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Regulation of the Transcriptional Activity of the IRF7 Promoter by a Pathway Independent of Interferon Signaling*

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Genes containing an interferon (IFN)-stimulated response element (ISRE) can be divided into two groups according to their inducibility by IFN and virus infection: one induced only by IFN and the other induced by both IFN and virus infection. Although it is now clear that IFN regulatory factor 7 (IRF7) is a multifunctional gene essential for induction of type I IFNs, regulation of the IRF7 promoter (IRF7p) is poorly understood. The IRF7 gene includes two IFN responsive elements, an IRF-binding element (IRFE) in the promoter region and an ISRE in the first intron, and is induced by the IFN-triggered Jak-STAT pathway by binding of the IFN-stimulated gene factor 3 (ISGF3) complex to the ISRE. In this study, we demonstrate that IRF3 and IRF7, which with the coactivators CREB-binding protein and P300 form the virus-activated factor (VAF) complex upon Sendai virus infection, bind to the IRF7 ISRE and IRFE and can directly activate IRF7 transcription. Promoter reporter assays show that both the ISRE and IRFE are responsive to activation by IRF7 and IRF3. In cells transiently expressing IRF7 or/and IRF3, the VAF level and binding of VAF are clearly increased after Sendai virus infection. Studies with Jak1 kinase inactive 293 cells that were stably transfected with a Jak1 kinase dead dominant negative construct, and the mutant cell lines SAN (IFNα^-/β^-), U2A (IRF9$^-$), U4A (Jak1$^-$), and DKO (IRF1$^-$/IRF2$^-$) show that the IRF7 transcription activated directly by VAF is distinct from and independent of the IFN signaling pathway. Thus, IRF7 transcription is autoregulated by binding of the IRF7-containing VAF to its own ISRE and IRFE. The results show two distinct mechanisms for the activation of the IRF7 promoter, by IFN and by virus infection. A regulatory network between type I IFNs and IRF7 is proposed. The distinct pathways may reflect special roles for an efficient antiviral response at different stages of virus infection.

In immune and inflammatory responses, virus infection results in activation or direct induction of a set of transcription factors, the interferon (IFN)[1] regulatory factors (IRFs), which are a family involved in the regulation of IFNs and IFN inducible genes. IFNs participate in antiviral defense, cell growth regulation, and immune activation by inducing a set of IFN inducible genes (1–5). IRF3 and IRF7 have been demonstrated to play an essential role in virus-dependent signaling, whereas IRF1, IRF-2, and IRF9 are critical for proper IFN-dependent gene expression (6–9).

All cellular IRFs have an N-terminal DNA-binding domain that is capable of binding to IFN-stimulated response elements (ISREs) with the consensus sequence 5′-(A/G)NGAAANN-GAAACT-3′ in the IFN gene promoters (6, 8–13). Besides IFN genes, many other genes such as the tumor suppressor p53 (14), the transporter associated with antigen processing 2 (Tap2) (15), and the Epstein-Barr virus (EBV) latent membrane oncoprotein 1 (LMP1) (16) possess ISRE motifs and are regulated by IRFs. Because these genes are involved in cell cycle regulation, apoptosis, and tumor suppression, IRFs are also involved in regulation of these processes in addition to their roles in immune regulation (17–19). Two such sequence motifs, 5′-GGAAAGCGAAACC-3′ (ISRE) and 5′-CAAAAGC-GAAACT-3′ (IRF-binding element, IRFE), have been identified in the IRF7 first intron and in its promoter, respectively (20). IRF7 has a wider DNA-binding specificity (GAAWNYG-AAANY, W = A or T, Y = C or T) compared with IRF3 (GAAASSGAAANY, S = G or C) (21). Studies on murine IFNA promoters showed that the core sequence GAAANN for IRF binding can be divided into four groups according to the identity of the NN: 1, the NC (N = G, C, A, or T) and ST bases inducible by both IRF3 and IRF7; 2, the NG and CA preferentially responsive to IRF3; 3, the GAAAAT repeats responding only to IRF7; and 4, the DA (D = T, A, or G) unresponsive to both factors (7, 22). Interestingly, both the IRF7 ISRE and IRFE match the first group, which is inducible by both IRF3 and IRF7.

The ISRE-containing genes are regulated by IRFs typically through the Jak-STAT pathway. In this pathway, type I or II IFN stimulation results in the phosphorylation of STAT1 and STAT2. The phosphorylated STAT1 and STAT2, together with IRF9, which is constitutively expressed in most cell types, form the complex, interferon-stimulated gene factor 3 (ISGF3) (23). This complex binds to the ISREs in the ISRE-containing genes and activates their transcription. A single copy of ISRE is sufficient for gene activation, but ISRE-containing genes are

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[1] The abbreviations used are: IFN, interferon; IRF, interferon regulatory factor; ISRE, IFN-stimulated response element; EBV, Epstein-Barr virus; LMP1, EBV latent membrane protein 1; IRFE, IRF-binding element (or IRF regulatory element); STAT, signal transducer and activator of transcription; ISGF3, interferon-stimulated gene factor 3; Jak, Janus kinase; VAF, virus-activated factor; DN, dominant negative; SeV, Sendai virus; EMSA, electrophoretic mobility shift assay; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; RT-PCR, reverse transcription PCR; ISG, interferon-stimulated gene; VRE, virus responsive element; HAU, hemagglutinin unit.

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not inducible in mutant cell lines in which any Jak-STAT pathway component has been knocked out (10, 24). Expression of IRF7 is typically mediated by the IFN-triggered Jak-STAT pathway in which ISGF3 directly binds to the IRF7 ISRE (20, 25).

Although virus infection or synthetic double-stranded RNA results in expression of type I IFNs, which induce expression of IFN-inducible genes, some of these IFN-inducible genes (such as ISG15/54/56) are also directly induced upon virus infection or by double-stranded RNA independent of the IFN-triggered Jak-STAT pathway (24, 26–30). The IRF family member, IRF1, is also induced by both IFN and direct virus infection independent of IFN signaling (9, 31). Other IFN-inducible genes such as ISG9–27 and ISG6–16, however, are only induced by IFNs but not by virus infection or double-stranded RNA (3, 32). Thus, it seems that virus infection and IFN trigger two distinct cellular pathways, which sometimes converge on the same cis-acting elements. The slight differences in the ISRE motifs in the promoters of these ISRE-containing genes confer their different specificity in inducibility by either IFN or virus through unknown mechanisms (3).

IRF7 functions as either an activator or a repressor in regulation of some ISRE-containing genes. In addition to induction of type I IFN expression (33, 34), IRF7 also represses EBNA1 transcription in EBV type III latency (35), activates Tap2 transcription via EBV LMP1 (15), and induces LMP1 expression (16).

Although extensive studies have shown that IRF7 is a multifunctional protein with transcriptional activity that depends on C-terminal phosphorylation, regulation of the IRF7 promoter (IRF7p) is poorly understood. Previous data show that ISGF3 is a potent factor in the induction of IRF7 expression in mouse (25) and human cells (20). In this study, in addition to induction by type I IFNs, we demonstrate that IRF7 transcription can be regulated by binding of both IRF7 and IRF3 proteins in the virus-activated factor (VAF) complex to the IRF7p ISRE and IRFE and that this regulation is independent of the IFN pathway.

EXPERIMENTAL PROCEDURES

Plasmids—The IRF7 promoter construct p(−1123/+575)-Luc, which contains the wild type ISRE and IRFE motifs as well as its three mutants, IRFEm-Luc, (ISRE+IRFE)m-Luc, and ΔISRE-Luc, were gifts from Paul Pitha (20). ISREm-Luc and ΔIRFE-Luc were made by site-directed mutation (Stratagene) using p(−1123/+575)-Luc as template. The IFNβ promoter construct, pGL3/IFNβp-Luc, was described previously (36). The expression plasmids IRF7 (35), FLAG-IRF7 and its mutants (36), IRF3 and IRF3(5D), were described previously (29). The zinc inducible kinase inactive Jak1 (Jak1 K896R) cDNA cloned in MTCB6+ vector was kindly provided by Paul Rothman (37). Both pMTCB6+-Jak1DN and EGFP-C1 vector contain a neomycin resistance gene that allows transfected cells to be selected using G418. FLAG-IRF7DN, FLAG-IRF3, FLAG-IRF3DN, and FLAG-IRF3(5D) were constructed by subcloning corresponding PCR products into pCMV2-FLAG vector (Sigma), which was kindly provided by John Hiscott.

Cell Lines and Establishment of Stable Transfectants—293 cells are derived from human kidney epithelial cells. HeLa cells are derived from cervical carcinoma. Mutant cell lines, U2A cells lacking functional IRF9, U4A cells lacking Jak1, and the parent cells 2fTGH (human fibroblasts) were gifts from George Stark (10, 38), and SAN cells (human glioblastoma) lacking type I IFN genes were the gift of Marc Wathelet and Tom Maniatis (39). The DKO cell line used in this study is a mouse embryonic fibroblast line with targeted disruption of both the IRF1 and IRF2 genes (40). All the cell lines were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and antibiotics.

293 cells were transfected with an Effectene kit following the manufacturer's instructions (Qiagen). Cells lines stably expressing 1) Jak1-DN, 2) EGFP-C1, FLAG-IRF7DN, and FLAG-IRF3DN, and 3) Jak1-DN, FLAG-IRF7DN, and FLAG-IRF3DN were generated. EGFP-C1 empty vector cotransfected in 2) was for selection with G418. Expression of Jak1-DN was induced by adding $ZnCl_2$ at a final concentration of 50 μM for 8 h before G418 was added. One day after transfection, cells were cultured in medium containing 800 μg/ml of G418 (Invitrogen) for 2 weeks. Single stable clones were screened and expression of FLAG-IRF7 and FLAG-IRF3 in some single clones was detected by Western blot using anti-FLAG (Sigma). Stably expressing cells were maintained in 400 μg/ml G418-containing medium.

Reporter Assays—For luciferase assays, cells in 12-well plates were transfected with 0.2 μg of reporter plasmid and 0.1 μg β-galactosidase expression plasmid with the use of an Effectene kit. After 12 h, cells were infected with 200 HAU SeV for 24 h or IFNα2 for 12 h after transfection. Cell lysates were combined with Luciferase Assay Reagent (Promega), and the relative light units were measured in an Autolumat LB953 (PE, Inc.). The activity of β-galactosidase was used as internal control.

Electrophoretic Mobility Shift Assay (EMSA)—Cells in 60-mm dishes were transfected with 2 μg of expression plasmids with an Effectene kit. 42 h after transfection, cells were infected with 200 HAU/ml Cantell Sendai viruses for 6 h before harvest (3). Cells were lysed in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 2 mM dithiothreitol, 5% glycerol, 0.5% Nonidet P-40, 10 μg/μl bovine serum albumin) with protease inhibitors. The whole cell lysates were used for EMSA, as described previously (36). Poly(dI-dC) (Amersham Biosciences) was added to reduce nonspecific binding at a final concentration of 62.5 μg/ml. Probes with 5′-GATC or 5′-GTAC adhesive ends were annealed and labeled with [α-^{32}P]dCTP in Klenow reactions. For each binding reaction, about 40,000 cpm of probe were used. For supershift assays, cell lysates were incubated with 0.2 μg FLAG antibody M2 (Sigma), 2 μg of IRF3 antibody SL-12 (BD Biosciences), CBP antibody A-22, or p300 antibody N-15 (Santa Cruz Biotechnology) before probe was added. Protein·DNA complexes were separated on 5% 60:1 acrylamide/bis-acrylamide gels.

Chromatin Immunoprecipitation Assay—HeLa cells in 100-mm dishes were transfected with 5 μg of expression plasmids or empty vector. Cells were infected with SeV for 6 h and then subjected to cross-linking by adding formaldehyde to a final concentration of 1% for 30 min at room temperature with slow rotation. Cross-linking was stopped by adding glycine to a final concentration of 125 mM for 5 min. Chromatin was sheared to ~300 bp by sonication. Immunoprecipitation, washing, and the recovery of bound DNA were performed following the protocol provided by Upstate Biotechnology. After extraction and precipitation, DNA pellets were dissolved in 60 μl of H_2O, and 15 μl of each were used for PCR. All PCR reactions were performed for 35 cycles with a high fidelity PCR system (Roche Applied Science) at an annealing temperature of 60 °C. PCR products were run on 1.5% agarose gels.

RT-PCR—Cells were infected with 200 HAU/ml of SeV or 500 units/ml IFNα2 (Sigma) for indicated time periods. RT was performed for 60 min in a volume of 20 μl with 1 μg of total RNA with an RT-PCR system kit (Promega). The cDNA products were diluted 5 times. PCR was run in the linear range (30 cycles) at an annealing temperature of 60 °C in a high fidelity PCR system (Roche Applied Science). Primers for β-actin were 5′-GGCATCGTGATGGACTCCG-3′ and 5′-GCTGGAAGGTGGACAGCGA-3′. PCR products were run on 1.5% agarose gels.

RESULTS

Virus Infection Induces Endogenous IRF7 mRNA—IRF7 possesses an ISRE and an IRFE in its promoter and first intron (20). IRF3 and IRF7 can potentially bind to these elements after activation by virus infection and therefore directly induce expression of IRF7 independently of the Jak-STAT pathway. To test this hypothesis, we first performed RT-PCR in SAN cells lacking type I IFN genes and in 293 cells stably transfected with a Jak1 kinase dominant negative construct (pMTCB6+/Jak1-DN). Total mRNAs were isolated for quantitative RT-PCR for detection of IRF7 expression (41–45).

SAN cells were infected with SeV for the indicated time periods (Fig. 1*A*). As shown in Fig. 1*A*, SeV infection increases IRF7 mRNA detectable after 6 h, and further increases can be detected up to 72 h. Because type II IFN (IFN γ) is produced by activated T-lymphocytes (T-cells) and natural killer cells (46, 47), the time course data from the human glioblastoma SAN cells indicate that IRF7 can be induced by SeV infection independently of type I and II IFNs.

Because the recently discovered IFNλ also induces an anti-

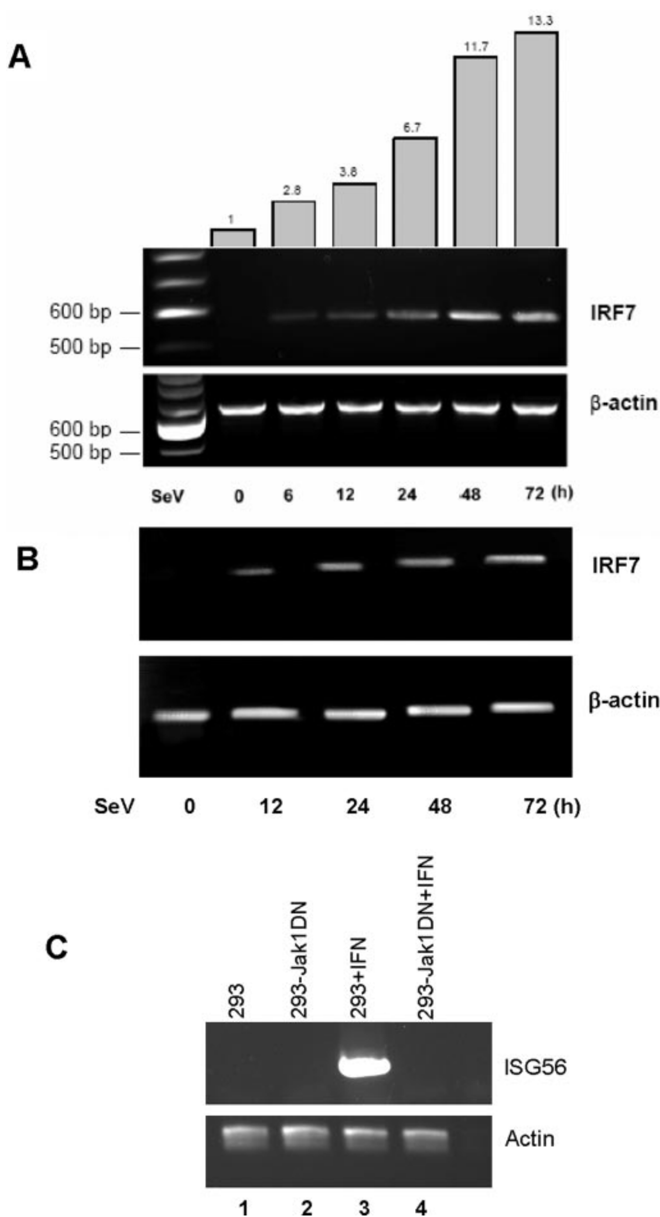


FIG. 1. **Virus infection induces expression of endogenous IRF7 mRNA.** SAN cells ($IFN\alpha^-/\beta^-$) (*A*) and Jak1 kinase inactive 293 cells (*B*) in 6-well plates were infected with 200 HAU/ml Cantell SeV for the indicated times. The histogram at the *top* represents quantification of the IRF7 mRNA intensity normalized to β-actin mRNA intensity. Fold is relative to the basal level of IRF7 mRNA in SAN cells. *C*, RT-PCR results for ISG56 gene expression show the inactivity of Jak1 kinase in these Jak1 kinase inactive 293 cells. Total RNA was extracted, and equal amounts of total RNA were used to prepare cDNA. PCR was performed with specific primers for IRF7 and β-actin. Primers for ISG56 are 5′-CCAGCGCTGGGTATGCGATCTCTGCC-3′ and 5′-GGGCCCGCTCATAGTACTCCAGGGC-3′, and primers for IRF7 are 5′-CGCGGCACTAACGACAGGCGAG-3′ and 5′-GCTGCCGTGCCC-GGAATTCCAC-3′.

viral state and expression of interferon-stimulated genes (ISGs) and works through the Jak-STAT pathway (48, 49), we used a Jak1 kinase dead, dominant negative stably transfected 293 cell line (293-Jak1DN) to exclude the possibility that SeV induction of IRF7 involves IFNλ and/or the Jak1-STAT pathway. Analysis of RNA from SeV-infected 293-Jak1DN cells revealed a pattern of IRF-7 induction similar to that observed in SAN cells (Fig. 1*B*). That the Jak1DN blocked endogenous Jak1 activity was confirmed by examining the expression of the ISG56 gene, a target of Jak1 signaling (50) (Fig. 1*C*, compare *lanes 3* and *4*).

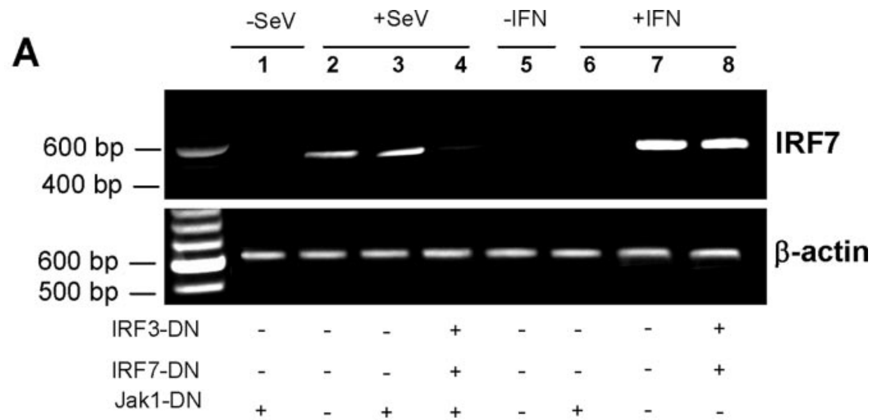
Also, we performed promoter reporter assays in 293 cells, HeLa cells, and in several mutant cell lines, SAN ($IFN\alpha^-/\beta^-$), U2A ($IRF9^-$), U4A ($Jak1^-$), and DKO ($IRF1^-/IRF2^-$). The results disclosed that IRF7 and IRF3 can activate a 1.6-kb IRF7 promoter construct, p(−1123/+575)-Luc which contains both the IRFE and ISRE, and SeV infection can increase the IRF7 promoter activity significantly in these cells (data not shown).

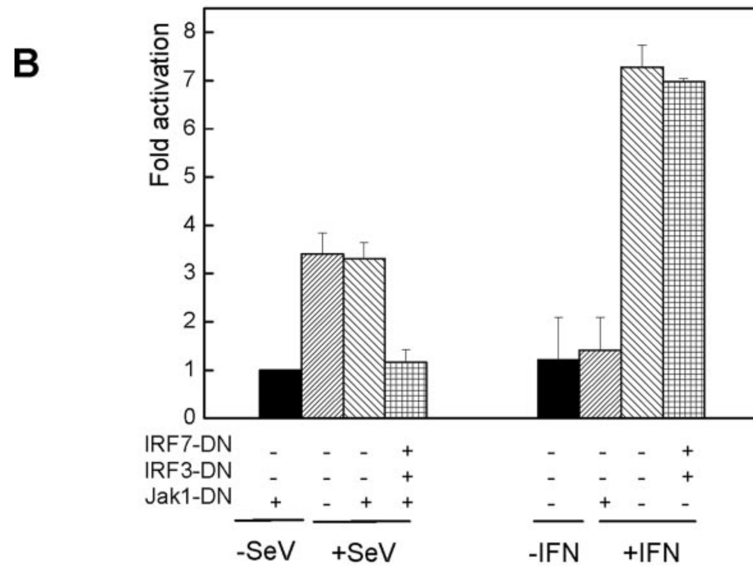
Thus, because Jak1 is required for signaling pathways triggered by all IFNs including IFN α/β (type I), IFNγ (type II), and IFNλ (10, 48, 49), the data from the mutant cell line SAN and Jak1DN cells indicate that IRF7 can be induced by SeV infection independently of all types of IFNs.

IRF7 and IRF3 Are Required for SeV Infection-induced IRF7 Expression—Because SeV infection induced IRF7 expression independently of IFNs, we next asked whether IRF7 and IRF3 are important for this induction. To answer this question we generated 293 stable cell lines expressing 1) dominant negative IRF7 (IRF7DN) and IRF3 (IRF3DN) (51) or 2) IRF7DN, IRF3DN, and Jak1-DN. We infected these cells, the 293-Jak1DN cells, and wild type 293 cells with SeV and analyzed IRF-7 mRNA by RT-PCR. SeV infection of 293-Jak1DN cells induced substantial amounts of IRF7 mRNA as expected (Fig. 2*A*, compare *lanes 1* and *3*). However, SeV infection did not induce significant IRF7 expression in the cells expressing the IRF3DN, IRF7DN, and Jak1DN mutants (Fig. 2*A*, compare *lanes 3* and *4*). These results indicate that the induction of IRF7 by SeV requires IRF3 and IRF7 but does not require Jak1. In contrast, in the absence of Jak1-DN, IFN treatment induced IRF7 expression (Fig. 2*A*, compare *lanes 5* and *7*), and IRF3-DN and IRF7-DN did not affect IRF7 levels (Fig. 2*A*, compare *lanes 7* and *8*), implying that IRF3 and IRF7 are not required for this induction. IRF7 was not induced by IFN in the presence of Jak1-DN (Fig. 2*A*, compare *lanes 5* and *6*), indicating that Jak1 is required for induction of IRF7 by IFN.

For the promoter reporter assays, the IRF7 promoter construct p(−1123/+575)-Luc was transfected in these stably transfected cells, and the cells were infected with SeV or treated with IFNα. Fig. 2*B* shows that SeV infection resulted in a significant increase of IRF7 promoter reporter activity in cells with or without Jak1 kinase activity. However, no significant increase was detected in the presence of IRF3-DN and IRF7-DN mutants. In contrast, IFN treatment resulted in greater IRF7 promoter activity in the absence of Jak1-DN regardless of the presence of IRF7-DN and IRF3-DN, but could not activate the IRF7 promoter construct in cells transfected with Jak1-DN. Thus, both the RT-PCR and promoter reporter results show that IRF3 and IRF7 are required for the direct induction of IRF7 by SeV infection.

Both IRFE and ISRE Contribute to IRF7 Promoter Activation—The ISRE but not the IRFE is responsive to IFN in transient transfection assays (20). To check if both the elements are responsive to IRF7 and IRF3, we compared the responses of a panel of mutants of p(−1123/+575)-Luc to transiently expressed IRF7 and IRF3. The mutants are depicted in Fig. 3*A* (20). Mutation in the ISRE core sequence (GGGAAAGCGAAACC to GGGAAAGCatAACC) or deletion of the ISRE dramatically reduced the basal activity to ~20% in empty vector-transfected cells, but mutation in the IRFE core sequence (CAAAAGCGAAACT to CAAAAGCttAACT) or deletion of the IRFE did not clearly affect the basal activity (Fig. 3*B*), implying that the ISRE but not the IRFE may account for the high constitutive activity. Similar to the wild type construct p(−1123/+575)-Luc, activation was still detected with mutation or deletion of either the ISRE or IRFE motif. However, mutation in both the ISRE and IRFE led to complete loss of the





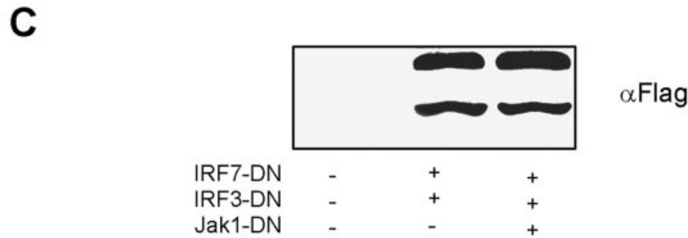
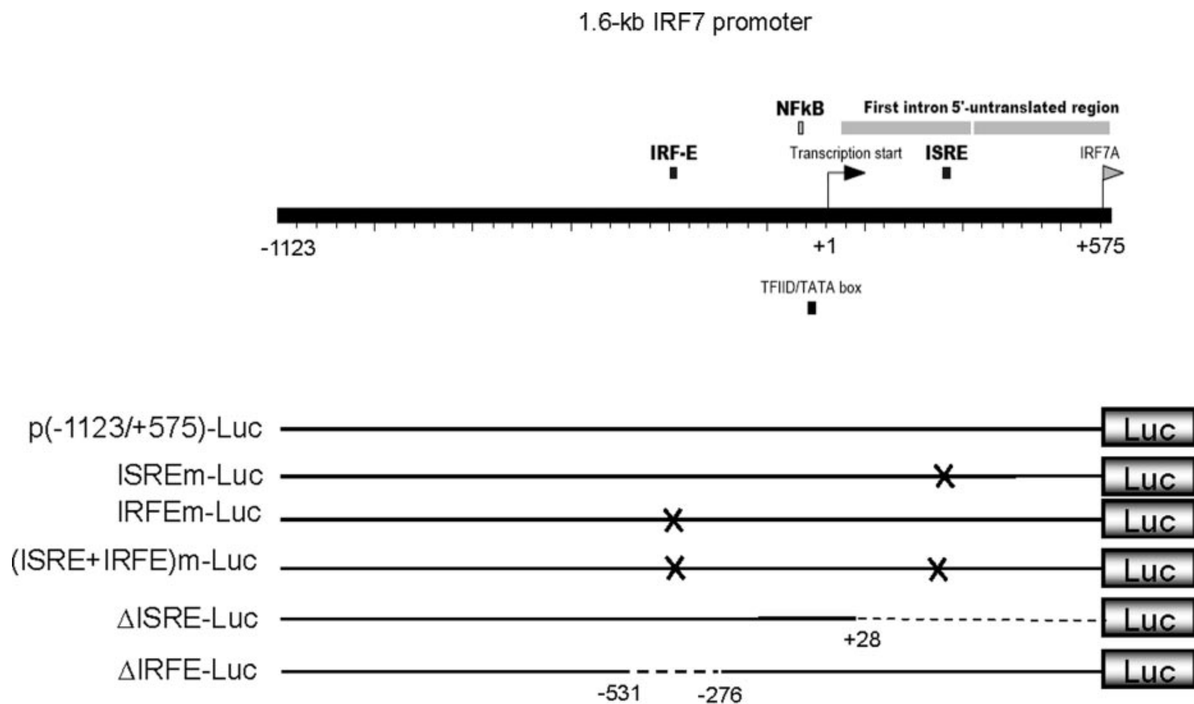


FIG. 2. **IRF3 and IRF7 but not Jak1 are required for induction of IRF7 by SeV infection.** *A*, 293 cells or Jak1 kinase inactive 293 cells were infected with 200 HAU/ml SeV for 48 h or treated with 500 units/ml IFNα2 (Sigma) for 12 h. Total RNA was used for RT-PCR. Primers for IRF7 are 5′-ATGGCCTTGGCTCCTG-AGAGGGCAGCCCC-3′ and 5′-GGGGG-CCTGGGGCTTGGAGTCC-3′. The IRF7 PCR product is located in the sequence corresponding to the DNA-binding domain, which is absent in FLAG-IRF7DN. *B*, 293 cells or 293 Jak1-DN stable cells were transfected with 0.2 μg of p(−1123/+575)-Luc and 0.1 μg of β-galactosidase construct. After 12 h, cells were infected with 200 HAU/ml SeV for 24 h or treated with IFNα2 for 12 h. Relative light units were measured and normalized by β-galactosidase activities. Reporter assay results are representative of two independent experiments, for each of which two duplicates for each sample were tested. *Error bars* represent the mean ± S.E. of the two duplicates. *C*, expression of FLAG-IRF3DN and FLAG-IRF7DN in stable cell lines expressing 1) FLAG-IRF7DN, FLAG-IRF3DN, and EGFP-C1 and 2) FLAG-IRF7DN, FLAG-IRF3DN, and Jak1-DN.

response to IRF3 and IRF7 (Fig. 3*B*). These data indicate that both the ISRE and IRFE contribute to activation of IRF7p by IRF3 and IRF7 but that the ISRE is chiefly responsible.

IRF3 and IRF7 Bind to the IRF7 Promoter as Constituents of the Complex VAF—To show whether IRF7 and IRF3 bind directly to the ISRE and IRFE of IRF7p, EMSA was performed with the use of synthesized IRF7p ISRE and IRFE as probes. As shown in Fig. 4*A*, in vector-transfected cells no specific band was detected (Fig. 4*A*, *lane 1*), but after virus infection a very weak specific band appeared (Fig. 4*A*, *lane 2*). The specificity of the band was demonstrated by competition assays with AP1 oligomers (data not shown). In all other cell lysates transfected with IRF7 or IRF3 or both, more intense bands of the same size were detected (Fig. 4*A*, *lanes 3–10*). The bands became much stronger after SeV infection (Fig. 4*A*, *lanes 4*, *6*, *8*, and *10*, compared with *lanes 3*, *5*, *7*, and *9*, respectively). An interesting feature of the specific band detected by EMSA is that regardless of the plasmid(s) transfected and whether the cells were infected with SeV, all of the bands are of the same size. Thus, it is most likely that IRF7 and IRF3 bind to the ISRE as part of the VAF complex in both uninfected and SeV-infected cells because VAF forms upon virus infection and includes both IRF3 and IRF7 (3).

To be sure that the bands represent VAF·DNA complex, the sample transfected with FLAG-IRF7 and IRF3 was subjected to supershift analyses with antibodies against FLAG, IRF3, p300, and CBP. The results show that besides containing just IRF7 and IRF3 these bands also contain p300 and CBP (Fig. 4*A*, *lanes 11*, *12*, *15*, and *16*). We also compared binding and supershift of the VAF complex with the PRD31 probe from



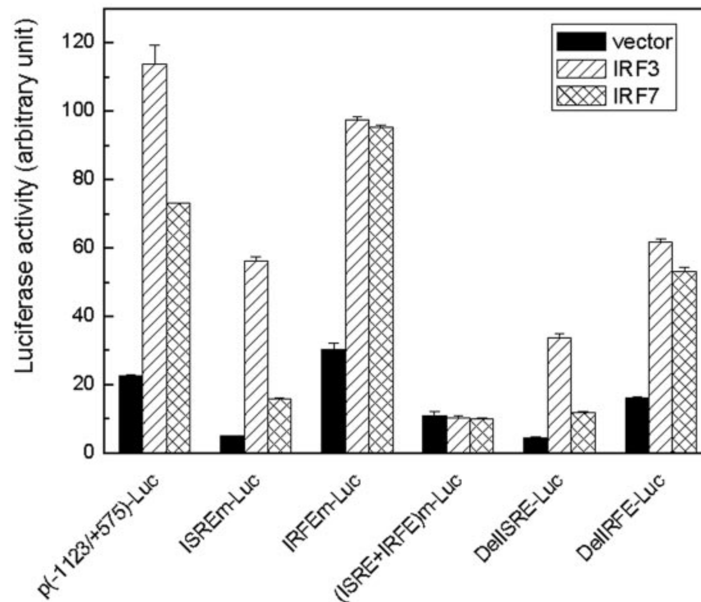


FIG. 3. **Both the IRFE and ISRE contribute to IRF7 promoter activation by IRF7 and IRF3.** *A*, reporter constructs of the 1.6-Kb IRF7 promoter and its mutants. *B*, HeLa cells in 12-well plates were transfected with expression plasmids IRF3 or IRF7 and the IRF7 promoter construct p(−1123/+575)-Luc or a panel of its mutants, ISREm-Luc, IRFEm-Luc, (ISRE+IRFE)m-Luc, ΔISRE-Luc, and ΔIRFE-Luc. Cells were harvested after 24 h for luciferase and β-galactosidase assays; β-galactosidase activity was used as internal control. Experiments were repeated three times; a typical result is shown. *Error bars* represent the mean ± S.E. of the two duplicates in one experiment.

IFNβp. As expected, for both the ISRE of IRF7p and PRD31 of IFNβp, the bands detected were the same size, were completely shifted by FLAG antibody, and completely blocked by the IRF3 antibody. The CBP and p300 antibodies are not as robust but still shifted most of the complex (Fig. 4*B*, *lanes 3–10*). The polyclonal IRF7 antibody H-246 (Santa Cruz Biotechnology) does not have access to IRF7 in this complex because it cannot produce a shift. The increase of VAF binding may be caused by virus-induced phosphorylation of IRF3 and IRF7, which results in formation of more VAF complexes (3). Also, we found that

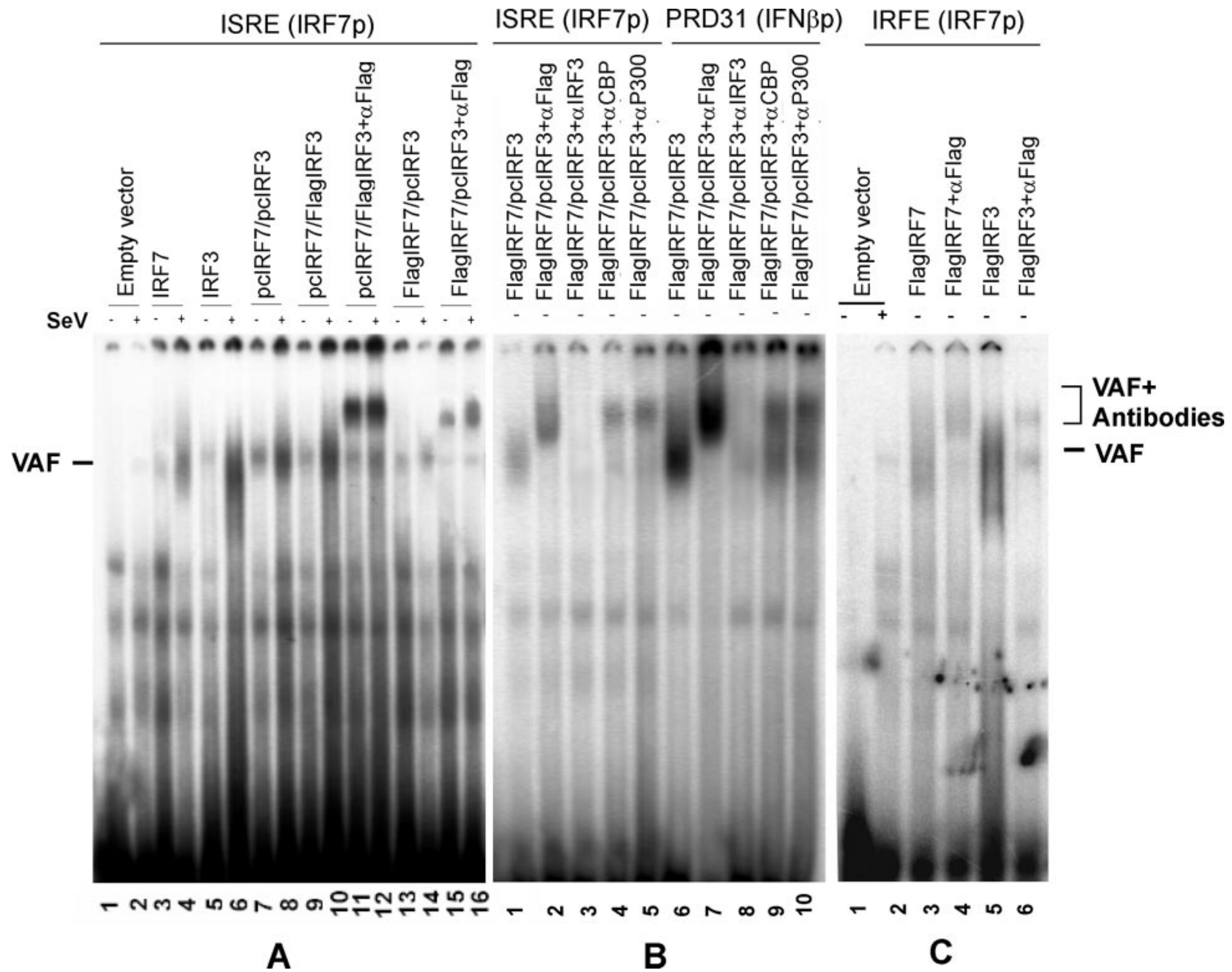


FIG. 4. **VAF binds to the ISRE and IRFE of the IRF7 promoter.** 293 cells in 60-mm dishes were transfected with 2 μg of expression plasmids as indicated. Cells were infected as indicated with 200 HAU/ml Cantell Sendai virus for 6 h before harvest. Cells were lysed in 60 μl of lysis buffer. 10 μl of whole cell lysate were used for each binding reaction. The synthesized double-stranded sequences containing IRF7 ISRE are 5′-GATCCTCCCGGGAAAGCGAAACCTAAACA-3′ and 5′-GATCTGTTTAGGTTTCGCTTTCCCGGGAG-3′. The sequences containing IRF7 IRFE are 5′-GTACGGTAACAAAAGCGAAACTCCATC-3′ and 5′-GATCGATGGAGTTTCGCTTTTGTTACC-3′. The sequences containing IFNβ positive regulatory domain 31 (PRD31) are 5′-GTACGAAAACTGAAAGGGAGAAGTGAAAGTG-3′ and 5′-GTACCACTTTCACTTCTCCCTTTCAGTTTTC-3′. The synthesized probes of IRF7p ISRE and IFNβp PRD31 were radioactively labeled; 40,000 cpm were used for each reaction. For supershift studies, 0.2 μg of FLAG antibody M2 or 2 μg of IRF3 antibody SL-12, CBP antibody A-22, or p300 antibody N-15 were incubated with the cell lysates for 20 min before the probes were added. Protein·DNA complexes were separated on 5% 60:1 Acr/bis-Acr gels.

both IRF7 and IRF3 can bind to the IRF7p IRFE (Fig. 4*C*). Thus, these results indicate that IRF3 and IRF7 bind directly to the IRF7p ISRE and IRFE as part of the complex VAF.

IRF7 and IRF3 Bind to the Endogenous IRF7 Promoter—The promoter reporter, RT-PCR, and EMSA results suggested that induction of IRF7 by SeV infection results from direct binding of VAF to the endogenous IRF7 promoter, which is embedded in chromatin. To confirm these observations, chromatin immunoprecipitation assays were performed. HeLa cells were transfected with the empty vector pCMV2-FLAG, FLAG-IRF7, or FLAG-IRF3, separately, and the cells were infected with SeV. Cell lysates were immunoprecipitated with monoclonal FLAG antibody M2, and the precipitates were extensively washed. PCR was performed with IRF7 ISRE- and IRFE-specific primers. As a positive control, PCR was also performed with IFNβ virus responsive element (VRE)-specific primers. As shown in Fig. 5, we detected a band using each of the paired primers in FLAG-IRF7 or FLAG-IRF3 transfected cells after immunoprecipitation with FLAG antibody. This band is very weak or not present in vector-transfected cells and in FLAG-IRF7 or FLAG-IRF3 transfected cells, extracts of which were immunoprecipitated with normal mouse IgG, indicating that it is specific and that both IRF7 and IRF3 bind to either or both ISRE and IRFE of the endogenous IRF7 promoter in addition to VRE of IFNβp. Because the promoter reporter assays (Fig. 3) show that IRF3 and IRF7 can activate IRF7 ISRE and IRFE reporter constructs separately and the EMSA results (Fig. 4) show that IRF3 and IRF7 can bind to these two elements *in vitro* separately, and we sheared the DNA to ~300 bp, whereas these two elements are ~600 bp apart, it is most likely that IRF3 and IRF7 bind to both the endogenous ISRE and IRFE elements.

DISCUSSION

Our results clearly show that IRF7 is a gene that can be induced directly by binding of VAF to its ISRE and IRFE upon SeV infection, and this induction is independent of the IFN-triggered Jak-STAT pathway (Fig. 6*A*). Our previous reports showed that EBV LMP1 can induce IRF7 expression through an unknown mechanism in addition to promoting phosphorylation of IRF7 protein (52, 53). Also, tumor necrosis factor α has been reported to induce IRF7 expression through activation of NFκB, which can bind to the NFκB-binding site identified in IRF7p (41). Besides these factors, IRF7 transcription is also thought to be regulated by promoter methylation and chroma-

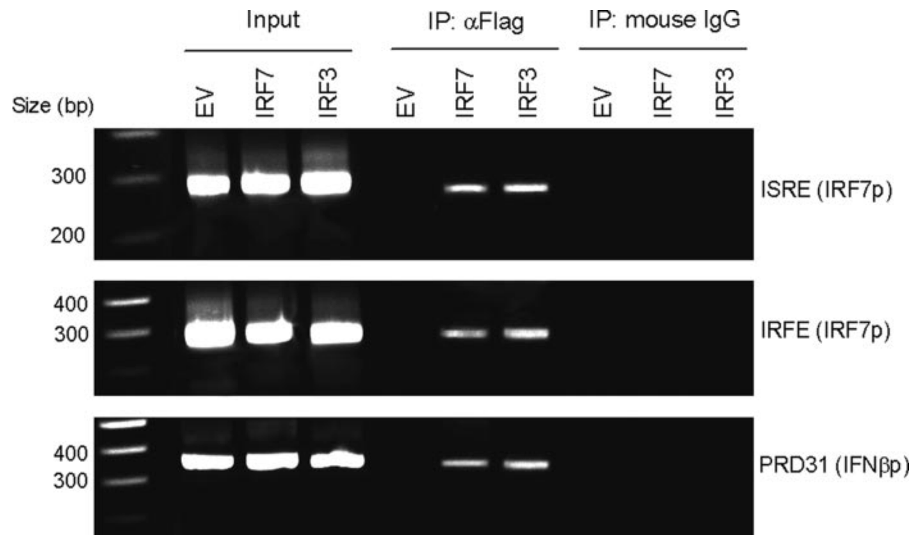


FIG. 5. **IRF7 and IRF3 bind to the endogenous IRF7 promoter.** HeLa cells in 100-mm dishes were transfected with 5 μg of FLAG-IRF7, FLAG-IRF3, or empty vector (*EV*). Cells were infected with SeV for 6 h before harvest. Cross-linking and chromatin immunoprecipitation assays were performed as described under "Experimental Procedures." Inputs were 5% of lysates of each sample. The primers for amplifying IRF7p IRFE (−414/-128) were 5′-CTCGGGAGGCTTAGGTAGGAG-3′ and 5′-CGGAGTAGGGAGGAGTGGAGG-3′, for IRF7p ISRE (+28/+292): 5′-GTACTGGGGACCCCAGACCCAC-3′ and 5′-CTGCGGAGACGGGAAAGGCGAC-3′, and for the VRE of the IFNβ promoter (−273/+56) 5′-GGTCGTTTGCTTTCCTTTGCTTTCTCCC-3′ and 5′-GTCGCCTACTACCTGTTGTGCCAGAGC-3′. *IP*, immunoprecipitation.

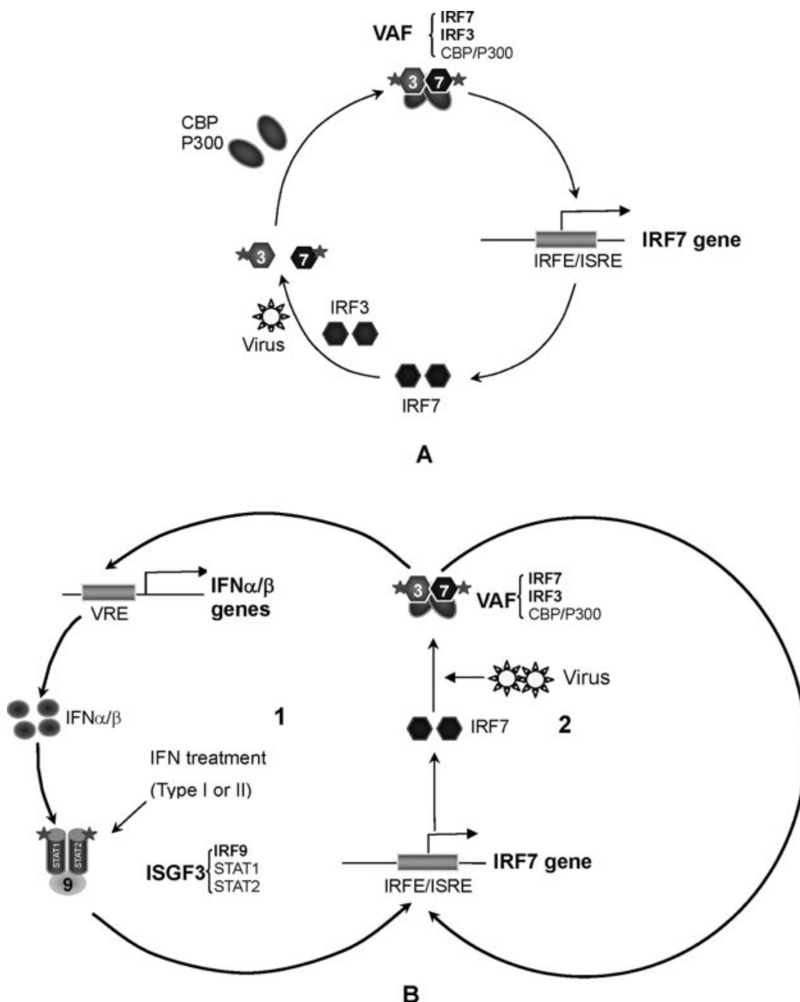


FIG. 6. **A proposed regulatory network between IFNα/β and IRF7.** *A*, virus infection activates IRF7 transcription independently of IFN signaling. Virus-induced formation of VAF binds to the IRF7 ISRE and IRFE and directly induces IRF7 transcription independently of IFN-triggered signaling. *B*, two regulatory circuits between IFNα/β and IRF7. **(1)** Virus infection results in phosphorylated IRF3 and IRF7, which form the VAF complex by recruiting CBP and P300. VAF binds to the VRE in the IFNβ promoter and strongly induces the expression of IFNβ (3). IFN (type I or type II) induces the phosphorylation of STAT1 and STAT2 that results in the formation of ISGF3 (23), an efficient inducer of IRF7 expression, by binding directly to the IRF7 ISRE (20, 25, 43). **(2)** VAF upon virus infection also binds to IRF7 ISRE and IRFE and activates IRF7 transcription.

tin accessibility based on the observations that reagents that loosen chromatin structure are capable of enhancing IRF7 expression (41, 52). Thus, regulation of IRF7 transcription is complex, and in addition to the identified functional ISRE, IRFE, and NFκB sites, there are many other potential transcription factor-binding sites in the IRF7 promoter.

Although we show here that IRF7 and IRF3 bind to the ISRE in the VAF complex, we cannot exclude the possibility that monomers and homodimers of IRF7 or IRF3 are also involved in the activation of IRF7p. In cells transfected with IRF7 alone or IRF3 alone, we sometimes detected two specific bands in EMSA, one of which ran faster than VAF·DNA complex (data not shown), suggesting that IRF7 or IRF3 homodimers may be involved in IRF7 regulation. Also, previous reports have shown that IRF3 or IRF7 homodimers are involved in the activation of their target genes (36, 54). IRF7 homodimers form in the absence of phosphorylation (55). Like VAF, formation of IRF3 homodimers requires phosphorylation of IRF3 and association with CBP/P300. IRF3 homodimers that form upon virus infection are even more stable than IRF3/IRF7 heterodimers (54).

These data imply that VAF may not be the only form that transactivates the IRF7 promoter. The possible presence of IRF7 or IRF3 homodimers may depend on the balance of IRF3 and IRF7 protein molecules. In cells transfected with IRF7 alone, IRF7 protein is present in greater amount than IRF3 protein, and so IRF7 homodimers probably form in addition to formation of VAF with IRF3 in response to virus infection (3).

Building on previous data on type I IFN regulation and IRF7 regulation, we propose a regulatory network between type I IFNs and IRF7 (Fig. 6*B*). This network includes two positive regulatory circuits. 1) VAF upon virus infection binds directly to the VRE of the IFNβ promoter and strongly induces IFNβ production (3). This pathway plays a central role in host-cell immune responses to virus infection (3, 56). Later, it was reported that IFN-triggered ISGF3 is an efficient inducer for IRF7 expression by binding directly to the IRF7 promoter (20, 25, 43) as confirmed in RT-PCR results (data not shown). 2) In this study, we found that IRF7 transcription is activated independently of circuit **1** by binding of VAF to IRF7 ISRE and IRFE. Thus, virus infection results in two distinct pathways: one leads to the IFN signaling pathway, and the other leads to direct induction of IRF7. However, because virus infection only results in weak activation of the IRF7 promoter but very strong activation of the IFNα/β promoters, virus infection is primarily responsible for IFNα/β induction.

The present results show that IRF7, like other IFN inducible genes such as ISG15/54/56 (24, 26–29), is inducible by virus infection independent of IFN signaling. Induction of ISRE-containing genes by either IFNs or both IFNs and virus through different pathways requires different IRF family members: the IFN signaling pathway involves IRF9, and the IFN-independent pathway activated by virus infection requires IRF3 and IRF7. The biological significance of the distinct pathways is obscure, but they do not perform redundant functions, may complement each other for an efficient antiviral response (30, 57, 58), and may reflect distinct requirements at different stages during virus infection. In addition, in contrast to IRF3, which is constitutively expressed in all cell types and is inducible neither by IFNs nor upon virus infection (59), IRF7 is not constitutively expressed in most cell types. Thus, the autoregulation of IRF7 may function to ensure that there is sufficient IRF7 for an efficient antiviral response.

Finally, these findings may impinge on the role of IRF7 in latent EBV infection. We have reported that IRF7 can induce the endogenous LMP1 promoter via its ISRE and restore LMP1 protein levels in a deficient cell line P3HR1 (16). The circuit proposed here may also indirectly affect levels of this important viral oncoprotein.

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