IFN Regulatory Factor-1 Bypasses IFN-Mediated Antiviral Effects through Viperin Gene Induction

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Viperin is an antiviral protein whose expression is highly upregulated during viral infections via IFN-dependent and/or IFN-independent pathways. We examined the molecular alterations induced by the transcriptional activator IFN regulatory factor (IRF)-1 and found viperin to be among the group of IRF-1 regulated genes. From these data, it was not possible to distinguish genes that are primary targets of IRF-1 and those that are targets of IRF-1-induced proteins, like IFN-β. In this study, we show that IRF-1 directly binds to the murine viperin promoter to the two proximal IRF elements and thereby induces viperin expression. Infection studies with embryonal fibroblasts from different gene knock-out mice demonstrate that IRF-1 is essential, whereas the type I IFN system is dispensable for vesicular stomatitis virus induced viperin gene transcription. Further, IRF-1, but not IFN type I, mediates the induction of viperin transcription after IFN-γ treatment. In contrast, IRF-1 is not required for IFN-independent viperin induction by Newcastle disease virus infection and by infection with a vesicular stomatitis virus mutant that is unable to block IFN expression and secretion. We conclude that the IRF-1 mediated type I IFN independent mechanism of enhanced viperin expression provides a redundant mechanism to protect cells from viral infections. This mechanism becomes important when viruses evade innate immunity by antagonizing the induction and function of the IFN system. The Journal of Immunology, 2010, 184: 5179–5185.
that the activation of viperin expression can result through IFN-independent mechanisms, depending on the infecting virus (8, 9, 12). In this study, we describe the transcription factor IRF-1 as an essential mediator of viperin induction, acting through an IFN-independent pathway in vesicular stomatitis virus (VSV) infected cells. We also investigated the molecular basis for the IRF-1 induced promoter activation. Furthermore, we show that an enhanced viperin expression, comparable to that in VSV infected cells, results in a reduction of VSV replication even in the absence of functional IFN signaling.

Materials and Methods

Cell culture and treatments

INFRN−/−, IFN−/−, and STAT1−/− mouse embryonic fibroblasts (MEFs) were kindly provided by R. Zawatsky (Deutsches Krebsforschungszentrum, Heidelberg, Germany), T. Decker (University of Vienna, Vienna, Austria), and M. Müller (Veterinary University of Vienna, Vienna, Austria). MEFs, NIH3T3, and myc/rapNIH3T3IH cells (19) were grown in DMEM supplemented with 10% FCS and antibiotics. IRF-1HER fusion protein was activated by 1 μM β-estradiol (Serva, Heidelberg, Germany). Cells were stimulated with 50 U/ml IFN-β or 100 U/ml IFN-γ (PeproTech, Rocky Hill, NJ) for the indicated time periods. Treatment with cycloheximide (CHX) (60 μg/ml) occurred 2 h before IRF-1HER activation and IFN-γ stimulation.

Viral infection

Cells were infected with Newcastle disease virus (NDV), VSV (Indiana strain), or AV2 for 1 h in DMEM without FCS and harvested at the indicated time points postinfection. AV2 is a VSV strain containing a mutated M-protein. For virus challenge, NIH3T3 cells were transiently transfected with pVBC-5vIGN or a control plasmid using Metatfectene (Biontex). Cells were infected 24 h after transfection, and the supernatant was collected after 24 h postinfection. LMTK− cells were infected with serial dilutions of the supernatants to determine viral titers (PFU/ml) by a plaque-forming assay.

Vector constructs

The −1814/+14 viperin-promoter reporter construct was created by amplifying 1800 bp of the murine viperin promoter from IB10 cells ( Primer: 5′-AAT TCC GCT CGA GCG GTA AAG GAC AGA CAA ACT GCA-3′ and 5′-ATT TCC CAA GGT TGG GTG ATA GCA ACA CAC CGT C-3′). Xhol/HindIII digestion, and cloning in pGL3basic (Promega, Mannheim, Germany). Cells were infected 48 h after transfection, and the supernatant was collected 24 h postinfection. LMTK− cells were infected with serial dilutions of the supernatants to determine viral titers (PFU/ml) by a plaque-forming assay.

Quantitative real-time PCR

Total RNA was extracted by using the RNasy kit (Qiagen, Hilden, Germany) and reversely transcribed with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Buckinghamshire, England). Quantitative RT-PCR analysis was performed by using a LightCycler (Roche, Mannheim, Germany) and the Quantitect SYBR Green PCR Kit (Qiagen). The data are represented as ratios relative to the results of a-actin. Standard curve analysis was performed for relative quantification. The following oligonucleotide primers were used: viperin, 5′-CTT CAA GTG GGA CGA ACA AGA CAA ACT GCA-3′ and 5′-GAC TCT CCA TGG TGT GTA AGA ATG TTT CTG-3′; XhoI/HindIII digestion, and cloning in pGL3basic (Promega, Mannheim, Germany). Cells were infected 24 h after transfection, and the supernatant was collected 24 h postinfection. LMTK− cells were infected with serial dilutions of the supernatants to determine viral titers (PFU/ml) by a plaque-forming assay.

Luciferase reporter assay

Viperin promoter constructs, murine IRF-1 expression plasmids pMT7-IRF-1 (20) or pMT-M6 (20) were cotransfected with pB Contruc (21) using Metatfectene (Biontex). Cell lysates were prepared 48 h after transfection. Activities of Firefly and Renilla luciferase were determined using the Dual Luciferase Kit (Promega, Madison, WI). Signals were normalized to the Renilla luciferase activity. All experiments were performed at least twice with triplicates before calculating mean values and standard deviations.

Electro mobility shift assay

Murine IRF-1 and firefly luciferase were in vitro translated using the TNT T7 Quick Coupled Transcription/Translation System (Promega), according to the manufacturer’s protocol. pMT7-IRF-1 and luciferase T7 control DNA served as templates. EMSA experiments were performed with 5 µl in vitro translated protein mix in the absence or presence of 1 μg anti-IRF-1 (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), as described in Klar and Bode (22). The used oligonucleotides were shown in the Supplemental Table II.

IFN assay

Type I IFN concentration in the cell culture supernatants was determined by an antiviral assay using LMTK− cells as described previously (23). Briefly, we seeded LMTK− cells in 96 wells and treated them for 24 h with serial diluted supernatants. Cells were challenged with VSV, IFN in the supernatant protects cells from the cytopathic effect through viral infection. Effects from supernatants were compared with standard type I IFN effect (PBL).

Promoter analysis

To predict potential transcription factor binding sites in the murine viperin promoter, the sequence from −1814 to +100 was analyzed by the web tool TESS (www.cbil.upenn.edu/teiss). This program uses sites or consensus strings and positional weight matrices from the TRANSFAC, IMDB, and the CBIL-GibbsMat database.

Western blot analysis and Abs

Western blot analysis was accomplished according to standard procedures using ECL detection (Amersham Munich, Germany). The following primary Abs were used: IFN-3P (Ser396) (4D4G), Cell Signaling (Danvers, MA); VSV-G (PSD4), Boehringer Mannheim (Mannheim, Germany); and actin (Ab-1), Oncogene (Cambridge, United Kingdom). IRRF-conjugated anti-rabbit and anti-mouse Abs (Amersham) were used as secondary Abs.

Results

Viperin transcription is upregulated by IRF-1

We previously established gene expression profiles to uncover the molecular alterations initiated by IRF-1 (24). Among several other genes with antiviral function, we found the viperin gene to be highly expressed in the presence of functional active IRF-1. This could be confirmed by transfection of a luciferase-based viperin promoter reporter in combination with an IRF-1 expression vector (Fig. 1A). Moreover, IRF-1 M6, a mutant that lacks the DNA binding domain, was incapable of inducing the promoter activity of the reporter construct (Fig. 1A), indicating that IRF-1 directly binds to the sequence and thereby activating the viperin promoter. To further verify this direct involvement of IRF-1 in the regulation of viperin, we analyzed viperin mRNA level in cells stably expressing an estradiol-activatable IRF-1hER fusion protein (19). Activation of the IRF-1hER fusion protein in the absence and presence of the translation inhibitor CHX strongly induced viperin mRNA synthesis (Fig. 1B, 1C). However, the presence of CHX prevented IFN-γ mediated upregulation of viperin supporting the hypothesis that under this condition IRF-1 gene expression must be induced to stimulate viperin transcription (Fig. 1C). Thus, activation of viperin transcription by sufficiently expressed IRF-1 is mediated directly and does not require de novo synthesis of proteins, enhancement of viperin expression can be mediated by elevated IRF-1 levels.

Bioinformatic promoter analyses of the murine viperin gene revealed several consensus response elements for NFκB, AP-1, NFAT, SP1, and three putative IRF elements (IRF-ES) (Fig. 2A). To determine the binding capacity of IRF-1 to these IRF-E sequences EMSAs were performed in vitro translated IRF-1. As expected, the control oligonucleotide (IFN stimulated regulatory element [ISRE]) was efficiently bound by the IRF-1 protein
Viperin is an IFN-1 regulated gene. A, NIH3T3 cells were transiently transfected with a plasmid containing the Firefly luciferase gene driven by the wt viperin promoter (-1814/+14) and an expression vector coding for IFN-1 or IFN-1 M6, a mutant that lacks the DNA binding domain. Transfection efficiency was normalized by cotransfection of a constitutive Renilla luciferase expression plasmid. B, NIH3T3 cells, stably expressing an IFN-1ER fusion protein (NIH3T3H), were treated with 1 μM β-estradiol to activate IFN-1. Relative viperin mRNA concentration was measured by quantitative real-time PCR at the indicated times. C, NIH3T3H cells were pretreated with 60 μg/ml CHX, 2 h before β-estradiol-induced activation of the IFN-1ER fusion protein (IFN-1 and CHX) or IFN-γ (100 U/ml) treatment (IFN-γ and CHX). All data represent mean values with SD from at least two independent experiments.

**FIGURE 1.** Viperin is an IFN-1 regulated gene. A, NIH3T3 cells were transiently transfected with a plasmid containing the Firefly luciferase gene driven by the wt viperin promoter (~1814/+14) and an expression vector coding for IFN-1 or IFN-1 M6, a mutant that lacks the DNA binding domain. Transfection efficiency was normalized by cotransfection of a constitutive Renilla luciferase expression plasmid. B, NIH3T3 cells, stably expressing an IFN-1ER fusion protein (NIH3T3H), were treated with 1 μM β-estradiol to activate IFN-1. Relative viperin mRNA concentration was measured by quantitative real-time PCR at the indicated times. C, NIH3T3H cells were pretreated with 60 μg/ml CHX, 2 h before β-estradiol-induced activation of the IFN-1ER fusion protein (IFN-1 and CHX) or IFN-γ (100 U/ml) treatment (IFN-γ and CHX). All data represent mean values with SD from at least two independent experiments.

**FIGURE 2.** IRF-1 directly binds to two proximal IRF-Es of the viperin promoter and thereby increases the promoter activity. A, Schematic map of the three IRF-1 binding sites (IRF-E-1, -2, and -3) in the 2000 bp upstream region of the murine viperin promoter. Their position in the promoter is represented with respect to the transcriptional start. B, Formation of IRF-1/DNA complexes was tested by EMSA using the sequence of the indicated IRF-E sites and in vitro translated IRF-1. A probe with the ISRE sequence of the IFN-β promoter was used as positive control. C, EMSA was performed as in B but with oligonucleotides representing mutated IRF-E sequences. D, NIH3T3H cells transiently transfected with a Renilla luciferase reporter plasmid (pBCRluc), a plasmid with the Firefly luciferase gene driven by the wt viperin promoter (~1814/+14) or reporter constructs containing mutations in one of the IRF-E sites. Activation of IRF-1 by β-estradiol (1 μM) treatment was performed 24 h before harvesting cells for the dual luciferase assay. Relative luciferase activities of Firefly luciferase were calculated by normalization to Renilla luciferase activity. The graph shows the fold increase of relative luciferase activities in cells treated with β-estradiol (IRF-1) compared with untreated cells (C).

IRF-1 is essential for IFN-γ and VSV induced viperin expression

Viperin is highly induced on IFN stimulation and by infection with a broad range of viruses. To investigate whether IRF-1 exerts an influence on these events, we tested wild-type (wt) and IRF-1–deficient (IRF-1−/−) MEFs for alterations in viperin mRNA levels caused by IFN-β, IFN-γ, VSV, and NDV, respectively. As expected, in wt MEFs, increased viperin mRNA levels could be detected as a consequence of treatment with all four stimuli (Fig. 3A). Stimulation of IRF-1−/− cells with IFN-β or infection with NDV leads to the induction of viperin (Fig. 3B) indicating that IRF-1 plays no role under these conditions. In contrast, IFN-γ failed to induce viperin expression in IRF-1−/− cells, suggesting an important role of IRF-1 in IFN-γ mediated viperin induction. A more revealing finding was that in the absence of the IRF-1 gene activity, the VSV-mediated viperin induction failed (Fig. 3B), indicating that the presence of the IRF-1 is essential to activate the viperin promoter under these conditions.

IFN-γ is known to be a potent IRF-1 inducer (25). Its binding to the IFN-γ receptor activates STAT1 and NFκB, which synergistically interacts to induce IRF-1 transcription (26). Therefore, we examined whether STAT1 also plays a role in the IRF-1–mediated viperin induction on virus infection. First of all, we investigated whether STAT1 itself or synergistically with IRF-1 influences the viperin promoter activity. Transfection of NIH3T3 cells with an IRF-1 expression vector induced the viperin promoter activity in a dose-dependent manner, whereas expression of STAT1 had no significant influence, both in the absence or presence of ectopic IRF-1 (Fig. 4A). Therefore, a direct influence of STAT1 on the viperin promoter activity is unlikely. In addition, performed quantitative RT-PCR analyses of STAT1−/− MEFs showed only weak induction of viperin by NDV but no induction after VSV infection (Fig. 4B). In contrast, the transient expression of IRF-1 significantly enhanced the amount of viperin mRNA in STAT1-deficient MEFs (Fig. 4C). This supports our hypothesis that an increase of cellular IRF-1 expression is sufficient to activate the viperin promoter and that STAT1 is essential for stimulation of IRF-1 transcription. These results also indicate a STAT1-dependent mechanism of viperin induction during NDV infection, that results from the involvement of STAT1 in other IRF-1 independent antiviral signaling pathways, namely, the type I IFN pathway.

**FIGURE 2.** IRF-1 directly binds to two proximal IRF-Es of the viperin promoter and thereby increases the promoter activity. A, Schematic map of the three IRF-1 binding sites (IRF-E-1, -2, and -3) in the 2000 bp upstream region of the murine viperin promoter. Their position in the promoter is represented with respect to the transcriptional start. B, Formation of IRF-1/DNA complexes was tested by EMSA using the sequence of the indicated IRF-E sites and in vitro translated IRF-1. A probe with the ISRE sequence of the IFN-β promoter was used as positive control. C, EMSA was performed as in B but with oligonucleotides representing mutated IRF-E sequences. D, NIH3T3H cells transiently transfected with a Renilla luciferase reporter plasmid (pBCRluc), a plasmid with the Firefly luciferase gene driven by the wt viperin promoter (~1814/+14) or reporter constructs containing mutations in one of the IRF-E sites. Activation of IRF-1 by β-estradiol (1 μM) treatment was performed 24 h before harvesting cells for the dual luciferase assay. Relative luciferase activities of Firefly luciferase were calculated by normalization to Renilla luciferase activity. The graph shows the fold increase of relative luciferase activities in cells treated with β-estradiol (IRF-1) compared with untreated cells (C).
A multiplicity of viruses, LPSs and dsRNA induce the viperin expression through IFN-dependent and IFN-independent mechanisms. The IFN-dependent viperin induction can be mediated by mechanisms. The IFN-dependent viperin induction can be mediated by

Discussion

A multiplicity of viruses, LPSs and dsRNA induce the viperin expression through IFN-dependent and IFN-independent mechanisms. The IFN-dependent viperin induction can be mediated by
type I IFNs, IFN-γ, and type III IFNs. Previous publications showed that activation of the type I IFN signaling pathway enhances the viperin transcription through the STAT1/STAT2/IRF-9 complex termed ISG factor 3 (ISGF3) by binding to the promoter response element ISRE (8, 12). In this article, we reveal IRF-1 as a mediator for the VSV and IFN-γ amplified viperin expression (Fig. 3). IRF-1 mediates its activity by binding to conserved IRF elements in the promoter of its target genes. Our data demonstrate that the viperin promoter contains two IRF-E sites, which are necessary for IRF-1 mediated promoter activation (Fig. 2). This overlap of gene induction by IRF-1 and type I IFNs is not astonishing, because the IRF binding sites and the one from IFN-induced ISGF3 complex are very similar.

Viperin limits replication of viruses like HCMV (10), VSV-pseudotyped HIV (14), and HCV (15). The exact mechanism by which viperin limits the viral replication is unknown. In HCMV infected cells the expression of viperin inhibits the expression of several structural proteins critical for viral assembly and maturation (10). In contrast, in influenza A virus infected cells, viperin inhibits the budding of the virus from the plasma membrane. This is a consequence of viperin-induced disruption of lipid-raft microdomains, which results from the interaction and inactivation of the enzyme farnesyl diphosphate synthase with viperin (11). VSV does not bud from lipid rafts, which is indicative for the presence of other antiviral mechanisms. Our data show, that viperin expression also inhibits the replication of VSV more than 1000-fold (Fig. 5C). This strong inhibition of virus replication by an intrinsic effect is only comprehensible in the unlikely case of 100% transfection efficiency. Transfection efficiency was determined by the cotransfection of a GFP expression vector and is 60% or higher (data not shown). This transfection efficiency is not sufficient for a 1000-fold reduction of viral replication. One possible explanation is that a very low expression level of viperin that might be induced by a transfection of nearly 100% of cells is sufficient to induce an antiviral response. Alternatively, paracrine effects could be responsible for this phenomenon. We checked the supernatant for further antiviral effects but could not detect a respective activity (data not shown).

Previous studies with IRF-1 deficient MEFs demonstrated an involvement of IRF-1 in the type I IFN-dependent antiviral response against ECMV infection (29) and in an IFN-γ mediated mechanism preventing vaccinia virus replication (30). In addition, it was found that IRF-1 exerts a direct antiviral response, which is consistent with

![FIGURE 5](image-url)  
**FIGURE 5.** The increase in viperin transcription during VSV infection is IRF-1 dependent and type I IFN independent. Relative (A) viperin and (B) IFN-1 mRNA amounts normalized to α-actin were determined by quantitative real-time PCR in IFNAR−/− MEFs treated with IFN-β (500 U/ml), IFN-γ (100 U/ml), VSV, or NDV at indicated times. B, *p* < 0.05; **p** < 0.01; ***p** < 0.001, Student *t* test. C. The wt and IFNAR−/− cells were transiently transfected with an empty vector (mock) or a viperin expression vector and infected with VSV (MOI 0.01) for 1 h. In addition, cells were treated with 500 U/ml IFN-β and/or Abs against 2000 U/ml type I IFN. Viral titers (PFU/ml) were determined 24 h postinfection by plaque formation assay. All data represent the mean value with SD of at least two independent experiments. MOI, multiplicity of infection.

![FIGURE 6](image-url)  
**FIGURE 6.** IRF-1 provides a redundant mechanism to bypass the type I IFN system. IRF-1−/− MEFs were infected for 1 h with NDV, VSV (MOI 0.01), or the mutated VSV strain AV2 (MOI 0.01). A. Secreted type I IFN was determined in the cell supernatant 24 h postinfection by an antiviral assay. B. At the indicated time periods after AV2 infection, total RNA was extracted, reversely transcribed, and analyzed for viperin mRNA amount by quantitative real-time PCR. All data represent the mean value with SD of at least two independent experiments. MOI, multiplicity of infection.

![FIGURE 7](image-url)  
**FIGURE 7.** Schematic presentation of viperin induction. Virus infection could lead to the induction of the type I IFN pathway. The secreted IFN acts in an autocrine and paracrine manner, leading to the activation of the heterotrimeric STAT1/STAT2/IRF-9 complex ISGF3. By its function as a transcriptional activator ISGF3 mediates the induction of ISGs, like viperin. Independently, virus infection can lead to the transcriptional induction of IRF-1, which directly activates the viperin promoter. Viruses, like NDV, can activate both pathways on infection, which leads to viperin induction in the absence of IFNAR or IRF-1. Many viruses, like VSV, developed strategies to block the expression or function of type I IFN. VSV infection inhibits the function of type I IFNs but activates the IRF-1 pathway that enables the infected cells to induce an antiviral response.
the finding that enforced expression of IRF-1 prevents VSV, EMCV, and HCV replication in an IFN-independent way (31, 32). Because our analysis revealed IRF-1 as being an essential factor for the increase of viperin gene expression postinfection with VSV, a virus that is extremely efficient in blocking the type I IFN function (Fig. 6A), we conclude that the IFN-independent induction of viperin is mediated by IRF-1 and propose a model that is depicted in Fig. 7. Virus infection (e.g., NDV) can lead to the production and secretion of IFN-β. In turn, IFN-β interacts with type I IFN receptors in an autocrine and paracrine manner, inducing ISGs via the Jak/STAT pathway. The independently induced IRF-1 induces the viperin expression as well (Fig. 1). Because both pathways are in place at the same time induction of viperin by NDV can neither be blocked by the deficiency of IFNAR nor the lack of IRF-1 (Figs. 3B, S4A). This model also explains the role of STAT1 that is essential for NDV and VSV infection because it is involved in both pathways (Fig. 4B). VSV infection blocks the production of IFN-β in wt- and IRF-1−/− cells (Fig. 6A and data not shown). Thus, viperin expression cannot be induced by the Jak/STAT pathway but is dependent on the alternative IRF-1 pathway (Fig. 3D). If the block of the IFNAR signaling in VSV can be overcome by using the VSV mutant strain A V2, which lacks the inhibitory IRF-3 pathway are involved in VSV defense. The failure of an electrophilic virus to promote an efficient VSV-neutralizing immune response because ISGs are induced by an IFN independent, but IRF-3 receptors (33). The entry of UV inactivated VSV particles results in the production of IFN-β and IFN-γ, which induce viperin by PTGS and regulated by proinflammatory agents. Arterioscler. Thromb. Vasc. Biol. 25: e13-e116. 7. Severa, M., E. M. Coccia, and K. A. Fitzgerald. 2006. Toll-like receptor-dependent and -independent viperin gene expression and counter-regulation by PRDI-binding factor-1/Blimp1. J. Biol. Chem. 281: 26188–26195. 8. Boudinot, P., S. Riffault, S. Salihi, C. Carrat, C. Sedlik, N. Mahmoudi, B. D. Hauser, and A. Moghimi. 2000. Vascular stomatitis virus and pseudorabies virus induce a vig1/cig5 homologue in mouse dendritic cells via different pathways. J. Gen. Virol. 81: 2675–2682.

References


Supplementary figure 1. Viperin expression limits VSV replication

NIH3T3 cells were transiently transfected with empty vector control (mock) or viperin expression vector and infected with VSV (MOI 0.01) for 1 h. 24 h post infection cells were harvested. (A) Western blot analysis of VSV-G protein from mock and viperin transfected cells. (B) Relative VSV mRNA amounts normalized to α-actin were determined by quantitative real-time PCR. All data represent the mean value with SD of at least two independent experiments.

Supplementary figure 2. Effects of VSV infection on IFN induction

W.t. IRF-1/- and IFNAR-/- MEFs were infected with VSV or the AV2 mutant (MOI 0.01) for 1 h. (A) Western blot analysis of activated IRF-3 12 h post infection by a phospho-specific IRF-3 antibody. 24 h post infection cells were harvested. Relative IFN-β mRNA (B) and IRF-7 mRNA (C) amounts normalized to α-actin were determined by quantitative real-time PCR. All data represent the mean value with SD of at least two independent experiments.
Supplementary figure 1

A

mock viperin

VSV-G

actin

B

relative VSV mRNA

0 200 400 600 800

mock viperin
**Supplementary table I. Oligos used for cloning of mutant viperin promoter constructs**

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<tr>
<td>IRF-Emu#1</td>
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<td>IRF-Emu#3</td>
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<sup>a</sup> Potential IRF-1 binding sites are underlined
<sup>b</sup> deleted nucleotides are hyphenated
Supplementary table II. *Oligos used for EMSA*

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<thead>
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<td>IRF-Emu#2/3</td>
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a Potential IRF-1 binding sites are underlined
b deleted nucleotides are hyphenated