; gene expression regulation; signal transduction; transcription factors

Introduction

Exposure of cells to interferons results in the induction of multiple genes important for the general host response to virus infection. Through various intracellular mechanisms, the actions of these interferon-inducible genes both promote cellular anti-viral immune responses by induction of MHC and accessory adhesion molecules in the infected tissue and establish an antiviral state in which viral replication is inhibited (Landolfo *et al*, 1995; Sen and Ransohoff, 1993; Hovanessian, 1991). Many interferon-inducible genes including MHC class I, (2′-5′) oligoadenylate synthetase (OAS), the double-stranded RNA-dependent protein kinase (PKR), ISG15, guanylate binding protein (GBP), and ISG54 contain *cis*-acting interferon stimulated response elements (ISREs) in their promoter regions (Benech *et al*, 1987; Cohen *et al*, 1988; Lew *et al*, 1991; Levy *et al*, 1988; Saunders *et al*, 1985; Rutherford *et al*, 1988). ISREs, characterized by repeats of the hexamer AAGTGA and a consensus motif of GGYAAAY (A/T) GAAACTY (Levy *et al*, 1988), are the primary promoter elements responsible for regulation of antiviral state genes. ISREs bind the transcription factor interferon stimulated gene factor 3 (ISGF3) (Fu *et al*, 1992), as well as

Correspondence: PT Massa, Department of Neurology, SUNY Health Science Center, 750 East Adams St., Syracuse, New York, 13210, USA
[4]Present address: Neuroimmunology Branch, National Institutes of Health, Bethesda, Maryland 20892, USA
Received 15 July 1998; revised 11 August 1998; accepted 18 August 1998

interferon regulatory factors 1 and 2 (IRF-1 and IRF-2) (Harada *et al*, 1989; Coccia *et al*, 1995). ISGF3 is a transcriptional activation factor consisting of tyrosine phosphorylated STAT1α and STAT2 complexed as a heterotrimer with IRF family member ISGF3γ/p48 (Fu *et al*, 1992). ISGF3γ is so named because it is substantially increased in cells after pre-treatment with IFN-γ and therefore allows much greater induction of ISGF3 upon subsequent treatment with either IFN-α/β. IRF-1 is also a positive regulator of interferon-inducible gene transcription (Tanaka *et al*, 1993), whereas IRF-2 acts as either a competitive inhibitor for binding of IRF-1 (Harada *et al*, 1990) or directly as a transcriptional repressor, consequently blocking induction of genes by IRF-1 (Yamamoto *et al*, 1994). IRF-1 induction by interferons is transcriptionally regulated and depends mainly on an enhancer (γ-activation site, GAS) that binds to STAT1α homodimers (γ-activated factor, GAF). Thus, the obligate role of STAT1α in antiviral responses (Horvath and Darnell, 1996; Durbin *et al*, 1996; Ihle, 1996; Bandyopadhyay *et al*, 1995) is reflected in its presence in transcription factor complexes that act on antiviral state genes.

It has previously shown that in neurons, unlike in other cell types, neither ISGF3 nor IRF-1 is induced in response to interferons when probed with the MHC class I ISRE (Ward and Massa, 1995). The lack of IRF-1 in neurons was found to be transcriptionally regulated and most likely accounted for the lack of MHC class I expression (Massa and Wu, 1995). The latter was in agreement with related studies on the regulation of MHC class I molecules and IRF-1 (Drew *et al*, 1993; 1995a; Kumar *et al*, 1997; Hobart *et al*, 1997). Moreover, upon exposure to double stranded RNA, the transcriptional repressor IRF-2 was uniquely induced to high levels in neurons and probably further contributed to suppresssion of the MHC class I genes (Ward and Massa, 1995; Drew *et al*, 1995b). Surprisingly, however, we have found that antiviral state was efficiently induced in neurons in response to interferons, suggesting that the molecular mechanisms for induction of antiviral state genes may be selective for this particular subset of genes. The latter suggested the distinct possibility that interferons were able to induce transcription factors that selectively acted on genes responsible for blocking viral replication but not on MHC class I genes.

The expression of antiviral state genes that interfere with viral replication in neurons may be particularly important because neurons do not express MHC class I molecules. Thus, whereas lack of MHC class I molecules on neurons allows these nonregenerative cells to escape immunopathological damage during viral infection (Joly *et al*, 1991; Joly and Oldstone, 1992; Massa *et al*, 1993; Sedgwick and Dorries, 1991), induction of antiviral state by IFNs or other distinct mechanisms may be critical for protecting these cells against direct cytopathic effects of the virus and reduction of virus spreading (Levine *et al*, 1991; 1996; Mokhtarian *et al*, 1996; Tucker *et al*, 1996; Schijns *et al*, 1991; Ward and Massa, 1995; Mucke and Oldstone, 1992). The latter is in accordance with findings that neurons produce large amounts of IFN-β upon viral infection and that this IFN-β acts in an autocrine and paracrine fashion to promote antiviral state but not MHC class I molecules (Ward and Massa, 1995). This finding was of interest because MHC class I, IFN-β, and antiviral state genes are commonly coregulated by trans-acting factors acting on related ISREs (Massa *et al*, 1993; Benech *et al*, 1987; Lenardo *et al*, 1989; Fan and Maniatis, 1989; Korber *et al*, 1988; Israel *et al*, 1989) and because type I interferons including IFN-β have been shown to be critical in controlling neurotropic virus infections in the brain (Fiette *et al*, 1995; Steinhoff *et al*, 1995). Therefore, we wanted to determine how particular antiviral state genes could be induced by IFN-β in neurons in a way that would not induce MHC class I molecules. To address this issue, we chose to study the expression of the OAS gene because of its regulation, like MHC class I genes, by an important ISRE in response to IFN-β and its well characterized role in suppressing replication of viruses of particular neuropathological significance (Sriram *et al*, 1989; Kumar *et al*, 1988).

Results

Efficient induction of antiviral state by IFN-β in neurons and astrocytes

To determine whether recombinant IFN-β was able to induce antiviral state in neurons and protect against cytopathic effects (CPE), cultures were treated with IFN-β and then infected with encephalomyocarditis virus (EMCV). Astrocytes were treated in parallel cultures for comparison. Without IFN-β pretreatment, EMCV caused extensive CPE in both neurons and astrocytes by 18 h post-infection at a multiplicity of infection of 1 (Figure 1) and resulted in the production of relatively high levels of infectious virions (Figure 1). However, treatment of neurons and astrocytes with IFN-β for 4 h prior to infection protected the cells against both CPE and viral replication (Figure 1). Interestingly, IFN-β-treated neurons appeared more resistant than astrocytes to both virus replication and CPE following EMCV infection if treated with IFN-β after, rather than before, infection (Figure 1). Compared to neurons, astrocytes showed little change in virus production relative to treatment timing but showed a profoundly increased susceptibility to CPE when treated after infection. Thus, protection from virus replication and CPE may be at least partly separable events in these cells. This possibility was supported by the observation that even though neurons and astrocytes were equally protected from CPE in the pre-treatment group,

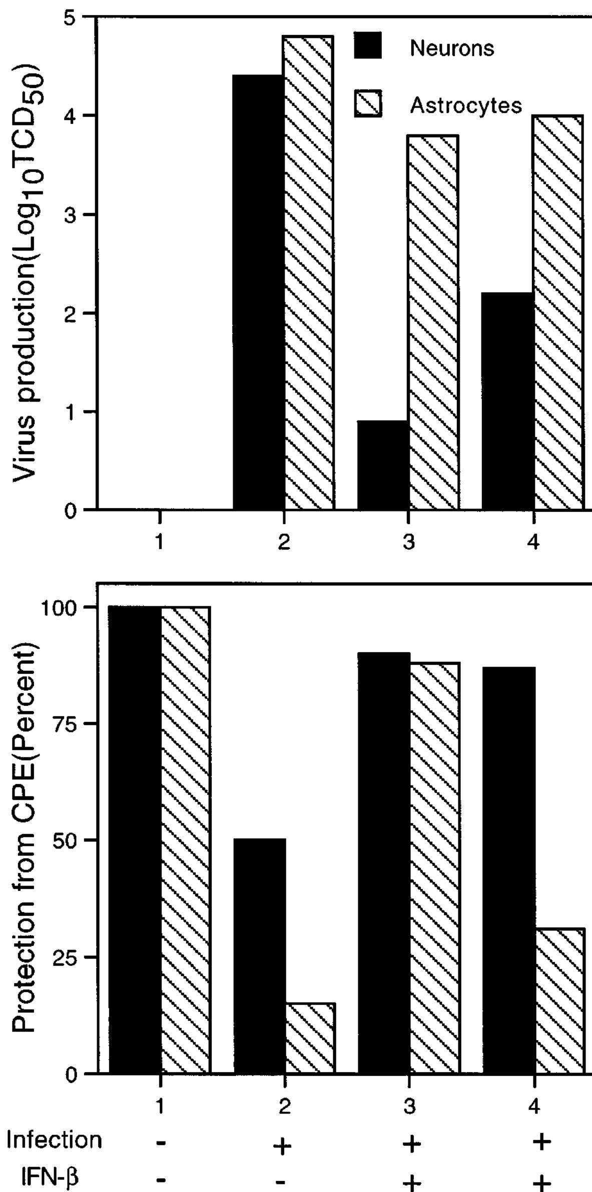


Figure 1 Induction of antiviral state and protection from CPE in neurons and astrocytes infected with EMCV. In groups 1–3, neurons and astrocytes were treated with 0 and 1000 U/mL of recombinant murine IFN-β for 4 h and then infected with EMCV. In group 4, cultures were first infected with EMCV then subsequently treated with 1000 U/mL of IFN-β to assess the relative pace of anti-viral state induction in the cells. Eighteen hours after infection, both virus production and CPE were quantified by the MTS assay.

virus replication was nearly three orders of magnitude higher in astrocytes than in neurons in this group. In sum, these data indicated a relatively rapid onset of anti-viral state in neurons.

Selective OAS gene induction in neurons

A number of antiviral state genes induced by IFN-β are probably responsible for resisting EMCV replication and cell death in the neuronal cultures. However, analysis was initially focused on the regulation of the OAS gene because of its critical role in resisting EMCV replication (Coccia *et al*, 1990; Rutherford *et al*, 1996; Kumar *et al*, 1988; Gribaudo *et al*, 1991). As expected from previous studies, RT–PCR analysis revealed little induction of MHC class I genes in RNA extracts from neurons compared to that seen from astrocytes (Figure 2). However, despite the lack in both constitutive and inducible expression of MHC class I genes in neurons, OAS mRNA was induced to about the same level in neurons and astrocytes (Figure 2). These data indicated that IFN-β preferentially affected OAS gene promoter activity in neurons and transcriptional regulation most likely accounted for differential induction of the OAS and MHC class I genes in these cells.

Selective responsiveness of ISRE-containing promoters to IFN-β in neurons

It has been shown that the region from −109 to −89 bp upstream of both the human and mouse OAS gene promoters, which contains a functional ISRE, is responsible for interferon-inducible expression of these genes (Benech *et al*, 1987; Ghosh *et al*, 1991; Rutherford *et al*, 1991). We therefore utilized CAT constructs (Figure 3A) to assess the role of these sequences in neurons. Primary cultures of neurons were transfected with either CAT constructs containing the endogenous OAS promoter region with the OAS-ISRE enhancer or with an identical construct lacking the ISRE. An MHC class I promoter construct containing the related ISRE was transfected in parallel in neurons and astrocytes. Transfected cells were treated with either 1000 U/ml of IFN-β or medium alone and processed for CAT activity 24 h after treatment. While the full length OAS promoter CAT construct was strongly inducible by IFN-β by approximately tenfold (Figure 3B), the construct lacking the OAS-ISRE was not induced. Also, the MHC class I construct containing the MHC-ISRE was not induced by IFN-β in neurons (Figure 3B), however the same construct was induced by approximately fivefold in astrocytes in response to IFN-β (Massa and Wu, 1995). This indicated that the region from −159 to −83 of the OAS gene, which contains the functional ISRE, is responsible for the IFN-β-inducible expression of the OAS gene construct in neurons but that a related site in the MHC class I construct is specifically silent in neurons.

ISGF3 is induced in neurons treated with IFN-β

Because neither IRF-1 nor ISGF3 was detected in neurons in past studies using the MHC class I gene ISRE as the probe, it was initially unclear how the OAS gene was induced in neurons in response to IFN-β. Therefore gel shift assays utilizing radiolabeled oligonucleotides corresponding to the OAS

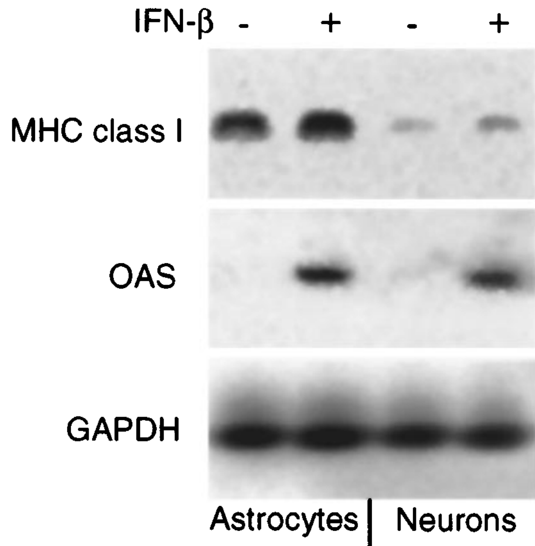
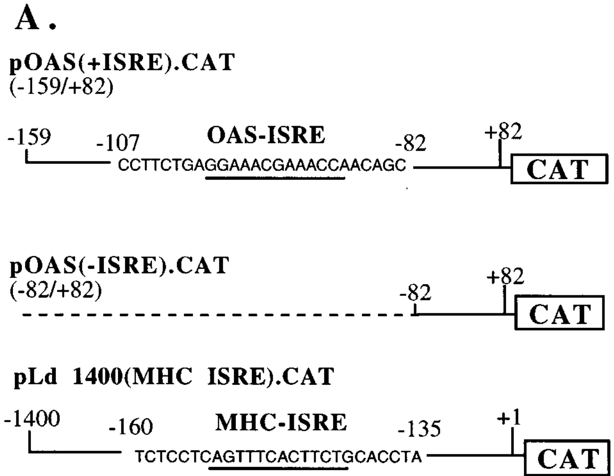


Figure 2 Comparison of induction of MHC class I and OAS gene mRNA in neurons and astrocytes treated with 1000 U/mL of IFN-β for 8 h. RT–PCR was performed on the same RNA extracts using primers for either OAS, MHC class I, or GAPDH as constitutive control.

and MHC class I ISREs were performed to identify possible site-specific factor binding. Both the OAS and MHC class I ISREs bound equally to IRF-2 which was slightly increased following IFN-β treatment and reacted specifically with an antiserum to IRF-2 (Figure 4). However, the OAS-ISRE bound preferentially to a highly inducible activity that was barely detectable with the MHC-ISRE probe (Figure 4). This activity had characteristics consistent with ISGF3 including relatively low mobility compared to IRF-2 and specific reactivity to antisera to STAT1α but not with antisera to STAT3, IRF-1, or IRF-2 (Figure 4). Also consistent with the ISGF3-like nature of the complex was the independence of protein synthesis for induction by IFN-β. ISGF3 binding to the OAS-ISRE was rapidly induced to approximately the same levels by IFN-β in the presence or absence of the protein synthesis inhibitor cycloheximide (Figure 4). However, as expected, cycloheximide effectively blocked the increase of IRF-2 in the same extracts (Figure 4), consistent with its dependence on transcription and translation for induction.

Figure 1 demonstrated that induction of antiviral state was efficient in neurons despite the lack of MHC class I induction after treatment with IFN-β. Therefore, we proposed that MHC class I induction may relate to IRF-1 expression in astrocytes whereas OAS induction may depend on ISGF3 activation in both astrocytes and neurons. Therefore, neurons and astrocytes were treated with IFN-β and induction of both IRF-1 and ISGF3 was



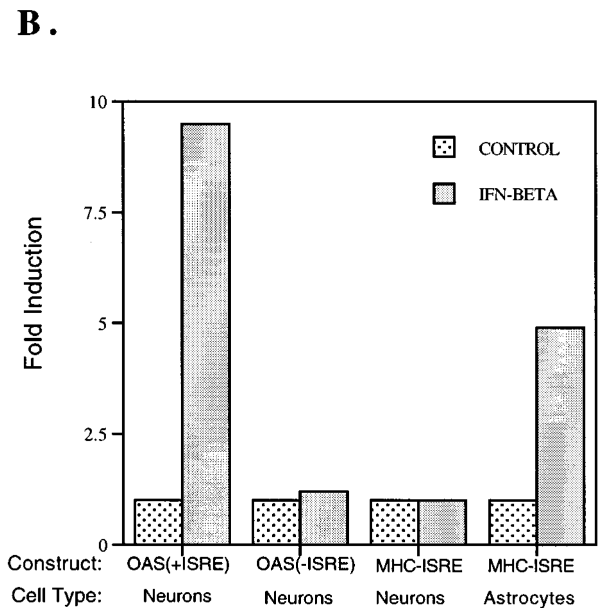


Figure 3 Induction of the (2′-5′) oligoadenylate synthetase (OAS) gene requires a promoter region containing an ISRE sequence (underlined). (**A**) OAS CAT constructs that either contain the ISRE sequence [OAS (+ISRE)], or do not [OAS (−ISRE)]. The pOAS (+ISRE). CAT plasmid construct contains sequences between +82 and −159 relative to the mRNA start site of the OAS gene (see Materials and methods). The pOAS (−ISRE). CAT construct is so designated with the '−' (negative) as it lacks the sequences between −82 and −159 including the ISRE. A CAT construct containing the related ISRE (underlined) of an MHC class I gene promoter is also shown. (**B**) Primary cultures of neurons and astrocytes were transfected with the CAT constructs shown above and treated with either 1000 U/mL IFN-β or media alone for 24 h. CAT enzyme activity was then analyzed by thin layer chromatography and quantified on a scintillation counter. Fold induction is relative to control cultures not treated with IFN-β with an induction ratio of approximately 1 compared to a promoterless CAT construct. Absolute CAT activity in control cultures was equal between CAT constructs and cells. CAT induction represents the average of two independent experiments.

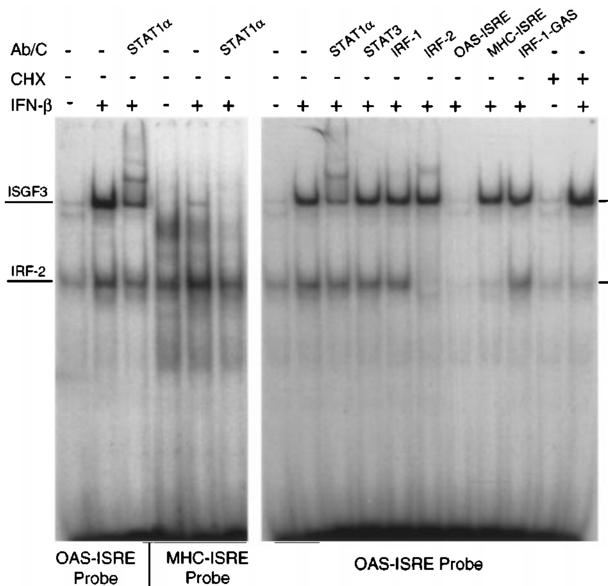


Figure 4 Induction, characterization, and specific binding of ISGF3 activity to the OAS-ISRE analyzed by gel mobility shift. Left panel: Neurons were treated with 1000 U/mL IFN-β for 2 h then processed for gel mobility shift analysis using either the OAS- or MHC class I- ISRE probe. Antibodies to STAT1α were added to some of the reactions as indicated to identify STAT1α-containing activities. Right: Same extracts and treatments as on the left but probed with the OAS-ISRE oligonucleotide and various antibodies and unlabeled oligonucleotide competitors as indicated at the top of the gel. Bands corresponding to either ISGF3 or IRF-2 are indicated. Ab/C: Antibody or unlabeled competitor oligonucleotides added to individual reactions as shown at the top of the gel. CHX: 50 μg/ml cycloheximide was added to some of the cultures with IFN-β to test dependence of induction of the ISGF3 and IRF-2 on protein synthesis.

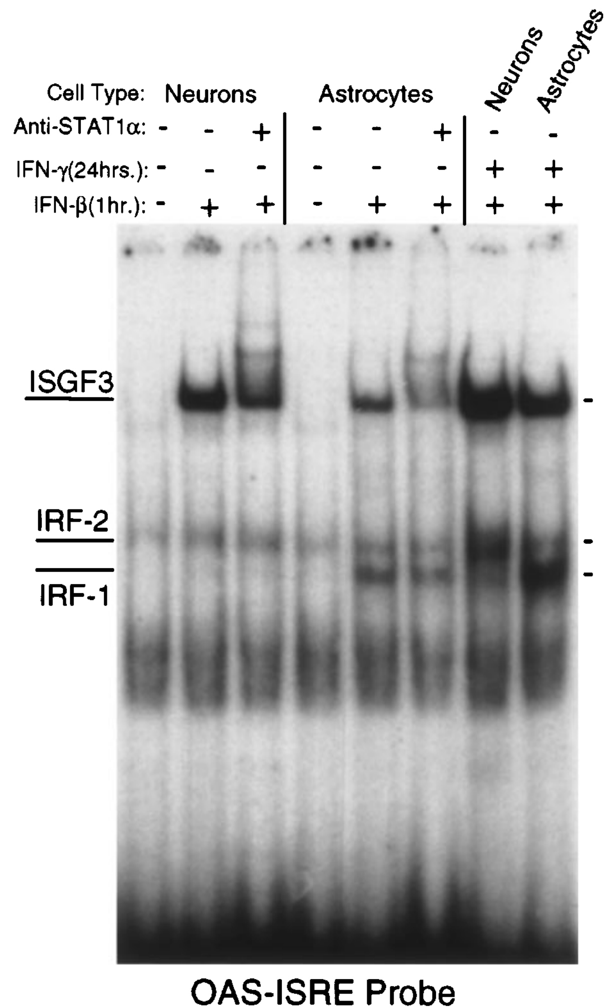


Figure 5 Analysis of relative levels of ISGF3 in neurons and astrocytes induced with IFN-β. Neurons and astrocytes were treated with 1000 U/mL IFN-β for 1 h then probed using the OAS-ISRE. ISGF3 is reactive to the antiserum to STAT1α as labeled. IRF-2 and IRF-1 in extracts are also labeled. In the last two lanes, neurons and astrocytes were treated with 100 Units/mL recombinant murine IFN-γ for 24 h prior to the 1 h stimulation with 1000 U/mL IFN-β to increase both IRF family members IRF-1 and ISGF3γ/p48.

analyzed. After IFN-β treatment, astrocytes expressed somewhat lesser amounts and ISGF3 than neurons. However, astrocytes, pretreated for 24 h with IFN-γ, followed by a 1 h pulse with IFN-β expressed about the same level of ISGF3 as neurons (Figure 5) indicating that IFN-γ was able to prepare astrocytes to respond to IFN-β perhaps by increasing ISGF3γ. IFN-γ pretreatment also further increased ISGF3 binding activity in response to IFN-β in neurons (Figure 5). However, IFN-γ pretreatment also led to much higher expression of IRF-1 in astrocytes compared to neurons (Figure 5). Higher expression of IRF-1 in astrocytes was also seen with cultures treated with IFN-β alone. The data taken together are consistent with the preferential activation of ISGF3 binding to the OAS-ISRE in neurons and of IRF-1 in astrocytes in response to IFN-β.

Relatively high binding activity of ISGF3 to the OAS-ISRE

As shown in Figure 4, ISGF3 bound to the OAS-ISRE with apparent high affinity compared to the MHC-ISRE. Also, while ISGF3 was readily competed from the OAS-ISRE probe with unlabeled OAS-ISRE, little competition was seen with excess unlabeled MHC class I-ISRE oligonucleotide (Figure 4). To further verify the selective binding of ISGF3 to the OAS-ISRE, competition assays were performed using increasing concentrations of ISRE competitors (Figure 6). As expected, the OAS-ISRE competed well at all concentrations for ISGF3 activity whereas the MHC class I site did not compete well even at a 500-fold excess to the probe. A related ISRE from the ISG15 gene also was able to compete for ISGF3 as previously reported (Reich and Darnell, 1989) further indicating a specific deficiency of the

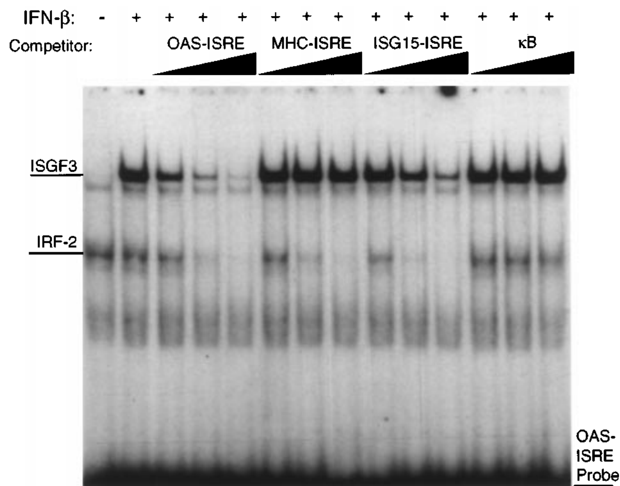


Figure 6 Analysis of differential binding activity of ISGF3 and IRF-2 in neurons by titration against various unlabeled competitor ISRE oligonucleotides as indicated at the top of the gel. The OAS-ISRE was used as the labeled probe. Extracts were produced from neurons treated for 1 h with 1000 U/mL IFN-β. The ramp above the lanes indicates increasing unlabeled competitor oligonucleotide to probe ratios in the order of 20-fold, 100-fold, and 500-fold excess competitor. Bands corresponding to ISGF3 and IRF-2 are labeled. The κB site of the IRF-1 gene promoter region is used as a non-specific competitor oligonucleotide.

MHC-ISRE site to bind to ISGF3. However, the MHC class I site competed as well as the other ISREs for IRF-2 binding (Figure 6) indicating that the sequence requirements for IRF-2 and ISGF3 factor binding were distinct and highly selective.

The data taken together indicated that neurons expressed relatively high levels of ISGF3 in response to IFN-β while astrocytes expressed more IRF-1. The latter is in accordance with the relative expression of the OAS and MHC class I genes in neurons and astrocytes, respectively (Figure 1) (Massa *et al*, 1993; Ward and Massa, 1995). In summary, it is proposed that strong preferential interaction of IFN-β-inducible ISGF3 with the OAS-ISRE relative to MHC class I ISRE is responsible for selective activation of the OAS gene and anti-viral state in neurons.

Discussion

OAS gene regulation was chosen for study because OAS has been shown to be active against EMCV replication and therefore may be relevant for antiviral state to neurotropic picornavirus infections in neurons (Williams *et al*, 1979; Rutherford *et al*, 1996; Coccia *et al*, 1990). Further, because both OAS and MHC class I genes are regulated by related ISREs, these studies were important to uncover possible mechanisms for differential activity of the ISREs in the two genes in neurons. In the current study it was found that neurons strongly expressed the OAS but not MHC class I genes when exposed to IFN-β and it was confirmed that the regulation of the OAS gene in neurons, as in other cells, occurs through the promoter region containing the ISRE. Furthermore, it was found that neurons utilized a mechanism for OAS gene induction in whch ISGF3 was induced by IFN-β and bound selectively to the OAS-ISRE thereby allowing induction of anti-viral state and silence of the MHC class I genes.

In accordance with the present observations, ISGF3 displays different binding specificities to various ISREs and related sequences (Veals *et al*, 1993). Whereas IRFs including IRF-1, IRF-2, and ISGF3γ/p48 generally require only the central nine nucleotide core of the ISRE (Tanaka *et al*, 1993; Veals *et al*, 1993), heightened binding affinity of ISGF3γ/p48 to the ISRE can be achieved through flanking sequences with apparent affinities for ISGF3γ/p48-associated STATs (Veals *et al*, 1993). In this way, the inherent weak binding of p48 relative to other IRFs to the core element is increased dramatically by the binding of STATs to these flanking sequences (Bluyssen *et al*, 1997). In particular, STAT1α appears to have high binding affinity for sequences containing the GAS half-site trinucleotide TTC located outside and at the border of the core IRF binding site (Decker *et al*, 1997; Bluyssen *et al*, 1997). Interestingly, the two oligonucleotide sequences, OAS-ISRE and ISG15-ISRE that bound to ISGF3 in the present studies contained a TTC trinucleotide just adjacent and 5′ to the core IRF element but not seen in either strand of the MHC class I ISRE flanking sequences (Figure 7). Preliminary studies using a probe with the TTC sequence deleted but the core OAS-ISRE intact, showed that ISGF3 binding was entirely lost but that IRF-2 binding remained intact and similar to that seen with the MHC class I ISRE (unpublished observations, PT Massa).

Therefore, a tentative model is proposed in which ISGF3 binds with high affinity and specificity to the OAS-ISRE but not the MHC-class I ISRE. This binding is imparted by synergistic interactions of both STAT1α with the TTC sequence of the GAS-like half site of the OAS-ISRE and of ISGF3γ/p48 with the core IRF-element (IRF-E) (Figure 7). Rather, the MHC-class I ISRE is presently considered to be a highly selective interferon regulatory factor element (IRF-E) to denote its selective affinity for IRFs similar to the PRDI site of the IFN-β gene which binds solely to IRFs-1 and -2 but not to ISGF3 (Veals *et al*, 1993). Thus, the selectivity of ISGF3 binding to the OAS-ISRE over that of the MHC class I site is not entirely unexpected but has to our knowledge not been previously characterized nor its functional importance elucidated. The preferential expression of ISGF3 and IRF-2 in neurons and the strong binding of ISGF3 to the OAS-ISRE in response to

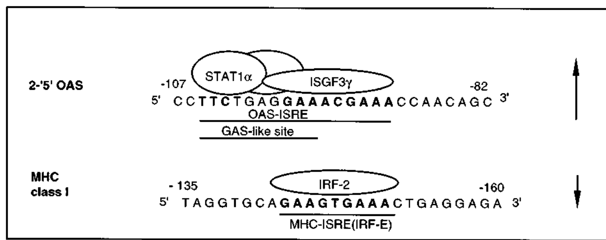


Figure 7 Summary of selective transcription factor/ISRE interactions consistent with present observations and previous reports. It is proposed that the OAS-ISRE and MHC class I-ISRE have identical binding affinity for IRFs including IRF-1, IRF-2, and perhaps ISGF3γ/p48 monomers. The ability of ISGF3γ/p48 to complex with STAT1α imparts selective high affinity for the OAS-ISRE that is most likely related to flanking sequences at the 5′ and/or 3′ border of the core IRF-E(9-base bold type sequence) as previously reported (see discussion). It is hypothesized that STAT1α binds to both ISGF3γ/p48 and to a TTC trinucleotide GAS half-site (bold trinucleotide) located immediately 5′ to the IRF-E site to which ISGF3γ/p48 binds. At the MHC class I-ISRE, IRF-2 is the predominant factor bound in neurons, whereas IRF-1 predominates in astrocytes. The latter is consistent with a mechanism for cell-specific transcriptional repression and activation of MHC class I and OAS genes, respectively (arrows), in neurons, and activation of both genes in astrocytes. It is proposed that the MHC site may not provide appropriate flanking sequences for ISGF3 binding beyond the core IRF-E (MHC-IRF-E) responsible for sole IRF-1/2 binding.

IFN-β suggests a specialized utilization of these specificities to achieve selective gene expression in response to interferons that is theoretically advantageous.

Accordingly, IFN-β treatment of neurons led to a slight increase in IRF-2 binding to the MHC class I-ISRE but not of the ISGF3 binding activity. In cells besides neurons that express MHC class I genes in response to IFN-β (e.g., astrocytes), the outcome is distinctly different in that induction of IRF-1 rather than IRF-2 occurs (Figure 5) (Massa and Wu, 1995; Ward and Massa, 1995). Consistent with the latter, it has been shown that induction of IRF-1 is critical for expression of MHC class I genes in parenchymal epithelial cells (Hobart *et al*, 1997; Chang *et al*, 1992) including those of neural origin, such as astrocytes (Massa and Wu, 1995). Therefore, the expression of IRF-2 and binding to the MHC class I-ISRE in neurons may be of extreme importance in the selective repression of MHC class I genes in these cells in response to interferons (Figure 7). This selective repression is thought to be important for maintaining immunoprivilege during viral infections of the central nervous system (Oldstone, 1997; Oldstone *et al*, 1986; Joly *et al*, 1991; Joly and Oldstone, 1992; Massa *et al*, 1993; Rall *et al*, 1995). Additionally, cell-specific expression of ISRE binding factors and sequence specific ISRE affinities for these factors is likely to be the basis for differential expression of ISRE-regulated genes in response to IFN-β in neural cells.

Materials and methods

Cell culture

Primary cultures of neurons were derived from the cerebella of 8-day-old NIH Swiss mice (Harlan-Sprague Dawley, Indianapolis, IN, USA) as previously described (Massa *et al*, 1993; Meier and Schousboe, 1982). Briefly, cerebella were dissected, minced, trypsinized, and subsequently plated onto tissue culture dishes. Neurons were transfected at 1 day following plating and/or treated with IFN-β at 2 days post-plating. The cells were then utilized in assays as indicated. Astrocytes were prepared from newborn NIH Swiss mice (Harlan-Sprague Dawley, Indianapolis, IN, USA) as previously described (Massa *et al*, 1992, 1993) and the cultures were fed at 5 days post-plating. At 6–8 days after plating the cultures were transfected with CAT constructs, treated with interferon-β, and processed to either extract mRNA for Northern blots or nuclear proteins for gel mobility shift assays.

Culture reagents

Recombinant murine interferon-β (Calbiochem, San Diego, CA, USA) and IFN-γ (R+D Systems, Minneapolis, MN, USA) was used at either 10 100, or 1000 U/mL as indicated. Encephalomyocarditis virus (EMCV) (American type culture collection, Rockville, MD, USA) was used at 2.5×10^3 PFU/mL.

RNA extraction and PCR analysis

Total cellular RNA was extracted from neurons and astrocytes by a guanidine isothiocyanate technique (Chomczynski and Sacci, 1987). Five micrograms of RNA were used in reverse transcriptase polymerase chain reactions (RT–PCR) utilizing the Superscript preamplification system (Life Technologies/Gibco-BRL, Gaithersburg, MD, USA) and PCR primers listed below. cDNA from RT–PCR reactions were resolved on a 1% agarose gel, stained with ethidium bromide, and quantified from digitized photographs (Un-scan-it, Silk Scientific, Orem, UT, USA). GAPDH primers were used for internal control standards (Clontech Laboratories, Inc., Palo Alto, CA, USA). Mouse OAS gene (Ichii *et al*, 1986) primers used for RT–PCR of the OAS mRNA (corresponding to the 43 kD form of OAS) (Ichii *et al*, 1986; Benech *et al*, 1985) were purchased (Biosynthesis, Inc., Lewisville, TX, USA): forward 5′-GGTGGAGTTTGATGTGCTG-3′; reverse 5′-GGTCCAGGATCACAGGCCTG-3′. MHC class I gene primers were: forward 5′-GAGCTTGTGGAGACCAGGC-3′ and reverse 5′-GCTCAGGCAGCCCCTTATG-3′.

CAT constructs and assay

MHC class I and OAS promoter CAT constructs shown in Figure 3A have previously been described (Segars *et al*, 1993; Benech *et al*, 1987; Cohen *et al*, 1988). The promoter sequences of these constructs

are connected to a chloramphenicol-acetyl-transferase (CAT) gene used to measure promoter activity (Gorman *et al*, 1982). Neurons were transfected with 3 μg of either construct using the DEAE-dextran method (McCutchan and Pagano, 1968). Cultures were also transfected with pCH-110, an SV40-β-galactosidase gene construct (Pharmacia, Inc., Piscataway, NJ, USA) as a control for transfection efficiency. Samples were analyzed using thin layer chromatography and CAT activity was measured using a scintillation counter.

Gel shift assays and oligonucleotide probes
Nuclear proteins were prepared from neurons and astrocytes as previously described (Massa *et al*, 1992; Lee *et al*, 1988). Protein concentrations were determined using the Biorad protein assay (Biorad Laboratories, Richmond, CA, USA). The following duplex oligonucleotide probes and competitors were purchased (Bio-synthesis, Inc., Lewisville, TX, USA): OAS-ISRE 5′-CCTTCTGAGGAAACGAAACCAA-3′ (Benech *et al*, 1987; Cohen *et al*, 1988); MHC-ISRE 5′-GTTAGGTGCAGAAGTGAAACTGA-3′ (Miyazaki *et al*, 1986); ISG15-ISRE 5′-AAGGGAAACCGAAACTGAA-3′ (Reich and Darnell, 1989); IRF-1-κB 5′-TGGGGAATCCCGC-3′ (Harada *et al*, 1994).

DNA probes for transcription factor binding reactions were prepared by end-labeling DNA with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). Binding of nuclear proteins to duplex oligonucleotide probes was analyzed using a gel shift mobility assay (Fried and Crothers, 1981; Garner and Rezvin, 1981; Massa *et al*, 1993). Radiolabeled DNA probes (30 000 c.p.m./ngDNA/reaction) were incubated with 2.5–15 μg of nuclear extract in the presence of 0.5 μg of poly(dI:dC) (Pharmacia, Inc., Piscataway, NJ, USA) for 40 min. To further characterize proteins, some reaction mixtures also had either competitor oligonucleotides or antibodies to transcription factors added before the labeled probe, and were incubated for an additional 30 min. Reaction mixtures were electrophoresed on a 4% polyacrylamide gel and autoradiographs of the gels were photographed.

Antibodies to transcription factors
Antibodies to IRF-1 and IRF-2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and antibodes to STAT1α and STAT3 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA) and used at a concentration of 2 μg IgG/reaction.

MTS antiviral assays
For antiviral assays, neurons and astrocytes were treated with the indicated amounts of IFN-β or medium alone for 4 h, washed, and subsequently inoculated with encephalomycarditis virus for 1 h. The inoculum was removed by repeated rinses, plain medium was reapplied, and cell supernatants containing virus were harvested 18 h after infection. In some experiments, cultures were infected without prior IFN-β treatment then exposed to exogenous IFN-β after infection to analyze the relative effectiveness of post-infection treatment. Cytopathic effects on neurons and astrocytes was analyzed directly on these cells by the MTS assay (Khabar *et al*, 1996). The MTS assay measures the number of viable hydrogenase-containing cells remaining in cultures as the result of CPE that convert MTS, a tetrazolium dye, to a water-soluble formazan as quantified by optical density. Released virus in supernatants (virus production) of neural cell cultures was assayed on Vero cells also by MTS assay. The latter was performed by inoculating Vero cells for 1 h with either astrocyte or neuronal virus supernatants at multiple dilutions, washing the inoculum from the cells, and then measuring viral cytopathic effects the next day with a MTS cell viability assay (Promega, Madison, WI, USA) and computing the tissue culture dose that give 50% cell death (TCD_{50}) (Reed and Muench, 1983). Twenty microliters of the MTS solution was added to each well of the microtiter plate and plates were incubated 3 h at 37°C and, subsequently, optical densities of cell cultures were measured using an ELISA reader. In some wells no cells were plated and color change in these wells was subtracted from each reading as a measure of background activity.

Acknowledgements

This work was supported by a grant from the National Multiple Sclerosis Society.

References

Bandyopadhyay SK, Leonard Jr, GT, Bandyopadhyay T, Stark GR, Sen GC (1995). Transcriptional induction by double-stranded RNA is mediated by interferon-stimulated response elements without activation of interferon-stimulated gene factor 3. *J Biol Chem* **270:** 19624–19629.

Benech P, Mory Y, Revel M, Chebeth J (1985). Structure of two forms of the interferon-induced (2′-5′) oligo A synthetase based on cDNAs and gene sequences. *EMBO J* **4:** 2249–2256.

Benech P, Vigneron M, Peretz D, Revel M, Chebath J (1987). Interferon-responsive regulatory elements in the promoter of the human 2′, 5′-oligo(A) synthetase gene. *Mol Cell Biol* **7:** 4498–4504.

Bluyssen HAR, Durbin JE, Levy DE (1997). ISGF3γ, p48, a specificity switch for interferon activated transcription factors. *Cytokine and Growth Factor Reviews* **7:** 11–17.

Chang CH, Hammer J, Loh JE, Fodor WL, Flavell RA (1992). The activation of major histocompatibility complex class I genes by interferon regulatory factor-1 (IRF-1). *Immunogenetics* **35:** 378–384.

Chomczynski P and Sacci N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159.

Coccia EM, Romeo G, Nissim A, Marziali G, Albertini R, Affabris E, Battistini A, Fiorucci G, Orsatti R, Rossi GB, Chebath J (1990). A full-length murine 2-5A synthetase cDNA transfected in NIH-3T3 cells impairs EMCV but not VSV replication. *Virol* **179:** 228–233.

Coccia EM, Marziali G, Stellacci E, Perrotti E, Ilari R, Orsatti R (1995). Cells resistant to interferon-beta respond to interferon-gamma via the Stat1-IRF-1 pathway. *Virol* **211:** 113–122.

Cohen B, Peretz D, Vaiman K, Benech P, Chebath J (1988). Enhancer-like interferon responsive sequences of the human and murine (2′-5′) oligoadenylate synthetase gene promoters. *EMBO J* **7:** 1411–1419.

Decker T, Kovarik P, Meinke A (1997). GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. *J Interferon and Cytokine Res* **17:** 121–134.

Drew PD, Lonergan M, Goldstein ME, Lampson LA, Ozato K, McFarlin DE (1993). Regulation of MHC class I and β2-microglobulin gene expression in human neuronal cells. *J Immunol* **150:** 3300–3310.

Drew PD, Franzoso G, Becker KG, Bours V, Carlson LM, Siebenlist U, Ozato K (1995a). NFκB and interferon regulatory factor 1 physically interact and synergistically induce major histocompatability class I gene expression. *J Interferon and Cytokine Res* **15:** 1037–1045.

Drew PD, Franzoso G, Carlson LM, Biddison WE, Siebenlist U, Ozato K (1995b). Interferon regulatory factor-2 physically interacts with NF-kappa B in vitro and inhibits NF-kappa B induction of major histocaompatability class I and beta 2-microglobulin gene expression in transfected human neuroblastoma cells. *J Neuroimmunol* **63:** 157–162.

Durbin JE, Hackenmiller R, Simon MC, Levy DE (1996). Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84:** 443–450.

Fan C-M, Maniatis T (1989). Two different virus-inducible elements are required for human β-interferon gene regulation. *EMBO J* **8:** 101–110.

Fiette L, Aubert C, Muller U, Huang S, Aguet M, Brahic M, Bureau J-F (1995). Theiler's virus infection of 129Sv mice that lack the interferon α/β or interferon γ receptors. *J Exp Med* **181:** 2069–2076.

Fried M, Crothers DM (1981). Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res* **9:** 6505–6525.

Fu X-Y, Schindler C, Improta T, Aebersold R, Darness Jr, JE (1992). The proteins of ISGF-3, the interferon α-induced transcriptional activator, define a gene family involved in signal transduction. *Proc Natl Acad Sci USA* **89:** 7840–7843.

Garner M, Rezvin A (1981). A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: applications to components of the E. coli lactose operon regulatory system. *Nucleic Acids Res* **9:** 3047–3060.

Ghosh SK, Kusari J, Bandyopadhyay SK, Samanta H, Kumar R, Sen GC (1991). Cloning, sequencing, and expression of two murine 2′-5′-oligoadenylate synthetases. Structure-function relationships. *J Biol Chem* **266:** 15293–15299.

Gorman C, Moffat L, Howard B (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2:** 1044–1051.

Gribaudo G, Lembo D, Cavallo G, Landolfo S, Lengyel P (1991). Interferon action: binding of viral RNA to the 40-kilodalton 2′-5′-oligoadenylate synthetase in interferon-treated HeLa cells infected with encephalomyocarditis virus. *J Virol* **65:** 1748–1757.

Harada H, Fukita T, Miyamoto M, Kimura Y, Maruyama M, Furia A, Miyata T, Taniguchi T (1989). Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell* **58:** 729–739.

Harada H, Willison K, Sakakibara J, Miayamoto M, Taniguchi T (1990). Absence of the type I IFN system in EC cells: Transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. *Cell* **63:** 303–312.

Harada H, Takahashi E, Itoh S, Harada K, Hori TA, Taniguchi T (1994). Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. *Mol Cell Biol* **14:** 1500–1509.

Hobart M, Ramassar V, Goes N, Urmson J, Halloran PH (1997). IFN regulatory factor-1 plays a central role in the regulation of the expression of class I and II MHC genes in vivo. *J Immunol* **158:** 4260–4269.

Horvath CM, Darnell Jr, JE (1996). The antiviral state induced by alpha interferon and gamma interferon requires transcriptionally active Stat1 protein. *J Virol* **70:** 647–650.

Hovanessian AG (1991). Interferon-induced and double-stranded RNA-activated enzymes: a specific protein kinase and 2′, 5′-oligoadenylate synthetases. *J Interferon Res* **11:** 199–205.

Ichii Y, Fukunaga R, Shiojiri S, Sokawa Y (1986). Mouse 2-5A synthetase cDNA: nucleotide sequence and comparison to human 2-5A synthetase. *Nucleic Acids Res* **14:** 10117.

Ihle JN (1996). STATS: Signal transducers and activators of transcription. *Cell* **84:** 331–334.

Israel A, Le Bail O, Hatat D, Piette J, Kieran M, Logeat F, Wallach D, Fellous M, Kourilsky P (1989). TNF stimulates expression of mouse MHC class I genes by inducing an NF kappa B-like enhancer binding activity which displaces constitutive factors. *EMBO J* **8:** 3793–3800.

Joly E, Mucke L, Oldstone MBA (1991). Viral persistence in neurons explained by lack of major histocompatibility class I expression. *Science* **253:** 1283–1285.

Joly E, Oldstone MB (1992). Neuronal cells are deficient in loading peptides onto MHC class I molecules. *Neuron* **8:** 1185–1190.

Khabar KSA, Al-Zoghaibi F, Dzimiri M, Taha M, Al-Tuwaijri A, Al-Ahda N (1996). MTS interferon assay: A simplified cellular dehydrogenase assay for interferon activity using a water-soluble tetrazolium salt. *J Interferon and Cytokine Res* **16:** 31–33.

Korber B, Mermod N, Hood L, Stroynowski I (1988). Regulation of gene expression by interferons: control of H-2 promoter responses. *Science* **239:** 1301–1306.

Kumar A, Yang Y-L, Flati V, Der S, Kadereit S, Deb A, Haque J, Reis L, Weissman C, Williams BRG (1997). Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: role of IRG-1 and NF-κB. *EMBO J* **16:** 406–416.

Kumar R, Choubey D, Lengyel P, Sen GC (1988). Studies on the role of the 2′-5′-oligoadenylate synthetase-RNase L pathway in beta interferon-medaited inhibiton of encephalomyocarditis virus replication. *J Virol* **62:** 3175–3181.

Landolfo S, Gribaudo G, Angeretti A, Gariglio M (1995). Mechanisms of viral inhibition by interferons. *Pharmac Ther* **65:** 415–442.

Lee KA, Bindereif A, Green MR (1988). A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene Anal Tech* **5:** 22–31.

Lenardo MJ, Fan C-M, Maniatis T, Baltimore D (1989). The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell* **57:** 287–294.

Levine B, Hardwick JM, Trapp BD, Crawford TO, Bollinger RC, Griffin DE (1991). Antibody-mediated clearance of alphavirus infection from neurons. *Science* **254:** 856–860.

Levine B, Goldman JE, Jiang HH, Griffin DE, Hardwick JM (1996). BCL-2 protects mice against fatal alphavirus encephalitis. *Proc Natl Acad Sci USA* **93:** 4810–4815.

Levy DE, Kessler DS, Pine R, Reich N, Darnell Jr, JE (1988). Interferon-induced nuclear factors that bind a shared promoter element correlate with psoitive and negative transcriptional control. *Genes & Development* **2:** 383–393.

Lew DJ, Decker T, Strehlow I, Darnell JE (1991). Overlapping elements in the guanylate-binding protein gene promoter mediate transcriptional induction by alpha and gamma interferons. *Mol Cell Biol* **11:** 182–191.

Massa PT, Hirschfeld S, Levi B-Z, Quigley LA, Ozato K, McFarlin DE (1992). Expression of major histocompatibility complex (MHC) class I genes in astrocytes correlates with the presence of nuclear factors that bind to constitutive and inducible enhancers. *J Neoroimmunol* **41:** 35–42.

Massa PT, Ozato K, McFarlin DE (1993). Cell type-specific regulation of major histocompatibility complex (MHC) class I gene expression in astrocytes, oligodendrocytes, and neurons. *Glia* **8:** 201–207.

Massa PT, Wu H (1995). Interferon regulatory factor element and interferon regulatory factor 1 in the induction of major histocompatibility complex class I genes in neural cells. *J Interferon and Cytokine Res* **15:** 799–810.

McCutchan JH, Pagano JG (1968). Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylamino-ethyl-dextran. *J Natl Cancer Inst* **41:** 351–356.

Meier E and Schousboe A (1982). Differences between GABA receptor binding to membranes from cerebellum during postnatal development and from cultured cerebellar granule cells. *Dev Neurosci* **5:** 546–553.

Miyazaki J-I, Appella E, Ozato K (1986). Negative regulation of the major histocompatibility class I gene in undifferentiated embryonal carcinoma cells. *Proc Natl Acad Sci USA* **83:** 9537–9541.

Mokhtarian F, Wesselingh SL, Choi S, Maeda A, Griffin DE, Sobel RA (1996). Production and role of cytokines in the CNS of mice with acute viral encephalomyelitis. *J Neuroimmunol* **66:** 11–22.

Mucke L, Oldstone MB (1992). The expression of major histocompatibility complex (MHC) class I antigens in the brain differs markedly in acute and persistent infections with lymphocytic choriomeningitis virus (LCMV). *J Neuroimmunol* **36:** 193–198.

Oldstone MBA, Blount P, Southern PJ, Lampert PW (1986). Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. *Nature* **321:** 239–243.

Oldstone MBA (1997). How viruses escape from cytotoxic T lymphocytes - molecular parameters and players. *Virol* **234:** 179–185.

Rall GF, Mucke L, Oldstone MBA (1995). Consequences of cytotoxic T lymphocyte interactions with major histocompatibility complex class I-expressing neurons in vivo. *J Exp. Med* **182:** 1201–1212.

Reed LJ, Muench M (1983). A simple method for estimating fifty percent endpoints. *Amer J Hyg* **27:** 493–497.

Reich NC, Darnell Jr JE (1989). Differential binding of interferon-induced factors to an oligoncuelotide that mediates transcriptional activation. *Nucleic Acids Res* **17:** 3415–3424.

Rutherford MN, Hannigan GE, Williams BR (1988). Interferon-induced binding of nuclear factors to promoter elements of the 2-5A synthetase gene. *EMBO J* **7:** 751–759.

Rutherford MN, Kumar A, Nissim A, Chebath J, Williams BR (1991). The murine 2-5A synthetase locus: three distinct transcripts from two linked genes. *Nucleic Acids Res* **19:** 1917–1924.

Rutherford MN, Kumar A, Coulombe B, Skup D, Carver DH, Williams BRG (1996). Expression of intracellular interferon constitutively activates ISGF3 and confers resistance to EMC viral infection. *J Interferon and Cytokine Res.* **16:** 507–510.

Saunders ME, Gewert DR, Tugwell ME, McMahon M, Williams BRF (1985). Human 2-5A synthetase: characterization of a novel cDNA and corresponding gene structure. *EMBO J* **4:** 1761–1768.

Schijns VE, Van der Neut R, Haagmans BL, Bar DR, Schellekens H, Horzinek MC (1991). Tumor necrosis factor-alpha, interferon-gamma and interferon-beta exert antiviral activity in nervous tissue cells. *J Gen Virol* **72:** 809–815.

Sedgwick JD, Dorries R (1991). The immune system response to viral infection of the CNS. *Sem Neurosci* **3:** 93–100.

Segars JH, Nagata T, Bours V, Medin JA, Franzoso G, Blanco JC, Drew PD, Becker KG, An J, Tang T, Stephany DA, Neel B, Siebenlist U, Ozato K (1993). Retinoic acid induction of major histocompatibility complex class I genes in NTera-2 embryonal carcinoma cells involves induction of NF-κB (p50–p65) and retinoic acid receptor-beta-retinoid X receptor beta heterodimers. *Mol Cell Biol* **13:** 6157–6169.

Sen GC, Ransohoff RM (1993). Interferon-induced antiviral actions and their regulation. *Adv Virus Res* **42:** 57–102.

Sriram S, Topham DJ, Huang SK, Rodriguez M (1989). Treatment of encephalomyocarditis virus-induced central nervous system demyelination with monoclonal anti-T-cell antibodies. *J Virol* **63:** 4242–4248.

Steinhoff U, Muller U, Schertler A, Hengartner H, Aguet M and Zinkernagel RM (1995). Antiviral protection by vesicular stomatitus virus-specific antibodies in alpha/beta interferon receptor-deficient mice. *J Virol* **69:** 2153–2158.

Tanaka N, Kawakami T, Taniguchi T (1993). Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. *Mol Cell Biol* **13:** 4531–4538.

Tucker PC, Griffin DE, Choi S, Bui N, Wesselingh S (1996). Inhibition of nitric oxide synthesis increases mortality in sindbis virus encephalitis. *J Virol* **70:** 3972–3977.

Veals SA, Santa Maria T, Levy DE (1993). Two domains of ISGF3γ that mediate protein-DNA and protein-protein interactions during transcription factor assembly contribute to DNA-binding specificity. *Mol Cell Biol* **13:** 196–206.

Ward LA, Massa PT (1995). Neuron-specific regulation of major histocompatibility complex class I, interferon-β, and anti-viral state genes. *J Neuroimmunol* **58:** 145–155.

Williams BRG, Golgher RR, Brown RE, Gilbert CS, Kerr IM (1979). Natural occurrence of 2-5A in interferon-treated EMC virus-infected L cells. *Nature* **282:** 582–586.

Yamamoto H, Lamphier MS, Fujita T, Taniguchi T, Harada H (1994). The oncogenic transcription factor IRF-2 possesses a transcriptional repression and a latent activation domain. *Oncogene* **9:** 1423–2428.