ABSTRACT

SMOAK, KATHLEEN A. The Cardiac Interferon Response To Reovirus Infection.
(Under the direction of Dr. Barbara Sherry)

Viral myocarditis may be caused by a number of different viruses, and the mechanisms of pathogenicity can be both immune mediated and/or due to direct cytopathic effect. While extensive research has been done regarding immune-mediated mechanisms, the mechanisms of direct cytopathic effect remain largely unexplored. Reovirus induced murine myocarditis is not immune mediated, providing for an excellent disease model. Reovirus induction of, and sensitivity to, interferon-beta (IFN-β) has previously been found to be an important determinant for protection against viral myocarditis. There are many interferon regulatory factors that can both regulate, and be regulated by, IFN-β. In the first part of this dissertation, transient transfections of primary cardiac myocyte cultures (PCMCs) were used to investigate the role of interferon regulatory factor-1 (IRF-1) in regulation of IFN-β. We found that while IRF-1 is downstream of the IFN response, it still plays an important protective role against viral myocarditis. In the next chapter, reovirus induction of Interferon Stimulated Genes (ISGs) was investigated in PCMCs and compared with a more generalized cell type, primary murine embryonic fibroblasts (PMEFs). Here we found that reovirus induction of ISGs is cell type-specific and interferon-mediated, yet levels of induction are discordant with viral induction of IFN. These results suggested that cell types that are prone to induction of IFN are resistant to induction of ISGs. In appendix I, reovirus induction of an Interferon Stimulated Response Element (ISRE) was investigated in the above mentioned cell types, providing insight into reovirus induction of ISGs and the cell
type-specific response. In appendix II, the effect of IRF-1 on ISG induction was further examined by overexpressing IRF-1. These data indicated that IRF-1 could regulate ISGs and ISRE sequences, but that IFN may induce or activate a product that inhibits IRF-1 induction of these genes. In the final appendix, reovirus induction of the ISG, IRF-7, was investigated in PCMCs and PMEFs using real-time PCR. Here, we found that reovirus induction of IRF-7 is discordant with reovirus induction of IFN. Possible mechanisms of differential ISG induction are discussed in the summary of this dissertation to provide an overall picture of the cardiac IFN response to virus infection.
THE CARDIAC INTERFERON RESPONSE TO REOVIRUS INFECTION

by

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DEDICATION:

To my parents, whose undying support has seen me through graduate school and who have always believed I would “make it,” even if I wasn’t so sure. To my sister, who was always there when I needed her and taught me to keep an open mind. To my brothers, for growing up and becoming my friends. To my husband, Chip, for helping me to realize how lucky I am and for always knowing what I needed, even if it was just a trip to the Waffle House. To all of my friends, who helped me keep things in perspective. And to Catsby, for making me laugh when I needed it the most.
PERSONAL BIOGRAPHY

Kathleen Mary Azzam was born in Utica, New York in 1972, but soon after, her family moved to Ohio, where she spent most of her childhood in the small towns of Galion and Marion. In 1982, she and her family moved to Raleigh, North Carolina, where she met the friends she would keep for life. After graduation from high school, Kathleen began her undergraduate studies at The University of North Carolina at Chapel Hill where she worked in a lab investigating herpes simplex virus. After receiving her Bachelor of Science degree in biology with a minor in chemistry in 1995, Kathleen worked as a technician at both the Lineberger Cancer Research Center and UNC hospitals and volunteered as an EMT at the South Orange Rescue Squad. During this time, she took several microbiology courses that guided her decision to pursue graduate school. In the fall of 1998, Kathleen entered North Carolina State University as a graduate student in the Microbiology Department under the direction of Dr. Barbara Sherry. Here she continued her studies of virus-host interactions and pursued a minor in biotechnology. On June 2, 2001, Kathleen married Chip Smoak, who was pursuing his Ph.D. degree in English at the University of North Carolina at Greensboro. Kathleen and Chip moved to Carrboro, North Carolina, and presently live happily with their lovable and amazing cat, Catsby. After her defense, Kathleen and Chip will enjoy a long awaited vacation to Europe, after which Kathleen will begin working the NIEHS in Research Triangle Park, N.C.
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LITERATURE REVIEW

REOVIRUS

The observation that reovirus-infected cells stain with the dye acridine orange led to
the discovery that reovirus RNA is double-stranded, and this was the first evidence for the
occurrence of dsRNA in nature (56). The reovirus dsRNA genome exists in the form of 10
discrete segments (reviewed (127)). The large (L) dsRNA segments encode three large (λ)
proteins, and the three medium sized (M) segments encode two structural proteins (µ1, µ2)
and one nonstructural protein (µNs). The four small (S) dsRNA segments encode three
structural proteins (σ1, σ2, σ3) and two nonstructural proteins (σns, σ1s). Each segment
encodes a single protein with the exception of the S1 gene segment. The S1 mRNA is
functionally dicistronic, and encodes two proteins in separate reading frames (43).

The reovirus virion is organized into both an inner and outer capsid, and proteolytic
cleavage of the outer capsid is important for activation and replication. The outer capsid
proteins involved in attachment and entry are µ1C, σ3, and σ1. Most (95%) of the µ1 outer
capsid protein exists in the form µ1C, generated by an assembly-related cleavage. During
receptor-mediated endocytosis, the µ1C fragment undergoes additional cleavage to produce δ
and φ, and this cleavage has been found to be important for penetration of cell membranes
(175). The outer capsid protein, σ3 is thought to play a role in assembly, but must be
removed by proteolysis for infection to proceed. The σ1 protein is the cell–attachment
protein (90).
The core proteins involved in replication are \( \mu_2 \), \( \lambda_2 \), and \( \lambda_3 \) (127). \( \mu_2 \) is an RNA binding protein (16) associated with the viral core RNA transcription complex (127). The \( \lambda_3 \) protein and \( \lambda_2 \) protein are both also involved in RNA synthesis (reviewed in (127). The \( \lambda_2 \) protein is a guanylyl transferase (105), and forms pentameric channels from the reovirus capsid core for extrusion of newly synthesized RNA (41, 145). The \( \lambda_3 \) protein is an RNA polymerase (39).

Reoviruses can target a variety of organs in the mouse, including the gastrointestinal tract, brain, pancreas, muscle, heart, lungs, and liver (reviewed in (192)). Interestingly, the tropism is virus-strain specific, with serotypes 1 (Lang) and serotype 3 (Dearing) localizing to different areas. In the gut, reovirus type 1 grows well in the intestinal tissue after oral inoculation, whereas reovirus serotype 3 does not (15). In the brain, type I is localized to ependymal cells, while type 3 is localized to neurons (204). In the endocrine part of the pancreas, serotype 3 infects the insulin-producing beta cells, while serotype 1 produces focal necrosis of the pancreatic islets causing animals to develop autoantibodies to insulin (132).

The segmented genome allows generation of reassortant reoviruses, containing a mixture of two parent viruses, allowing the identification of viral genes that determine phenotypes \textit{in vitro} and \textit{in vivo}. Reassortant reoviruses containing various combinations of genes derived from serotype 1 (Lang) and serotype 3 (Dearing), indicate that the S1 and L2 genes determine the difference in the capacity of reovirus type 1 and type 3 to survive in the gut (15). Also using these reassortants, it can be shown that the capacity of reoviruses to infect neural cells (type 3 pattern) or ependymal cells (type 1 pattern) in culture is a property of the S1 gene (204). The S1 gene has also been found to be the primary determinant for the
production of anti-insulin antibodies, which have been detected with serotype 1 but not serotype 3 (132). Studies using one efficiently myocarditic reovirus variant (designated 8B) suggest that its phenotype is determined predominantly by the M1 gene, coding for the μ2 viral core protein, although other genes can modify the disease (see viral myocarditis) (171).

VIRAL MYOCARDITIS

Viral myocarditis affects an estimated 5-20% of the human population (210). It is often fatal in infants (20, 110), and although it is usually resolved in adults, it can progress to chronic myocarditis, dilated cardiomyopathy, and cardiac failure (38, 111, 121, 178). Many viruses have been implicated in this disease, including picornaviruses, orthomyxoviruses, paramyxoviruses, togaviruses, rhabdoviruses, arenavirus, poxvirus, herpesviruses, and adenoviruses (210). Recently, with the re-administration of the smallpox vaccine, seven cases of cardiac adverse events have arisen (two cases of myocarditis), leading to a growing concern amongst vaccinees with pre-existing heart problems (71).

Enteroviruses including coxsackie viruses, are responsible for up to 50% of all cases of human viral myocarditis. Enterovirus-induced myocarditis is predominantly immune cell-mediated, and is characterized by inflammatory infiltrates of macrophages and lymphocytes (25, 27, 100, 148, 210). Focal lesions of the coronary vessels and the endocardium are also evident in mouse models, but when infected mice are depleted of CD4⁺T lymphocytes, these myocardial lesions are substantially decreased in number and intensity (27). There is also evidence, however, that the pathogenesis is also due to direct viral cytopathicity for myocardial cells. For instance, in SCID (severe combined immunodeficiency) mice,
enteroviruses can still induce myocarditis (21), and immunosuppressive agents have been found to be ineffective in clinical trials (112). Interestingly, while immune-mediated mechanisms of pathogenesis have been studied extensively (25, 148, 210), the mechanisms of direct cytopathic effect remain largely unexplored. Importantly, rubella and yellow fever virus infection both result in myocarditic lesions in the absence of primary inflammation in mice (reviewed in (210)). Moreover, in adenovirus (110) and human immunodeficiency virus (HIV)-associated myocarditis (34), the degree of cardiac inflammation does not correlate with the severity of cardiac dysfunction. Therefore, further studies investigating the viral mechanisms of direct cytopathic effect would likely expand our knowledge of many forms of virally-induced myocarditis.

In contrast to enterovirus-induced myocarditis (144), multiple lines of evidence suggest that reovirus-induced myocarditis in mice is not immune-mediated, and that lymphocytes protect against reovirus-induced myocarditis. Hearts infected with 8B, a myocarditic reovirus, exhibit marked necrosis and extensive tissue damage with little inflammatory infiltrate (171). Also, 8B infection of nude (T-cell deficient) (173) or SCID (T and B cell deficient) mice (172) still results in cardiac lesions (172, 173), indicating that neither T nor B-cells are required for reovirus-induced myocarditis. On the contrary, T cells protect against reovirus-induced damage, as demonstrated by adoptive transfer of reovirus-immune spleen cells, depleted of either CD4+ or CD8+ T-cells using cell specific antibodies (172). Monoclonal antibodies specific for 8B capsid proteins were also protective against myocarditis, suggesting that B cells also protect against, rather than aggravate cardiac damage (172). Reovirus induces apoptosis in cardiac cells, and inhibition of this apoptosis
prevents reovirus-induced cardiac damage (36). Further, in primary cardiac myocyte cultures, cumulative cell death correlates with viral myocarditic potential (9). Therefore, reovirus-induced myocarditis is not immune-mediated; instead reflecting virally induced apoptosis, and thus provides for a unique disease model to study the cardiac response to the direct cytopathic effect of viruses.

The reovirus genome is composed of 10 segments of double-stranded RNA, and with one exception, each gene segment encodes one protein (127). The generation of “reassortant viruses”, containing a mixture of genes derived from two parent viruses, has allowed for the identification of viral genes that determine pathogenesis in the mouse. Genetic analysis of reassortant reoviruses has identified the M1, L1, and L2 genes as determinants of reovirus-induced myocarditis (170, 171). The M1 gene product, µ2, is an RNA binding protein (16) associated with the viral core RNA transcription complex (127), and the M1 gene has been genetically identified as a determinant of RNA synthesis (26, 169, 218). The L1 encoded λ3 protein and the L2 encoded λ2 protein are both also involved in RNA synthesis (reviewed in (127). The λ2 protein is a guanylyl transferase (105), and forms pentameric channels from the reovirus capsid core for extrusion of newly synthesized RNA (41, 145). The λ3 protein is an RNA polymerase (39). µ2, λ2, and λ3 together form a structural unit at each core vertex (127).

The fact that these three proteins are involved in RNA synthesis implicates virally induced RNA synthesis as a determinant of myocarditis. Indeed, the rate of viral RNA synthesis correlates with viral myocarditic potential (169). However, viral RNA synthesis does not correlate with yield of infectious virus from initially-infected cells in the culture,
with the yield being equivalent between all viruses (169). The yield of infectious virus after multiple rounds of infection in culture, however, does correlate with both viral RNA synthesis and viral myocarditis (169). These data implicate a possible role for virus-induced IFN in determining reovirus spread.

Addition of anti-IFN-α/β antibody to infected cardiac cultures benefits the spread of nonmyocarditic reoviruses more so than that of myocarditic reoviruses, and this benefit is associated with the M1 and L2 genes (174). This response to IFN-α/β is cell type-specific, as it is not observed in a differentiated skeletal muscle line (C2C12). Also, in mice depleted of IFN-α/β by addition of IFN-α/β antibody, a nonmyocarditic reovirus induces cardiac lesions, emphasizing the importance of type I IFNs in protection against this disease. Finally, IFN bioassays indicate that while all reoviruses induce IFN-β in primary cardiac cultures, nonmyocarditic reoviruses induce more IFN-β than do myocarditic reoviruses. (174). These data together suggest that reovirus induction of, and sensitivity to, IFN-β is an important determinant for protection against myocarditis.

For treatment of human cases of viral myocarditis, IFN-α (which binds to the same receptor as IFN-β) and thymic hormones which stimulate IFN production have been found to lower viral titer and improve cardiac function (117, 118, 181). Indeed, a clinical trial for dilated cardiomyopathy comparing conventional treatment with IFN-α treatment found that IFN-α was more effective (118). However, complete restoration of cardiac function has not yet been achieved with current therapies. IFN infusion is also used as an antiviral therapy for chronic hepatitis B and C (195), and modifications of IFN-α to increase stability have recently been found to improve the potency of drug therapy (78). Therefore, further studies
into the mechanisms of viral pathogenesis and viral response to IFN are important for development of more effective treatments.

As will be discussed below, IFN inhibits viral replication by inducing specific antiviral genes through the JAK-STAT pathway. As will also be discussed below, the SOCS proteins can inhibit this pathway. Therefore, it is of particular interest that small-molecule antagonists of SOCS, or tissue-specific vector delivery of SOCS inhibitors, have been shown to increase myocyte resistance to enterovirus-induced cardiac injury (206). This strategy of SOCS inhibition might be useful for other viral diseases or cancers in which patients have been found to be resistant to IFN therapy (58). Cytokine-induced JAK-STAT activation is markedly reduced in IFN-resistant leukemia cell lines where SOCS proteins are highly expressed without cytokine stimulation, suggesting that reduced activation of Jaks by aberrant SOCS induction could be the mechanism of IFN resistance (151). Therefore, strategies for inhibiting SOCS may be effective for early stages of myocarditis as well as for patients that are resistant to IFNs.

**SIGNALING OF INTERFERON STIMULATED GENES (ISGs)**

Interferons (IFNs) were so named because of their ability to inhibit, or interfere, with viral replication (194). There are two types of IFN, type I IFN (which includes both IFN-α and IFN-β) and type II IFN (IFN-γ). Type I IFN is produced by a variety of infected cells, whereas type II IFN is produced by activated T lymphocytes and natural killer cells (194). Cells produce type I IFN in response to infection by a variety of viruses, an event known to be central to the innate immune response of the host. The ability of reoviruses to induce
IFN-β is an important determinant for protection against myocarditis (174), especially since cardiac cells are not replenished. The antiviral effects of IFN-β are mediated by the induction of interferon-stimulated genes (ISGs) (152, 164, 179).

IFN-α/β induction of ISGs is mediated by activation of the JAK-STAT pathway (Figure 1). After IFN-α/β binds to its receptor, receptor-associated cytoplasmic Janus tyrosine kinases (Jaks), Jak1 and Tyk2, are activated (137, 159). This triggers site-specific tyrosine phosphorylation of Stat1 and Stat2 (32). The phosphorylated Stats dimerize and enter the nucleus (73, 137, 138) with p48 (also known as IRF-9) to form the IFN-stimulated gene factor 3 (ISGF3) complex (33, 122, 160). This complex binds to the Interferon Stimulated Response Element (ISRE) sequence of the ISG to stimulate transcription (163). This pathway, involving ISGF3-mediated induction of ISGs, is the best characterized of the IFN-α/β stimulated pathways. However, Stat1/Stat2 heterodimers may also form in response to IFN treatment, leading to transcriptional regulation of certain ISGs without IRF-9 (53). In addition, Stat-1 independent IFN signaling has also been cited (54), as have the phosphorylation of Stat3 (214), and Stat5 and Stat6 (45) in response to IFN-α/β stimulation, suggesting additional possible mechanisms for regulation of ISGs.

While multiple IFN-regulated pathways induce ISGs, some viruses, such as vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV), Sendai virus, Newcastle disease virus (NDV), or even dsRNA have been shown to induce certain ISGs directly, not through the synthesis of IFN (7, 61, 198). This induction is mediated by interferon regulatory factors (IRFs), which will be discussed later in this chapter, and which are capable of inducing ISGs through sequence similarities between their binding site, the IRF-E, and the
ISRE (124). A consensus ISRE sequence has been identified, A(G)NGAAANNGAAACT (31), and while variations do exist, recurring GAAA motifs characterize most ISGs (29). The ISRE is the predominant determinant of IFN, dsRNA, and virus induction of ISGs (7, 30, 31).

Over 100 interferon-induced genes have now been identified (35, 37). Some of these ISGs have well characterized antiviral functions, such as PKR, 2’-5’oligo (A) synthetase, and MHC class I and II genes. The functions of others are unknown, however, investigations into their regulation can provide valuable information for determining new sequences that confer IFN inducibility.

PKR

IFN inhibits reovirus replication (75), and has been shown to inhibit the translation of reovirus proteins to a greater extent than it inhibits viral transcription (203). In interferon-treated and virus-infected cells, the double stranded RNA activated kinase, PKR, is an important regulator of translation. After being upregulated by IFN and activated by dsRNA, PKR autophosphorylates and subsequently catalyzes the phosphorylation of the α subunit of eukaryotic protein synthesis initiation factor (eIF)-2 on serine residue 51 (153). This phosphorylation results in the sequestration of the guanine nucleotide exchange factor, eIF-2B, preventing the exchange of GDP for GTP on eIF, and inhibiting mRNA translation initiation (69). HeLa cells infected with reovirus have reduced protein synthesis and increased PKR activity (128). In addition to dsRNA, PKR can also be activated by the stem
loop structures of some single stranded RNAs (63). These structures are characteristic of the reovirus S1mRNA, which is a potent activator of the kinase (14).

PKR has other antiviral functions, and can also phosphorylate the NFκB inhibitor, IκB (106), which leads to activation and nuclear translocation of NFκB (188), thereby regulating IFN-β and other cytokines. PKR is critical for the reovirus induction of IFN-β in cardiac myocytes and has been shown to protect against reovirus-induced myocarditis (180). PKR also regulates the transcriptional activation mediated by the p53-tumor suppressor (28), and modulates cytokine signaling and transcriptional activation via Stat factors (209).

The promoter region of PKR contains multiple positive and negative regulatory domains for transcription factor binding (86) (Figure 2). The 13-bp ISRE confers inducibility by IFN, and upregulation of the PKR ISRE is thought to be mediated only by ISGF3 (124), however, overexpressing IRF-1 does lead to an increase in PKR expression (126). Located 4 bp upstream from the ISRE is a kinase conserved sequence (KCS) that is exactly conserved between mouse and human promoters and which is required for transcriptional activity (184). A single gamma IFN-activated sequence (GAS)-like element and multiple recognition sites for factors involved in responses to various cytokine and hormone signals are also present in the PKR promoter. The negative regulatory domain (NRD) negatively affects PKR transcription by a mechanism found to be dependent upon the KCS element. Therefore, both the ISRE and the NRD function through the KCS to regulate PKR transcription (184).
2′5′ OLIGO (A) SYNTHETASE

2′5′ oligo (A) synthetase is transcriptionally upregulated by IFN and activated by dsRNA (120, 128). The synthetase catalyzes the synthesis of a family of oligonucleotides of the structure pppA (2′p5′A)_n, also called 2′-5′ oligoadenylate (120). 2′5′ oligoadenylate activates a ribonuclease, RNase L, that is present in latent form in the cytoplasm of mammalian cells (176). RNase L then degrades dsRNA, halting translation of viral proteins. Reovirus infection increases production of 2′-5′ oligoadenylate (130), with concomitant cleavage of reovirus mRNA (6, 120, 129). While a decrease in protein synthesis is not detected (129), a degradation of reovirus mRNA results in a decrease in reovirus transcripts (6).

The ISRE element in the promoter of the 2′5′-oligo (A) synthetase gene is located between 155 bp and 72 bp upstream of the translation initiation codon (24). This region also contains three nuclear factor binding sequence elements, including a constitutive enhancer, an interferon-activated enhancer, and a region that binds a putative repressor (10). Like PKR, oligo (A) synthetase induction appears to be mediated exclusively through ISGF3 (124), although IRF-2 (113) and IRF-1 (23) have both been shown to bind to the ISRE.

MHC class I and II

MHC molecules are cell surface glycoproteins that can bind a wide variety of different peptides (77). The MHC molecule binds the peptide in an intracellular location and delivers it to the cell surface, where the combined ligand can be recognized by a T cell. MHC class I molecules present viral proteins to CD8+ T cells, which kill the infected cell
upon recognizing the foreign peptide:MHC class I complex on the surface. MHC class II molecules present peptides to CD4⁺ T cells. IFN upregulates MHC class I and II antigen presenting proteins (12). Reovirus induction of IFN enhances expression of MHC class I molecules on cultured human thyroid follicular cells (2). Reovirus binding to the cell surface also leads to enhanced class I and II expression (51).

IRF-8 binds to the ISRE motif in the promoter region of the MHC class I gene, H-2L<sup>D</sup>, which interacts with TAP molecules during peptide loading (205). IRF-1 is thought to be involved in regulation of MHC class I expression, as evidenced by reduction of CD8⁺T cell population in IRF-1<sup>−/−</sup> mice (115), while IRF-2 is implicated in downregulating MHC class I genes and β-2 microglobulin (40). MHC class I genes are induced in neurons, but not astrocytes, most likely due to cell type-specific differences in IRF-1 or IRF-2 (113). IRF-7 and IRF-9 have additionally been implicated in upregulation of MHC genes (221).

561

The 561 gene encodes P56, which was one of the first IFN-inducible proteins to have its cDNA cloned (18, 88). P56 belongs to a family of IFN-induced proteins, all of which have structural homologies, but unknown functions (200). The primary structure of P56 does not reveal any functional motifs other than the presence of eight tetratricopeptide repeats (TPRs) (61), which mediate protein-protein interactions and represent a recurring structural motif in many proteins involved in the IFN antiviral response (55). A yeast two-hybrid screen revealed that P56 interacts with the Int-6 protein (60), which is encoded by the Int-6 locus, whose disruption by the insertion of a mouse mammary tumor virus (MMTV) genome
causes breast cancer in mice (107). Later, it was found that Int-6 is identical to the P48 subunit of the translation initiation factor eIF-3 (1). P56 interacts with eIF-3 in the cytoplasm and inhibits its function (62). Cellular expression of P56 by transfection inhibited protein expression by 20-30% (61), and P56 has also been found to be a strong negative regulator of cell proliferation (52). However, in contrast to IFN treatment, P56 does not inhibit the replication of vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) (61). Further investigations into the effect of P56 on the replication of other viruses may determine the antiviral properties of P56.

The 561 regulatory region contains two ISREs, but no other known sites for binding transcription factors (199) (Figure 2). 561 can be induced by lipopolysaccharides (162), IFN (37) (191), virus (190) (61), and dsRNA (7, 52, 190, 191). In fact, recent microarray analyses have determined that 561 is the human gene most strongly induced in response to type I IFN (37) or dsRNA (52). Studies in a number of different cell lines suggest that dsRNA induction of 561 is direct, not mediated by IFN (190) (191). In GRE cells, which carry a deletion of the genomic locus containing the IFN-α and IFN-β gene, and therefore do not activate Stat1, Stat2, p48 (IRF-7), Tyk2, or Jak1 through IFN, dsRNA still induces 561 mRNA, suggesting that the dsRNA signaling pathway for 561 induction is distinct from the pathway used by IFN (7). Double-stranded RNA induction of 561 is inhibited in cells expressing the antisense of IRF-1, suggesting a possible mechanism for regulation (7), and studies overexpressing IRF-3 in cells defective in dsRNA signaling (P2.1 cells) indicate that IRF-3 is also involved in 561 induction. (139). Furthermore, immunofluorescence with P56 antibody in GRE cells reveals that EMCV, VSV, and Sendai virus can each directly induce
P56, but reovirus cannot (although the reovirus data was not shown in this report). More surprising is the observation that in the P2.1 cell line, where both the dsRNA-signaling pathway and IFN-induced pathway are nonfunctional (89), Sendai virus can still induce P56 (61), indicating that three distinct pathways (mediated by IFN, dsRNA, or lastly, virus) exist for P56 induction. It is still not clear what degree of overlap may exist for these three distinct pathways, however, since nuclear translocation of IRF-3 is found in P2.1 cells after Sendai virus infection, IRF-3 may be a mechanism for viral induction of P56 (61), however subsequent studies with a dominant negative form of IRF-3 did not completely inhibit 561 induction, indicating that other factors, such as IRF-1, may be involved (57).

6-16

The ISG, 6-16 has a completely unknown function, however, the regulatory region of 6-16 shares homology with that of IFN-β, 2’-5’ oligo (A) synthetase, and class I MHC genes (142). The 6-16 promoter region contains a tandem ISRE (Figure 2) with the sequence GGGAAAAAT, which is required for induction (142). A repressive domain (RD) (19) as well as a putative SP1 site have also been identified (142). The mechanisms governing expression of the 6-16 human gene have been shown to be conserved between human and murine cells (81).

Unlike 561 mRNA, 6-16 mRNA is induced efficiently by IFN, but poorly by dsRNA (7). Gel shift and detailed footprinting analysis indicate that IFN induction is mediated by binding of an “induced factor” to the ISRE, while suppression of transcription may involve competition for the ISRE by other factors (29). IFN-induced IRF-1 and IRF-2 both bind to
the 6-16 ISRE, and this binding is dependent on the central AT/TA hypervariable
dinucleotide of the ISRE (136), suggesting that one or both of these factors may be involved.

NEGATIVE REGULATION

Viral infection induces expression of IFN-β that activates JAK signaling at the early
stages of myocarditis (206). In addition to upregulation of ISGs, JAK-STAT activation also
results in induction of the suppressor of cytokine signaling (SOCS) family, particularly
SOCS1, SOCS3, and CIS (216). SOCS1 and SOCS3 can bind Jaks and inhibit their catalytic
activity, while CIS binds to cytokine receptors and blocks Stat recruitment to the receptor
(133) (Figure 3). Interestingly, in enterovirus-induced myocarditis, the JAK-STAT pathway
was found to be essential for signaling in cardiac myocyte antiviral defense, and a member of
the SOCS proteins was found to play a negative role in this defense (206). Specifically,
cardiac myocyte-specific transgenic expression of SOCS1 inhibits enterovirus-induced
signaling of Jaks and Stats, with accompanying increases in viral replication,
cardiomyopathy, and mortality in coxsackievirus-infected mice. Further, inhibition of SOCS
in cardiac myocytes through adeno-associated virus-mediated (AAV-mediated) expression of
a dominant-negative SOCS1 increased myocyte resistance to enterovirus-induced acute
cardiac injury (206). Therefore, activation of the JAK-STAT pathway leads to induction of
both positive, as well as potentially negative, regulators of ISGs in the heart.
IFN-β REGULATION

The ISGs described above are all stimulated by treatment with IFN-β (7, 13, 37, 120, 124). IFN-β is not constitutively expressed, but is induced by a number of different factors, including lipopolysacharide (LPS), tumor necrosis factor (TNF)-α, double stranded (ds) RNA, and viral infection (194). Transcriptional regulation is complex, involving multiple positive and negative regulatory factors. Interactions between these proteins and the IFN-β regulatory region help to convert the IFN-β gene from an inactive to an active state.

The IFN-β regulatory region has four positive regulatory domains (PRD1 through PRDIV) (Figure 4). Before transcriptional induction may take place, high mobility group (HMGY/I) proteins bind to the PRDII and PRDIV sites (189) to initiate conformational change in chromatin structure. This allows for the binding of factors necessary to form both the enhancesome and preinitiation complexes (217). Such factors include the activated form of ATF-2, which binds to the C-terminal domain of HMG1 (83), as well as NF-κB and IRF-1, which bind to the N-terminal region of HMG1 (217). While HMG1 is necessary for maximal IFN-β gene induction (42, 83), it is also important in disassembly of the enhancesome. The enhancesome complex is destabilized either by acetylation of HMG1 by CBP and/or p300 (123), or phosphorylation of HMG1 by casein kinase II (104, 161).

NF-κB binds to the PRDII of the IFN-β regulatory region (50) and interacts with both HMG1 (217) and IRF-1 (157). However, in unstimulated cells, NF-κB resides in the cytoplasm, bound to its repressor protein, IκB. IκB masks both its own nuclear localization signal, as well as those of NF-κB until it is degraded (76). Degradation occurs first by phosphorylation of IκB by either IκB kinases (220) or PKR (22). IκB may then bind to β-
TrCP, signaling its subsequent degradation by the 26S proteasome (116). After IκB is degraded, the nuclear localization signals are unmasked, and NF-κB translocates to the nucleus where it regulates IFN-β gene transcription. NF-κB may interact and synergize with IRF-1 to upregulate IFN-β (50) or it may bind to IRF-2, potentially causing repression of IFN-β transcription (40). NF-κB is also autoregulated by its own induction of IκB (74).

Upon induction, IκB is synthesized and translocates to the nucleus, where it removes NF-κB from DNA and causes export from the nucleus (72)

IFN-β transcription is also regulated by a growing family of interferon regulatory factors (IRFs). There are now 13 identified IRFs, 4 of which are virally encoded (reviewed in (187)). Some of these transcription factors have been found to bind to the Interferon Response Element (IRF-E) in the PRDI or PRDIII IFN-β regulatory region and regulate transcription. Studies indicate that these include IRF-1, IRF-2, IRF-3, and IRF-7, however much is still not known about the other IRFs. All of the cellular IRFs characterized thus far contain a highly conserved DNA binding domain with the consensus amino acid sequence FQ/RIP/VWKH within which lies five conserved tryptophan residues (141) (Figure 5). Also, IRFs have been found to bind to DNA via a characteristic winged helix-turn-helix motif (44). Interestingly, the IRF-E consensus sequence G(A)AAAG(C)T(C)GAAAG(C)T(C) (186) is virtually indistinguishable from the interferon-stimulated response element (ISRE): A(G)NGAAANNGAAACT (31), and IRFs have been found to bind to a number of different ISGs. Therefore, a more detailed description of the IRFs that bind both the IFN-β regulatory region and ISREs is provided below.
INTERFERON REGULATORY FACTOR-1 (IRF-1)

IRF-1 mRNA is not normally expressed in the brain, liver, or many other tissues, but is constitutively expressed in the heart (119). IRF-1 is induced by viral infection (65, 119), type I and type II IFNs (47), poly I:C, and TNF-α (48). The regulatory region has an SP-1 site, CAAT box, NF-κB site, and a GAS site (64) (Figure 6).

The IRF-1 transcription factor was originally identified as a regulator of virus-inducible enhancer elements of the human IFN-β gene (119), and has since been found to bind as a dimer (84) to the Interferon Regulatory Factor Element (IRF-E) in the PRDI of the IFN-β regulatory region (49). cDNA encoding mouse IRF-1 was cloned and characterized as a protein 329 amino acids in length with an approximate molecular weight of 37.3 kDa (119). The characteristic winged helix-turn-helix was first described for IRF-1 using x-ray crystallography (44). This motif contains three-α-helices, four-stranded antiparallel β-sheets, and three long loops. The third α-helix contacts the major groove of the GAAA sequence, while several other segments, including the three loops and three α-helices, contact its surrounding sequence (44). Mutational analysis of IRF-1 revealed that DNA binding activity resides in the N-terminal region, with five strictly conserved tryptophan residues (64) (Figure 5). Crystallography revealed that tryptophans 11, 38, and 58 bind to DNA (44). While the N-terminal region of IRF-1 is the DNA binding domain, the C-terminus is characterized by the abundance of acidic and serine or threonine residues, and constitutes the transcriptional activation domain of IRF-1 (64). The IRF-1 protein may be post-translationally modified by phosphorylation. Protein kinase A (PKA), protein kinase C (PKC), and casein kinase II (CKII) have been reported to serine phosphorylate IRF-1 at amino acids 138-150 and amino
acids 219-231 (96). Mutation of these tyrosine residues inhibited the transactivation of IRF-1, suggesting that phosphorylation by kinases may regulate IRF-1 transcriptional activity (96, 99). The IRF-1 protein has a relatively short half-life of about 20 minutes (197).

The role for IRF-1 in viral induction of IFN-β is complex. Overexpression of IRF-1 may induce IFN-α and IFN-β in the absence of viral infection (47), or may require virally induced phosphorylation for activation (96, 197). Studies suggest that while IRF-1 can modulate viral induction of IFN-β, it is not required. Specifically, depletion of IRF-1 decreases Newcastle disease virus induction of IFN-β (146). Newcastle disease virus, however, induces IFN-β in IRF-1−/− mice (147), IRF-1−/− embryonic stem cells (150), and IRF-1−/− embryonic fibroblasts (115, 147). Interestingly, in the same studies, dsRNA induction of IFN-β was impaired in IRF-1−/− embryonic fibroblasts (115), suggesting that viral infection and dsRNA may use distinct pathways for induction of IFN-β. This is of particular interest when investigating the role of reovirus, which is comprised of 10 segments of dsRNA (127).

IRF-1 is both virus and IFN inducible (48, 119). The similarity between IRF-Es and interferon-stimulated response elements (ISREs) would predict IRF-1 induction of interferon-stimulated genes. Indeed, overexpressed IRF-1 can provide antiviral protection even in the absence of IFN-β function (140). IRF-1 has been shown to upregulate several ISGs, such as PKR (126), 2’5’ oligo (A) synthetase (23), MHC class I and II (12), 561 (7), and 6-16 (136). IRF-1 has also been found to be critical for induction of the inducible nitric oxide synthetase (iNOS) gene (79), causing the production of the short-lived volatile gas, nitric oxide. In addition to antibacterial roles (79), nitric oxide has been shown to inhibit the
replication of coxsackie B-3 virus in macrophages (102) and protect against coxsackie B-3 virus-induced myocarditis (70).

IRF-1 also plays a role in immune response. IRF-1 influences development and function of natural killer cells. Analysis of spleen and liver of IRF-1−/− mice demonstrates a reduction in NK cell counts (182), indicating that NK cell development is impaired in these mice. Mice lacking IRF-1 also have reduced numbers of mature CD8+ T cells, even though maturation of CD4+ T cells is normal (115). This reduction in CD8+T cell population may be due to the low level of expression of MHC class I molecules, as LMP-2 and TAP-1 are both decreased in mice lacking IRF-1 (207).

In addition to antiviral and immunomodulatory roles, IRF-1 can also induce genes required for apoptosis. Caspases are important regulators of cell programmed death, and IRF-1 induces caspase-1 (183), caspase-7, and other members of the family of caspases (187). Importantly, reovirus-induced myocarditis reflects virally induced apoptosis (36), implicating a role for IRF-1.

In a wide variety of cell types, ectopic overexpression of IRF-1 leads to growth inhibition (185). While the absence of IRF-1 does not itself promote tumor development, IRF-1 belongs to a class of tumor suppressor genes that antagonize tumor development (reviewed in (85)). The mechanism of IRF-1 induced tumor suppression has been linked to its induction of antiproliferative genes, such as PKR (67), oligo (A) synthetase (66), the tumor suppressor protein, p53, and cell cycle dependent kinase inhibitor, p21 (185).
INTERFERON REGULATORY FACTOR-2 (IRF-2)

IRF-2 is inducible by IFN-α/β, albeit with slower kinetics of induction than IRF-1, and can also be induced upon viral infection (64). The IRF-2 regulatory region has an NF-κB site, SP1 sites, a CAAT box, and an IRF-E, and thus it can be induced by IRF-1. (64).

The primary structures of IRF-1 and IRF-2 showed 62% sequence similarity in their N-terminal regions (Figure 5), whereas the rest of the molecules showed only 25% sequence similarity (64). The crystal structure of the IRF-2 DNA binding domain bound to DNA shows cooperative binding to a tandem repeat of the GAAA core sequence induced by DNA structural distortions (46). Like IRF-1, the DNA binding activity resides in the N-terminal region of the protein, however, unlike IRF-1, the C-terminal region of IRF-2 is rich in basic amino acids. The C-terminus of IRF-2 contains a repression domain: following viral infection or dsRNA treatment, IRF-2 has been found to undergo proteolytic processing and is cleaved in its C-terminus, leading to IRF-2 repressor function (208). Interestingly, IRF-2 proteolytic processing has also been shown to decrease IRF-2 repressor function (134), suggesting the possibility for cell type-specific roles for IRF-2. Post-transcriptionally, the serine residues of IRF-2 can be phosphorylated by PKA, PKC, and casein kinase II (11).

IRF-2 has a half-life of 8 hours, which is relatively longer than that of IRF-1 (20 min.) (197). As mentioned above, IRF-2 can act to induce or repress IFN-β induction. Other than regulation through proteolytic processing, IRF-2 can also repress IFN-β by interacting with either NF-κB (40) or the IFN-β enhancesome transcription complex (196). In MEFs, IRF-2 represses gene expression by binding to IRF-Es (64), but in C2C12 cells skeletal muscle cells, IRF-2 induces IFN-β gene expression (168). Interestingly, however,
overexpressed IRF-2 was shown to repress reovirus induction of IFN-β in both C2C12 and primary cardiac myocytes, but not L929 cells (168). IRF-2 can also have effects on constitutive levels of IFN-β and represses these levels in primary cardiac myocytes, but induces them in C2C12 cells (168), suggesting a complex, cell type-specific role for IRF-2. IRF-2 may also be stimulus dependent, as demonstrated in IRF-2−/− mice, where IRF-2 was found to be critical for repression of NDV, but not dsRNA, -mediated induction of IFN-β (115).

IRF-2 was originally considered an antagonist of ISG induction (67), competing with IRF-1 for binding to the ISRE site. This mechanism of competitive repression has been proposed for a number of ISGs, including ISG15, 2′-5′ oligo (A) synthetase, and 6-16 (reviewed in (136). IRF-2 can also bind to NF-κB and repress MHC class I genes and β-2 microglobulin (40). Recently, however, IRF-2 has been reported to facilitate IRF-1 mediated induction of a responsive gene (211). Additionally, IRF-2−/− mice were shown to carry defects in NK cell development (101), suggesting an immunomodulatory role for IRF-2. Also contrasting the idea that IRF-2 is an antagonist of ISG induction is the fact that certain viruses are unexpectedly more lethal in IRF-2−/− mice compared to wildtype mice (59, 115). IL-12 production is also suppressed in IRF-2−/− macrophages, and IRF-2−/− mice are susceptible to Leishmania major infection due to a defect in Th1 cell differentiation. Thus, rather than acting as a negative regulator, IRF-2 may contribute to IL-12 gene expression in activated macrophages (101).
INTERFERON REGULATORY FACTOR-3 (IRF-3)

More recently, two structurally related IRF family members, IRF-3 and IRF-7 were found to be involved in IFN-α/β induction (Figure 5). IRF-3 is constitutively expressed in all tissues examined so far, from immortalized cell lines, to primary cells and tissues (reviewed in (165), thereby eliminating the need for de novo synthesis upon viral infection. In uninfected cells, IRF-3 is present in an autoinhibitory form (98). Viral infection or dsRNA can induce IRF-3 phosphorylation on serine residues (202) and homodimerization (95, 98, 219). This activation induces translocation of IRF-3 from the cytoplasm to the nucleus (87), and association with CBP/p300 (202, 215). IRF-3 interaction with CBP/p300 helps to stabilize the enhancesome complex, provides a link to the basal machinery (82), and allows p300 to hyperacetylate histones localized to the IFN-β promoter to promote IFN-β induction (135). IRF-3 also binds directly to the PRDIII of the IFN-β promoter (156, 158) to upregulate transcription.

The IRF-3 gene encodes a 427-amino acid protein of 55 kDa (4). IRF-3 has a nuclear import and export signal and an internal inhibitory region. The internal inhibitory region has homology to a region present in other IRF proteins shown to be involved in protein-protein interactions, thus acquiring the name IRF association domain (IAD) (166, 167). The IAD region is thought to cause the latent protein to fold as an inactive monomer and has been implicated in the formation of IRF-3/IRF-7 heterodimers (5).

IRF-3 is required for reovirus induction of IFN-β in primary cardiac myocytes, but not in L929 cells, suggesting that IRF-3 function can be cell type-specific (131). Similarly, Herpes simplex virus-1 has been shown to activate IRF-3 more efficiently in a human
foreskin fibroblast cell line than in a human fetal lung cell line, and ISG54 induction followed the same pattern (143).

IRF-3 has been shown to both bind to an ISRE oligonucleotide (3), and upregulate an ISRE-containing reporter in the absence of viral infection (30). In the absence of type I IFN, NDV can stimulate IRF-3 to induce some ISGs, such as ISG15, ISG54, and IP-10, but not others, such as 2’-5’ oligo (A) synthetase and 6-16 (30, 124). 561 is also induced directly by IRF-3, (57, 61, 139), and has been implicated in mediating IRF-3 induced apoptosis (57). The three nucleotides which flank the ISRE site of these genes have been found to be critical for IRF-3 binding (30, 124), and lack of either the upstream AAA or the downstream CTG sequences leads to a loss of IRF-3 binding activity (30). EMCV increases in virulence in IRF-3−/− mice, indicating IRF-3’s important antiviral role (155).

INTERFERON REGULATORY FACTOR-7 (IRF-7)

Although a basal level of expression has been detected in all tissues examined, IRF-7 is predominantly expressed in the spleen, thymus, and peripheral blood lymphocytes (PBL)(3, 222). Unlike IRF-3, IRF-7 is not expressed constitutively but rather is induced following treatment of cells with IFN, LPS, or viral infection (3, 165). The human IRF-7 gene encodes a 503-amino acid protein of ~67kDa (IRF-7A), although proteins translated from multiple splice variant mRNAs are also produced (IRF-7B, IRF-7C, and IRF-7H). Likewise, a 457 amino acid protein represents the full length murine IRF-7 (IRF-7α), but there are also murine proteins translated from splice variant mRNAs (IRF-7β, IRF-7 λ) (3, 222) (also see Appendix 3).
While IRF-3 is a primary activator of IFN-β transcription, studies in IRF-3−/− and IRF-7 expression-abolished mice suggested a critical role for IRF-7 as well. Specifically, the induction of IFN-α/β genes was completely abolished in IRF-3−/− and IRF-7 expression-abolished cells, however, ectopic expression of neither IRF-3 nor IRF-7 restored the normal induction profile of IFN-α/β genes unless the two factors were coexpressed (155). This work led to a more comprehensive model for both IFN-β and IFN-α induction, involving IRF-3 in early stages, and IRF-7 for amplification later (68, 94, 154, 155).

In the early phase, which is mostly IFN-independent, IRF-3 rapidly induces IFN-β and IFN-α4 (108). IFN is then secreted from cells and binds to the IFN-α/β receptor to activate ISGF3, a trimeric complex consisting of Stat1, Stat2, and IRF-9 (193). ISGF3 then induces IRF-7 via the ISRE sequence (108, 154, 155). Upon virally induced phosphorylation, IRF-7 dimerizes and translocates to the nucleus (3, 108, 154). IRF-7 then binds and upregulates “delayed” IFN-α genes (IFN-α2, IFN-α5, IFN-α6, and IFN-α8) as well as IFN-β gene, causing amplification of IFN-β production (108). This biphasic IFN-α/β gene induction mechanism, regulated by IRF-3 and IRF-7, ensures the transcriptional efficiency and diversity of the IFN genes for efficient antiviral response (92).

IFN-induced IRF-7 has a very short half-life (0.5 to 1 hr) compared to IRF-3, suggesting that this labile nature of IRF-7 represents a mechanism that is critical to make the whole IFN gene induction process transient (155). In addition, IRF-7 can be acetylated by PCAF (p300-CBP-associated factor), which can negatively regulate IRF-7 mediated IFN-β induction (17). The identity of the kinase responsible for both IRF-7 and IRF-3 phosphorylation remains to
be elucidated (177), but this phosphorylation may also provide an additional regulatory mechanism.

IRF-7, like IRF-3, is activated by virus-induced phosphorylation and dimerization (109). Serine phosphorylation occurs in its C-terminal region, which is highly homologous to the corresponding region of IRF-3 (Figure 5), and mutation or deletion in this region has been shown to cause inactivation of IRF-7 (108, 154). However, overexpressed IRF-7 does not require virus activation for function (97). The correlation between IRF-7 activity and induced dimerization as well as the ability of forced dimerization to substitute for virus-induced phosphorylation led to a model where the IRF-7 inhibitory (IAD) region is involved in internal, heterotypic protein-protein interactions, causing the latent protein to fold as an inactive monomer (5). This region has also been implicated in the formation of homodimers or heterodimers with IRF-3, which may affect IFN-β gene induction (98, 108, 155, 201).

In contrast to IRF-3 (with CBP), IRF-7 localization is not dependent on the association with known transcription coactivators, but rather has been attributed only to its nuclear export sequence (NES) in the C-terminal region between amino acids 416 and 467, and the nuclear localization signal (NLS) believed to be present in the N-terminal region of IRF-7 (97).

Interestingly, the roles of IRF-3 and IRF-7 in viral induction of IFN-β are cell type-specific. Constitutive IRF-7 levels are much lower in third passage MEFs than in fifth passage MEFs, and NDV induction of IFN-β requires IRF-3 in the former but not the later cells (68). The lack of IRF-7 in the fibrosarcoma cell line, 2fTGH, correlates with hypermethylation of the CpG island in the human IRF-7 promoter (103). Such
hypermethylation may represent one mechanism whereby differential cell type-specific responses to IFNs are generated.

Selection of optimum DNA binding domains from a pool of random sequences showed that IRF-3 prefers a more restricted target sequence than does IRF-7. IRF-3 preferred an accurate direct repeat of the motif GAANN, which is found in type I IFN promoters. IRF-7 also bound a similar direct repeat, but it was much more tolerant to sequence variations in the core sequence elements (94). Both IFN-α4 and IFN-β promoters contain perfect GAANN repeat motifs, allowing them to bind and be activated by IRF-3. Other IFN-α genes display more variability within their promoters, making them targets for IRF-7 but not for IRF-3 (94). Interestingly, despite the broader binding range of IRF-7, there have been no reports to date of direct, IRF-7 mediated induction of ISGs.

IRF-7 was originally cloned and identified based on its binding activity to the Epstein-Barr virus (EBV) Bam HI Q promoter (Qp) (222) and has since been found to play a role in regulation of EBV latency. In type I latency, Qp is used for transcription of EBNA-1 mRNA. EBNA-1 is the EBV viral protein needed for replication of the episome and maintenance of the latent infection state. In type III latency, however, it is thought that IRF-7 acts as a repressor of Qp, functioning to keep the promoter inactive and allow another promoter, C/wp to regulate EBNA-1 instead. Interestingly, the EBV LMP-1 protein, the principal oncoprotein required for immortalization and transformation, both induces IRF-7 expression and activates IRF-7 protein by phosphorylation and nuclear translocation. Activated IRF-7 may then mediate regulation of EBV target genes. One of these genes is the TAP-2 gene, which is induced along with HLA-1 and TAP-1 for antigen processing. IRF-7
may also regulate HLA and TAP-1 and thus serve to regulate the immune system during EBV latency (221). Because of IRF-7’s connection to EBV latency and LMP-1, much work is being done to determine whether it is involved in the pathogenesis of EBV-associated tumors.

**OTHER IRFs**

**IRF-9** was originally identified as the DNA-binding subunit of the transcription factor, ISGF3 (interferon stimulated gene factor 3), and termed p48/ISGF3γ (193). ISGF3 is IFN-inducible, and contains the signal transducers and activators of transcription (Stat) 1 and Stat2, as well as IRF-9 (91). IRF-9 is expressed in a variety of tissues and is essential for the antiviral interferon response by IFN-α/β and IFN-γ (31) (see Signaling of ISGs). Initially, studies suggested that ISGF3 binds to the IFN-β promoter to stimulate induction (80, 212), however, since then, compelling evidence suggests that ISGF3 participates indirectly through IRF-7 induction (155). Studies in knockout mice indicate that in addition to IRF-1 and IRF-7, IRF-9 may also contribute to the induction of MHC class I molecules, TAP-1, LMP-2, and β-2-microglobin by IFN-α/β stimulation (reviewed in (187)).

Information is scarce regarding the functions of **IRF-5** and **IRF-6**, but IRF-5 has recently been found to be activated by some viruses (but not others) to induce specific IFN-α genes (8). **IRF-8**, also termed Interferon Consensus Sequence Binding Protein (ICSBP), was originally identified as a protein that binds to the ISRE of the MHC class I gene H-2L^D^ (205). Expression of IRF-8 is restricted to lymphoid cell lineages and is induced only by IFN-γ, and not IFN-α/β (125). **IRF-4** is also only expressed in T and B cells but is not induced by IFNs
IRF-4 and IRF-8 form a complex that represses the expression of ISG-15 in macrophages (149). Kaposi sarcoma associated herpes virus (KSHV) encodes the remaining four IRF homologs (viral IRFs or vIRFs). These vIRFs bind cellular IRFs to inhibit IRF-mediated transcriptional activation (223). Expression of an antisense RNA for certain vIRFs results in repression of several viral genes, suggesting the vIRFs may also positively regulate other viral proteins (93).

**SUMMARY**

Reovirus-induced myocarditis is not immune-mediated, and thus provides for a unique disease model to study the direct effect of viruses on cardiac cells. Reovirus induction of, and sensitivity to, IFN is an important determinant for protection against reovirus-induced myocarditis. IFN signaling has been shown to induce over 100 ISGs of differing and sometimes unknown functions, and IFN inducibility is conferred through the ISRE site of the ISG.

IFN-β transcription is regulated by HMGY(I), NF-κB, and IRFs. IRF-1 and IRF-2 were the first to be identified as regulators of IFN-β transcription. The identification of IRF-3 and IRF-7 and their functional analysis have revealed the essential and distinct roles of these factors in the biphasic pathway for IFN-α/β induction, which may have evolved to make the host defense against viruses more efficient.

Other than regulation of IFN-β, some of the members of the IRF family of transcription factors, notably IRF-1, IRF-2, and IRF-3, also regulate induction of ISGs in the absence of IFN. This is achieved through sequence similarities between the IRE and the
ISRE, which both have recurring GAAA motifs. The ability of cells to respond to viruses by
directly activating ISG expression (without requiring the synthesis of interferon) is a defense
mechanism that may give cells an increased chance of survival during viral infections.
Lastly, cell type-specific differences in IRF expression or function may determine differential
antiviral responses.
REFERENCES CITED


42. **Du, W., and T. Maniatis.** 1994. The high mobility group protein HMGI(Y) can stimulate or inhibit DNA binding of distinct transcription factor ATF-2 isoforms. PNAS. 91:11318-11322.


120. Miyamoto, N. G., B. L. Jacobs, and C. E. Samuel. 1983. Mechanism of interferon action. Effect of double-stranded RNA and the 5'-O-monophosphate form of 2',5'-


137. **Pellegrini, S., and I. and Dusanter-Fourt.** 1997. The structure, regulation and function of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). Eur. J. Biochem. **248:**615-633.


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a transcription factor containing complex containing IRF-3 and CBP/p300. EMBO J. 17:1087-1095.


Figure 1. Induction of ISGs. IFN-α/β induction of ISGs is mediated by activation of the JAK-STAT pathway. After IFN-α/β binds to its receptor, receptor-associated cytoplasmic Janus tyrosine kinases (Jaks), Jak1 and Tyk2, are activated. This triggers site-specific tyrosine phosphorylation of Stat1 and Stat2. The phosphorylated Stats dimerize and enter the nucleus with p48 (also known as IRF-9) to form the IFN-stimulated gene factor 3 (ISGF3) complex. This complex binds to the Interferon Stimulated Response Element (ISRE) sequence of the ISG to stimulate transcription. This pathway, involving ISGF3-mediated induction of ISGs, is the best characterized of the IFN-α/β stimulated pathways, but other IFN-regulated pathways induce ISGs, and some viruses and dsRNA have been shown to induce certain ISGs directly, not through the synthesis of IFN. This induction is mediated by interferon regulatory factors (IRFs) which are capable of inducing ISGs through sequence similarities between their binding site, the IRF-E, and the ISRE. The ISRE is the predominant determinant of IFN, dsRNA, and virus induction of ISGs.
**Figure 2. ISG Regulatory Regions.** The regulatory regions of PKRlux, 561lux, and 6-16lux are indicated with 5’ and 3’ nucleotides in **bold**. The location of the Interferon Stimulated Response Element (ISRE) site noted, as well as and sequence conferring inducibility. Importantly, recurring GAAA motifs are found in many interferon-stimulated genes and are found in each ISRE above. The PKR TATA-less promoter contains a 13 nt ISRE, a kinase-conserved site (KCS), gamma IFN-activated sequence (GAS)-like site, sites for binding SP1, NF-kB, and NF-IL6, as well as a negative regulatory domain (NRD). The 561 regulatory region contains two ISREs, but no other known sites for binding transcription factors. The 6-16 promoter contains two 14 nt ISREs with the same sequence, GGGGAAAAT, found to be required for inducibility (142). A repressor domain (RD) as well as a putative site for binding SP1 have also been identified.
Figure 3. **SOCS Inhibition of the JAK-STAT pathway.** SOCS1 and SOCS3 can bind Jaks and inhibit their catalytic domain. CIS does not bind Jaks, but instead binds to cytokine receptors and blocks Stat recruitment to the receptor. Therefore, Stat-responsive genes, such as IFN-β and ISGs are inhibited.
Figure 4. Regulation of IFN-β. The IFN-β regulatory region has four positive regulatory domains (PRD1 through PRDIV). Before transcriptional induction may take place, high mobility group (HMGY/I) proteins bind to the PRDII and PRDIV sites to initiate conformational change in chromatin structure (not shown for simplicity). This allows for the binding of factors necessary to form both the enhancesome and preinitiation complexes. Such factors include the activated form of ATF-2, which binds to the C-terminal domain of HMG1, as well as NF-κB and IRF-1, which bind to the N-terminal region of HMG1. NF-κB binds to the PRDII of the IFN-β regulatory region and interacts with both HMGI and IRFs. IRFs bind to the Interferon Response Element (IRF-E) in the PRDII or PRDIV IFN-β regulatory region. The binding of ATF-2/c-Jun, NF-κB, and IRFs results in recruitment of CBP and subsequent association of the complex with the PolII holoenzyme, implicating CBP as a bridge between transcriptional machinery and the IFN-β enhancer.
Figure 5. Functional domains of some IRF family members. All IRFs have significant homology of the 115 amino acids in the N-terminal region. Five tryptophan (W) repeats are conserved among these family members. IAD (IRF association domain); NES (nuclear export signal); NLS (nuclear localization signal). Diagram taken, in part, from Taniguchi, et. al, 2001.
Figure 6. IRF-1 regulation and function. The pIRF-1 lux reporter contains 500 bp of the promoter region 5’ from the transcription start site. Putative binding sites for known transcription factors are depicted. IRF-1 is both virus and IFN-inducible, and has many functions. In addition to regulation of IFN-β, it may also regulate ISGs such as PKR, Oligo(A)Synthetase, MHC class I and II genes, 561, and 6-16. IRF-1 also regulates iNOS, genes for apoptosis, growth inhibition, immune response.
CHAPTER 2

INTERFERON REGULATORY FACTOR-1, INTERFERON-β, AND REOVIRUS-INDUCED MYOCARDITIS

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ABSTRACT

Viral myocarditis is an important human disease, and reovirus-induced myocarditis in mice provides an excellent model to study direct viral damage to the heart. Previously, we showed that reovirus induction of, and sensitivity to, interferon-β (IFN-β) is an important determinant of viral pathogenicity in the heart, and that the transcription factor interferon regulatory factor-3 (IRF-3) is required for reovirus induction of IFN-β in primary cardiac myocyte cultures. Given several lines of evidence suggesting a possible distinctive environment for IRFs in the heart, we have now focussed on IRF-1. Previous studies demonstrated that viruses, double stranded-RNA (dsRNA), and IFN-α/β can each induce IRF-1, and that IRF-1 plays a role in dsRNA, but not viral, induction of IFN-α/β. Importantly, none of these studies used a virus with a dsRNA genome (such as reovirus), none of them used a highly differentiated non-lymphoid cell type, and none of them addressed whether viral induction of IRF-1 is direct or is mediated through viral induction of IFN-β. Indeed, as recently as this year it has been assumed that viral induction of IRF-1 is direct. Here, we found that reovirus induced IRF-1 in primary cardiac myocyte cultures, but that IRF-1 was not required for reovirus induction of IFN-β. Surprisingly, we found that reovirus failed to induce IRF-1 in the absence of the IFN-α/β response. This provides the first evidence that viruses may not induce IRF-1 directly. Finally, nonmyocarditic reovirus strains induced more cardiac lesions in mice deficient for IRF-1 than they did in wild type mice, directly demonstrating a protective role for IRF-1. Together, the results indicate that while IRF-1 is downstream of the IFN-β response, it plays an important protective role against viral myocarditis.
INTRODUCTION

Viral myocarditis affects an estimated 5-20% of the human population (65). Many viruses have been implicated in this disease (65), with enteroviruses and adenoviruses most likely accounting for the majority of cases (31). Enterovirus-induced myocarditis is both immune-mediated (7, 48) and due to direct cytopathic effect (5, 21), while adenovirus-induced myocarditis is most likely not immune-mediated (31). Given that many viruses gain access to the heart, and that cardiac myocytes are not replenished, the innate response of cardiac cells to viral insult may well be a significant determinant of cardiac damage.

Reovirus-induced myocarditis is not immune-mediated (54, 55), but instead reflects virally induced apoptosis (9), and thus provides a unique model to investigate the cardiac response to viral infection.

Previously, we found that in primary cardiac myocyte cultures, nonmyocarditic reoviruses induce more interferon-β (IFN-β) and are more sensitive to the antiviral effects of IFN-α/β than myocarditic reoviruses (56). In addition, nonmyocarditic reoviruses induce myocarditis in mice pretreated with anti-IFN-α/β antibody (56). Thus, IFN-β is an important determinant of reovirus-induced myocarditis. Interestingly, addition of anti-IFN-α/β antibody benefits reovirus spread in primary cardiac myocyte cultures, but not in differentiated C2C12 (skeletal muscle) cells (56), suggesting a possible unique role for IFN in the heart.

IFN-β transcription is regulated by multiple positive and negative regulatory factors in the cell. Viruses can induce or activate many of these factors, and the critical roles for the transcription factors interferon regulatory factor-3 (IRF-3) and IRF-7 have been described
extensively (reviewed in (60). Interestingly, IRF-3 is required for viral induction of IFN-β in primary cardiac myocyte cultures but not in the undifferentiated L929 cell line (42). The related interferon regulatory factor, IRF-1, is not normally expressed significantly in the brain, liver, or many other tissues, but it is constitutively expressed in the heart (34). Given the critical role of IFN-β in mediating protection against reovirus myocarditis, and given these precedents suggesting a unique environment for IRFs in the heart, we investigated the interplay between IRF-1, IFN-β, and reovirus myocarditis.

IRF-1 binds to the Interferon Regulatory Factor Element (IRF-E) in the IFN-β regulatory region (13). Overexpression of IRF-1 may induce IFN-α and IFN-β in the absence of viral infection (11), or may require virally induced phosphorylation for activation (29, 62). Studies suggest that while IRF-1 can modulate viral induction of IFN-β, it is not required. Specifically, depletion of IRF-1 decreases Newcastle Disease Virus induction of IFN-β (45). Newcastle Disease Virus, however, induces IFN-β in IRF-1−/− mice (46), IRF-1−/− embryonic stem cells (49), and IRF-1−/− embryonic fibroblasts (32, 46). Interestingly, in these same studies, dsRNA induction of IFN-β is impaired in IRF-1−/− embryonic fibroblasts (32), suggesting that viral infection and dsRNA may use distinct pathways for induction of IFN-β. This is particularly relevant when investigating reovirus induction of IFN-β as the viral genome is comprised of dsRNA (38). Thus, the role of IRF-1 in viral induction of IFN-β may be virus-specific. Moreover, no previous studies addressed the role of IRF-1 in induction of IFN-β in differentiated cells.

IRF-1 is both virus and IFN inducible (12, 34). The similarity between IRF-Es and interferon-stimulated response elements (ISREs) would predict IRF-1 induction of
interferon-stimulated genes. Indeed, overexpressed IRF-1 can provide antiviral protection even in the absence of IFN-β function (44). Thus, IRF-1 may play critical roles both upstream and downstream of IFN-β transcription. Surprisingly, despite longstanding evidence that viruses induce IRF-1 (19, 34, 36, 47, 59, 61), no one has asked whether this is a direct effect of the virus or is instead mediated by virally induced IFN.

Here, we report that reovirus induces IRF-1 in primary cardiac myocyte cultures, but that IRF-1 is not required for reovirus induction of IFN-β in these cells. Surprisingly, reovirus fails to induce IRF-1 in the absence of IFN-α/β function. This provides the first evidence that viral induction of IRF-1 may be mediated through viral induction of IFN-β. Lastly, reovirus induces more cardiac damage in IRF-1⁻/⁻ neonatal mice. Together, the data indicate that while IRF-1 is downstream of IFN-β, it plays an important protective role against viral myocarditis.

MATERIALS AND METHODS

Viruses: Reovirus stocks were characterized previously for their myocarditic phenotypes (53, 55). Serotype 3 Dearing (T3D) is a prototype strain that is nonmyocarditic (55) and induces IFN-β (56). Reovirus 8B is a reassortant virus that is potently myocarditic (55) and induces IFN-β poorly (56). Reovirus DB95 is a reassortant virus that is nonmyocarditic (55) and induces IFN-β (56).

Mice and Inoculations: Timed pregnant Cr:NIH (S) mice were obtained from the National Cancer Institute. IRF-1⁻/⁻ mice (46) were generously provided by Jenny Ting, University of
North Carolina, Chapel Hill, NC. IFN-α/β receptor−/− (35) and wildtype parental 129Sv/Ev mice were generously provided by Dr. Herbert W. Virgin IV (Washington School of Medicine, St. Louis, Missouri). For inoculations with reovirus, indicated mice were mated, housed individually in isolator cages, and checked daily for births. Neonates (2 days old) were inoculated in the left hind limb with 20 µl of virus diluted with gel saline (8B: 1.0 x 10^3 PFU; DB95: 5.0 x 10^5 PFU). At 7 days postinjection, mice were euthanized. Hearts were removed and fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin (H&E). Blinded slides (≥ 3 hearts per virus dose, and ≥ 8 sections per heart) were scored for lesions by morphometric analysis utilizing a Olympus Vannox morphometric scope. Mice were housed according to AAALAC recommendations and all procedures were approved by NCSU IACUC.

**Cell Cultures:** To generate primary cardiac myocyte cultures from Cr:NIH(S), 129Sv/Ev, IRF-1−/−, or IFN-α/β receptor−/− mice, term fetuses or 1-day-old neonates were sacrificed and the apical two-thirds of the hearts were removed, minced, and trypsinized (3). Cells were plated at a density of 1.25 X 10^6 cells per well in six-well clusters (Costar, Cambridge, Mass.) and incubated for 1.5 to 2 h to remove rapidly adherent cells (predominantly fibroblasts). The remaining cells (predominantly myocytes) were resuspended in Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, Md.) supplemented with 7% fetal calf serum (HyClone, Logan, Utah), 0.06% thymidine (Sigma Co., St. Louis, Mo.), and 10 µg of gentamicin (Sigma Co.) per ml, (“supplemented media”) and plated as indicated for
each procedure. Myocyte cultures contained 5-20% fibroblasts (3), consistent with levels reported by others (18, 23, 33), and consistent with cell heterogeneity in the heart.

**Plasmids:** The pβlux reporter plasmid was constructed as previously described (42), and contains the murine IFN-β regulatory region inserted into pGL3-Basic (Promega). The pIRF-1 lux reporter plasmid was constructed by using polymerase chain reaction (PCR) to add Kpn I and Bgl II restriction sites to bases –1 to –514 upstream of the murine IRF-1 coding region, and inserting the product into pGL3-Basic. Murine IRF-1 inserted into pRc/CMV1 (66) was kindly provided by Dr. Jerry Doherty (Washington School of Medicine, St. Louis, MO). pRc/CMV2 was purchased (Invitrogen). pRL-SV40 (expressing renilla luciferase constitutively from an SV40 promoter), pGL3-Basic (lacking a promoter and therefore expressing baseline firefly luciferase), pGL3-Control (expressing firefly luciferase constitutively from an SV40 promoter) were purchased (Promega). DNA was purified for transfection using Qiagen’s Maxiprep system (Qiagen Inc., Valencia, Calif.).

**Transfections:** Primary cardiac myocyte cultures were plated at a density of $1.0 \times 10^5$ cells per well in 0.5 ml in 48-well tissue culture plates from Costar (Figures 4, 5A, 5B, 6A, and 7) or $3.5 \times 10^5$ cells per well in 1 ml in 12-well tissue culture plates (Figures 3, 5C, and 6B). All cultures were allowed to adhere for 1 day prior to transfection. Transfection was performed as previously described (41, 42) using FuGENE6 according to the manufacturer’s protocol (Boehringer Mannheim/Roche Molecular Biomedicals, Indianapolis, Ind.). Transfection conditions were optimized previously using a β-galactosidase reporter plasmid
that demonstrated that myocytes in primary cardiac myocyte cultures were transfected, although efficiency was low (41). Unless noted otherwise in figures, the amount of DNA added to the indicated wells was as follows in a 48-well cluster experiment: $p\beta$lux 0.5 µg; pIRF-1 lux 0.5 µg; pRL-SV40 0.01 µg; pGL3-Control 1.0 µg; pRc/CMV2 1.0 µg; murine IRF-1 (mIRF-1) 1.0 µg. DNA concentration was doubled for all plasmids in a 12 well cluster experiment. FuGENE6 was used in a volume equal to twice the total micrograms of plasmid DNA to be transfected per well (e.g., 2 µg of plasmid DNA per well required 4 µl of FuGENE6 per well).

**Infections:** Infections were performed 1 day postransfection or > 24 hours postplating cells (immunoprecipitation and RNA extraction). Primary cardiac myocyte cultures were washed twice with supplemented DMEM immediately prior to infection. For transfections, two wells were trypsinized and viable cells were counted using trypan blue exclusion. Myocyte cultures for immunoprecipitation or RNA extraction were assumed to be at 50% their plating densities, based on previous experience. For transfected cultures in 12-well plates and cultures plated for RNA and for immunoprecipitation, cells were infected with reovirus T3D (Dearing) at 25 PFU per cell in 300 µl supplemented media or were mock infected. Cultures were incubated for 1 h at 37°C in 5% CO$_2$. For transfected cultures in 12-well plates and cultures plated for RNA, 700 µl supplemented DMEM was then added. Transfected cultures plated in 48-well plates were treated identically but with 50% less volume (150 µl inoculum and 350 µl overlay volumes). Immediately following overlay, 1000 units/ml IFN-α/β (Sigma, Figure 6A; Lee Biomolecular, Figure 6B), or 1100 neutralizing units/ml anti-IFN-
α/β or preimmune antibody (Access Biomedical, Figure 7) was added if indicated. Cells were incubated at 37°C and 5% CO2 for times indicated.

**Dual-luciferase assay:** The dual-luciferase assay was performed according to the manufacture’s protocol (Promega) with the following exceptions: cells were washed twice with phosphate-buffered saline prior to the addition of lysis buffer, cells were allowed to remain in lysis buffer at 4 °C for at least 15 min, and the surfaces of the wells were then scraped with either a Teflon cell lifter from Costar (12-well clusters) or a rubber policemen from Fisher Scientific (48-well clusters). Measurements were made using a Lumat LB 9507 luminometer (EG&G Berthold, Oakridge, Tenn.) and autoinjection. Normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity. Normalized luciferase values varied minimally between replicate wells within an experiment, but varied significantly between experiments regardless of cell type, reporter, or stimulus. This was likely due to different stabilities of the renilla and firefly luciferase substrates. Therefore, all conclusions based on comparisons between experiments relied on comparisons between “fold changes” rather than normalized luciferase.

**Immunoprecipitation:** Cr:NIH(S) primary cardiac myocyte cultures (plated at 8 X 10⁵ cells per well in 12-well clusters and incubated for > 24 h) were infected as described above. After 1 h at 37° C in 5% CO₂, cultures were washed twice with methionine-free media and overlayed with 375 μl of supplemented methionine-free media containing 40 μCi ³⁵S-Methionine trans-label (specific activity 10.5 mCi/ml). At the indicated time postinfection,
cultures were washed twice with PBS and removed from plastic by scraping with a teflon cell lifter (Costar). Cells were then microfuged at 3000 RPM, 4°C for 5 min. Pellets were resuspended in 100μl cell lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40) supplemented with 1 mM phenyl-methyl sulfonyl chloride (PMSF), 10 ng/μl leupeptin, 10 ng/μl pepstatin, and 1 mM DTT and subjected to 2 freeze thaws. Samples were then microfuged at >13,000 RPM, 4°C, for 5 min. Resulting supernatant was incubated with 100 μl of a pre-equilibrated 50% Sepharose CL4B (Pharmacia) slurry at 4°C with gentle rocking for ≥3 h. Beads were allowed to settle by gravity or were microfuged for 3 min, 4°C at 6000 RPM. Supernatant was incubated with 100μl (50% slurry) of Protein A-sepharose beads (Pharmacia) preincubated with 10 μl murine IRF-1 antibody (Santa Cruz Biotech, cat. #SC-640 at 200 μg/ml) per sample (equivalent of 100 μl of a 50% slurry) at 4°C with gentle rocking, overnight. Beads were washed 4X with 6 packed bead volumes of lysis buffer supplemented with PMSF. Protein was eluted with 50 μl 2X Laemmli Sample Buffer and analyzed on a 10% SDS-polyacrylamide gel. Gel bands were quantified by scanning dried gels on a Packard Instant Imager.

**Northern Analysis:** Cr:NIH(S) primary cardiac myocyte cultures (plated at 2.0 x 10⁶ cells per well in 6 well clusters and incubated >24 h) were infected as described. At 14 h postinfection, supernatants were aspirated, and polyA⁺ mRNA was isolated directly from cells in culture plates according to the Oligotex Direct mRNA Protocol for Isolation of polyA⁺ mRNA from Animal Cells (Qiagen), with the exception that a syringe and needle was used instead of a QIAshredder for homogenizing samples. According to expected protocol
yields, approximately 0.3-0.6μg of T3D or Mock infected polyA⁺ mRNA was extracted and electrophoresed on a formaldehyde denaturing gel (52), using Oligotex 5x RNA loading buffer (Qiagen). RNA was transferred to nylon membrane and UV-crosslinked. The nylon was incubated in a rolling apparatus at 51°C for 1 h with 100μl/cm² prehybridization solution (5x SSPE, 50% formamide, 1% SDS, 5x Denhardt’s and 100 μg/ml denatured salmon sperm DNA). Riboprobe was generated from either the β-actin or murine IRF-1 template using Riboprobe Systems-T3 and -SP6 respectively (Promega). The blot was then hybridized at 51°C for approximately 20 h with 20μl/cm² hybridization buffer identical to prehybridization buffer but lacking salmon sperm DNA and containing 2000 cpm/ul of the IRF-1 riboprobe. The nylon was subjected to two room temperature 15 min washes with a 5x SSPE, 0.5% SDS solution to wash off unlabeled probe and bands were quantified on a Packard Instant Imager. The membrane was similarly hybridized with a β-actin riboprobe at 65°C and bands were again quantified. At this temperature, the IRF-1 riboprobe was removed.

Statistical analysis: A Student’s one-tailed $t$ test and pooled variance was used for statistical analysis. Results were considered significant at $P= 0.05$.

RESULTS

Reovirus induces IRF-1 in primary cardiac myocyte cultures

To determine if reovirus infection induces IRF-1 in the heart, de novo protein synthesis was monitored in primary cardiac myocyte cultures (Figure 1). Immunoprecipitation of $^{35}$S-Met labeled whole cell extracts revealed de novo synthesis of IRF-1 in mock infected cultures,
consistent with published data indicating constitutively high expression of IRF-1 in the heart (34). There was a statistically significant 1.3-fold increase in IRF-1 expression at 12 h post infection with T3D, a nonmyocarditic reovirus that induces IFN. To determine if reovirus could transcriptionally upregulate IRF-1, mRNA was examined by northern analysis (Figure 2). Primary cardiac myocyte cultures were either mock infected or infected with T3D. PolyA⁺ RNA was extracted and subjected to northern analysis using a riboprobe specific for IRF-1 or β-actin. As previously reported (34), IRF-1 mRNA was expressed constitutively in mock-infected cardiac cells. However, there was a 2.3-fold (quantification of gel in Figure 2) and a 1.5-fold (data not shown) induction of IRF-1 at 14 h postinfection when normalized to β-actin.

The mechanisms of IRF-1 transcriptional regulation were further examined using a luciferase reporter, pIRF-1 lux, which contains approximately 500 base pairs of the 5’-regulatory region of the murine IRF-1 gene inserted upstream of a firefly luciferase reporter gene. Primary cardiac myocyte cultures were transfected with pIRF-1 lux and a plasmid constitutively expressing renilla luciferase for normalizations. Cells were then either mock or virally infected, and normalized luciferase activity was analyzed. In contrast to control plasmid (pGL3-C) that showed no increase in activity (Figure 3A), T3D induced pIRF-1 lux at 8, 11, and 14 h postinfection 1.2 to 1.6-fold (Figure 3A, 3B) consistent with T3D induction of protein (Figure 1) and mRNA (Figure 2) in these cultures.

**IRF-1 induces IFN-β, but is not required for viral induction of IFN-β in primary cardiac myocyte cultures.** Since IRF-1 is a transcriptional activator of IFN-β (11), we next
determined whether IRF-1 induces IFN-β in primary cardiac myocyte cultures, using a luciferase reporter pβlux, which contains the IFN-β regulatory region upstream of a firefly luciferase reporter gene. We cotransfected primary cardiac myocyte cultures with pβLux, and either a plasmid constitutively expressing murine IRF-1 (mIRF-1) or a control plasmid (pRc/CMV2), and then analyzed normalized luciferase activity. In contrast to the control plasmid that showed no increase in activity, overexpressed IRF-1 induced pβlux 4.1-fold (Figure 4). Therefore, IRF-1 can induce IFN in these cardiac cells.

Using an IFN bioassay and RT-PCR, we demonstrated that reoviruses induce IFN-β in primary cardiac myocyte cultures (56). Consistent with our previous results (42), T3D induced an IFN-β reporter construct 3.3-fold in these cultures (Figure 5A). To determine whether IRF-1 was required for T3D induction of IFN-β in primary cardiac myocyte cultures, we compared T3D induction of pβlux in cultures generated from IRF-1−/− mice to cultures generated from wildtype parental C57BL/6J and 129Sv/Ev strains. T3D infection resulted in an approximate 3-fold induction of pβlux in both 129Sv/Ev and C57BL/6J primary cardiac myocyte cultures (Figure 5B) and a similar 4.1-fold induction in the IRF-1−/− cultures (Figure 5C). Therefore, IRF-1 is not required for viral induction of IFN-β in primary cardiac myocyte cultures.

Reovirus does not induce IRF-1 in primary cardiac myocyte cultures in the absence of IFN-α/β function. Viral infection or addition of IFN-α/β to certain cell types can induce IRF-1 (12, 34). Despite this association, no one has asked whether viral induction of IRF-1 is direct or is mediated through viral induction of IFN-α/β, in any cell type. We found that
addition of IFN-α/β to Cr:NIH (S) primary cardiac myocyte cultures did not induce a control plasmid (pGL3-C, Figure 6A), but induced IRF-1 at multiple time points (Figure 6B). T3D induced IRF-1 1.5-fold in 129Sv/Ev myocyte cultures as well (Figure 7A). While induction was apparent in cells treated with preimmune antibody, addition of anti-IFN-α/β antibody decreased viral induction of IRF-1 significantly. Some residual viral induction of IRF-1 remained, however, and similar results were obtained in Cr:NIH(S) cultures (data not shown). To determine whether this effect was due to insufficient antibody or due to direct induction by the virus, viral induction of IRF-1 was examined in primary cardiac myocyte cultures generated from IFN-α/β receptor−/− mice. While T3D induced IRF-1 1.5-fold in cultures generated from the parental wild type 129Sv/Ev mice, T3D failed to induce IRF-1 in cultures from mice lacking the IFN-α/β receptor (Figure 7B). Therefore, most, if not, all viral induction of IRF-1 is through IFN-β.

Nonmyocarditic reoviruses can induce myocarditis in IRF-1−/− neonatal mice. Lastly, to investigate the role of IRF-1 in vivo, neonatal mice were injected with two different reoviruses. Reovirus 8B is potently myocarditic in mice, while reovirus DB95 characteristically induces no or few cardiac lesions. Mice were injected with the dose of virus indicated, and at 7 days postinjection, cardiac sections were prepared, stained with hematoxylin and eosin, and examined for microscopic lesions (Figure 8). As expected, 8B induced a large number of lesions in 129Sv/Ev wild type and IRF-1−/− strains (experiments not performed in C57BL/6J mice). In contrast, while DB95 induced few or no lesions in both 129Sv/Ev and C57BL/6J wild type parental mice, the virus induced many lesions in
mice lacking IRF-1. Similar results were obtained using a higher dose of DB95 (3 x 10^6 PFU; data not shown). Thus, IRF-1 is protective against viral myocarditis.

**DISCUSSION**

IFN-β is a significant determinant of reovirus-induced myocarditis (56). IRF-3 and IRF-7 have been shown to be critical in the viral induction of IFN-β (42, 51, 60, 63). Evidence for IRF-1 regulation of IFN-β has been less straightforward, with data suggesting that IRF-1 can regulate (45), but is not required for (32, 46, 49) viral induction of IFN-β. Those studies, however, used a virus with a single-stranded RNA genome (Newcastle Disease Virus), and they investigated only undifferentiated cells. Moreover, the same studies demonstrated that dsRNA induction of IFN-β is impaired in the absence of IRF-1 (32). It was thus unclear whether a virus with a double-stranded RNA genome, reovirus, would utilize IRF-1 for IFN-β induction, and whether IRF-1’s role might be altered in differentiated cells. In addition, since the heart has higher constitutive levels of IRF-1 mRNA than other tissues examined (34), it was unclear whether cardiac cells would provide a unique environment for IRF-1 function. Finally, our previous evidence that IRF-3 functions differently in cardiac cells than in L929 cells for viral induction of IFN-β (42), suggests that the heart may provide a unique requirement for IRFs in general. We show here that reovirus induces IRF-1 in primary cardiac myocyte cultures and that overexpressed IRF-1 induces IFN-β in those cultures. Using primary cardiac myocyte cultures derived from IRF-1−/− mice, however, we show that reovirus infection induces IFN-β in the absence of IRF-
Together these data suggest that while IRF-1 is not required for viral induction of IFN-β in the heart, IRF-1 can modulate IFN-β expression there.

Using primary cardiac myocyte cultures derived from IFN-α/β receptor−/− mice, we have found that most, if not all, reovirus induction of IRF-1 is mediated through virally induced IFN-β. Despite numerous reports of virally induced IRF-1 (19, 34, 36, 47, 59-61), the possibility that this could be an indirect effect had not previously been addressed. While most investigators assumed that viral induction of IRF-1 is a direct effect of the virus, results here provide the first evidence that viruses may not induce IRF-1 directly. Recent studies describe the complexity of the IFN response, requiring both the initial direct virus activation of IRF-3 for initial IFN induction, and subsequent IFN induction of IRF-7 for a full IFN response (51, 60, 63). Our results indicate that virus induction of IRF-1 is not direct, but rather, like IRF-7, is mediated by viral induction of IFN. IRF-1 may have a second important similarity to IRF-7: maximal induction of IFN requires IFN-induced IRF-7, and maximal IFN-induction of interferon-stimulated genes may require IFN-induced IRF-1 (see below). Future studies will address whether the requirement for IFN-β in virus induction of IRF-1 is unique to reovirus or cardiac cells, or whether it is common to many viruses and cell types.

The IRF-1 regulatory region contains an IFN-γ activation sequence (GAS) (20, 57) and a binding site for NF-κB (57). The mechanism by which IFN-β induces IRF-1 is likely through the formation of Stat1/Stat2 heterodimers (28). While IFN-β induction of antiviral genes through the JAK-STAT pathway has been well-characterized, IFN-β induction of IRF-1 may provide additional critical induction of these same genes. Indeed, IFN’s antiviral
effects can be impaired in IRF-1−/− cells (25). IRF-1 has been shown to bind in monomeric and dimeric form (26, 58) to several ISREs, and IRF-1 has been shown to regulate transcription of the antiviral gene PKR (4, 37), the antiviral gene 2′-5′ oligo A synthetase (6, 45), and major histocompatibility genes (22, 27). These data suggest that IRF-1 may be important for maximal IFN induction of interferon-stimulated genes. Interestingly, reovirus infected human thyroid follicular cells show enhanced MHC I expression through virally induced IFN (1), and one possible mechanism could be through IRF-1. Given that reovirus infection activates PKR (17) and 2′-5′ oligo A synthetase (40), and that these genes have been implicated in inhibiting reovirus replication (8, 17, 39, 50) a possible antiviral role for IRF-1 as a direct inducer of these interferon stimulated genes is difficult to ignore.

In neonatal mice, reovirus-induced cardiac lesions were increased in the absence of IRF-1, demonstrating its protective role against myocarditis. IRF-1 has been shown to play a similar protective role against Venezuelan equine encephalitis virus (15) and encephalomyocarditis virus (25). Replication of vesicular stomatitis virus and herpes simplex virus in embryonic fibroblasts, however, is unaffected by the absence of IRF-1 (25). Thus the antiviral role of IRF-1 may be virus and cell type-specific. The increased cardiac lesions here in reovirus-infected IRF-1−/− mice most likely reflect increased viral replication and could be due to a number of possible mechanisms. These mice are deficient in natural killer (NK) cell function (10), an early component of the host response to infection which has been shown to protect against Coxsackie virus-induced myocarditis (14). IRF-1−/− mice also have decreased numbers of CD8+ T cells (64). Nonmyocarditic reoviruses, however, remain nonmyocarditic in mice lacking CD8+ T cells (54, 55), suggesting that increased cardiac
lesions in IRF-1−/− mice do not reflect deficient CD8+ T cell function. A similar argument can be made for the proposed critical role for IRF-1 in B-cell activation (16), in that nonmyocarditic reoviruses remained nonmyocarditic in mice lacking B cells (54). Finally, IRF-1 may play a critical role in induction of ISREs, as discussed above, and regulates other antiviral genes containing IRF-Es. For example, inducible nitric oxide synthase (iNOS) is regulated by IRF-1 (24) in many cells including cardiac myocytes (2) and has been shown to inhibit the replication of both avian reovirus (43) and Coxsackie B virus-induced myocarditis (30). Thus, IRF-1’s protective role may reflect regulation of antiviral genes (containing ISREs or IRF-Es) or immune cells, or both. Future studies will address the downstream effectors of the IRF-1 protective response.

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REFERENCES CITED


Figure 1. Reovirus induces IRF-1 in primary cardiac myocyte cultures. Cr:NIH(S) primary cardiac myocyte cultures were metabolically labeled with $^{35}$S-Met and either mock- or T3D-infected. Cell extracts were prepared at the indicated time postinfection. IRF-1 was immunoprecipitated and analyzed by gel electrophoresis. Duplicate wells are shown for T3D-infected cultures at 9 h.p.i., 10.5 h.p.i., and 12 h.p.i. or mock infected cultures at 12 h.p.i. The far right lane shows approximate molecular weights from prestained marker. Gels were dried and scanned for quantification of IRF-1-specific bands (bottom panel). Asterisk denotes a significant increase between mock- and virus-infected cultures ($P=0.05$)
Figure 2. Reovirus transcriptionally upregulates IRF-1 mRNA in primary cardiac myocyte cultures. Cr:NIH(S) primary cardiac myocyte cultures were either mock- or T3D-infected. Poly-A+ mRNA was extracted 14 h.p.i. and electrophoresed on a formaldehyde denaturing gel. Northern analysis revealed a band approximately 2.1 kB after hybridizing with IRF-1 riboprobe (left panel). IRF-1 probe was removed and the membrane was rehybridized with β-actin probe, revealing a band at approximately 1.9 kB and another at 1.3 kB, corresponding to β-actin and α-actin respectively (right panel). The far right lane shows approximate molecular weights. V=Viral, M=Mock.
Figure 3. Reovirus induces an IRF-1 reporter construct. Primary cardiac myocyte cultures generated from Cr:NIH(S) mice were transfected with the indicated plasmid and the normalization plasmid pRL-SV40. One day post-transfection, the cultures were mock infected ( ) or infected with reovirus T3D ( ). Luciferase activity was measured 14 h postinfection in panel A and at 8, 11, and 14 h postinfection in panel B. For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity. Each bar shows the mean of three wells (+ the standard error of the mean). Asterisks denote a significant increase between mock- and virus-infected cultures (panel A, $P<0.001$; panel B, at 8 h $P=0.006$, at 11 h $P=0.001$, at 14 h $P=0.003$).
Figure 4. Murine IRF-1 induces IFN-β. Primary cardiac myocyte cultures generated from Cr:NIH(S) mice were transfected with pβlux, the normalization plasmid pRL-SV40, and either control (pRc/CMV2) or murine IRF-1 (mIRF-1) effector plasmid. Cells were mock infected 1 day post-transfection, and luciferase activity was measured 14 h postinfection. For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity. Each bar shows the mean of three wells (+ the standard error of the mean). Similar results were obtained in replicate experiments. The asterisk denotes a significant increase between cultures transfected with pRc/CMV2 and murine IRF-1 (P=0.01).
Figure 5. IRF-1 is not required for viral induction of IFN-β. Primary cardiac myocyte cultures generated either from Cr:NIH(S) mice (panel A), from C57BL/6J mice (panel B, left), from 129Sv/Ev mice (panel B, right), or from IRF-1 (-/-) mice (panel C) were transfected with control plasmid (PGL3-C) or pβlux and the normalization plasmid pRL-SV40. One day post-transfection, the cultures were mock infected ( ) or infected with reovirus T3D ( ). Luciferase activity was measured 14 h postinfection. For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity. Each bar shows the mean of three wells (+ the standard error of the mean). Asterisks denote a significant increase between mock- and virus-infected cultures (panel A, \( P=0.001 \); panel B left, \( P=0.003 \); panel B right, \( P=0.048 \); panel C, \( P=0.001 \)).
B.

![Graph showing normalized luciferase activity for C57BL/6J and 129Sv/Ev strains.](image)

C.

![Graph showing normalized luciferase activity for IRF-1 (-/-) knockouts.](image)
Figure 6. Treatment with type 1 IFN induces IRF-1 in primary cardiac myocytes. 
Primary cardiac myocytes were transfected with the control plasmid pGL3-C (A) or pIRF-1 lux (B), and the normalization plasmid pRL-SV40. One day posttransfection, the cultures were mock treated ( ) or treated with IFN-α/β ( ). Luciferase activity was measured at 14 h posttreatment (Figure 6A) and at 8, 11, and 14 h posttreatment (Figure 6B). For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity. Each bar shows the mean of three wells (+ the standard error of the mean), with the exception of the pIRF-1 lux bar in panel 6A, which shows the mean of four wells (+ the standard error of the mean). Asterisks denote a significant increase between mock- and IFN-treated cultures (panel A, right $P=.036$; panel B, from left to right, $P<0.001$; $P=.001$; $P<0.001$).
Figure 7. Viral induction of IRF-1 is through virally induced IFN-β. Primary cardiac myocyte cultures generated from 129 Sv/Ev or IFN-α/β receptor (-/-) mice were transfected with pIRF-1 lux and the normalization plasmid pRL-SV40. The cells were mock infected ( ) or infected with reovirus T3D( ) 1 day postransfection. In panel A, 1100 neutralizing units/ml anti-IFN-α/β or preimmune antibody was added to the wells with overlay media immediately following infection. Luciferase activity was measured 14 h postinfection. For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity. Each bar shows the mean of three wells (+ the standard error of the mean). Similar results were obtained in replicate experiments. In panel A (left), the asterisk denotes a significant increase between preimmune-treated cultures mock or T3D infected; \( P=0.03 \). In panel A (middle), the asterisk denotes a significant decrease between infected cultures treated with preimmune or anti-IFN antibody; \( P=0.03 \). In panel A (right), the asterisk denotes a significant increase between anti-IFN-α/β antibody-treated cultures, mock- or T3D-infected; \( P=0.01 \). In panel B (left), the asterisk denotes a significant increase between cultures, mock- or T3D-infected; \( P=0.011 \).
Figure 8. **IRF-1 can protect against viral myocarditis.** C57BL/6J ( ), 129 Sv/Ev ( ), or IRF-1−/− ( ) neonatal mice were inoculated intramuscularly with either 1 x 10^3 PFU reovirus strain 8B (myocarditic; C57BL/6J not injected) or 5 x 10^5 - 1 x 10^6 PFU reovirus strain DB95 (nonmyocarditic). At 7 days postinjection, blinded H&E-stained cardiac sections were examined for microscopic lesions utilizing morphometric analysis. Percent lesion refers to the average area of lesion divided by the average total area, x 100, + standard error of the mean. The asterisk denotes a significant increase between DB95 infected C57BL/6J, 129 Sv/Ev, or IRF-1−/− mice; \( P=0.05 \) for C57BL/6J vs. IRF-1−/−; \( P=0.005 \) for 129 Sv/Ev vs. IRF-1−/−.
CHAPTER 3

REOVIRUS INDUCTION OF IFN AND IFN INDUCTION OF ISGS

IS CELL TYPE-SPECIFIC AND DISCORDANT

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Running title: Reovirus and IFN Induction of ISGs.
ABSTRACT

Viral myocarditis is an important human disease, but the mechanisms of direct viral damage to the heart remain largely unexplored. Reovirus-induced myocarditis in mice is not immune-mediated, providing an excellent model for the disease. In the past, we showed that reovirus induction of, and sensitivity to, interferon (IFN)-β are important determinants of protection against viral pathogenicity in the heart. The effects of IFN-β are mediated by the induction of multiple antiviral genes termed interferon-stimulated genes (ISGs). Here, we investigated reovirus induction of IFN-β and ISGs in two primary cell cultures: a highly differentiated cell type, primary cardiac myocytes (PCMC), and a more plastic cell type, primary embryonic fibroblasts (PMEFs). Reovirus induced IFN-β significantly in PCMCs, but poorly or not at all in PMEFs, despite equivalent infection efficiencies for the two cell types. Surprisingly, reovirus induced several ISGs (561, 6-16, and PKR) to significantly higher levels in PMEFs than in PCMCs. Moreover, IFN-α/β induction of the ISGs displayed these same patterns of cell type–specificity, and reovirus induction of these genes was primarily mediated by its induction of IFN-β. Thus, in contrast to several other RNA viruses, reovirus bypasses direct signaling of ISGs, delaying the cell’s antiviral response. Moreover, these data suggest that while ISGs are regulated by IFN-β, cell type-specific signals, rather than IFN-β quantity, are the primary determinants of viral and IFN-β induction of ISGs. Finally, these data suggest that cells prone to viral induction of IFN-β are resistant to induction of ISGs, indicating a possible cell type-specific feedback mechanism regulating the protective response to viral infection.
INTRODUCTION

Viral myocarditis affects an estimated 5-20% of the human population (77). Many viruses have been implicated in this disease (77), with enteroviruses and adenoviruses most likely accounting for the majority of cases (32). Enterovirus-induced myocarditis is both immune-mediated (10, 49) and due to direct cytopathic effect (8, 28), while adenovirus-induced myocarditis is most likely not immune-mediated (32). Given that many viruses gain access to the heart, and that cardiac myocytes are not replenished, the innate response of cardiac cells to viral insult may well be a significant determinant of cardiac damage. However, non-immune mediated mechanisms of viral myocarditis remain largely unexplored. Reovirus-induced myocarditis is not immune-mediated (62), but instead reflects virally induced apoptosis (15), and thus provides a unique model to investigate the cardiac response to viral infection.

The role of interferon-β (IFN-β) in providing cellular resistance to viral infection is well documented (6, 34, 50, 58, 70). Previously, we have found that in primary cardiac myocyte cultures (PCMCs), nonmyocarditic reoviruses induce more IFN-β and are more sensitive to the antiviral effects of IFN-α/β than myocarditic reoviruses (63). Addition of anti-IFN antibody benefits reovirus spread in PCMCs (63), and nonmyocarditic reoviruses induce myocarditis in mice pretreated with anti-IFN-α/β antibody. Therefore, IFN-β is an important determinant of protection against reovirus-induced myocarditis (63).

The effects of IFN-β are mediated by the induction of interferon-stimulated genes (ISGs) which can produce antiviral effects at different stages of viral replication (50, 58, 64). Over one hundred ISGs have been documented (14, 16), and while the antiviral effects of some are well characterized, many have functions that are still unknown. For example, the double stranded
RNA activated kinase, PKR, has been shown to play a role in the control of cell growth, apoptosis, differentiation, and the antiviral actions of IFNs (6, 9). Specifically, PKR phosphorylates the NFκB inhibitor, IκB (31), which leads to activation and nuclear translocation of NFκB (69), thereby regulating IFN-β and other cytokines. PKR also blocks initiation of translation by phosphorylating the α subunit of eukaryotic protein synthesis initiation factor (eIF) 2 (27, 51). While not as well characterized as PKR, the 561 encoded protein, p56, has been shown to interact with the p48 subunit of eIF3 to downregulate translation (24) and negatively regulate cell proliferation (18). In addition, structurally, p56 has tetratricopeptide repeat (TPR) motifs similar to those found in other antiviral proteins (23). In contrast to PKR and 561, the function of 6-16 is unknown. However, the 6-16 regulatory region shares homology with that of the IFN-β, 2’-5’-oligo (A) synthetase, and class I MHC genes (46). Regardless of function, every ISG promoter contains an interferon-α/β stimulated response element (ISRE) which may vary in sequence.

IFN induction of antiviral ISGs is mediated by activation of the JAK-STAT pathway. After IFN-α/β binds to their receptor, receptor-associated cytoplasmic Janus tyrosine kinases (Jaks) are activated (43, 54). This permits Stat1 and Stat2 to become phosphorylated, form heterodimers, and migrate to the nucleus, recently reviewed (30, 43, 54), where they become associated with p48 (IRF-9) to form the IFN-stimulated gene factor 3 (ISGF3) complex (13, 36, 55). This complex binds to the ISRE sequence, to stimulate the ISG (57). While the predominant IFN signaling pathway is through ISGF3, Stat1/Stat2 heterodimers may also form in response to IFN treatment, leading to transcriptional regulation of certain ISGs without IRF-9 (19). Stat1 independent IFN signaling has also been cited (20), as have the
phosphorylation of Stat3 (78), and Stat5 and Stat6 (17) in response to IFN-α/β stimulation, suggesting additional possible mechanisms for regulation of ISGs.

While multiple IFN-regulated pathways induce ISGs, some viruses, such as vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV), Sendai virus (23), Newcastle disease virus (NDV) (71), or even dsRNA (3) have been shown to induce certain ISGs in an IFN-independent manner, without the synthesis of IFN. This induction is likely through the action of interferon regulatory factor-3 (IRF-3) or IRF-1 (3, 12, 21, 23), which are capable of inducing ISGs through sequence similarities between their binding site, the IRF-E, and the ISRE (38).

IFN, viral infection, and dsRNA can use distinct signaling pathways to upregulate antiviral genes (3, 11, 12, 23, 71, 73, 75, 76), and antiviral pathways can vary between cell types. Surprisingly, no previous studies of the cell’s response to viral infection have used primary cultures other than murine embryonic fibroblasts (MEFs), and in most cases, the MEFs were passaged. Here we investigate reovirus induction of IFN-β and ISGs in two primary cell cultures, a differentiated cell type, primary cardiac myocytes (PCMCs), and a more generalized cell type, primary MEFs (PMEFs). Because evidence suggests that PMEFs change their phenotype with respect to IFN induction upon even limited passage (26), we used only freshly generated PMEFs.

We report here that reovirus induces IFN-β and ISGs differentially depending on the cell type, despite similar efficiencies of infection. Reovirus induced IFN-β significantly in PCMCs, but poorly or not at all in PMEFs. Surprisingly however, reovirus induced the ISGs 561, 616, and PKR to a significantly higher level in PMEFs than in PCMCs. IFN-α/β treatment induced
these ISGs in the same pattern as reovirus did, and reovirus failed to induce any of the ISGs in the absence of IFN-α/β function in either primary cell culture. Thus, in contrast to several other RNA viruses, reovirus bypasses IFN-independent signaling of ISGs, delaying the cell’s antiviral response. Moreover, these data suggest that while ISGs are regulated by IFN-β, other cell type-specific signals dictate differential responses. This provides the first report of cell type-specific IFN-α/β regulation of ISGs in a primary nonlymphoid differentiated cell culture. Finally, the discordance between viral induction of IFN-β and ISGs suggests a possible cell type-specific feedback mechanism governing the protective response to viral infection.

MATERIALS AND METHODS

**Mice:** IFN α/β receptor−/− mice generated by Muller et al. (37) and wildtype parental 129Sv/Ev mice were generously provided by Dr. Herbert W. Virgin IV (Washington School of Medicine, St. Louis, Missouri). Mice were housed according to AAALAC recommendations and all procedures were approved by NCSU IACUC.

**Cell Cultures:** To generate primary cardiac myocyte cultures (PCMCs) from 129Sv/Ev IFN-α/β receptor−/− mice, term fetuses or 1-day-old neonates were sacrificed and the apical two-thirds of the hearts were removed, minced, and trypsinized (4). Cells were plated at a density of 1.25 x 10^6 cells per well in 6-well clusters (Costar, Cambridge, Mass.) and incubated for 1.5 to 2 h to remove rapidly adherent cells (predominantly fibroblasts). The remaining cells (predominantly myocytes) were resuspended in Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD) supplemented with 7% fetal calf serum (HyClone, Logan, Utah), 0.06%
thymidine (Sigma Co., St. Louis, Mo.), and 50 µg of pipracil (Lederle Co.) per ml, (“supplemented media”) and plated as indicated for each procedure. Myocyte cultures contained 5-20% fibroblasts (4), consistent with levels reported by others (25, 29, 33), and consistent with cell heterogeneity in the heart. To generate primary murine embryonic fibroblasts (PMEFs), 13-15 day old embryos were removed and washed in DMEM F12 (Gibco BRL, Gaithersburg, MD) supplemented with 10 µg gentamycin per ml and 50 µg pipracil per ml, extruded through a 10 ml syringe, and trypsinized for 1 h. Cells were then filtered through a mesh cell strainer and centrifuged at 1200g for 8 min. The cell pellet was resuspended in DMEM F12 supplemented with 10% fetal calf serum (HyClone), 10% tryptose phosphate broth, and above antibiotics (“supplemented media”) and this process was repeated two times to wash. The cells were then plated at a density of 1.25 x 10⁶ cells per well in 6-well clusters (Costar, Cambridge, Mass.) and incubated for 1.5 to 2 h to separate rapidly adherent fibroblasts from all other non-adherent cells, which were washed away with DMEM F12 after incubation. Fibroblasts were trypsinized for 3-4 minutes after which time supplemented media was added to neutralize the trypsin. PMEFs were then plated as indicated for each procedure. These processes were repeated for each experiment to generate fresh cultures of PCMCs and PMEFs. Passaging MEFs has been shown to change their phenotype with respect to IRFs and IFN (26), and therefore, the PMEFs were never frozen or passaged.

**Plasmids:** The βlux reporter plasmid was constructed as previously described (41), and contains the murine IFN-β regulatory region inserted into pGL3-Basic (Promega). The 6-16lux reporter plasmid was constructed by amplifying upstream sequences (-603 to +437) of the human 6-16
gene from the GS203CAT plasmid (which contains these sequences 5′ to the CAT gene) and inserting them into the Xho I and Hind III sites of pGL3-Basic (Promega). Both GS203CAT and the 561lux reporter were generously provided by Dr. Ganes C. Sen (The Cleveland Clinic Foundation, Cleveland, Ohio). The 561lux reporter contains sequences (-654 to +3) of the 561 promoter transferred into the Sst I and Hind III sites of pGL3-Basic (Promega). The PKRlux reporter plasmid was constructed by amplifying the murine PKR promoter sequences (-777 to +132) using PCR and inserting them into the Kpn I and Bgl II sites of pGL3-Basic. pRL-SV40 (expressing renilla luciferase constitutively from an SV40 promoter), pGL3-Basic (lacking a promoter and therefore expressing baseline firefly luciferase), pGL3-Control (expressing firefly luciferase constitutively from an SV40 promoter) were purchased (Promega). DNA was purified for transfection using Qiagen’s Maxiprep system (Qiagen Inc., Valencia, Calif.).

Transfections: PCMCs or PMEFs were plated at a density of 1.0 × 10^5 cells per well in 0.5 ml in 48-well tissue culture plates from Costar. All cultures were allowed to adhere for 1 day prior to transfection. Transfection was performed as previously described (2, 40, 41) using FuGENE6 according to the manufacturer’s protocol (Boehringer Mannheim/Roche Molecular Biomedicals, Indianapolis, Ind.). Transfection conditions were optimized previously using a β-galactosidase reporter plasmid that demonstrated that myocytes in PCMCs were transfected, although efficiency was low (40). A comparison of raw renilla luciferase values for all reporter plasmids indicated that there was no significant difference in transfection efficiency regardless of reporter plasmid or cell type. Additionally, there was no difference in normalized pGL3-Control values between PCMCs and PMEFs, indicating that the assay normalizations did not
vary between cell type. Unless noted otherwise in figures, the amount of DNA added to the indicated wells was as follows in a 48-well cluster experiment: βlux 1.0 µg; 561Lux 1.0 µg; PKRlux 1.0 µg; 6-16lux 0.4 µg; pRL-SV40 0.01µg; pGL3-Control 1.0 µg. FuGENE6 was used in a volume equal to twice the total micrograms of plasmid DNA to be transfected per well (e.g., 2 µg of plasmid DNA per well required 4 µl of FuGENE6 per well).

Infections: Infections were performed 1 day postransfection. PCMCs or PMEFs were washed twice with supplemented DMEM immediately prior to infection. For infections, two wells were trypsinized and viable cells were counted using trypan blue exclusion. For PCMCs, cells were infected with either reovirus T3D (Dearing) or VSV (strain Indiana, generously provided by Dr. Ganes Sen) at 25 PFU per cell in 150µl supplemented media or were mock-infected. PMEF cultures were infected with reovirus T3D at 25 PFU per cell and with VSV at 1 PFU per cell since VSV was cytopathic to PMEFs at higher PFUs per cell. Cultures were incubated for 1 h at 37°C in 5% CO2. For transfected cultures, 350 µl supplemented DMEM was then added. Three hours prior to harvest, 5500 units/ml IFN-α/β was added if indicated (Lee Biomolecular Research Inc.). While this concentration of IFN-α/β was chosen in an attempt to mimic concentrations induced by reovirus (63), 1000 units/ml was found to give similar results. Cells were incubated at 37°C and 5% CO2 for 15 h before harvesting. The nonmyocarditic reovirus, T3D, was carefully chosen for these experiments as it has proven to be a consistent IFN inducer in PCMCs, and thus a likely inducer of ISGs. Immunohistochemistry of mock- and T3D-infected cells indicated that at 10 h postinfection, PCMCs and PMEFs were infected with the same efficiency (4-7% and 5-10% respectively). The low percentage of
infection can be attributed to the early timepoint (10 h), chosen to ensure detection of only primary infection. Using an alternative method to prevent secondary infection and harvesting at a later time point, we have found that similar reoviruses (having the same σ1 attachment protein) infected 20-45% of PCMCs (60), and we would expect a similar frequency for PMEFs.

_Dual-luciferase assay:_ The dual-luciferase assay was performed according to the manufacturer’s protocol (Promega) with the following exceptions: cells were washed twice with phosphate-buffered saline prior to the addition of lysis buffer, cells were allowed to remain in lysis buffer at 4 °C for at least 15 min. Measurements were made using a Lumat LB 9507 luminometer (EG&G Berthold, Oakridge, Tenn.) and autoinjection. Normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity.

_Statistical analysis:_ A Student’s one-tailed _t_ test and pooled variance was used for statistical analysis. Results were considered significant at _P_≤ 0.05.

**RESULTS**

_Reovirus induces more IFN-β in PCMCs than PMEFs._ We have previously demonstrated that reoviruses induce IFN-β in PCMCs using an IFN bioassay (63), RT-PCR (63), and reporter constructs (2, 41). Here, to determine whether reovirus also induces IFN-β in PMEFs, PCMC and PMEF cultures were cotransfected with an IFN-β reporter construct, βlux, and a plasmid constitutively expressing renilla luciferase for normalizations, and then either mock- or virally-infected. Normalized luciferase activity was analyzed and fold induction was determined for
multiple experiments (Figure 1). Consistent with previous results, T3D induced βlux to a statistically significant level in all 15 experiments performed in PCMCs. However, reovirus induced βlux only 1.2-fold in the PMEFs, with the induction being statistically significant in only 2 experiments out of 9. A comparison of all experiments demonstrated a statistically significant difference between reovirus induction of βlux in PCMCs and PMEFs ($P<0.001$).

**Reovirus and IFN-α/β induce ISGs more in PMEFs than in PCMCs.** Given that reovirus induced more IFN-β in PCMCs than in PMEFs, one might expect similar cell type-specificity for reovirus induction of ISGs. Transcriptional regulation of the ISGs 561, 6-16, and PKR were examined in PCMCs and PMEFs using luciferase reporters. The 561lux, 6-16lux, or PKRlux plasmids were cotransfected with the normalization plasmid into PCMCs and PMEFs. Normalized luciferase activity was analyzed for multiple experiments and the fold induction due to virus infection or IFN-α/β treatment was determined. As expected, control plasmid (pGL3-C) showed no increase in activity in either cell type following virus or IFN-α/β stimulation (data not shown). Surprisingly, however, while reovirus induced each of the ISG reporters in both cell types, multiple experiments demonstrated that this induction was significantly higher in PMEFs than in PCMCs for each of the three ISGs (Figure 2). Thus, T3D induction of ISGs was greater in PMEFs compared to PCMCs despite higher induction of IFN-β in PCMCs relative to PMEFs. IFN-α/β induction of ISGs followed the same cell type-specific pattern as reovirus induction of ISGs (Figure 2). This suggested that the quantity of IFN induced by T3D did not determine the extent of ISG induction, but instead, the cell type was the primary determinant both in reovirus and IFN-α/β induction of ISGs.
Reovirus requires IFN-α/β function for induction of ISGs in PMEFs. To determine whether reovirus induction of ISGs is direct or is mediated through induction of IFN-α/β, we compared viral induction of reporter plasmids in PMEFs derived from IFN-α/β receptor−/− and 129Sv/Ev mice. As a positive control, we included VSV, which has been previously demonstrated to induce ISGs directly in the absence of IFN-α/β (23). As expected, VSV induced ISGs in IFN-α/β receptor−/− PMEFs with no statistically significant reduction relative to 129Sv/Ev PMEFs (Figure 3). In contrast, multiple experiments revealed that for T3D there was a statistically significant reduction in the average fold induction of ISGs in the IFN-α/β receptor−/− PMEFs relative to the 129Sv/Ev PMEFs (Figure 3). Furthermore, while T3D significantly induced each of the ISGs in every experiment in cultures generated from the parental wildtype 129Sv/Ev mice, it failed to significantly induce any of the ISGs in any of the experiments in the IFN-α/β receptor−/− PMEFs, indicating that most, if not all, T3D induction of the ISGs tested is through the IFN signal. In addition, while VSV induction of IFN-β was not reduced in IFN-α/β−/− PMEFs, the significant reduction in IFN-β induction by T3D in IFN-α/β receptor−/− PMEFs indicated that most T3D induction of IFN-β is through IFN-induced amplification.

Reovirus requires IFN-α/β function for induction of ISGs in PCMCs. In the PCMCs (Figure 4), VSV induced the ISG reporters to the same extent in the 129Sv/Ev and IFN-α/β receptor−/− cultures, indicating that as in PMEFs, VSV can directly induce ISGs in the absence of IFN-α/β. Interestingly, VSV induction of ISGs was lower in PCMCs than in PMEFs (compare Figures 3 and 4), displaying the same cell type-specificity as reovirus and IFN (Figure
2). Given that reovirus induced the ISG reporters only poorly in wildtype PCMCs (Figure 2), a statistically significant reduction in induction of ISGs in IFN-α/β/−/− PCMCs was not expected. Indeed, while reovirus induced the ISG reporters statistically significantly in fewer experiments in IFN-α/β receptor −/− PCMCs than in wildtype PCMCs, and while the average induction was lower (Figure 4), the difference between cultures was statistically significant only for 561.

While VSV induction of IFN-β was not reduced in IFN-α/β −/− PCMCs, the significant reduction in IFN-β induction by T3D in IFN-α/β receptor −/− PCMCs was, again, an indication that most T3D induction of IFN-β is through IFN-induced amplification.

**DISCUSSION**

Reovirus induces IFN-β in a variety of cell lines (52), PCMCs (60), and primary cultures of human thyroid follicular cells (1). Here we show that poor reovirus induction of IFN-β in PMEFs and robust induction in PCMCs is significantly reduced in the absence of the IFN-α/β receptor (Figures 3, 4), indicating that most of the IFN-β measured in these cell types reflects further amplification of IFN-β upon IFN-β dependent-signaling. This suggests that the initial IRF-3 dominated phase of IFN-β induction in both PCMCs and PMEFs is minimal, relative to the later induction phase dominated by IRF-7 (53, 68, 74). We have found that the requirement for IRF-3 in reovirus induction of IFN-β is cell type-specific (41), and others have found that in the context of NDV infection, this requirement may depend on cell type-specific constitutive levels of IRF-7 (26). Together, these data suggest that the cell type-specific role of IRF-3 and/or possible differences in the cell type-specific levels of IRF-7 may be responsible for the differential induction of IFN-β in PCMCs and PMEFs.
Using PCMCs or PMEFs derived from IFN-α/β receptor−/− mice, we found that most, if not all, reovirus induction of ISGs (PKR, 561, and 6-16) is mediated through virally induced IFN-β (Figures 3, 4). This suggests that reovirus, unlike VSV (23), EMCV (23), Sendai virus (23), and NDV (71) bypasses IFN-independent signaling of ISGs. Interestingly, Sendai virus induction of similar ISGs is predominantly through IFN-β in Vero cells (48), suggesting possible cell type-specificity. Reovirus induction of ISGs predominantly through IFN-β (in both PCMCs and PMEFs) is also supported by the observation that reovirus and IFN displayed similar cell type-specific induction of ISGs (Figure 2). Furthermore, because IFN induced these ISGs in the absence of viral infection, it is unlikely that reovirus provides additional regulatory signals. While IFN treatment alone did not induce IFN-β in PCMCs or PMEFs (data not shown), likely because IRF-7 requires virally induced phosphorylation for its amplification effects, IFN treatment did induce ISGs, most likely because the added IFN was sufficient to directly induce ISGs with no need for further amplification.

Importantly, while our results suggest that the pathways for viral and IFN induction of ISGs likely overlap, the cell type-specific pattern of ISG induction is discordant with T3D induction of IFN-β. Specifically, reovirus induces IFN-β in PCMCs, likely through the JAK-STAT pathway, however, none of the ISGs are significantly and consistently induced by T3D in this cell type. Instead, reovirus induces all three ISGs in PMEFs, despite poor induction of IFN-β, in comparison to PCMCs. These results suggest that the quantity of IFN-β induced by T3D does not determine ISG induction, but instead, that the cell type is the primary determinant of both reovirus and IFN-α/β induction of ISGs. Moreover, these data suggest that cells prone to viral induction of IFN-β are resistant to induction of ISGs. Induction reflects both initial basal
expression and subsequent signaling, and future investigations identifying absolute levels of ISGs will provide further insight into these cell type-specific phenotypes. A recent report of cell type-specific differences in IFN-α induction of ISGs in primary T cell and dendritic cell cultures indicates that differential responses also occur in professional immune cells (56). Those studies, however, did not address viral induction of IFN or ISGs, nor did they address IFN-dependent amplification of IFN. Possible similarities between immune and non-immune cells in these cell type-specific responses remain to be explored.

It is interesting that the three ISGs behaved similarly, despite their distinct regulatory regions (see Figure 2 of Chapter1). PKR contains multiple regions for transcription factor binding, including among others: a KCS site, ISRE sequence, GAS element, NF-κB binding site, and an NF-IL6 binding site (65). In contrast, the 561 promoter contains ISREs, but no other predicted sites for transcription factor binding (21, 45, 72). Similarly, aside from a predicted SP1 site, and tandem ISRE, the 6-16 regulatory region is largely devoid of predicted sites for transcription factor binding (7). Indeed, there are no predicted transcription factor binding sites that are constant between all three ISGs, other than the ISRE. Notably, IRFs can bind ISREs (38) due to sequence similarities with IRF-Es, and may thus provide the missing link between these three ISGs. IRF-1 (3, 5, 39, 42, 66), IRF-2 (42), and IRF-3 (21) have all been implicated in regulation of these ISGs.

Antiviral pathways can vary between cell types, and cell type-specific variations in IRF-1, IRF-2, and IRF-3 have previously been identified. While IRF-1 mRNA is not normally expressed in the brain, liver, or many other tissues, it is constitutively expressed in the heart (35). We have found that IRF-2 has opposite effects on constitutive IFN-β expression in
skeletal muscle cells and cardiac muscle cells (59). And finally, we have found that IRF-3 is required for viral induction of IFN-β in primary cardiac myocyte cultures but not in the undifferentiated L929 cell line (41). Others have confirmed cell type-specific variations in IRF-3 function in other cell types (47). Given these precedents suggesting a unique environment for IRFs in the heart, cell type-specific differences in ISG induction may well be explained by cell type-specific variations in constitutive or induced IRF levels. For example, IRF-2 can act as a competitive antagonist of ISG induction because it has the same binding specificity as IRF-1 (67), and thus others have suggested that ISG regulation may reflect the ratio of IRF-1 to IRF-2 (44, 67). Indeed, cell type-specific regulation of ISGs has been observed in astrocytes and neurons, where MHC class I genes are induced in neurons, but not astrocytes, most likely through cell type-specific differences in IRF-1 or IRF-2. Therefore, it is possible that IRF-2 or another IRF may inhibit ISG induction in PCMCs. IRF-1 has been shown to regulate 561 (3), 6-16 (42), and PKR (5, 39) albeit through different stimuli. Given that IRF-1 is constitutively expressed in the heart (35), it is possible that IRF-1 cannot be induced significantly, leading to low levels of subsequent ISG induction. In the same respect, IRF-1 regulated baseline levels of PKR, 561, and 6-16 levels may be high in PCMCs, leading to poor induction of these ISGs.

An alternative hypothesis is that ISG induction depends on ISGF3 formation and binding, but that the poor ISG induction in the PCMCs might be explained by an ISGF3 signaling deficiency in this cell type (Figure 5). While this model may explain differential induction of the ISGs in the two cell types, it does not explain the high level of IFN-β amplification in PCMCs, presumably mediated by ISGF3 induced IRF-7. This aspect will be further investigated in Appendix 3. Since cardiac myocytes do produce significant levels of
IFN-β in response to viral infection, it is puzzling that these unreplenishable cells do not also induce ISGs to significantly high levels. Perhaps cells prone to viral induction of IFN-β evolved a deficiency such as the one described above as a feedback mechanism to regulate the protective response to viral infection.

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REFERENCES CITED


adenosinetriphosphatase and myosin content in cultured heart cells. Arch. Biochem. 
Biophys. 240:312-318.

34. Mittnacht, S., P. Straub, H. Kirchner, and Jacobsen. 1988. Interferon treatment
inhibits onset of herpes simplex virus immediate-early transcription. Virology.

Miyata, and T. Taniguchi. 1988. Regulated expression of a gene encoding a nuclear
factor, IRF-1, that binds specifically to IFN-beta gene regulatory elements. Cell.
54:903-913.

36. Muller, M., C. Laxton, J. Briscoe, C. Schindler, T. Improta, J. E. J. Darnell, G.
of the 91 kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal 
transduction pathways. EMBO J. 12:4221-4228.

and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral 

38. Nakaya, T., M. Sato, N. Hata, M. Asagiri, H. Suemori, S. Noguchi, N. Tanaka,
and T. Taniguchi. 2001. Gene Induction Pathways Mediated by Distinct IRFs during 
Viral Infection. Biochemical and Biophysical Research Communications. 283:
1150-1156.

genes following inducible expression of IRF-1 or IRF/RelA fusion proteins. 
Oncogene. 15:1425-35.

myocyte cultures with DNA and anti-sense oligonucleotides using FuGENE 6 

required for viral induction of beta interferon in primary cardiac myocyte cultures. J. 


Figure 1. Reovirus induces more IFN-β in PCMCs than in PMEFs. PCMCs and PMEFs generated from 129Sv/Ev mice were transfected with the βlux plasmid (firefly luciferase) and the normalization plasmid pRL-SV40 (constitutive renilla luciferase). One day post-transfection, the cultures were mock-infected or infected with reovirus T3D. Luciferase activity was measured 15 h postinfection. For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity, and fold induction for each experiment was determined by dividing the normalized luciferase readout from three T3D-infected wells by that from three mock-infected wells. Each bar indicates the mean fold induction of multiple experiments (+ the standard error of the mean). Fraction of experiments with statistically significant induction indicates the fraction of experiments where induction in the three mock-infected wells was statistically significantly different ($P<0.05$) from that in the three T3D-infected wells. The asterisk denotes a significant difference between PCMCs and PMEFs ($P<0.001$).
Figure 2. Reovirus and IFN-α/β induce ISGs more in PMEFs than in PCMCs. PCMCs and PMEFs generated from 129Sv/Ev mice were transfected with the indicated plasmid and the normalization plasmid pRL-SV40. One day post-transfection, the cultures were mock infected, infected with reovirus T3D ( ), or treated with IFN-α/β ( ). Three hours post-IFN-α/β-stimulation, or 15 hours post-infection, cultures were harvested for luminometry. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Fraction of experiments with statistically significant induction is as for Figure 1. Asterisks denote a significant difference between PCMCs and PMEFs (from left to right, 616: $P<0.001$; $P=0.002$; 561: $P=0.002$; $P=0.017$; PKR: $P=0.049$; $P=0.047$).
Figure 3. Reovirus requires IFN-α/β function for induction of ISGs in PMEFs. PMEFs generated from 129Sv/Ev ( ) or IFN-α/β-receptor -/- ( ) mice were transfected with the indicated plasmid and the normalization plasmid pRL-SV40. One day post-transfection, the cultures were infected with either VSV strain Indiana, or reovirus T3D. Luciferase activity was measured 15 h post-infection. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Data from T3D-infected 129Sv/Ev PMEFs included here are from Figures 1 and 2. Fraction of experiments with statistically significant induction is as for Figure 1. Asterisks denote a significant decrease between 129Sv/Ev and IFN-α/β receptor -/- PMEFs (from left to right, $P=0.049$; $P<0.001$; $P=0.001$; $P=0.028$).
Figure 4. Reovirus requires IFN-α/β function for induction of ISGs in PCMCs. PCMCs generated from 129Sv/Ev (■) or IFN-α/β-receptor−/− (□) mice were transfected, infected, and measured for luciferase activity as in Figure 3. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Data from T3D-infected 129Sv/Ev PCMCs included here are from Figures 1 and 2. Fraction of experiments with statistically significant induction is as for Figure 1. Asterisks denote a significant decrease between 129Sv/Ev and IFN-α/β receptor−/− PCMCs (from left to right, P<0.001; P<0.001).
Figure 5. Possible mechanism for differential ISG induction in PMEFs and PCMCs. While PMEFs may regulate ISGs through the classic JAK-STAT pathway, PCMCs may have evolved a negative feedback mechanism that regulates large scale induction of ISGs. One possibility may be a deficiency in the formation of ISGF3. The deficiency could lead to over-production of Stat1/Stat2 heterodimers (19), Stat1 homodimers (22), or some other complex.
APPENDIX 1
REOVIRUS INDUCTION OF AN ISRE REPORTER

INTRODUCTION
In Chapter 3, we found that reovirus induces ISGs to a much higher extent in PMEFs than in PCMCs. No binding sites were similar between the ISGs except for the ISRE site, and thus we constructed an ISRE reporter to further investigate reovirus induction of ISGs. Surprisingly, reovirus and IFN-α/β treatment induced the reporter significantly in PCMCs, but poorly or not at all in the PMEFs. While these data contrast the full-length reporter data from Chapter 3, reovirus failed to induce the ISRE in the absence of IFN-α/β function in either cell type, again indicating that reovirus bypasses direct signaling of ISREs, delaying the cell’s antiviral response.

MATERIALS AND METHODS
MICE: IFN α/β receptor−/− mice generated by Muller et al (10) and wildtype parental 129Sv/Ev mice were generously provided by Dr. Herbert W. Virgin IV (Washington School of Medicine, St. Louis, Missouri). Mice were housed according to AAALAC recommendations and all procedures were approved by NCSU IACUC.

CELL CULTURES: PCMC and PMEF cultures were generated as described in Chapters 2 and 3. Briefly, to generate primary cardiac myocyte cultures (PCMCs) from 129Sv/Ev or IFN-α/β receptor−/− mice, term fetuses or 1-day-old neonates were sacrificed and the apical two-thirds
of the hearts were removed, minced, and trypsinized (1). Cells were then pre-plated and incubated for 1.5 to 2 h to remove rapidly adherent cells (predominantly fibroblasts). The remaining cells (predominantly myocytes) were resuspended in Dulbecco’s modified Eagle medium (DMEM) with supplements and were plated at $1.0 \times 10^5$ cells/well in 48-well clusters. Myocyte cultures contained 5-20% fibroblasts (1), consistent with levels reported by others (3, 6, 8), and consistent with cell heterogeneity in the heart. To generate primary murine embryonic fibroblasts (PMEFs) from 129Sv/Ev mice, 13-15 day old embryos were removed and washed in DMEM F12 with supplements, homogenized, and trypsinized for 1 h. Cells were then filtered through a mesh cell strainer and centrifuged. The cell pellet was resuspended in DMEM F12 with supplements and this process was repeated two times to wash. The cells were then pre-plated and incubated for 1.5 to 2 h to separate rapidly adherent fibroblasts from all other non-adherent cells, which were washed away with DMEM F12 after incubation. Fibroblasts were trypsinized for 3-4 minutes after which time supplemented media was added to neutralize the trypsin. PMEFs were then plated as above for PCMCs. These processes were repeated for each experiment to generate fresh cultures of PCMCs and PMEFs. Passaging MEFs has been shown to change their phenotype with respect to IRFs and IFN (5), and therefore, the PMEFs were never frozen or passaged.

**Plasmids:** The βlux reporter plasmid was constructed as previously described (12), and contains the murine IFN-β regulatory region inserted into pGL3-Basic (Promega). The PKRLux reporter plasmid was constructed by amplifying the murine PKR promoter sequences (-777 to +132) using PCR and inserting them into the *Kpn I* and *Bgl II* sites of
pGL3-Basic. The ISRElux reporter plasmid was constructed by using polymerase chain reaction (PCR) to add *Kpn I* and *Bgl II* restriction sites to a tandem copy of a murine PKR ISRE sequence (GGAAAACGAAACA) in order to insert the sequence into pGL3-Basic. This ISRE sequence corresponds to the murine PKR promoter sequences –172 to –160.

pRL-SV40 (expressing renilla luciferase constitutively from an SV40 promoter) pGL3-Basic (lacking a promoter and therefore expressing baseline firefly luciferase), and pGL3-Control (expressing firefly luciferase constitutively from an SV40 promoter) were purchased (Promega). DNA was purified for transfection using Qiagen’s Maxiprep system (Qiagen Inc., Valencia, Calif.).

**Transfections:** Transfections were performed as previously described for Chapters 2, and 3. One day postplating, PCMCs or PMEFs plated at a density of 1.0 × 10⁵ cells per well in a 48-well cluster were transfected using FuGENE6 according to manufacturer’s protocol. A comparison of raw renilla luciferase values for all reporter plasmids indicated that there was no significant difference in transfection efficiency regardless of reporter plasmid or cell type. Additionally, there was no difference in normalized pGL3-Control values between PCMCs and PMEFs, indicating that the assay normalizations did not vary between cell type.

The amount of DNA added to the indicated wells was as follows in a 48-well cluster experiment: βlux 1.0 µg; PKRlux 1.0 µg; ISRElux 0.75 µg; pRL-SV40 0.01µg; pGL3-Control 1.0 µg. FuGENE6 was used in a volume equal to twice the total micrograms of plasmid DNA to be transfected per well (e.g., 2 µg of plasmid DNA per well required 4 µl of FuGENE6 per well).
Infections: Infections were performed 1 day postransfection. PCMCs or PMEFs were washed twice with supplemented DMEM immediately prior to infection. For transfections, two wells were trypsinized and viable cells were counted using trypan blue exclusion. For PCMCs, cells were infected with either reovirus T3D (Dearing) or VSV (strain Indiana, generously provided by Dr. Ganes Sen) at 25 PFU per cell in 150µl supplemented media or were mock-infected. PMEF cultures were infected with reovirus T3D at 25 PFU per cell and with VSV at 1 PFU per cell since VSV was cytopathic to PMEFs at higher PFUs per cell. Cultures were incubated for 1 h at 37°C in 5% CO₂. For transfected cultures, 350 µl supplemented DMEM was then added. Three hours prior to harvest, 5500 units/ml IFN-α/β was added if indicated (Lee Biomolecular Research Inc.). While this concentration of IFN-α/β was chosen in an attempt to mimic concentrations induced by reovirus (13), 1000 units/ml was found to give similar results. Cells were incubated at 37°C and 5% CO₂ for 15 h before harvesting. The nonmyocarditic reovirus, T3D, was carefully chosen for these experiments as it has proven to be a consistent IFN inducer in PCMCs, and thus a likely inducer of ISGs. Immunohistochemistry of mock and T3D infected cells indicated that at 10 h postinfection, PCMCs and PMEFs were infected with the same efficiency (4-7% and 5-10% respectively). The low percentage of infection can be attributed to the early timepoint (10 h), chosen to ensure detection of only primary infection.

Dual-luciferase assay: The dual-luciferase assay was performed according to the manufacturer’s protocol (Promega) with the following exceptions: cells were washed twice with phosphate-buffered saline prior to the addition of lysis buffer, cells were allowed to remain
in lysis buffer at 4 °C for at least 15 min. Measurements were made using a Lumat LB 9507 luminometer (EG&G Berthold, Oakridge, Tenn.) and autoinjection. Normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity.

Statistical analysis: A Student’s one-tailed $t$ test and pooled variance was used for statistical analysis. Results were considered significant at $P \leq 0.05$.

RESULTS

Reovirus and IFN-α/β induce an ISRE reporter more in PCMCs than PMEFs. We have previously demonstrated that reoviruses induce IFN-β more in PCMCs than in PMEFs (Chapter 3, and Figure 1 here). To determine whether reovirus induces an isolated ISRE sequence, PCMC and PMEF cultures were cotransfected with an ISRE reporter construct, ISRELux, and a plasmid constitutively expressing renilla luciferase for normalizations. Wells were then either mock or virally infected, or treated with IFN-α/β, and normalized luciferase activity was analyzed. As with $\beta$lux, T3D induced the isolated ISRE sequence to a much higher level in the PCMCs (4.0-fold) than in the PMEFs (1.2-fold) (Figure 1), and this differential induction between PCMCs and PMEFs was statistically significant ($P<0.001$). Treatment with IFN-α/β displayed the same cell type-specific response, again with a statistically significant differential induction ($P=0.015$). Together these results suggested that ISREs respond to IFN-β and that reovirus induction of isolated ISRE sequences is cell type-specific, with more induction in PCMCs than PMEFs. These data were in sharp contrast to the full-length PKR ISG reporter
data, where reovirus and IFN induced the PKR reporter to a statistically significant higher level in the PMEFs than the PCMCs (Chapter 3, and Figure 1, here).

**Reovirus requires IFN-α/β function for induction of isolated ISRE sequences.** To determine whether T3D induction of ISREs is direct or is mediated through reovirus induction of IFN-α/β, we compared viral induction of the ISRE reporter plasmid in IFN-α/β receptor−/− and 129Sv/Ev PCMCs. Data for βlux and PKRlux are taken from Chapter 3 for comparison. As a positive control, we included VSV, which has been previously demonstrated to induce ISGs directly in the absence of IFN-α/β (2). As expected, VSV induced the ISRE reporter in both the IFN-α/β-receptor−/− and 129Sv/Ev PCMCs. In contrast, multiple experiments revealed that there was a statistically significant reduction (P<0.001) in the average T3D fold induction of the ISRE in the IFN-α/β receptor−/− PCMCs relative to that in the 129Sv/Ev PCMCs (Figure 2). Furthermore, while T3D significantly induced the ISRE in 8 out of 9 experiments in cultures generated from the parental wildtype 129Sv/Ev mice, T3D failed to induce the ISRE in most experiments in the IFN-α/β receptor−/− PCMCs (Figure 2). Together these data indicated that like T3D induction of βlux, T3D induction of the ISRE reporter is through the IFN signal. Since T3D induced ISRElux poorly in the wildtype PMEFs, there was no significant reduction in T3D induction in the IFN-α/β receptor−/− PMEFs, as expected (Figure 3). Interestingly, VSV induced the ISRE reporter 2-3-fold in PCMCs (Figure 2), but less than 2-fold in the PMEFs (Figure 3), displaying the same cell type-specificity as reovirus and IFN (Figure 1).
DISCUSSION

Reovirus induces IFN-β more in PCMCs than in PMEFs, and interestingly, reovirus and IFN-α/β both induce an ISRE reporter in the same cell type-specific pattern (Figure 1). These data contrast reovirus induction of PKR and other ISGs, which are induced to a higher level in PMEFs, suggesting that induction of the ISRE and ISG reporters occurs by different mechanisms. One hypothesis might include the possibility that the tandem ISRE is induced preferentially by ISGF3, while the ISG reporter is induced by IRF-1. Why might this be? Nucleotide sequences upstream and downstream of the ISRE have been found to be critical for activated IRF-3 binding (Daly, 1995), and while these critical sequences have not been determined for IRF-1, it may be that they do not exist in the tandem ISRElux reporter, but do exist in the PKRlux reporter. Therefore, if the tandem ISRE is induced by ISGF3, but the PKR promoter is induced preferentially by IRF-1, then IFN induction of the ISGF3 complex in PCMCs would subsequently induce ISRElux to a high level. The high constitutive level of IRF-1 (9) previously found in PCMCs would not affect ISRElux, but would affect PKRlux, leading to a low level of induction in these cells.

However, other hypotheses exist for the differential induction between the ISRElux and PKRlux reporters. The PKR promoter has previously been found to be induced primarily through ISGF3 (11), and the PKRlux reporter contains the same sequences used in these studies, while the ISRElux reporter contains only the PKR ISRE in tandem. Therefore, the PKR reporter could be regulated by ISGF3, while the ISRElux reporter may be regulated by another factor. IRF-1 has a DNA sequence specificity partially overlapping the binding of ISGF3, and may be the primary inducer of the tandem ISRE reporter (ISRElux). In Chapter 2, we found
that reovirus induction of IRF-1 is primary through IFN. The mechanism by which IFN-β induces IRF-1 is likely through the formation of Stat1/Stat2 heterodimers (7), which bind to the IFN-γ activation sequence (GAS) (4, 14) of IRF-1. If there is a deficiency in PCMCs that prevents ISGF3 from forming, as suggested in Chapter 3, Figure 5, Stat heterodimers would be produced instead, and may upregulate IRF-1 to high level in PCMCs (Figure 4). T3D induction of the ISRElux reporter may thus reflect a high level of IRF-1 induction in PCMCs, leading to a high level of ISRElux induction. In the PMEFs, which have no such deficiency, ISGF3 would be produced efficiently, leading to a high level of PKRlux induction, but a low level of ISRElux induction, due to its binding preference for IRF-1 (Figure 4). IRF-1 induction of the ISRE and ISG reporters will be further investigated in the following appendix. Regardless of the mechanism of induction between the ISRE and the ISGs, both are mediated by IFN, as evidenced by the lack of induction in the IFN-α/β receptor−/− mice (Figures 2, 3).
REFERENCES CITED


Figure 1. **Reovirus and IFN-α/β induce an isolated ISRE more in PCMCs than in PMEFs.** PCMCs and PMEFs generated from 129Sv/Ev mice were transfected with the indicated plasmid and the normalization plasmid pRL-SV40. One day post-transfection, the cultures were mock-infected, infected with reovirus T3D ( ), or treated with IFN-α/β ( ). Three hours post-IFN-α/β-stimulation, or 15 hours post-infection, cultures were harvested for luminometry. For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity, and fold induction for each experiment was determined by dividing the normalized luciferase readout from three T3D-infected wells by that from three mock-infected wells. Each bar indicates the mean fold induction of multiple experiments (+ the standard error of the mean). Fraction of experiments with statistically significant induction indicates the fraction of experiments where induction in the three mock-infected wells was statistically significantly different ($P<0.05$) from that in the three T3D-infected wells. The asterisk denotes a significant difference between PCMCs and PMEFs (from left to right, $\beta$lux $P<0.001$; ISRElux: $P<0.001$; $P=0.015$; PKR: $P=0.049$; $P=0.047$).
**Figure 2.** Reovirus requires IFN-α/β function for induction of ISRE sequences in PCMCs. PCMCs generated from 129Sv/Ev (■) or IFN-α/β-receptor −/− (□□□) mice were transfected with the indicated plasmid and the normalization plasmid pRL-SV40. One day post-transfection, the cultures were infected with either VSV strain Indiana, or reovirus T3D. Luciferase activity was measured 15 h post-infection. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Data from T3D-infected 129Sv/Ev PCMCs included here are from Figure 1 (ISRElux) or Chapter 3 (βlux, PKRlux). Fraction of experiments with statistically significant induction is as for Figure 1. Asterisks denote a significant decrease between 129Sv/Ev and IFN-α/β receptor −/− PCMCs (from left to right, βlux: *P*<0.001; ISRElux: *P*≤0.000)
Figure 3. Reovirus requires IFN-α/β function for induction of ISRE in PMEFs. PCMCs generated from 129Sv/Ev ( ) or IFN-α/β-receptor −/− ( ) mice were transfected, infected, and measured for luciferase activity as in Figure 2. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Data from T3D-infected 129Sv/Ev PMEFs included here are from Figure 1 (ISRElux) or Chapter 3(βlux and PKRlux). Fraction of experiments with statistically significant induction is as for Figure 1. Asterisks which denote a significant decrease between 129Sv/Ev and IFN-α/β receptor −/− PMEFs (from left to right, βlux P=.049; PKRlux P=0.028)
Figure 4. Possible mechanism for differential ISRE/ISG induction. IRF-1 is the primary inducer of ISRElux, and the Stat dimers that are formed in the PCMCs can upregulate IRF-1, leading to enhanced induction of the ISRE reporter in this cell type. The PMEFs, on the other hand, produce a majority of ISGF3 molecules through the JAK STAT pathway, which compete with any IRF-1 that is formed for binding to the ISRE promoter.
APPENDIX 2

IRF-1 INDUCTION OF IFN-β AND ISGS

INTRODUCTION

IRF-1 is a transcriptional activator of IFN-β (6), and in Chapter 2 we found that overexpressed murine IRF-1 (mIRF-1) can induce IFN-β in wildtype PCMCs. IRF-1 has also been shown to bind in monomeric and dimeric form to several ISGs (7, 13, 22), and can upregulate PKR (3, 18), 2’-5’ oligo(A)synthetase (4), MHC class I and II (14), 561 (1), and 6-16 (20). Importantly, in IRF-1⁻/⁻ mice, we found a higher level of reovirus induced myocarditic lesions (Chapter 2), suggesting that IRF-1 may be a determinant of the antiviral response. While reovirus induction of IRF-1 is mediated by reovirus induced IFN (Chapter 2), overexpressed IRF-1 has been found to provide antiviral protection even in the absence of IFN-β function (21). Here we find that overexpressed mIRF-1 can induce IFN-β, ISRE sequences, and ISGs in IFN-α/β receptor⁻/⁻ cultures, and that surprisingly, this induction is to a higher level than that found in wildtype cells, suggesting that IFN-β may normally inhibit IRF-1 induction of antiviral genes.

MATERIALS AND METHODS

Mice: IFN α/β receptor⁻/⁻ mice generated by Muller et al (17) and 129Sv/Ev mice were generously provided by Dr. Herbert W. Virgin IV (Washington School of Medicine, St. Louis, Missouri). Mice were housed according to AAALAC recommendations and all procedures were approved by NCSU IACUC.
Cell Cultures: Cell cultures were generated as previously described (Chapters 2, 3, Appendix 1). Briefly, to generate primary cardiac myocyte cultures (PCMCs) from 129Sv/Ev or IFN-α/β receptor−/− mice, term fetuses or 1-day-old neonates were sacrificed and the apical two-thirds of the hearts were removed, minced, and trypsinized (2). Cells were then pre-plated and incubated for 1.5 to 2 h to remove rapidly adherent cells (predominantly fibroblasts). The remaining cells (predominantly myocytes) were resuspended in Dulbecco’s modified Eagle medium (DMEM) with supplements and were plated at 1.0 x 10^5 cells/well in 48-well clusters. Myocyte cultures contained 5-20% fibroblasts (2), consistent with levels reported by others (8, 12, 15), and consistent with cell heterogeneity in the heart. To generate primary murine embryonic fibroblasts (PMEFs) from 129Sv/Ev mice, 13-15 day old embryos were removed and washed in DMEM F12 with supplements, homogenized, and trypsinized for 1 h. Cells were then filtered through a mesh cell strainer and centrifuged. The cell pellet was resuspended in DMEM F12 with supplements and this process was repeated two times to wash. The cells were then pre-plated and incubated for 1.5 to 2 h to separate rapidly adherent fibroblasts from all other non-adherent cells, which were washed away with DMEM F12 after incubation. Fibroblasts were trypsinized for 3-4 minutes after which time supplemented media was added to neutralize the trypsin. PMEFs were then plated as above for PCMCs. These processes were repeated for each experiment to generate fresh cultures of PCMCs and PMEFs. Passaging MEFs has been shown to change their phenotype with respect to IRFs and IFN (11), and therefore, the PMEFs were never frozen or passaged.
**Plasmids:** The luciferase reporter plasmids were constructed as previously described in Chapters 2, 3, and Appendix 1. Briefly, the βlux reporter plasmid (19) contains the murine IFN-β regulatory region inserted into pGL3-Basic (Promega). The 6-16lux reporter plasmid was constructed by amplifying upstream sequences (-603 to +437) of the human 6-16 gene and inserting them into the XhoI and Hind III sites of pGL3-Basic. The 561lux reporter, provided by Dr. Ganes C. Sen (The Cleveland Clinic Foundation, Cleveland, Ohio), contains sequences (-654 to +3) of the 561 promoter transferred into the SstI and Hind III sites of pGL3-Basic. The PKRlux reporter plasmid was constructed by amplifying the murine PKR promoter sequences (-777 to +132) using PCR and inserting them into the KpnI and BglII sites of pGL3-Basic. The ISRElux reporter plasmid was constructed by using polymerase chain reaction (PCR) to add KpnI and BglII restriction sites to a tandem copy of a murine PKR ISRE sequence (GGAAAACGAAACA) in order to insert the sequence into pGL3-Basic. Murine IRF-1 inserted into pRc/CMV1 (Yim *et al.*, 1997) was kindly provided by Dr. Jerry Doherty (Washington School of Medicine, St. Louis, MO). pRc/CMV2 was purchased (Invitrogen). pRL-SV40 (expressing renilla luciferase constitutively from an SV40 promoter), pGL3-Basic (lacking a promoter and therefore expressing baseline firefly luciferase) were perchased (Promega). DNA was purified for transfection using Qiagen’s MaxiPrep system (Qiagen Inc., Valencia, Calif.).

**Transfections:** Transfections were performed as previously described for Chapters 2, 3, and Appendix 1. One day postplating, PCMCs or PMEFs plated at a density of $1.0 \times 10^5$ cells per well in a 48-well cluster were transfected using FuGENE6 according to manufacturer’s
protocol. A comparison of raw renilla luciferase values for all reporter plasmids indicated that there was no significant difference in transfection efficiency regardless of reporter plasmid or cell type. Additionally, there was no difference in normalized pGL3-Control values between PCMCs and PMEFs, indicating that the assay normalizations did not vary between cell type. The amount of DNA added to the indicated wells was as follows: βlux 1.0 µg; 561lux 1.0 µg; PKRlux 1.0 µg; 6-16lux 0.4 µg; ISRElux 0.75 µg; pRc/CMV2 0.5µg; murine IRF-1 (mIRF-1) 0.5 µg; pRL-SV40 0.01µg. FuGENE6 was used in a volume equal to twice the total micrograms of plasmid DNA to be transfected per well (e.g., 2 µg of plasmid DNA per well required 4 µl of FuGENE6 per well).

_Dual-luciferase assay_: The dual-luciferase assay was performed as previously described for Chapters 2, 3 and Appendix 1. Briefly, exceptions to the manufacturer’s protocol included washing the cells with PBS prior to the addition of lysis buffer and incubating cells at 4 °C for at least 15 min before harvest. Measurements were made using a Lumat LB 9507 luminometer (EG&G Berthold, Oakridge, Tenn.) and autoinjection. Normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity.

_Statistical analysis_: A Student’s one-tailed _t_ test and pooled variance were used for statistical analysis. Results were considered significant at _P_ ≤ 0.05.

**RESULTS**

**IRF-1 can induce IFN-β in the absence of the IFN-α/β receptor.** Previously, we found that IRF-1 could induce IFN-β in PCMCs. (Chapter 2). To determine whether IRF-1 could
induce IFN-β in cells lacking the IFN-α/β receptor, we cotransfected 129Sv/Ev or IFN-α/β receptor-/- PCMCs with βlux, and either a plasmid constitutively expressing murine IRF-1 (mIRF-1) or control plasmid (pRc/CMV2), and analyzed normalized luciferase activity. In contrast to control plasmid (not shown), and consistent with our previous data, mIRF-1 induced βlux in the 129Sv/Ev parental wildtype cultures approximately 3-fold (Figure 1). Surprisingly, however, mIRF-1 induced βlux to a much higher level, 27-fold, in cultures null for the IFN-α/β receptor. The differential induction between wildtype and IFN-α/β receptor-/- cultures was statistically significant (P=0.019), indicating that mIRF-1 can induce IFN-β better without the effects of IFN that the receptor provides.

**IRF-1 can induce ISRE and ISGs in PCMCs null for the IFN-α/β receptor.**

To determine whether mIRF-1 could induce ISRE and ISG reporters, we first cotransfected mIRF-1 or control vector and the ISRElux reporter into 129Sv/Ev PCMCs, and found that mIRF-1 could induce the reporter, although this induction was not always significant (Figure 2). More compelling was the induction in the IFN-α/β receptor-/- PCMCs, where induction was on average, 20-fold and consistently significant (Figure 2). mIRF-1 could not induce any of the ISG reporters in the wildtype PCMCs, but induced these reporters significantly in almost all experiments null for the IFN-α/β receptor (Figure 3). This induction was less than 10-fold, however, indicating that in PCMCs, the ISRE reporter was much more sensitive to IRF-1 than the ISG reporters (Compare figures 2 and 3).
IRF-1 can induce IFN-β, ISRE, and ISGs in both wildtype and IFN-α/β receptor −/− PMEFs. Similar experiments were performed in the wildtype and IFN-α/β receptor −/− PMEFs. In the wildtype PMEFs, mIRF-1 induced both the IFN-β (Figure 4) and the ISRE sequence (Figure 5) approximately 10-fold, and this induction was further enhanced in PMEFs null for the IFN-α/β receptor. mIRF-1 induced the ISG reporters in the wildtype PMEFs to a significant level (Figure 6), and mIRF-1 induction appeared to be enhanced for the 561 and 6-16 reporters in the IFN-α/β receptor −/− mice. As in the PCMCs, mIRF-1 induction of the ISRElux reporter in PMEFs was to a much higher level than that of the PKRlux reporter (Compare figures 5 and 6).

DISCUSSION

These data together indicate that in both PCMCs and PMEFs, mIRF-1 can induce IFN-β, ISRE sequences, and ISGs. Interestingly, mIRF-1 induction of all of the promoters was greater in the wildtype PMEFs compared to the wildtype PCMCs. This might be explained by the higher constitutive levels of IRF-1 in the PCMCs (16), which may be reducing the extent of induction in this cell type. One thing to note, however, is that overexpressed factors can regulate certain genes even if they do not regulate these genes under normal physiologic conditions. For instance, we found in Chapter 2, that mIRF-1 regulates, but is not required for, reovirus induction of IFN-β.

What role does IRF-1 play in reovirus induction of ISGs? In Chapter 3 and Appendix 1, we found that ISGs and ISRE sequences are likely regulated differentially. Here we find that in both PCMCs and PMEFs, mIRF-1 induction of the ISG reporters is lower than that of the ISRE reporter, indicating that the ISRE sequence is more sensitive to induction by IRF-1.
than are the ISGs. This might reflect a differential mechanism of regulation. If ISRE sequences are induced by IRF-1, the enhanced induction in the IFN-α/β receptor−/− cultures might be due to a lack of competition between ISGF3 and IRF-1 for binding to the ISRE. This hypothesis, however, does not explain why there would be more induction of the ISGs, which are conversely regulated by ISGF3, in the IFN-α/β receptor−/− cells than in the wildtype.

In general, IRF-1 induction of βlux, ISRELux, and ISG reporters was enhanced in cultures null for the IFN-α/β receptor. Therefore, it seems that in both cell types, IFN-β may induce or activate a product that normally inhibits IRF-1 induction of IFN-β, ISRE sequences, and ISGs. IRF-2 is induced by IFN-β and has been shown to repress both IFN-β (9) and ISGs (5, 10, 20). However, since IRF-1 induces IRF-2 (9), one would expect inhibition in the IFN-α/β receptor−/− cultures as well, and therefore, IRF-2 is not the most likely candidate. It’s not clear from these data what this factor might be, but PCMCs may have more of this inhibiting factor than PMEFs, as evidenced by the higher mIRF-1 induction of all plasmids in the wildtype PMEFs compared to the wildtype PCMCs. In Chapter 3, we found that cell types that induce a lot of IFN-β are resistant to induction of ISGs, and the fact that IFN induces or activates a factor that inhibits IRF-1 induction of IFN-β, ISRE sequences, and ISGs may be related to this resistance.
REFERENCES CITED


3. **Beretta, L., M. Baggay, R. Berger, S. M. Hanash, and N. Sonenberg.** 1996. Expression of the protein kinase PKR is modulated by IRF-1 and is reduced in 5q-associated leukemias. Oncogene. **12:**1593-1596.


Figure 1. IRF-1 can induce IFN-β in the absence of the IFN-α/β receptor. PCMCs generated from 129Sv/Ev mice were transfected with β lux, the normalization plasmid pRL-SV40, and either control (pRc/CMV2) or murine IRF-1 (mIRF-1) effector plasmid. Cells were mock infected 1 day post-transfection, and luciferase activity was measured 15 h postinfection. For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity, and fold induction for each experiment was determined by dividing the normalized luciferase readout from three mIRF-1 transfected wells by that from three control transfected wells. Each bar indicates the mean fold induction of multiple experiments (+ the standard error of the mean). Fraction of experiments with statistically significant induction indicates the fraction of experiments where induction in the three control-transfected wells was statistically significantly different (P<0.05) from that in the three mIRF-1 transfected wells. The asterisk denotes a significant difference between 129Sv/Ev and IFN-α/β receptor−/− PCMCs (P=0.019).
**Figure 2. IRF-1 can induce ISRE in PCMCs null for the IFN-α/β receptor.** PCMCs generated from 129Sv/Ev mice were transfected with ISRElux, the normalization plasmid pRL-SV40, and either control (pRc/CMV2) or murine IRF-1 (mIRF-1) effector plasmid. Cells were mock infected 1 day post-transfection, and luciferase activity was measured 15 h postinfection. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Fraction of experiments with statistically significant induction is as for Figure 1. While the two experiments in the wildtype gave 5.2- and 4.5-fold induction, the experiments in the IFN-α/β receptor−/− PCMCs gave 9- to 40-fold, resulting in a differential induction which was not statistically significant ($P=0.138$).
Figure 3. IRF-1 can induce ISGs in PCMCs null for the IFN-α/β receptor. PCMCs generated from 129Sv/Ev mice were transfected with the ISG reporters (561lux, 6-16lux, or PKRlux), the normalization plasmid pRL-SV40, and either control (pRc/CMV2) or murine IRF-1 (mIRF-1) effector plasmid. Cells were mock infected 1 day post-transfection, and luciferase activity was measured 15 h postinfection. Normalized luciferase activity and fold induction were determined as in Figure 1. Fraction of experiments with statistically significant induction is as for Figure 1. The asterisks denotes a significant difference between 129Sv/Ev and IFN-α/β receptor−/− PCMCs (6-16: P=0.039; PKR: P=0.047). The wells transfected with 561lux gave 0.9- to 1.6-fold induction in the wildtype PMEFs, and ranged from 1.8- to 8-fold in the IFN-α/β receptor−/− PCMCs, and therefore the differential induction between the two cell types was not statistically significant.
Figure 4. IRF-1 can induce IFN-β in PMEFs null for the IFN-α/β receptor. PMEFs generated from 129Sv/Ev mice were transfected with βlux, the normalization plasmid pRL-SV40, and either control (pRc/CMV2) or murine IRF-1 (mIRF-1) effector plasmid. Cells were mock-infected 1 day post-transfection, and luciferase activity was measured 15 h postinfection. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Fraction of experiments with statistically significant induction is as for Figure 1. The fold induction for the one experiment in the wildtype was approximately 12-fold, while the five experiments in the IFN-α/β receptor−/− ran from 12- to 32-fold, and therefore the differential induction between the two cell types was not statistically significant.
Figure 5. IRF-1 can induce ISRE in PMEFs null for the IFN-α/β receptor. PMEFs generated from 129Sv/Ev mice were transfected with ISRElux, the normalization plasmid pRL-SV40, and either control (pRc/CMV2) or murine IRF-1 (mIRF-1) effector plasmid. Cells were mock infected 1 day post-transfection, and luciferase activity was measured 15 h postinfection. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Fraction of experiments with statistically significant induction is as for Figure 1. The asterisk denotes a significant difference between 129Sv/Ev and IFN-α/β receptor null PMEFs (P=0.037).
Figure 6. IRF-1 can induce ISGs in PMEFs null for the IFN-α/β receptor. PMEF cultures generated from 129Sv/Ev mice were transfected with the ISG reporters (561lux, 6-16lux, or PKRlux), the normalization plasmid pRL-SV40, and either control (pRc/CMV2) or murine IRF-1 (mIRF-1) effector plasmid. Cells were mock infected 1 day post-transfection, and luciferase activity was measured 15 h postinfection. Normalized luciferase activity and fold induction were determined as in Figure 1. Each bar shows the mean fold induction of multiple experiments (+ the standard error of the mean). Fraction of experiments with statistically significant induction is as for Figure 1. The wells transfected with 561lux gave 5- to 8-fold in the wildtype PMEFs, and 9.5- to 13-fold in the IFN-α/β receptor−/−, yielding a differential induction which was not statistically significant. Statistics for differential induction between wildtype and IFN-α/β receptor−/− cultures require that more than one experiment be performed for both cell types, therefore, these statistics could not be calculated for the 6-16lux and PKRlux reporters.
APPENDIX 3

EVALUATION OF THE ROLE OF IRF-7 IN REOVIRUS INDUCTION OF IFN-\(\beta\) USING REAL-TIME PCR.

INTRODUCTION

While IRF-1 was originally thought to be a primary regulator of IFN-\(\beta\) induction, conflicting data have arisen regarding the requirement for IRF-1 in viral induction of IFN-\(\beta\). We have found that in primary cardiac myocyte cultures, IRF-1 is not required for viral induction of IFN-\(\beta\) (Chapter 2). It is now thought that IRF-7 and IRF-3 are possibly the more critical players in IFN-\(\beta\) regulation. In IRF-3\(^{-/}\) and IRF-7 expression-abolished MEFs, the induction of IFN-\(\alpha/\beta\) genes did not occur (12). The normal induction profile of IFN-\(\alpha/\beta\) was restored only when the two IRFs were co expressed, but not when either one was expressed without the other (12). Therefore, it is thought that IRF-7 and IRF-3 each have essential but distinct roles. A biphasic model has been proposed which delineates an early and late phase of IFN-\(\alpha/\beta\) regulation (Figure 1). In the early phase, constitutively expressed IRF-3 is phosphorylated, interacts with CBP/p300 and translocates to the nucleus where it binds to the IFN-\(\beta\) IRF-E. IFN-\(\beta\) is secreted from the cell, binds IFN-\(\alpha/\beta\) receptor, and signals through ISGF3 to induce the IRF-7 ISRE sequence. IRF-7 is not constitutively expressed like IRF-3, but is expressed at very low levels in most cell types (13). Upon virally induced phosphorylation, IRF-7 then interacts with PCAF (p300-CBP-associated factor) (3), and translocates to the nucleus where it binds to IFN-\(\alpha/\beta\) genes, causing amplification of IFN-\(\beta\) production. This latter phase of IFN-\(\beta\) regulation is strictly interferon dependent and dominated largely by IRF-7.
In Chapter 3, we found that reovirus induction of IFN-β is significantly reduced in IFN-α/β-receptor−/− PCMCs. The poor induction of IFN-β in PMEFs was reduced further in IFN-α/β-receptor−/− PMEFs. The positive feedback loop governed by IRF-7 in viral induction of IFN-β has been described for a number of virus/cell systems (5, 7, 11, 12). Therefore, these data together suggest that most of the IFN-β signal is IFN-β dependent and thus may likely involve the transcription factor IRF-7. Here we use real-time PCR SYBR Green assays to evaluate the role of IRF-7 in reovirus induction of IFN-β. Despite a higher level of IFN-β induction in PCMCs, induced IRF-7 levels were surprisingly equivalent in the two cell types, suggesting that the role of IRF-7 in IFN-β dependent IFN-β synthesis is likely cell type-specific. Further, our results indicate that the extent of viral induction of IFN-β may be governed by basal levels of IFN-β rather than induced levels of IRF-7.

MATERIALS AND METHODS

Mice: Timed pregnant Cr:NIH(S) mice were obtained from the National Cancer Institute. 129Sv/Ev mice were generously provided by Dr. Herbert W. Virgin IV (Washington School of Medicine, St. Louis, Missouri). Mice were housed according to AAALAC recommendations and all procedures were approved by NCSU IACUC.

Cell Cultures: Cell cultures were generated as previously described (Chapters 2, 3, Appendices 1, 2). Briefly, to generate primary cardiac myocyte cultures (PCMCs) from NIH(S) mice, term fetuses or 1-day-old neonates were sacrificed and the apical two-thirds of the hearts were removed, minced, and trypsinized (2). Cells were then pre-plated and
incubated for 1.5 to 2 h to remove rapidly adherent cells (predominantly fibroblasts). The remaining cells (predominantly myocytes) were resuspended in Dulbecco’s modified Eagle medium (DMEM) with supplements and were plated either at 1.25 x 10^6 cells/well in 24-well clusters or at 6 x 10^5 cells/well in 48-well clusters. Myocyte cultures contained 5-20% fibroblasts (2), consistent with levels reported by others (4, 6, 9), and consistent with cell heterogeneity in the heart. To generate primary murine embryonic fibroblasts (PMEFs) from 129Sv/Ev mice, 13-15 day old embryos were removed and washed in DMEM F12 with supplements, homogenized, and trypsinized for 1 h. Cells were then filtered through a mesh cell strainer and centrifuged. The cell pellet was resuspended in DMEM F12 with supplements and this process was repeated two times to wash. The cells were then pre-plated and incubated for 1.5 to 2 h to separate rapidly adherent fibroblasts from all other non-adherent cells, which were washed away with DMEM F12 after incubation. Fibroblasts were trypsinized for 3-4 minutes after which time supplemented media was added to neutralize the trypsin. PMEFs were then plated as above for PCMs. These processes were repeated for each experiment to generate fresh cultures of PCMCs and PMEFs. Previous studies investigating IRF-7 have used primarily passaged MEFs or immortalized cell lines. However, passaging MEFs has been shown to change their phenotype with respect to IRFs and IFN (5), and immortalized cell lines have been shown to exhibit silencing of the IRF-7 gene, possibly due to hypermethylation (8).

**Infections:** Infections were performed 2 days post plating. PCMCs or PMEFs were washed twice with supplemented DMEM immediately prior to infection. Cells were either mock
infected or infected with reovirus T3D (Dearing) at 25 PFU per cell in 300µl supplemented media for cultures plated in 24-well clusters. Cultures were incubated for 1 h at 37°C in 5% CO₂. 700 µl supplemented DMEM was then added. Cultures plated in 48 well clusters were treated identically, but with 50% less volume (150-µl inoculum and 350-µl overlay volumes). Cells were incubated at 37°C and 5% CO₂ for 15 h before harvesting. Immunohistochemistry of mock and T3D infected cells indicated that at 10 h post infection, PCMCs and PMEFs were infected with the same efficiency (4-7% and 5-10% respectively). The low percentage of infection can be attributed to the early time point (10 h), chosen to ensure detection of only primary infection.

**RNA harvest and Reverse Transcription:** At 15 h post infection, supernatants were aspirated. Cells were lysed directly from the culture plates, homogenized using Qiashredders (Qiagen), and total RNA was isolated using an RNeasy kit (Qiagen). Contaminating genomic DNA was removed by DNase treatment using an RNase-free DNase Set (Qiagen). According to expected protocol yields, ~0.6µg (from 6 x 10⁵ cells/well)-12.5 µg (from 1.25 x 10⁶ cells/well) total RNA was extracted. RNA from each well was then subjected to reverse transcription using the following components at final concentrations for a total reaction volume of 75 µl: 5 µM oligo-dT (Promega), 1X Taq buffer, 7.5 mM MgCl₂, 1 mM DTT, 1 mM each dNTP, 0.5 U/µl RNA guard (Pharmacia), 0.22 U/µl avian myeloblastosis virus reverse transcriptase (AMV-RT) (Promega).
Real-time PCR: Approximately 5% (4.4%) of the RT reaction was then amplified by real-time PCR. Initial PCR reactions were performed with a total volume of 50ul containing the following components at final concentrations: 1X Taq buffer (Promega), 3 mM MgCl₂, 0.3 mM ea. dNTP, 0.3 uM forward primer, 0.3 uM reverse primer, 1.25 U/µl Platinum Taq (Invitrogen), 1X SYBR Green (1:10,000 dilution of Molecular Probes stock), and 10 nM fluorescein (dilution of BioRad 1 uM stock). Later experiments were performed in a 25µl reaction using Quantitech master mix (Qiagen) spiked with 10 nM fluorescein with similar results. Spiking the master mix with fluorescein provides an additional fluorescence that is necessary for the system to collect well factors. Well factors are collected at the beginning of each experiment and are used by the software to compensate for any system or pipetting non-uniformity in order to optimize fluorescent data quality and analysis (1). Duplicates or triplicates of PCR were carried out in 96-well plates with optical sealing tape (Bio-Rad).

Primers for amplicons: The sequences for the primers were selected using online software designed by Steve Rosen and Helen J. Skaletsky (1998): http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. Primers were checked for base complementarity using http://oligos.qiagen.com/oligos/toolkit.php (software provided by Qiagen, 1996-2003). Primers were then designed as follows:

IRF-7 forward primer: 5’ CAGTGCTACAGGCAGTGGTG 3’ (Sherry lab oligo #253)
IRF-7 reverse primer: 5’ AGCAGGACTGGGAAAGATCAC 3’ (Sherry lab oligo 254)
IFN-β forward primer: 5’ GGAGATGACGGAGAAGATGC 3’ (Sherry lab oligo #241)
IFN-β reverse primer: 5’ CCCAGTGCTGGAGAAATTGT 3’ (Sherry lab oligo #242)
GAPDH forward primer: 5’ CAACTACATGGTCTACATGTTC 3’ (Sherry oligo #239)
GAPDH reverse primer: 5’ CTCGCTCCTGGAAGATG 3’ (Sherry lab oligo #240)

Note that the sequence of the IRF-7 amplicon is present in all three IRF-7 spliceoforms (α,β,γ) in exon 7 (Figure 2), and therefore we could ensure that all spliced variants would be detected.

**Real-time thermal cycling conditions:** Amplification, quantification, and melt curve analysis (detection of specific products) were performed on an iCycler iQ Fluorescence Thermocycler (Bio-Rad) with the following cycle profile: 95°C (PCR initial activation step, dwell time dependent on polymerase used; 13.5 min. for Quantitech, and 1.5 min. for platinum taq), followed by 50 cycles of 95°C for 10 sec (denaturation) and 59°C for 60 sec (annealing, synthesis, and fluorescent data collection step), followed by a melt curve protocol designed for decrement temperatures of 0.5°C with a starting temperature of 95°C and ending at 50°C (melt curve data collection step).

**Standard curves and PCR Quantification:** Standard curves were generated for both relative and absolute quantification. For relative quantification, serial 10-fold dilutions of cDNA were used to generate a four point standard curve for each primer pair. Mock-infected cDNA was used for GAPDH standard curves, while T3D-infected cDNA was used for target genes to better ensure that experimental samples would fall within range of the curve. The relative expression of the gene of interest was determined by calculating the ratio of the extrapolated
concentration of that gene to the extrapolated concentration of the GAPDH gene using the following equation(s) (10):

$$\text{Fold induction} = \frac{E(\text{target gene})^{\Delta C_t (\text{mock-reovirus})}}{E(\text{housekeeping gene})^{\Delta C_t (\text{mock-reovirus})}}$$

$C_t=$ threshold cycle

$E =$ Efficiency$ = 10^{-1/slope}$

An E of 2 indicates that the reaction was 100% efficient. For reliable data analysis, only experiments yielding $>90\%$ efficiency for all three standard curves were used for quantification.

For absolute quantification, standard curves of known concentration of DNA had to be generated in order to determine exact copy number of experimental samples. Therefore, for each gene of interest, primers for PCR product standards were designed to flank the sequences of the amplicon fragments:

IRF-7 forward primer: 5’ TGGGTTTCCTGGATGTGAC 3’ (Sherry lab oligo #255)
IRF-7 reverse primer: 5’ TTCACCAGGATCAGGGTC 3’ (Sherry lab oligo #256)
IFN-β forward primer: 5’ GCACCTGCTGTGCTTC 3’ (Sherry lab oligo 249)
IFN-β reverse primer 5’ CCATCCAGGCGTAGCTG 3’ (Sherry lab oligo #250)
GAPDH forward primer: 5’ GTGAAGGTCGGTGTGAACGG 3’ (Sherry lab oligo #251)
GAPDH reverse primer 5’ GTGGCAGTGAGCATGGAC 3’ (Sherry lab oligo #252)
PCR products to be used for standards were generated using a standard thermocycler (MJ Research, Inc.), with the following thermocycling protocol: 2’ 99°C; 1’ (52°C for IRF-7 and IFN-β, 54°C for GAPDH); 1’ 72°C; 1’ 94°C; 1’ 50°C; 1’ 72°C; repeat from 94°C step 35 times; 5’ 72°C. Products were then analyzed by agarose gel electrophoresis, and if only one band was apparent, the reaction was PCR purified using QIA quick PCR purification kit (Qiagen), or if any other bands were also detected, the reaction was gel purified using QIA quick Gel Extraction Kit (Qiagen). Concentrations of PCR products were then determined by fluourometry. Copy number of each PCR product was determined (using the equation(s): copies=moles x 6.0228 x 10^23, where moles=bp x 610 Daltons). Concentration (ng/µl) was based on a standard curve. These PCR products (each approximately 500 bp long) containing IRF-7, IFN-β, or GAPDH cDNA sequences, were then diluted to generate a 4 point standard curve, which fit assay parameters. These standard curves were used to estimate copy number from sample threshold cycle (Ct) values.

Statistical analysis: A Student’s one-tailed t test and pooled variance were used for statistical analysis. Results were considered significant at P≤ 0.05.

RESULTS

Detection of specific products using heat dissociation (melt) curves. SYBR Green I binds to the minor groove of double stranded DNA and fluoresces 50-100 times brighter than when not bound to DNA. However, since SYBR Green intercalating dyes bind to any double-stranded DNA molecule, it is not possible to distinguish between multiple products (i.e.,
primer dimer formation or amplification of other non-specific products) without performing additional analyses. Therefore, melt curves were used to measure the melting temperature, Tm, of the IFN-β, IRF-7, and GAPDH amplicons (Figure 3). Fluorescence is brightest when the two strands of DNA are annealed. At the Tm, the DNA denatures and releases the SYBR Green I, causing a dramatic decline in fluorescence. This decrease in fluorescence is plotted as Fluorescence vs. Temperature (Figure 3A). Plotting the negative first derivative of this data versus temperature change (-dF/dT vs. temperature) results in a melting peak and Tm for each amplified product. The Tm of each amplicon was determined (product size in parenthesis): GAPDH: 88.5°C (122 bp); IFN-β: 85°C (106bp); IRF-7: 90.5°C (110 bp). The presence of only one peak per reaction validates primer specificity (Figure 3B). Reactions containing PCR master mix without template cDNA were also run as negative controls (Figure 3C). These reactions yielded no products (Figure 3C), or, alternatively, yielded products that were at nearly undetectable levels, occurring 10 or more cycles after the lowest concentration of the standard curves. Negative controls containing RNA instead of DNA were also run with similar results (data not shown). Due to the high sensitivity of the real-time assay, a clean workplace was required to prevent environmental contamination.

**Reovirus induction of IRF-7 is discordant with reovirus induction of IFN-β.**

In Chapter 2, with the use of IFN-α/β receptor−/− cultures, we found that reovirus induction of IFN-β in both PCMCs and PMEFs is primarily through IFN. Given the significant role of IRF-7 in interferon dependent IFN-β synthesis, we sought to determine whether reovirus induction of IRF-7 would correlate with IFN-β induction in PCMCs and PMEFs. PCMCs
and PMEFs were mock-or T3D-infected, and total RNA was extracted, subjected to reverse transcription, and amplified by real-time PCR to determine fold induction by relative quantification. Consistent with our reporter data (Chapter 3), reovirus induced IFN-β to a much higher extent in PCMCs than PMEFs (Figure 4). Interestingly, however, reovirus induction of IRF-7 was similar in both cell types. Together, these data suggested that reovirus induction of IRF-7 is discordant with reovirus induction of IFN-β. Thus, the role of IRF-7 in IFN-β dependent IFN synthesis is likely cell type-specific.

Detection of IRF-7 and IFN-β mRNA expression levels in PCMCs and PMEFs. Other than increased sensitivity, another advantage to using real-time PCR is the ability to detect and quantify constitutive levels of target genes and express these levels in terms of copy number. Here, basal mRNA expression levels of IFN-β and IRF-7 in PCMCs and PMEFs were determined using absolute quantification. Total RNA was extracted from uninfected PCMCs and PMEFs and subjected to reverse transcription and real-time PCR. Basal levels of IRF-7 were found to be equivalent in PCMCs and PMEFs (Figure 5, left), correlating with similar viral induction of IRF-7 (Figure 4). In contrast, basal IFN-β mRNA levels were significantly higher in PMEFs than in PCMCs (Figure 5, right). These data indicated that basal IFN-β mRNA expression levels are not determined by basal IRF-7 mRNA expression levels in PCMCs and PMEFs. Importantly, however, since a higher level of basal IFN gene expression is accompanied by a lower level of viral induction in the PMEFs (and vice-versa in PCMCs), basal IFN-β levels may determine the extent of viral induction of IFN-β.
DISCUSSION

IFN-β gene induction is regulated in a biphasic manner involving critical roles for IRF-3 (early phase) and IRF-7 (late phase). The roles of IRF-3 and IRF-7 in viral induction of IFN-β are cell type-specific. Specifically, constitutive IRF-7 levels are much lower in third passage MEFs than fifth passage MEFs, and NDV induction of IFN-β requires IRF-3 in the former but not the latter cells, consistent with the latter already employing IRF-7 mediated amplification of IFN-β. Since these two cell types differ by only two passages, these results indicated a need for studies in primary cell culture.

Using absolute quantification of real-time PCR, we find that basal levels of IFN-β were 2-3 times higher in PMEFs than in PCMCs, while basal IRF-7 cDNA copy numbers were similar in each of the two cell cultures (Figure 5). These data indicate either that basal IRF-7 does not regulate constitutive IFN-β in one or both of these cell types, or that it does so differentially, having more of an effect in PMEFs. Perhaps PMEF IRF-7 is activated to an efficiently higher level under unstimulated conditions, or other factors, such as IRF-3, govern basal levels of IFN-β in PMEFs or PCMCs. Since IRF-7 is an ISG, basal levels of IRF-7 can be maintained by spontaneous secretion and signaling of IFN-β. However, higher basal levels of IFN-β do not lead to higher basal IRF-7 in PMEFs, indicating that IFN-β baseline levels do not regulate basal IRF-7 or that they instead provide a negative regulatory signal to keep basal IRF-7 levels low in this cell type.

Consistent with the reporter data from Chapter 3, relative quantification of real-time PCR revealed that viral induction of IFN-β is higher in the PCMCs than in the PMEFs (Figure 4). Given that basal IRF-7 levels are equivalent in the two cell types, but basal IFN-β
is lower in PCMCs, the greater viral induction of IFN-β in the PCMCs than in PMEFs may reflect the lower basal level of IFN-β in the former but not the latter cell type. This observation contradicts that of Hata et al. (5) who found that higher constitutive levels of IFN-β determined an enhanced signal upon viral induction in passaged MEFs (5). However, the role of IRF-7 in viral induction of IFN-β has never been described in any primary differentiated cell type.

In Chapter 3 we found that reovirus induction of IFN-β was IFN-dependent in PCMCs, implicating a role for IRF-7 in amplification of IFN-β. Interestingly, however, here we find that IRF-7 induction does not mirror IFN-β induction in PCMCs and PMEFs (Figure 4). Despite a higher level of IFN-β induction in PCMCs, induced levels of IRF-7 were surprisingly equivalent in the two cell types, suggesting that the role of IRF-7 in IFN-β dependent IFN-β synthesis is likely cell type-specific. Induced IRF-7 levels do correlate with basal levels of IRF-7, which are also equivalent in PCMCs and PMEFs (Figure 5). This indicates that there are equal opportunities for viral phosphorylation in the two cell types. Alternatively, other steps in the induction process, such as IRF-7 activation via dimerization or nuclear translocation may be the varying factor between these cell types. For example, IRF-7 has been found to be acetylated by PCAF in both mock and virus-infected cultures, and acetylation has been postulated to provide a negative regulatory signal to control IRF-7 induction of IFN-β (3). Regardless, these data suggest that IRF-7 is not the primary determinant of viral amplification of IFN-β in PCMCs. The lack of high baseline levels of IFN-β in PCMCs (Figure 5) may instead determine the extent of viral induction in this cell type.
The real-time PCR assays described in this report have allowed us to further investigate reovirus induction of IRF-7 and determine constitutive levels of IRF-7 and IFN-β. Absolute quantification, along with increased sensitivity, are advantages to using real-time PCR instead of the reporter system used in previous studies (Chapters 2 and 3). The trends reported here are reproducible. However, one limitation of this assay is the interassay variability that has been apparent from analyzing the IFN-β data in particular. SYBR green assays have been found to have less reproducibility when detecting genes exhibiting a low copy number, we have found that copy numbers are much lower for IFN-β than IRF-7 for both cell types (Figure 5). Therefore, assay conditions may require further optimization for continued investigation of IFN-β using real-time PCR.
REFERENCES CITED


Figure 1. Biphasic mechanism for IFN-β gene induction The early phase of IFN-β induction is largely dominated by IRF-3, which is constitutively expressed and virally activated to induce IFN-β. Upon induction, IFN-β is secreted and binds to the IFN-α/β receptor, stimulating a phosphorylation cascade (the JAK STAT pathway) which induces IRF-7. Upon virus induced activation, IRF-7 further upregulates IFN-β. Therefore, IRF-7 dominates this later phase of IFN- β synthesis.
Figure 2. Structure of mIRF-7 spliced variants. IRF-7 sequence amplicon is present in exon 7, and thus we could ensure that all spliced variants (α, β, γ) would be detected.

*Diagram modified from Marie et. al., 2000 and Levy et al., 2002*
Figure 3: Melt Curve Analysis for Detection of Specific Products: A: Plots of Fluorescence (y-axis) vs. Temperature (x-axis) for each primer set. B) Plotting the -dF/dT (y-axis) vs. temperature (x-axis) allows the Tm of each amplicon to be determined: GAPDH: 88.5°C; IFN-β: 85°C; IRF-7: 90.5°C. The presence of only one peak per reaction validates primer specificity C) -dF/dT (y-axis) vs. temperature (x-axis): Negative Controls yielded no peaks.
Figure 4. Reovirus induction of IRF-7 is discordant with reovirus induction of IFN-β. PCMC and PMEFs were mock- or reovirus-infected. 15 h postinfection, RNA was harvested for reverse transcription and relative quantification of real-time PCR using IRF-7, IFN-β, and GAPDH specific primers. Fold induction is based on mock-and reovirus-infected wells (3 wells each) normalized to GAPDH (see M&M for calculation). Error could not be calculated from a representative experiment since each fold induction is a single ratio of two averages.
Figure 5. Constitutive levels of IFN-β and IRF-7 mRNA. mRNA was harvested from PCMCs or PMEFs for reverse transcription and absolute quantification of real-time PCR. cDNA was amplified using primers specific for GAPDH, IFN-β and IRF-7, along with standards of known concentrations to allow calculation of copy numbers. The range for standard curves were as follows: GAPDH and IRF-7: 10^8-10^5 copies; IFN-β: 10^6-10^3 copies. Results are expressed as the ratio of copies of the gene of interest (IFN-β or IRF-7) relative to copies of GAPDH. Bars represent triplicate wells (+ standard error of the mean). The asterisk designates a statistically significant difference between PCMCs and PMEFs (P=0.02).
SUMMARY

The data from this dissertation have further defined the cardiac IFN response to reovirus infection. First, reovirus induction of IRF-1 is primarily through IFN in PCMCs. However, in IRF-1$^{-/-}$ mice, nonmyocarditic reovirus strains induce more cardiac lesions than in wild type mice, directly demonstrating a protective role for IRF-1. With this in mind, transcriptional regulation of IFN-$\beta$ and ISGs was also investigated.

Regarding transcriptional regulation of IFN-$\beta$, we found that IRF-1 regulates IFN-$\beta$ but is not required for reovirus induction of IFN-$\beta$ in PCMCs. Reovirus induction of IFN-$\beta$ is IFN-dependent in PCMCs, and possibly PMEFs. However, reovirus induction of IRF-7 is discordant with reovirus induction of IFN-$\beta$. Finally, reovirus induction of IFN-$\beta$ is cell type-specific, with more induction in PCMCs than PMEFs.

Regarding transcriptional regulation of ISGs, reovirus induction of both ISGs and an ISRE sequence is primarily through IFN in PCMCs and PMEFs. Reovirus and IFN induction of ISRE sequences correlates with reovirus induction of IFN-$\beta$, and is thus cell type-specific, with more induction in PCMCs than PMEFs. However, reovirus and IFN induction of ISGs is discordant with reovirus induction of IFN-$\beta$, with more induction in PMEFs than PCMCs, indicating that cells which produce a lot of IFN are resistant to induction of ISGs. VSV induction of both the ISGs and the ISRE sequence follows the same cell type-specific patterns as reovirus, suggesting that similar regulatory mechanisms may be at work, despite the fact that VSV induces these genes directly, while reovirus induction is mediated by IFN. Finally, while overexpressed IRF-1 regulates both the ISRE sequence and the ISGs, it is not clear from this data whether IRF-1 is required for reovirus induction of
these genes. The mechanisms by which IFN-β, ISGs, and ISRE sequences are differentially regulated cannot be explained by any one hypothesis described in this dissertation. However, each of the three hypotheses described below, which include aspects inconsistent with each other, are supported by a subset of data.

Hypothesis #1

While IFN-β may normally induce ISREs and ISGs through ISGF3, other factors can also induce ISGs and ISREs. This hypothesis relates to the role of IRF-1 as the mediator of an additional path, and here we assume that the IRF-1 pathway is the primary mode of induction for ISREs and ISGs (including IRF-7) in PCMCs and PMEFs. However, IFN-β induces or activates a product, “X”, that inhibits IRF-1 induction of ISRE, ISGs, and IFN-β (Figure 1). This hypothesis is supported by the following data:

1) Overexpressed IRF-1 induces IFN-β, ISRE sequences, and ISGs to a higher level in cultures null for the IFN-α/β receptor (Appendix 2).

2) In response to T3D infection, PCMCs induce a lot of IFN but are resistant to induction of ISGs (Chapter 3). The hypothesis that IFN induces or activates a factor that inhibits IRF-1 induction of IFN-β, ISRE sequences, and ISGs relates to this resistance. Similarly, induction of the ISG, IRF-7, is discordant with IFN-β induction in PCMCs (Appendix 3). We hypothesize that in PCMCs, reovirus/IFN induction of “X” is more efficient than induction of IRF-1.

3) In response to T3D infection, PMEFs induce very little IFN but ISGs are induced to a
high level (Chapter 3). Similarly, induction of the ISG, IRF-7, is discordant with IFN-β induction in PMEFs (Appendix 3). We hypothesize that in PMEFs, reovirus/IFN induction of IRF-1 is more efficient than induction of “X”.

4) Higher baseline IFN-β levels in PMEFs do not correlate with higher constitutive levels of IRF-7 or higher induction of IRF-7, but instead correlate with lower IFN-β induction (Appendix 3). Here, we hypothesize that baseline IFN induction of “X” is more efficient than induction of IRF-1 in PMEFs.

It is unclear how IFN-β could induce IRF-1 in the presence of this inhibitory factor, “X”, or what this factor might be, but PCMCs may have more of it than PMEFs, as evidenced by the higher mIRF-1 induction of all reporters (IFN-β, ISGs, and ISRE sequences) in the wild type PMEFs compared to the wild type PCMCs (Appendix 2). This factor may simply out compete IRF-1 for binding to the ISRE, and could therefore be something as simple as ISGF3 or another IRF, such as IRF-7.

While IRF-2 has been shown to repress both IFN-β (2) and ISGs (1, 3, 6), IRF-1 induces IRF-2 (2). Therefore, if IRF-2 were factor “X” then we would have expected IRF-2’s repressive effects in the IFN-α/β receptor ^ cultures as well. Regardless of the mechanism of inhibition, the above-mentioned hypothesis, by itself, does not explain differential induction of ISGs and ISREs in PCMCs and PMEFs, but may work in conjunction with one of the alternative hypotheses described below.
Hypothesis #2 (Figure 2)

ISGs are preferentially induced by IRF-1, while the ISRE sequence is preferentially induced by ISGF3, and cell type-specific induction is due to a high constitutive level of mIRF-1 in PCMCs (4) This hypothesis is supported by the following data:

1) T3D and IFN induction of ISGs is lower in PCMCs than PMEFs (Chapter 3). High constitutive levels of IRF-1 in PCMCs leads to low induction of ISGs, either because IRF-1 is not induced to high levels, or because high constitutive levels of IRF-1 keep ISGs at high constitutive levels in unstimulated conditions. The high constitutive IRF-1 present in PCMCs does not affect the ISRElux reporter because it is induced by ISGF3 (Appendix 1).

2) Reovirus induction of IRF-7 is discordant with reovirus induction of IFN-β in PCMCs (Appendix 3) because IRF-7, like other ISGs, is preferentially induced by IRF-1, but is resistant to induction in PCMCs due to the high constitutive levels of IRF-1 in this cell type.

3) Overexpressed IRF-1 induces all of the reporters tested (IFN-β, ISG reporters, and the ISRE reporter) to a higher level in PMEFs than PCMCs (Appendix 2), indicating that the lack of high constitutive IRF-1 in PMEFs allows enhanced induction.

4) Overexpressed IRF-1 induces ISGs to a higher level in IFN-α/β receptor⁻/⁻ (Appendix 2), possibly due to a lack of interference with ISGF3.

It should be noted that this hypothesis does not explain why reovirus/IFN induction of the ISRElux reporter would be higher in the PCMCs than the PMEFs, unless there were more efficient ISGF3 signaling in PCMCs. This hypothesis also does not explain the higher
ISRElux induction in the IFN-α/β receptor−/− cultures, where there is no activated ISGF3. One would have to assume that in the absence of other stimuli, IRF-1 induces the ISRE sequence.

**Hypothesis #3 (Figure 3)**

The ISRE sequence is preferentially induced by IRF-1, while the ISGs are preferentially induced by ISGF3, and there is an ISGF3 deficiency in PCMCs.

**This hypothesis is supported by the following data:**

1) T3D and IFN induction of the ISRElux reporter is higher in PCMCs, and because of an ISGF3 deficiency in this cell type, a majority of Stat heterodimers are produced instead of ISGF3. These Stat dimers induce IRF-1 to a high level, leading to higher ISRElux induction in PCMCs. In PMEFs, normal levels of ISGF3 are produced, which out competes IRF-1 for binding to the ISRE reporter, leading to a low level of ISRElux induction in this cell type (Appendix 1).

2) T3D and IFN induction of ISGs is higher in PMEFs than PCMCs (Chapter 3). IFN induces the formation of ISGF3 in PMEFs, leading to a high level of ISG induction. Others have found that the PKR promoter, in particular, is regulated by ISGF3 (5), lending further credence to this hypothesis. In PCMCs, IFN produces only Stat dimers (which induce IRF-1), and IRF-1 competes with ISGF3 for binding to the ISG, leading to a low level of ISG induction in this cell type.
3) IRF-7 induction is discordant with IFN-β induction in PCMCs (Appendix 3). IRF-7 is induced by ISGF3 (like the other ISGs), but the majority of Stat dimers compete for binding, keeping induction low in this cell type.

4) Overexpressed IRF-1 induces the ISRE reporter to a higher level than the ISG reporters in both PCMCs and PMEFs (Appendix 2).

5) Overexpressed IRF-1 induces the ISRE sequence in wild type PMEFs, but even better in PMEFs null for the IFN-α/β receptor (Appendix 2). In the wild type PMEFs, IRF-1 induces IFN, which in turn, induces the formation of ISGF3. ISGF3 competes with IRF-1 for binding the ISRE. In cultures null for the receptor, IRF-1 can induce the ISRE without ISGF3 competition because IFN cannot induce the formation of ISGF3.

This hypothesis does not fully explain why T3D induction of IFN-β is higher in PCMCs, since amplification is presumably mediated by ISGF3 induced IRF-7. Also, this data does not account for the higher ISG induction in the IFNα/β receptor−/−, where there is no ISGF3. One would have to assume that in the absence of other stimuli, IRF-1 induces ISGs.
REFERENCES CITED


Figure 1. Hypothesis #1
While IFN-β may normally induce ISREs and ISGs through ISGF3, other factors can also induce ISGs and ISREs. This hypothesis relates to the role of IRF-1 as the mediator of an additional path, and here we assume that the IRF-1 pathway is the primary mode of induction for ISRE sequences and ISGs (including IRF-7) in PCMCs and PMEFs. However, IFN-β induces or activates a product, “X”, that inhibits IRF-1 induction of ISRE, ISGs, and IFN-β. *Virus does not induce IRF-1 in the absence of IFN-α/β.
**Figure 2. Hypothesis #2**

ISGs are preferentially induced by IRF-1, while the ISRE sequence is preferentially induced by ISGF3, and cell type-specific induction is due to a high constitutive level of mIRF-1 in PCMCs (4).
Figure 3. Hypothesis #3 (A) The ISRE sequence is preferentially induced by IRF-1, while the ISGs are preferentially induced by ISGF3, and (B) there is an ISGF3 deficiency in PCMCs.