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Viral Infections Activate Types I and III Interferon Genes through a Common Mechanism*

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Viral infections trigger innate immune responses, including the production of type I interferons (IFN-α and -β) and other proinflammatory cytokines. Novel antiviral cytokines IFN-λ1, IFN-λ2, and IFN-λ3 are classified as type III IFNs and have evolved independently of type I IFNs. Type III IFN genes are regulated at the level of transcription and induced by viral infection. Although the regulatory mechanism of type I IFNs is well elucidated, the expression mechanism of IFN-λs is not well understood. Here, we analyzed the mechanism by which IFN-λ gene expression is induced by viral infections. Loss- and gain-of-function experiments revealed the involvement of RIG-I (retinoic acid-inducible gene I), IPS-1, TBK1, and interferon regulatory factor-3, key regulators of the virus-induced activation of type I IFN genes. Consistent with this, a search for the *cis*-regulatory element of the human *ifnλ1* revealed a cluster of interferon regulatory factor-binding sites and a NF-κB-binding site. Functional analysis demonstrated that all of these sites are essential for gene activation by the virus. These results strongly suggest that types I and III IFN genes are regulated by a common mechanism.

Interferon (IFN)[2] plays a critical role in innate as well as adaptive immune responses against viral infections (1, 2). Viral infections trigger the activation of type I IFNs (IFN-α and IFN-β). The mechanism behind the virus-induced expression of type I IFNs is well documented (3, 4). Plasmacytoid-dendritic cells, which are responsible for a high level of IFN-α in serum, detect virus-associated molecular patterns via Toll-like receptor-7 or -9 receptors. The signal is transduced to IRAK1/IRAK4/IKK-α kinases in a MyD88-dependent manner resulting in the activation of interferon regulatory factor 7 (IRF-7) through its specific phosphorylation. In other cells, extracellular double-stranded RNA is detected by Toll-like receptor-3, transducing a signal through the TRIF/TICAM-1 adaptor in a MyD88-independent manner and then activating TANK-binding kinase 1 (TBK1) or IκB kinase I (IKK-i) kinase and subsequently IRF-3 through its phosphorylation. The third class of receptors for virus-associated molecular patterns resides in the cytoplasm and detects replicating viral RNA. The retinoic acid inducible-gene I (RIG-I) family of RNA helicases are shown to recognize viral double-stranded RNA by their helicase domain and transmit signals to downstream molecules via their caspase recruitment domain (CARD) (5, 6). Although the precise mechanism involved has not been elucidated, IFN-β promoter stimulator 1 (IPS-1), a mitochondrial protein containing CARD, is thought to further transmit the signal to IRF-3 kinases, TBK1, and IKK-i (7–10).

Recently, a study using mice with disrupted genes for key molecules of the three different pathways of type I IFN gene activation was reported (11, 12). Interestingly, RIG-I family helicases RIG-I and melanoma differentiation-associated gene 5 play key protective roles in infections of Japanese encephalitis virus and encephalomyelitis virus, respectively. It appeared that disruption of either of the Toll-like receptor pathways did not have a strong effect on protection against these viruses, suggesting a central role for the RIG-I family in host protection *in vivo*.

In recent years, several novel interleukin-10-related cytokines have been identified (13–15). Although IFN-λs, a family of interleukin-10-related cytokines, are evolutionarily distantly related to type I IFNs, they exhibit antiviral activity similar to type I IFNs. Thus, they are collectively known as type III IFNs. Type III IFNs interact with cell surface receptors composed of interleukin-10 receptor β and interleukin-28 receptor α, distinct from those for types I or II IFNs. The binding of a type III IFN to its receptor results in the intracellular activation of Janus kinase 1 and signal transducers and activators of transcription STAT1 and STAT2 and then the subsequent formation of the IFN-stimulated gene factor 3 complex. Similar to type I IFNs, type III IFNs are induced to express by viral infection or treatment with poly(I·C) or lipopolysaccharide (16). However, the regulatory mechanisms involved are not well understood.

Here we analyzed how type III IFN genes are regulated by

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[2] The abbreviations used are: IFN, interferon; IRF, interferon regulatory factor; IRAK, interleukin-1 receptor-associated kinase; MEF, mouse embryonic fibroblast; RIG-I, retinoic acid-inducible gene I; IPS-1, IFN-β promoter stimulator 1; CARD, caspase recruitment domain; RACE, rapid amplification of cDNA ends; EMSA, electrophoresis mobility shift assay; NDV, Newcastle disease virus; TRIF, Toll/IL-1 receptor domain-containing adapter inducing IFN-β; TICAM-1, Toll/IL-1 receptor-containing adapter molecule 1; TANK, Traf family member-associated NF-κB activator.

viral infections. We took advantage of a comparative approach using the well established molecular mechanism regulating type I IFN genes. We looked at the effect of loss and gain of function of the signaling molecules critical for type I IFN genes. We mapped and analyzed *cis*-regulatory elements that function as virus-inducible enhancers. Our results demonstrate a central role for a pathway involving RIG-I, IRF-3, and NF-κB.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, Preparation of Cell Extracts, and Luciferase Assay—Mouse embryonic fibroblasts (MEFs) and 293T cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin-streptomycin. L929 cells were maintained in minimum essential medium with 5% fetal bovine serum and penicillin-streptomycin. 293T and L929 cells were transiently transfected with the calcium-phosphate method and FuGENE 6 (Roche Applied Science), respectively. For the preparation of cell extracts, cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) and centrifuged at 245,000 × *g* for 10 min. The supernatant was used for immunoblotting. The Dual-Luciferase reporter assay system (Promega) was used for luciferase assays. As an internal control for the Dual-Luciferase assay, the *Renilla* luciferase construct pRL-TK (Promega) was used.

Plasmid Constructs—We used pEF-FLAG-IRF-3 5D and pEF-FLAG-RIG-I CARD as described previously (6, 17). IPS-1 cDNA was purchased from the Biological Resource Center of the National Institute of Technology and Evaluation of Japan. We amplified this cDNA with an oligonucleotide for the N-terminal FLAG tag by PCR and inserted it into the XbaI-SpeI site of pEF-BOS(+) (pEF-FLAG-IPS-1). The promoter region of human *ifnλ1* was amplified by genomic PCR (upper primer, 5′-CTAGGTCGACGGGCAACAAGAGCAAAACTA-3′, lower primer, 5′-GTGGATCCGCTAAATCGCAACTGCTTCC-3′), and the PCR fragment was inserted into the SalI-BamHI site of luciferase vector (pλ1-(−554/+14)Luc). The nucleotide sequence was confirmed with the BigDye DNA sequencing kit (Applied Biosystems). We chemically synthesized fragments encompassing the promoter sequence of the *ifnλ1* between −225 and −36 and inserted them into the SalI-BamHI site of p-55Luc (p-55λ1-(−225/−36)Luc). Synthesized fragments of p-55λ1mut.IRF-Luc, p-55λ1mut.NF-κBLuc, and p-55λ1mut.IRF/mut.NF-κBLuc were designed as shown in Fig. 4*A* and inserted into the SalI-BamHI site of p-55Luc.

Quantitative Real Time-PCR—Total RNA was prepared with TRIzol reagent (Invitrogen), treated with DNase I (Roche Diagnostics), and amplified by real time-PCR with the ABI PRISM 7500 sequence detection system (Applied Biosystems). TaqMan reverse transcription reagents and the TaqMan Universal PCR mix (Applied Biosystems) were used for cDNA synthesis and PCR, respectively. We used commercial TaqMan primer-probe sets (Applied Biosystems) for mouse *ifnλ2*, human *ifnλ1*, and human *ifnλ2*. As an internal control for the comparative threshold cycle method, a primer-probe set for eukaryotic 18 S rRNA (Applied Biosystems) was used. The results were normalized to the abundance of internal 18 S rRNA and were reproducibly obtained in two independent transfection experiments.

Rapid Amplification of cDNA Ends (RACE)—RACE analysis was performed using the FirstChoice RNA ligase-mediated RACE (Ambion) according to the manufacturer's instructions. Total RNA was prepared as for quantitative real time-PCR. Nested PCR reaction was performed using recombinant TaqDNA polymerase (TaKaRa), *ifnλ1*-specific outer primer (5′-GGCCACATATTTGAGGTCTC-3′), and *ifnλ1*-specific inner primer (5′-CGCGGATCCAGAAGCCTCAGGTCCCAATT-3′) according to the manufacturer. The RACE product was cloned into pBluescript II vector, and 10 random clones were sequenced.

Electrophoresis Mobility Shift Assay (EMSA)—The oligonucleotides containing self-complementary sequences for NF-κB (5′-GGGAAATTCTCTTAGCTTGAGAATTTCC-3′) or mutated NF-κB (5′-GCCGAATTCTCTTAGCTTGAGAATTCGG-3′) of human *ifnλ1* were used as a probe. The method for EMSA was described previously (18). We used commercial anti-NF-κB antibodies for human p50, p65, and IRF-3 (IBL; catalog numbers 18661, 18667, and 18781, respectively).

RESULTS

The Effect of Loss of Function of IRF-3, TBK1, and RIG-I on Virus-induced Activation of ifnλ2—It has been reported that the expression of IFN-λ is induced by stimuli such as viral infections and treatment with poly(I·C) or lipopolysaccharide in a parallel fashion to that of type I IFN genes (16). We investigated the role of the transcription factor IRF-3 and its regulatory kinase, TBK1, using MEFs derived from respective knock-out mice (19, 20). Because mice have no ortholog gene of the human *ifnλ1*, we examined the expression level of the mouse *ifnλ2*.

MEFs were mock-treated or infected with Newcastle disease virus (NDV), and the endogenous mouse *ifnλ2* mRNA level was determined by quantitative real time-PCR. Although a significant level of expression of the mouse *ifnλ2* gene was observed in NDV-infected wild-type cells, IRF-$3^{-/-}$ cells were totally deficient in *ifnλ2* (Fig. 1*A*). TBK$1^{-/-}$ cells exhibited a significantly suppressed expression of the *ifnλ2* (Fig. 1*B*). The residual induction is likely due to IKK-i (IKK-ε). Viral infections are shown to activate type I IFN genes through the activation of the RNA helicase RIG-I, which acts as a sensor for replicating viral RNA in cells (6, 21). We investigated the involvement of RIG-I in the NDV-induced activation of the *ifnλ2* by using RIG-I knock-out MEFs. As shown in Fig. 1*C*, RIG-I-deficient cells failed to activate the *ifnλ2*, suggesting RIG-I is critical for this signaling. Essentially the same result was obtained in small interfering RNA-mediated knockdown experiments using transformed mouse L929 cells (data not shown). We do not know the reason why gene expression levels somehow vary between +/+ cells. Although the genetic background of the mice is the same, MEFs were prepared at different embryonic stages for IRF-3, TBK1, and RIG-I knockouts. To compare the effect of knock-out, each wild-type/knock-out pair was prepared at the same embryonic stage or from littermates. Also, cell culture histories are different between different gene knockouts but are identical between each wild-type/knockout

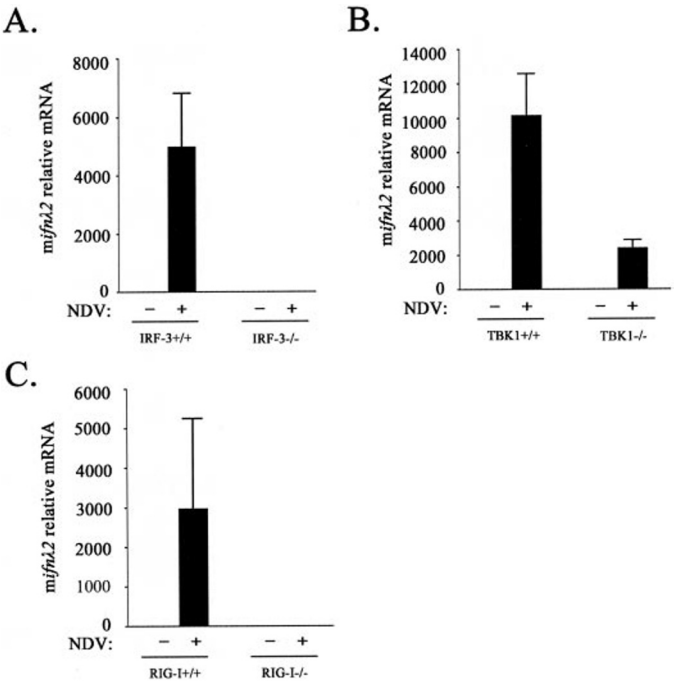

FIGURE 1. **Critical role of IRF-3, TBK1, and RIG-I in NDV-induced activation of the *ifnλ2*.** MEFs derived from mice with wild-type or knocked out IRF-3 (*A*), TBK1 (*B*), and RIG-I (*C*) genes were mock-treated (−) or infected (+) with NDV as indicated. Twelve hours after infection, cells were harvested and mouse *ifnλ2* mRNA levels were determined by quantitative real time-PCR. *Error bars* indicate the S.E. of triplicate measurements.

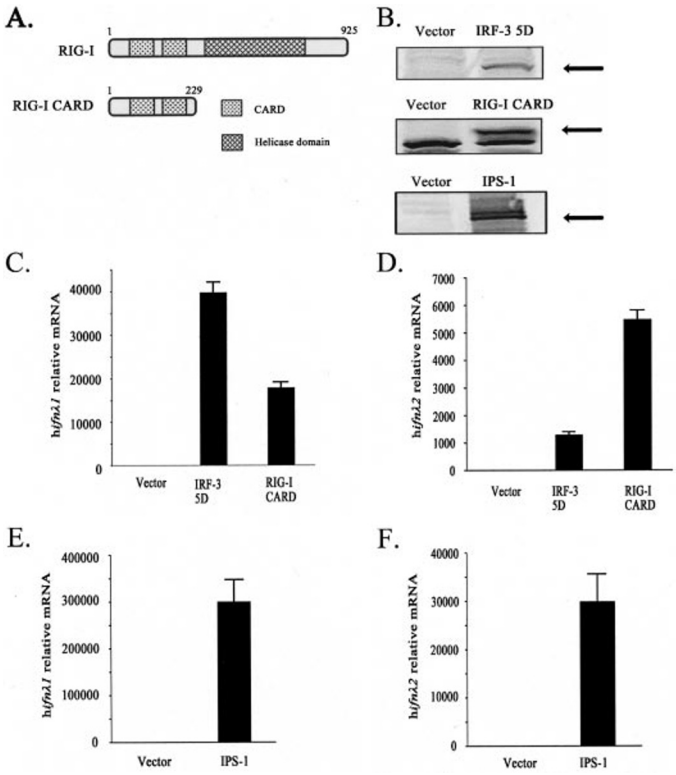

FIGURE 2. **Expression of a constitutively active IRF-3, RIG-I, and IPS-1 results in the activation of human *ifnλ1* and *ifnλ2* .** *A*, structure of full-length RIG-I and RIG-I CARD. *B*, transiently expressed IRF-3 5D, RIG-I CARD, and IPS-1 in 293T cells were monitored by SDS-PAGE followed by Western blotting. Shown is induction of endogenous human *ifnλ1* mRNA (*C* and *E*) and human *ifnλ2* mRNA (*D* and *F*) by ectopic expression of IRF-3 5D, RIG-I CARD, and IPS-1, respectively. The mRNA levels were determined as in Fig. 1.

pair. Thus, comparison is best made between each +/+ and −/−.

Gain of Function of IRF-3 and RIG-I Results in the Activation of Human IFN-λ Genes—As an alternative approach, we expressed dominant active mutants of IRF-3 and RIG-I in 293T cells and monitored endogenous human *ifnλ1* and *ifnλ2* mRNA by quantitative real time-PCR. IRF-3 5D is a mutant with five Ser/Thr residues replaced with Asp. IRF-3 5D is constitutively phosphorylated at Ser-386 by some unknown kinase(s) in human cells and is capable of activating targets including type I IFN genes (17). Overexpression of a deletion mutant of RIG-I, which contains CARD alone (RIG-I CARD, Fig. 2*A*), results in the constitutive activation of type I IFN genes (6). Ectopic expression of constitutively active IRF-3 and RIG-I in 293T cells (Fig. 2*B*) resulted in the constitutive activation of human *ifnλ1* and *ifnλ2* (Fig. 2, *C* and *D*). These results strongly suggest that IFN-λ genes are regulated by a mechanism common to type I IFN.

IFN-λ Genes are Regulated by IPS-1-mediated Signaling—Recently, IPS-1 (also known as MAVS, VISA, or Cardif) was identified as an adaptor molecule of RIG-I signaling (7–10). It has been hypothesized that activated RIG-I interacts with IPS-1 between respective CARD domains, resulting in the activation of IRF-3, NF-κB, and type I IFN genes. Interestingly, overexpression of full-length IPS-1 can induce the expression of type I IFN genes without a viral infection. We therefore expressed full-length IPS-1 in 293T cells (Fig. 2*B*) and monitored human *ifnλ1* and *ifnλ2* mRNA. Transfection of the IPS-1-encoding vector but not empty vector induced the expression of endogenous human *ifnλ1* and *ifnλ2* (Fig. 2, *E* and *F*), suggesting that

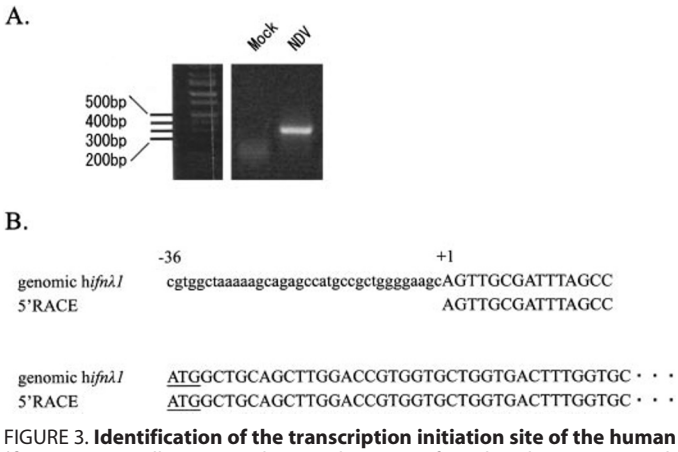

FIGURE 3. **Identification of the transcription initiation site of the human *ifnλ1*.** *A*, HeLa cells were mock-treated or NDV-infected, and RACE PCR product was visualized by agarose electrophoresis. *B*, the RACE PCR fragment from *A* was cloned and sequenced to determine the transcription initiation site. The sequence of the RACE PCR product is aligned with the genomic sequence of the human *ifnλ1*. *Underbars* indicate the translation initiator ATG.

IFN-λ genes are regulated by IPS-1-mediated signaling, the major pathway triggered by viral replication.

Identification of the Transcription Initiation Site of the Human ifnλ1—The above results suggest the presence of one or more virus-inducible enhancer elements within the IFN-λ genes. To identify these elements, the transcription initiation site(s) of human *ifnλ1* was investigated by RACE. The RACE product was

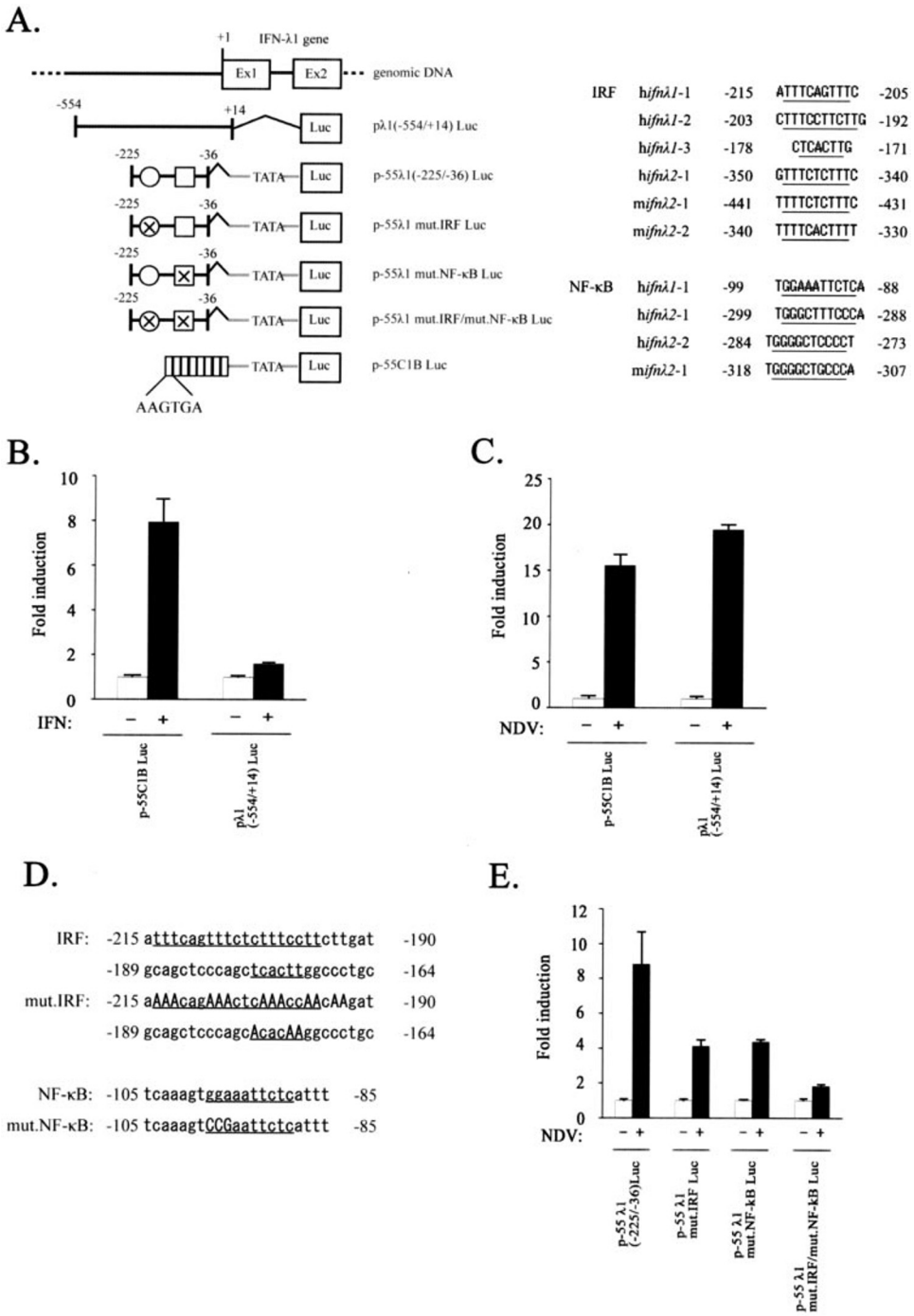

FIGURE 4. **Determination of *cis*-acting elements within the promoter of the human *ifnλ1*.** *A*, *left*, schematic representation of reporter constructs. This figure does not indicate the exact position of the elements. *Right*, binding sites for IRF and NF-κB (*underline*) in human *ifnλ1*, human *ifnλ2*, and mouse *ifnλ2* genes. The positions for the human *ifnλ1* are indicated as relative to the transcription initiation site (Fig. 3), whereas the positions of human *ifnλ2* and mouse *ifnλ2* are related to the translation initiation ATG. The reporters were transfected into L929 cells with pRL-TK as an internal control. The cells were mock-treated (−) or treated with 1000 units/ml of IFN-β for 12 h (+) (*B*) or infected with NDV for 12 h (+) (*C* and *E*) and were subjected to the Dual-Luciferase assay. *D*, nucleotide sequences of IRF and NF-κB binding sites present between −225 and −36 (*underline*); mutated nucleotides are indicated as *capital letters*. *Error bars* indicate the S.E. of triplicate transfections.

exclusively detected with NDV-infected RNA (Fig. 3*A*). The product was subcloned and sequenced. A random 10 clones had identical 5′ ends as shown in Fig. 3*B*. There was no TATA-like sequence at the expected position, thus we conclude that *ifnλ1* is a TATA-less gene.

Identification of the cis-Acting Regulatory Element of the Human ifnλ1—Next, we analyzed virus-inducible enhancer elements within the *ifnλ1*. We isolated the HeLa cell genomic fragment encompassing the human *ifnλ1* −554 to +14 relative to the transcription initiation site (see "Experimental Procedures") (Fig. 4*A*). The fragment was cloned into a firefly luciferase reporter gene (pλ1-(−554/+14)Luc) (Fig. 4*A*). For comparison, a reporter containing eight tandem repeats of the IRF-binding motif (p-55C1BLuc) (Fig. 4*A*, *bottom*) was used. Although p-55C1BLuc was significantly activated by IFN-β treatment, pλ1-(−554/+14)Luc was not significantly affected by IFN-β treatment, consistent with the observation that IFN-λ is barely inducible (1/1000 of viral induction) by type I IFN *per se* (Fig. 4*B*) (22). In contrast, NDV infection strongly induced the activation of both p-55C1BLuc and pλ1-(−554/+14)Luc, showing that the isolated genomic fragment contains a virus-inducible enhancer (Fig. 4*C*).

Critical Function of IRF and NF-κB-binding Sites within the ifnλ1 Enhancer—Within the above fragment, we noted putative binding sites for IRF (−214 to −172) and NF-κB (−98 to −89) (Fig. 4*A*). It is worth noting that multiple IRF-binding sites and a NF-κB motif exist similar to the *ifnβ* enhancer. Also, corresponding motifs were found in the human *ifnλ2* (Fig. 4*A*, *right*). To examine the function of these motifs, we chemically synthesized the region (−225 to −36) of human *ifnλ1* and cloned it into the upstream of the basal promoter of human *ifnβ* fused to a firefly luciferase reporter gene (p-55Luc (18)). Fig. 4*E* shows that the synthetic fragment functions as a virus-inducible *cis*-element, suggesting that the synthetic fragment encompasses the regulatory element. Disruption of the binding sites for either IRF or NF-κB reduced the level of virus-induced gene expression, and simultaneous disruption of these binding sites resulted in further impaired viral induction (Fig. 4, *D* and *E*). These results indicate that these binding sites function as components of a virus-inducible enhancer.

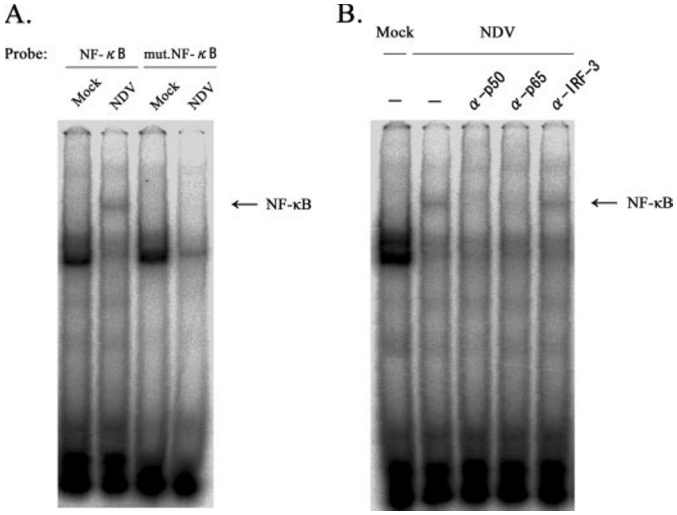

FIGURE 5. **p50/p65 NF-κB binds to the *ifnλ1* NF-κB motif.** *A*, HeLa cell extracts prepared from mock-treated or NDV-infected cells were subjected to EMSA using labeled *ifnλ1* NF-κB or mutated NF-κB oligonucleotide probes (see "Experimental Procedures"). *B*, NDV-infected extracts were each treated with the indicated antibody and were subjected to EMSA, as in *A*.

NF-κB Binds to the NF-κB Motif of ifnλ1—The above reporter assay suggested that the NF-κB motif is critical for IFN-λ gene induction. We tested NF-κB binding to the motif by EMSA (Fig. 5). NDV infection specifically induced binding activity to the wild-type but not to the mutated probe, suggesting that the motif is a functional NF-κB site (Fig. 5*A*). In addition, anti-p50 or -p65 antibody blocked the specific complex (Fig. 5*B*), suggesting that p50/p65 heterodimer binds to the *ifnλ1* NF-κB site.

DISCUSSION

The type I and III IFNs exhibit little structural conservation and are thought to have different origins. Indeed, types I and III IFNs elicit biological effects through interaction with distinct cell surface receptors. We found that, irrespective of their evolutional origin, IFN-λ genes are regulated by a mechanism common to type I IFNs. Our loss-of-function analyses revealed the involvement of IRF-3, TBK1, and RIG-I. Ectopic activation of IRF-3, RIG-I, and IPS-1 all activated type III IFN genes. These signaling molecules play a critical role in the virus-induced activation of type I IFN genes. Our search for a *cis*-element in the promoter of the human *ifnλ1* revealed a cluster of IRF-binding sites and a NF-κB-binding site as components of the virus-inducible enhancer. This is reminiscent of type I IFN enhancers, because multiple IRF sites critically regulate virus-inducible enhancers of IFN-α and -β, and a single κB site plays a critical role in the activation of IFN-β. Although IRF sites are potentially subject to regulation by IFN through IFN-stimulated gene factor 3, the expression of types I or III genes is little induced by IFN treatment *per se*. Thus, the common expression patterns of types I and III IFN genes are determined by the arrangements of these transcription factor-binding sites, which may be independently acquired through the evolution of the respective genes. Consistent with our finding, Osterlund *et al.* (23) report that IRF-3 binds to the IRF site predicted by a DNA affinity-binding assay. They also report the binding of NF-κB to the promoter region (at nucleotide position −264 to −256 from the transcription initiator site); however, removal of the predicted binding site did not affect the enhancer activity.[3] Instead, disruption of the other proximal NF-κB site (−98 to −89) significantly reduced the enhancer activity. Disruption of both IRF and NF-κB sites significantly reduced the transcriptional activity, but the mutant promoter exhibited weak activation, suggesting that unidentified *cis*-elements exist within the DNA fragment. In addition, they reported differential activation of *ifnλ1* and *ifnλ2*. Influenza A virus induces the expression of *ifnλ1* (but not *ifnλ2*), although Sendai virus induced production of high levels of both *ifnλ1* and *ifnλ2*. It is worth noting that, although putative binding sites for IRF and NF-κB are found in the promoter region of the *ifnλ2* (Fig. 4*A*, *right*), DNA sequence conservation to that of *ifnλ1* is low. Detailed analyses are necessary to elucidate the mechanism behind the virus-specific activation of type III IFN genes.

Because of the use of distinct receptors, types I and III IFNs likely do not signal identical biological outcomes in anti-viral and anti-cancer activities. Type I IFNs have been used to treat viral infections and certain cancers. The present study will help to establish a new strategy using IFN inducers. Developing inducers that commonly or differentially regulate types I and III IFN genes may enable us to manipulate the IFN system for therapeutic purposes.

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