Promoter Organization of the Interferon-A Genes Differentially Affects Virus-induced Expression and Responsiveness to TBK1 and IKKe*

Received for publication, June 22, 2005, and in revised form, November 28, 2005 Published, JBC Papers in Press, December 27, 2005, DOI 10.1074/jbc.M506812200

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Virus-induced expression of interferon (IFN)-A genes is regulated by two members of the IFN regulatory factor (IRF) family, IRF-3 and IRF-7, which are activated by phosphorylation during viral infection by the IKK-related serine/threonine kinases TBK1 and IκB kinase ε (IKKe). In this study, we demonstrate that three IRF-binding sites located in the virus-responsive element mediate the transcriptional activation of the IFN-A4 promoter by IRF-3. The precise arrangement of these IRF elements is required for synergistic activation of the IFN-A4 promoter following Newcastle disease virus infection or activation by TBK1 or IKKe. The ordered assembly of IRF-3 multimers on the promoter also determines cooperative recruitment of IRF-3 and CREB-binding protein and differential virus-induced expression of IFN-A4 gene promoter compared with IFN-A11. Naturally occurring nucleotide substitutions disrupt two of the IRF elements in the IFN-A11 gene promoter, leading to a dramatic decrease in IRF-3 and CREB-binding protein recruitment and in IRF-3-dependent transcription. Transcription of the IFN-A4 promoter by IRF-7 is mediated by two IRF elements; promoter mutants that carry a reversed IRF element retain the ability to respond to IKKe or TBK1 expression in the presence of IRF-7 but lose the capacity to respond to virus or kinase-induced IRF-3. Interestingly, IKKe or TBK1 stimulates the IRF-7-mediated transcription of IFN-A11, although at a lesser extent compared with IFN-A4. Our data indicate that virus-induced expression of IFN-A genes is dictated by the organization of IRF elements within the IFN-A promoters and that the differential IFN-A gene expression, based on the IRF-3 responsiveness, is partially compensated in the presence of IRF-7 when both factors are activated by IKKe or TBK1.

The immediate cellular response to virus infection is characterized by the transcriptional activation of type I IFN4 genes involved in the host antiviral defense program (1). Multiple IFN-A gene family members are differentially expressed in cultured cells or embryonic fibroblasts infected by virus (2–4), and in vivo studies indicate that the virus species as well as the natural route of infection determines the magnitude of type I IFN production and the nature of the producer cell. For example, plasmacytoid dendritic cells are identified as the major IFNα/β-producing cells that rapidly express high levels of IFN-A during systemic infection and acute viremia, whereas local viral infections stimulate delayed and lower IFN-A expression, requiring a positive feedback amplification mechanism dependent on IFN-β production in cell types other than IFNα/β-producing cells (5–8). These studies also indicated that differential IFN-A gene regulation is critical for the development, magnitude, and duration of innate and adaptive antiviral responses in the course of viral infection.

Different members of the interferon regulatory factor (IRF) family participate in the IFN-A gene regulation, either positively or negatively, although virus-induced differential IFN-A gene expression is essentially mediated by IRF-3 and IRF-7 (9–12). These factors possess overall structural and biochemical similarities, including their virus-induced activation by C-terminal serine phosphorylation, dimerization, nuclear transport, and DNA binding to different IRF elements in the promoter of target genes (13–18). IRF-3 is constitutively expressed in all cell types, whereas the expression of IRF-7, constitutive in lymphoid cells and certain subsets of dendritic cells, is induced by IFNα/β during viral infection in other cell types (19, 20). The ubiquitous constitutive expression of IRF-3 implies that this factor is required for the primary activation of IFN-A, IFN-B, and a subset of IFN-stimulated genes, yet generation of mice deficient for IRF-3 or IRF-7 indicated that IRF-7 functions even in the absence of IRF-3 and attributed a critical role for both factors in the activation of the initial phase of IFN-A/B gene induction (21). Once the initial activation of genes is achieved, according to a multistep IFN-A/B gene expression model established for mouse embryonic fibroblasts, IRF-7 cooperates with IRF-3 to amplify IFN-A4 and IFN-B gene transcription and induce a set of delayed mouse IFN-A genes, including IFN-A2, -A5, -A6, and -A8 (18, 22, 23). Among the different members of mouse IFN-A multigenic family, the IFN-A4 gene is the only immediate early gene expressed following virus infection in different cell lines and exhibits higher expression levels compared with other IFN-A genes (2, 3, 22, 24, 25). In this regulatory network, IRF-3 and IRF-7 are potent activators of virus-induced IFN-A gene transcription, although the mechanisms of their cooperative interaction with target sequences remain to be elucidated.

IRF-3 phosphorylation is initiated by binding of double-stranded RNA, a product of viral replication considered as an early inducer of type I IFN synthesis, on membrane-associated Toll-like receptor-3 that leads to the recruitment of the specific TIR domain-containing adaptor
TRIF and the activation of two serine/threonine kinases, TBK1 (TANK-binding kinase) and IKKe, that phosphorylate IRF-3 [26, 27]. IRF-3 activation also occurs upon recognition of double-stranded RNA by RIG-1, a retinoic acid-inducible RNA helicase involved in cytoplasmic immune surveillance [28]. TBK1 or IKKe is required for IRF-3 activation by RIG-1, the mitochondrial antiviral signaling protein MAVS functioning as an adaptor between RIG-1 and both kinases [29–33]. IRF-3 phosphorylation by TLR4, a receptor for LPS from Gram-negative bacteria, also requires the recruitment of TBK1 or IKKe via the adaptor proteins TRAF6 and TRAM [34]. Ligand binding on TLR-7 and -8 that deliver signals following their interaction with viral single-stranded RNA or TLR-9 that recognizes unmethylated CpG-containing DNA and senses infection by cytomegalovirus or herpes simplex virus type-1 leads directly to IRF-7 activation [35–37]. TLR7- and TLR9-dependent pathways require the general TLR adaptor, MyD88 (myeloid differentiation factor) associating with TRAF6 and the serine-threonine kinase IRAK1 that induces IRF-7 phosphorylation [38–40]. Whether IRF-7 mediates differential activation of IFN-A genes when TLR7 or TLR9-dependent signaling is triggered in plasmacytoid dendritic cells infected by virus is unknown. TBK1 and IKKe are not involved in these signalings, and the relative transcriptional capacity of IRF-7 activated by IRAK1 or these kinases has to be determined. Studies with TBK1- and IKKe-deficient mice have confirmed the critical role of TBK1 for IFN-A/B or IFN-stimulated gene expression in mouse embryonic fibroblasts following virus infection [41–44], although TBK1 is not pivotal for IRF-3 or IRF-7-mediated gene transcription in bone marrow-derived macrophages or thymocytes where high levels of constitutive IKKe are detected. These studies have clearly demonstrated the involvement of TBK1 and IKKe in virus-induced type I IFN expression but did not determine the individual role of these kinases in IFN-A gene regulation.

To determine the individual effects of IKKe and TBK1 on the differential expression of IFN-A genes and elucidate the mechanism of the cooperative transcription of IFN-A gene promoters by IRF-3 or IRF-7, the effect of each kinase on IRF3- or IRF7-mediated transcription of mouse IFN-A4 and IFN-A11 gene promoters was evaluated. We demonstrate that the precise arrangement of multiple IRF elements within the IFN-A4 gene promoter determines in vivo recruitment of IRF-3 and CBP in correlation with virus inducibility. This organization is also critical for the differential stimulation of IFN-A4 gene promoter activity by IKKe and TBK1. Our data indicate that naturally occurring nucleotide substitutions that alter the IRF-3 response of IRF elements on different promoters, upstream of the chloramphenicol acetyltransferase (CAT) or luciferase reporter gene have been previously described [45].

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Virus Infection**—Human embryonic kidney 293 (ATCC CRL 1573), mouse L929 (ATCC CCL 1), and human HeLa S3 cells (ATCC CCL 2.2) were grown in α-minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, glucose, and antibiotics. Infection of L929 and HeLa cells with Newcastle disease virus (NDV; La Jolla strain) and HEK293T cells with Sendai virus were performed at a multiplicity of infection of 1–5. After a 1-h incubation in serum-free medium, cells were washed and placed in growth medium containing 5% fetal bovine serum for 7 h.

**Plasmids**—Reporter plasmids pIF4T and pIF11T carrying the −470 to +19 fragment of the mouse IFN-A4 gene promoter and the −457 to +19 fragment of the mouse IFN-A11 gene promoter, respectively, and the pIF4J and pIF11J constructs containing the −120 to +19 fragment of both promoters, upstream of the chloramphenicol acetyltransferase (CAT) or luciferase reporter gene have been previously described [45]. Constructs carrying −78A/G (pIF4J-M78), −57G/C (pIF4J-M57), or both substitutions (pIF4J-M57/78) in the mouse IFN-A4 gene promoter were also described earlier [46]. Promoters containing NN/TA substitutions in different GAAANN motifs were obtained by two-step PCR mutagenesis of the pIF4J plasmid. Similar two-step PCR mutagenesis of the pIF4J plasmid containing the −120 to +19 fragment of the mouse IFN-A4 promoter was used to obtain IFN-A4 promoters with reversed orientation of B or C module (pIF4J[Brev]-CAT and pIF4J[Crev]-CAT, respectively). The sequence of each construct was confirmed by DNA sequence analysis. Plasmids expressing human IRF-3, IRF-3(5D), IRF-7A, MAVS, RIG-I, dRIG-I, IKKe, and TBK1 as well as the kinase-dead mutants of the kinases (K38A constructs) have been previously described [17, 26, 47].

**Transfections and Reporter Assays**—Human HEK 293T cells were seeded at 7 × 10⁴ cells in 12-well plates in minimal essential medium supplemented with 5% fetal calf serum. After 18–24 h, 0.5 µg of reporter plasmid and 62.5 ng of reference plasmid pIEF-LacZ in the presence of various amounts of IFR- or kinase-expressing plasmids (see the legends to Figs. 7 and 9) were transfected by the calcium phosphate method [48]. 48 h after transfection, cells were harvested to monitor the enzymatic activities. The CAT or luciferase values, normalized according to the β-galactosidase levels, were obtained in at least three independent transfection experiments, each performed in triplicate with two or three different clones. Transcription levels were expressed as relative transcription (RT) and inducibility values correspond to either the ratio between RT values obtained in overexpression conditions (with IRF- and/or kinase-expressing plasmids) to values obtained by cotransfection of the empty vector pcDNA3 or to the ratio between RT values obtained in virus-induced and in mock-induced conditions.

**Electrophoretic Mobility Shift Assays**—The electrophoretic mobility shift assay was performed with probes corresponding to the 4J, 4J[Brev] and 4J[Crev] fragments generated by PCR of the corresponding plasmid using a [γ-³²P]ATP (Amersham Biosciences; 3000 Ci/mmol) 5’-labeled upstream primer corresponding to the −119 to −103 region of IFN-A4 promoter and an unlabeled downstream primer corresponding to the −53 to −31 region of the IFN-A4 promoter. The recombinant glutathione S-transferase proteins fused with the DNA-binding domain (DBD) of IRF-3 (residues 1–133) and IRF-7 (residues 1–150) were previously described [47]. Binding reactions performed in the presence of 20 µg/ml IRF-3(DBD), 400 µg/ml IRF-7(DBD), or both recombinant proteins together for 30 min at room temperature and electrophoresis were described previously [46]. The DNA-protein complexes were resolved on a 7% polyacrylamide (30:1) gel in 25 mM TEB, pH 8.3. The bound DNA was quantitated using a Storm PhosphorImager (Amersham Biosciences).

**Chromatin Immunoprecipitation Assay**—HeLa cells (5 × 10⁶) were transfected with 10 µg of pIF4T, pIF4T-M57, pIF4T-M78, or pIF4T-M78/57 plasmids. 48 h after transfection, cells were infected with NDV for 6 h. HEK293T cells (2 × 10⁶) were plated and transfected 24 h later with 7.5 µg of plasmids pIF4T, pIF11T, pIF4J[Brev], or pIF4J[Crev] together with 3 µg of pcDNA3-IKKε or pcDNA3-TBK1 in the absence or presence of 1.5 µg of pcDNA3-IRF-3. Chromatin immunoprecipitation assays were performed according to the protocol described by Orlando et al. [49] and modified by Whatelet et al. [14]. Protein-bound cross-linked chromatin was extracted from cells after sonication and purified by isopycnic centrifugation. 20 µg of chromatin were immunoprecipitated with 5 µg of polyclonal anti-IRF-3 (s-9082) or anti-CBP (sc-583 and sc-369) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). DNA, isolated from immunoprecipitated material after reversion of cross-link, was used as a template for PCR amplification...
with the primers sense (5′-ACAAGCAGAGTGGAAG-3′), corresponding to the −119 to −103 fragment of the IFN-A4 or IFN-A11 gene promoter, and antisense (5′-CCCATACTACAGACTAC-CGTCCTTT-3′), annealing with the reporter CAT gene.

**Immunoblotting Assays**—HEK293T cells were seeded at 3 × 10⁶ cells/100-mm plate and transfected after 24 h by the calcium phosphate method with 1.5 µg of pIF4T-CAT reporter construct, 0.75 µg of pIEF-βGal control plasmid, and 6 µg of pcDNA3-FLAGIKKε or pcDNA3-FLAGTBK1 either alone or together with 0.75 µg of pcDNA3-IRF-3wt or pcDNA3-IRF-7A. 20 µg of nuclear or cytoplasmic extracts, prepared according to Dignam et al. (50), were separated on 8% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using standard procedures (51). FLAG-tagged proteins were detected with monoclonal anti-FLAG M2 antibodies (F-3165; Sigma) and antibodies directed against phosphorylated IRF-7 were generated using a C-terminal peptide (amino acids 471–489) carrying phosphoserine substitutions at serine 477 and 479 residues. Anti-poly(ADP-ribose) polymerase (sc-75, sc-8392), and anti-α-tubulin (sc-5286) were purchased from Santa Cruz Biotechnology.

### RESULTS

**Preferential Binding Sites for IRF-3 and IRF-7 Define the Virus-responsive Element of the Mouse IFN-A4 Gene Promoter**—The IFN-A4 gene is the only immediate early IFN gene expressed following virus infection in different murine cell lines (25). Maximal virus-induced transcription of the mouse IFN-A4 gene requires the presence of the virus-responsive element, VRE-A4, delimited to the −120 to −40 region of IFN-A4 gene promoter (45, 52). IRF-3 and IRF-7 are potent activators of virus-induced IFN-A gene transcription, although their mechanism of interaction with target IFN-A sequences remains to be elucidated. To define the specificity of IRF-3 and IRF-7 for the three IRF-elements (B, C, and D) located in the VRE-A4, mutated IFN-A4 promoters were generated by substituting these motifs with the GAAATA motif (Fig. 1), which was previously shown to be unresponsive to both IRF-3(5D) and IRF-7A ectopic expression (46). The capacity of these mutated VRE-A4 constructs to respond to virus or to activation by IRF-3(5D) or IRF-7A was then examined (Table 1).

Transcription mediated by IRF-3 was mainly affected by −81,−80TG/TA pair substitution (7-fold decrease of transcription with MC1 construct compared with pIF4T), and to a lesser extent by −94,−93GT/TA, −75,−74GC/TA, or −46,−45CT/TA substitutions (4–5-fold decrease observed in MB1, MC2, and MD2 constructs). Among the doubly mutated versions of the IFN-A4 promoter, IRF-3-dependent transcription was impaired more than 20-fold in the MC1-2 construct carrying the −81,−80TG/TA and −75,−74GC/TA substitutions. Inhibition was due to the −81,−80TA pair, since IRF-3-mediated transcription was not significantly affected in the MC2D1 mutant also carrying the −75,−74GC/TA pair substitution. The 7-fold decrease observed with MB2C1 was also due to the −81,−80TA pair, since the same inhibition was obtained with MC1. Thus, the TG residues in the GAAATTGGAAGC sequence (C element) were pivotal for the transcription of the IFN-A4 promoter by IRF-3. Comparison of the data obtained with MB1, MB2, and MB1-2 further indicated that the 5-fold decrease in activation observed with the MB1-2 construct was due to −94,−93GT/TA pair substitution and indicated that the sequence GAAATGGAAG (B element) in VRE-A4 defines another binding site for IRF-3. In contrast, the GG/TA and CT/TA substitutions in the GAAAGGAGAAACT sequence (D element) only slightly affected the IRF-3 response, suggesting that IRF-3 weakly recognized the D element.

Nucleotide substitutions in any IRF element had minor effects on IRF-7-mediated transcription of the IFN-A4 promoter (Table 1); IRF-7-mediated activation was affected by the −94,−93GT/TA pair substitution in the B element that decreased activation of MB1 and MB1-2 mutants more than 3-fold compared with pIF4T. As observed with IRF-3, the GAAATA motif in the B element appeared dispensable for IRF-7-mediated transcription (1.5–2-fold decrease with MB2 and MB2C1 compared with pIF4T). IRF-7-mediated transcription was only slightly affected by substitutions in the C element. The 3.3-fold inhibition observed with MD2 compared with the 2.6- and 2-fold inhibition observed with MD1 and MD1-2, respectively, indicated that

### Table 1

| Promoter | A | B | C | D |
|----------|---|---|---|---|
| pIF4T    | 103| 98| 87| 74|
| MA       | TA| TA| TA| TA|
| MB1      | TA| TA| TA| TA|
| MB2      | TA| TA| TA| TA|
| MB1-2    | TA| TA| TA| TA|
| MB2C1    | TA| TA| TA| TA|
| MC1      | TA| TA| TA| TA|
| MC2      | TA| TA| TA| TA|
| MC1-2    | TA| TA| TA| TA|
| MC2D1    | TA| TA| TA| TA|
| MD1      | TA| TA| TA| TA|
| MD2      | TA| TA| TA| TA|
| MD1-2    | TA| TA| TA| TA|
| Mct      | TA| TA| TA| TA|

**FIGURE 1. Sequence of the IFN-A4 promoter**

| −103 | −98 | −87 | −74 |
|------|-----|-----|-----|
| pIF4T| MA  | MB1 | MB2 |
|      |     | MB1-2| MB2C1|
|      |     | MC1 | MC2 |
|      |     | MC1-2| MC2D1|
|      |     | MD1 | MD2 |
|      |     | MD1-2| Mct |

**TABLE 1**

| Virus responsive element of IFN-A4 (VRE-A4) | A | B | C | D |
|---------------------------------------------|---|---|---|---|
| A  | B  | C  | D  |
| 103| 98 | 87 | 74 |
| TA | TA | TA | TA |
| TA | TA | TA | TA |
| TA | TA | TA | TA |
| TA | TA | TA | TA |
| TA | TA | TA | TA |
| TA | TA | TA | TA |
| TA | TA | TA | TA |
| TA | TA | TA | TA |

**References**

1. **et al.** (45). The half-sites constituted by GAAANN motifs present in each IRF element (8–D) and the IRF-like element A are boxed. Nucleotides that are mutated in different constructs are indicated. The Mct construct used as control in transfection experiments carries the −60,−59TC/TA substitution outside of a GAAANN motif.
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TABLE 1
Responses of NN/TA IFN-A4 promoter mutants to IRF3, IRF7A, and virus infection

Specificity of IRF-3 and IRF-7 for the three IRF-elements of the IFN-A4 gene promoter is shown. Transfection experiments were performed in HEK293T cells with pIF4T or the NN/TA-mutated promoters in the presence of 200 ng of plasmid expressing either the constitutively active IRF-3(5D) or IRF-7A. RT values and -fold inhibition are indicated in comparison with IFN-A4 promoter construct pIF4T. The 2-fold inhibition observed in mutants MA, MB2, and MD1, comparable with the 1.4-fold decrease observed with Mct (control mutant containing a NN/TA substitution outside of an IRF element), indicated that the NN residues in the corresponding DNA motifs were not critical for IRF-3-mediated transcription. The NDV inducibility was determined in L929 cells. S.D. values were calculated by computing the results of three independent transfection experiments. Lowest values are indicated in boldface type.

| Construct | IRF-3(5D) | IRF-7A | NDV/L929 |
|-----------|-----------|---------|----------|
| pIF4T     | 100       | 100     | 1208     |
| MA        | 46 ± 6    | 44 ± 12 | 2.5      |
| MB1       | 25 ± 10   | 29 ± 7  | 3.5      |
| MB2       | 50 ± 12   | 57 ± 9  | 1.8      |
| MB1-2     | 18 ± 10   | 27 ± 8  | 3.7      |
| MB2C1     | 13 ± 12   | 65 ± 15 | 1.5      |
| MC1       | 14 ± 8    | 56 ± 17 | 1.8      |
| MC2       | 20 ± 12   | 34 ± 5  | 2.9      |
| MC1–2     | 5 ± 5     | 44 ± 10 | 2.3      |
| MC2D1     | 56 ± 6    | 34 ± 5  | 2.9      |
| MD1       | 58 ± 6    | 38 ± 10 | 2.6      |
| MD2       | 25 ± 13   | 30 ± 6  | 3.3      |
| MD1–2     | 45 ± 13   | 53 ± 5  | 1.9      |
| Mct       | 74 ± 9    | 72 ± 7  | 1.4      |

Reversal of IRF Element B or C Abolishes IRF-3-mediated Cooperative Transcription and Virus Inducibility without Affecting IRF-7-dependent Transactivation of IFN-A4 Gene Promoter—Virus-induced expression of the IFN-B gene requires correct combination of different factors (ATF-2/c-Jun, IRF-3, IRF-7, and NF-kB) with the architectural factor HMGI(Y) and their alignment on the same face of the DNA helix. The assembly of an enhanceosome on the IFN-B gene promoter has been well characterized (53–55), whereas formation of IFN-A enhancesomes involving IRF-3 or IRF-7 has not been documented. To determine whether the orientation of an IRF element in the IFN-A promoter is critical for transcription by IRF-3 or IRF-7, IFN-A4 promoter mutants pIF4J[Brev] and pIF4J[Crev] carrying the B or C element in reverse orientation were generated (Fig. 2A); these mutants were tested for responsiveness to IRF3(5D), IRF7A, virus infection, or IKKe/TBK1 expression. Reversal of the B element in pIF4J[Brev] abolished IRF3(5D)-mediated transcription without affecting the transcription by IRF-7A (Fig. 2B). The increase in inducibility (260-fold compared with 80-fold) was due to the 3–4-fold decrease in the constitutive expression of the reporter construct. The electrophoretic mobility shift assay performed with 4J and 4J[Brev] probes indicated that reversal of the B element did not affect the c1 complex resulting from the binding of IRF-3(DBD) homodimers to the B, C, or D element of the IFN-A4 promoter (Fig. 2D, lanes 1 and 2). Reversal of the B element affected only the formation of high stoichiometry electrophoretic mobility shift assay

FIGURE 2. Activation of the IFN-A4 promoter requires a precise arrangement of IRF elements. A, diagram of the IFN-A4 gene promoter. The 120 to +19 fragment in the pIF4J construct. The orientation of the B or C element was reversed in pIF4J[Brev] and pIF4J[Crev], respectively. B, the relative transcription levels were determined in Fig. 1. C, virus inducibility (Ind) was determined in L929 cells infected with NDV. D, DNA binding activities of IRF-3(DBD) or IRF-7(DBD)-glutathione S-transferase fusion proteins (20 μg/ml for IRF-3 and 400 μg/ml for IRF-7) were obtained by electrophoretic mobility shift assay using as probe the −119 to −31 fragment of pIF4J, pIF4J[Brev], or pIF4J[Crev] construct. Complexes of 2:1 stoichiometry (C1, C4, and C5) corresponding to the binding of two glutathione S-transferase-IRF(DBD) molecules on DNA and complexes of 4:1 and 6:1 stoichiometries C2 and C3 obtained with IRF-3(DBD) as well as C6 and C7 obtained when IRF-3 and IRF-7 DBDs were used together are indicated. Bound and free DNA were quantitated by PhosphorImager analysis and plotted as percentage relative to the total amounts of DNA.
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Complexes c2 and c3, hallmarks of cooperative binding of IRF-3(DBD), representing IRF3(DBD) homodimers that interact with two and three IRF elements, respectively. Similarly, when both IRF-3 and IRF-7 DBD's were used, only the high stoichiometry complexes c6 and c7 containing multimers of IRF-3 and IRF7 were affected (Fig. 2D, lanes 7 and 8). As expected, IRF-7 binding was not altered by reversal of the B element.

Reversal of the C element in pIF4J[Crev] had the same differential effect on IRF-3 and IRF-7 transcriptional and binding activities (Fig. 2, B and D, lanes 3, 6, and 9), indicating again the critical role of the orientation of IRF elements in cooperative transcription mediated by IRF-3. Most importantly, this particular arrangement was also crucial for virus-induced transcription, since reversal of the B or C element totally abolished the NDV responsiveness of IFN-A4 promoter, as indicated by the 108-fold inducibility of pIF4J compared with the 5–8-fold inducibility values of pIF4J[Brev] or pIF4J(Crev) in L929 cells (Fig. 2C). These results clearly indicated that cooperative transcription mediated by IRF-3 dimers bound to three IRF-elements on the IFN-A4 promoter correlates with virus-induced transcription and requires the ordered assembly of protein-DNA complexes in the proximal region of the IFN-A4 promoter. In contrast, transactivation by IRF-7 homodimers, which does not necessarily correlate with the virus responsiveness of the promoter, may require a distinct organization.

Differential Transcription of Mouse IFN-A4 and IFN-A11 Gene Promoters—We previously showed that the weak virus-induced expression of the IFN-A11 gene compared with IFN-A4 was due to the presence of two nucleotide substitutions in the IFN-A11 gene promoter (45). These substitutions, −78A/G and −57G/C, are located in the second GAAA core motif of the C element (GAATTGGAAGGC sequence) and in the first half of the D element (GAAAGGAGAAGGACT), respectively (Fig. 3). IRF-3- or IRF-7-mediated transcription and the synergism between these factors were dramatically affected in the case of IFN-A11 (Fig. 4A). Interestingly, when IKKε or TBK1 were expressed, IKKε exerted a strong stimulatory activity on IFN-A4 promoter transcription (57-fold), compared with the 3-fold enhancement displayed by TBK1 (Fig. 4B); kinase-dead versions of IKKε or TBK1 did not stimulate transcription. When each kinase was expressed together with IRF-3, IFN-A4 gene promoter transcription was stimulated more efficiently (Fig. 4C). No stimulatory effect on IFN-A11 promoter transcription was observed when IKKε or TBK1 was expressed alone or in the presence of IRF-3, although a 4–5-fold increase was obtained in the latter case (Fig. 4, B and C), confirming that the IFN-A11 gene promoter was unresponsive to IRF-3. Moreover, only the IFN-A4 promoter transcription was induced in HEK293T cells infected by Sendai virus or in L929 cells infected by NDV (Fig. 4D). These results indicated that the differential virus-induced transcription of IFN-A4 and IFN-A11 promoters correlated with their differential response to IRF-3 stimulated by IKKε or TBK1. Interestingly, differential activation was also observed when RIG-I, an intracellular receptor for viral RNA or the mitochondrial antiviral signaling protein MAVS that functions downstream of RIG-I (28, 30) was expressed in 293T cells (Fig. 4D). The IFN-A11 gene promoter did not respond to IRF-3 activation even when cells expressed a constitutively active N-terminal form of RIG-I (∆RIG-I), whereas the IFN-A4 promoter was stimulated 15-fold in similar conditions.

Chromatin immunoprecipitation experiments were performed to assess whether the differential transactivation observed with IKKε or TBK1 expression may be due to differential recruitment of IRF-3. Anti-IRF3 and anti-CBP antibodies were used to immunoprecipitate nuclear proteins cross-linked to chromatin in IRF-3- and kinase-expressing cells. Specific amplification of the IFN-A4 promoter was easily observed when IKKε was coexpressed with IFN-A4 and IFN-A11 gene promoters. Interestingly, reversal of the C element in the IFN-A4 promoter did not affect the IRF-3 recruitment but completely abolished the recruitment of CBP (lanes 11 and 13). These results, together with the data obtained with DNA-binding assays (presented in Fig. 2D), demonstrated that CBP recruitment to the IFN-A4 promoter requires the ordered assembly of IRF-3 dimers bound to three IRF elements on the proximal promoter region.

We have then examined whether the −78A/G and −57G/C substitutions that naturally occur in the C and D elements of IFN-A11 also affected IFN-A4 transcription by introducing the −78A/G and −57G/C substitutions into the IFN-A4 promoter (Fig. 6A). The
with the detection of a small fraction of nuclear IRF-3 in unstimulated cells (15, 56); no amplification of the IFN-A4 promoter was observed in the absence of immunoprecipitating antibodies (lane 2). Furthermore, the −78G/C substitution strongly reduced IRF-3 recruitment, compared with −57A/G substitution (lanes 4 and 5). These results illustrated the critical role of the C element in recruitment of IRF-3 to the IFN-A4 gene promoter and indicated that efficient IRF-3-mediated cooperative transcription was due to the number, precise arrangement, and specificity of the IRF elements.

IKKε and TBK1 Differentially Affect the IRF-3-mediated Transcription of IFN-A4 Gene Promoter—The robust response of the IFN-A4 gene promoter to IKKε expression, compared with a weak stimulation by TBK1 (Fig. 4, B and C, and Fig. 6C) was an intriguing and unexpected observation. Actually, transfection of 12.5 ng of the IKKε-expressing plasmid in HEK 293T cells was sufficient to obtain threshold levels of transcriptional activity (up to 5–6 relative units), whereas 25 times higher amounts of pcDNA3-TBK1 were required to observe a similar effect (Fig. 7A and Table 2). The 20-fold induction of the IFN-A4 gene promoter observed by expressing 75 ng of pcDNA3-IKKε was only slightly augmented by further increasing the amount of kinase up to 300 ng. In this case, transcriptional activity was 10-fold higher than observed in the presence of 300 ng of TBK1-expressing plasmid. A 4–8-fold difference between IKKε and TBK1 was also observed when each kinase was expressed in the presence of exogenous IRF3. In this case, threshold promoter activity required 3 ng of pcDNA3-IKKε and 10 times higher amounts of pcDNA3-TBK1; a further increase in the amount of exogenous kinase resulted in RT values 3–6-fold and 5–8-fold higher with IKKε and TBK1, respectively (Fig. 7B, Table 2). The differential effect of IKKε or TBK1 on IRF-3-mediated transcription was less pronounced (2–3-fold) when each kinase was expressed in the presence of high concentrations of exogenously added substrate IRF-3 (Fig. 7C and Table 2). Immunoblot analysis with anti-FLAG antibodies monitoring the expression of IKKε and TBK1 indicated surprisingly that, although IKKε displayed higher stimulatory activity on IRF-3-mediated transcription, this kinase was barely detected in cytoplasmic extracts compared with TBK1 (Fig. 7D). Furthermore, in nuclear and cytoplasmic cell extracts, IKKε was only detected in the nuclear extracts of 293T cells when expressed alone, whereas co-expression of exogenous IRF-3 resulted in IKKε cytoplasmic localization (Fig. 8, lanes 2 and 7 and lanes 3 and 8).
contrast, TBK1 was detected in the cytoplasmic extracts of cells expressing exogenous IRF-3 but not in nuclear extracts (Fig. 8, lanes 4 and 5 and lanes 9 and 10). However, expression of IKKe or TBK-1 together with IRF-3 resulted in IRF-3 phosphorylation, detected by the phosphoserine 396 phosphospecific antibodies (Fig. 8, lanes 3, 5, 8, and 10) and correlated with IFN-A4 promoter activity. Detection of poly-

FIGURE 6. Disruption of IRF elements in the IFN-A4 gene promoter affects activation and IRF-3 DNA binding. The reporter plasmid plF4T and the constructs that contain point-mutated versions of the IFN-A4 promoter at position −78A/G (plF4T-M78), −57G/C (plF4T-M57), or both (plF4T-M78/57) schematized in A were tested for their transcriptional activity in the presence of 50 ng of plasmid expressing IRF-3(5D) or IRF-7A (B) or in the presence of 12.5 ng of pcDNA3-Ikke or pcDNA3-TBK1 (C). Virus inducibility (Ind.) of these constructs was determined in HEK293T cells infected with Sendai virus or in L929 cells infected with NDV (D). The virus-induced in vivo recruitment of IRF-3 on the IFN-A4 gene promoter was analyzed by chromatin immunoprecipitation experiments (E) in HeLa cells transfected with plF4T (lanes 1–3) or mutated IFN-A4 promoters plF4T-M57 (lane 4), plF4T-M78 (lane 5), or plF4T-M78/57 (lane 6). 48 h post-transfection, cells were mock-induced (lane 1) or infected by NDV (lanes 2–6) and fixed by formaldehyde 5 h later. DNA was sonicated following extraction and immunoprecipitated with polyclonal IRF-3 antibodies (lanes 1 and 3–6) or treated with protein A-Sepharose in the absence of antibodies (lane 2). Purified DNA was then subjected to a PCR using specific primers for plF4J, M57, M78, or M57/78. The PCR products were analyzed on a 1% agarose gel.

FIGURE 7. IKKe and TBK1 differentially affect IRF3-mediated activation of IFN-A4. The stimulatory effect of IKKe or TBK1 overexpression on the IFN-A4 promoter transcription was determined by transient transfection of HEK293T cells with increasing amounts of kinase-expressing plasmid, either alone (A) or in the presence of 25 ng of pcDNA3-IRF-3 (B), or 250 ng of the same construct (C). The RT values are presented in Table 2. Expression levels of IKKe and TBK1 proteins was monitored in cytoplasmic extracts from 293T cells transfected with increasing amounts of kinase-expressing plasmid by Western blot experiments performed with monoclonal anti-FLAG antibodies (D).
that IRF-3-mediated transcription is activated at low concentrations of IKK due to the predominant nuclear localization of this kinase, whereas higher levels of cytoplasmic TBK1 are required.

IKKe and TBK1 Stimulate the IRF-7-mediated Transcription of IFN-A11 Gene Promoter—Surprisingly, IKKe or TBK1 strongly stimulated IRF-7-mediated transcription of IFN-A11, 133- and 36-fold, respectively, compared with 408- and 56-fold stimulation of IFN-A4 induc- tion of the IFN-A4 gene promoter by IRF-7 or IRF-3 requires these distinct binding sites that contribute to different extents to cooperative transcription. The first IRF element corresponds to the −98 to −87 GAAAGTGAAAAG sequence (B element), where the GT residues determine the specificity for IRF-7 and IRF-3. The −86 to −75 GAAXXTCGAAAAGC sequence (C element) corresponds to the second IRF element and is a selective target for IRF-3, whereas the −57 to −45 GAAAGGAGAAAAC sequence (D element) is weakly recognized by both IRF-3 and IRF-7. Our data suggest that when both factors are simultaneously activated during viral infection, synergistic activation of the IFN-A4 gene promoter is achieved by preferential recognition of B and C elements by IRF-7 and IRF-3 homodimers, respectively, whereas the D site may be occupied by either IRF-7 or IRF-3. We also demonstrate that the in vivo recruitment of IRF-3 and CBP to the IFN-A4 gene promoter following virus- or kinase-dependent activation is dictated by the precise arrangement of the IRF elements in the promoter. These results are in agreement with previous in vitro binding studies demonstrating that disruption of the C or D elements decreased IRF-3 affinity by 6- and 3-fold, respectively, whereas disruption of both elements almost completely abolished the IRF-3 binding (46).

DNA distortions in the GAAA core sequences of cognate binding sites have been proposed as a determinant for cooperativity in IRF binding (57, 58). Accordingly, transcriptional cooperativity might be favored by IRF-3 binding to the C element by similar distortions, thus preparing the IFN-A4 template for the cooperative IRF-3 interaction with the B and D modules. Virus-induced transcription of IFN-A gene promoters also depends on the ability of IRF-3 or IRF-7 homodimers to interact with CBP/p300 coactivators (18, 59, 60). Our results showed that reversal of the B or C module abolishes IRF-3-mediated transcription of IFN-A4 promoter in correlation with a dramatic decrease of in vivo CBP recruitment to the promoter, indicating that ordered assembly is required for IRF-3-mediated cooperative transcription. Furthermore, the absence of a single IRF element significantly disturbs the interaction of IRF-3 with the promoter and destabilizes the assembly required for

**DISCUSSION**

The present study demonstrates that three IRF elements (designated as B, C, and D) constitute binding sites for IRF-3 and IRF-7 and define the virus-responsive element (VRE-A4) of the murine IFN-A4 gene promoter. Transactivation of the IFN-A4 gene promoter by IRF-3 or IRF-7 requires these distinct binding sites that contribute to different extents to cooperative transcription. The first IRF element corresponds to the −98 to −87 GAAAGTGAAAAG sequence (B element), where the GT residues determine the specificity for IRF-7 and IRF-3. The −86 to −75 GAAXXTCGAAAAGC sequence (C element) corresponds to the second IRF element and is a selective target for IRF-3, whereas the −57 to −45 GAAAGGAGAAAAC sequence (D element) is weakly recognized by both IRF-3 and IRF-7. Our data suggest that when both factors are simultaneously activated during viral infection, synergistic activation of the IFN-A4 gene promoter is achieved by preferential recognition of B and C elements by IRF-7 and IRF-3 homodimers, respectively, whereas the D site may be occupied by either IRF-7 or IRF-3. We also demonstrate that the in vivo recruitment of IRF-3 and CBP to the IFN-A4 gene promoter following virus- or kinase-dependent activation is dictated by the precise arrangement of the IRF elements in the promoter. These results are in agreement with previous in vitro binding studies demonstrating that disruption of the C or D elements decreased IRF-3 affinity by 6- and 3-fold, respectively, whereas disruption of both elements almost completely abolished the IRF-3 binding (46).

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cooperative IRF-3 binding and transcription, alone or together with IRF-7. Formation of an IRF-3-dependent transcriptional complex on the virus-responsive enhancer of the IFN-B promoter. Enhanceosome formation requires the correct combination of different transcription factors (ATF-2/c-Jun, IRF-3, IRF-7, and NF-κB) and the architectural factor HMGI(Y), which alters the curvature of the DNA, thus facilitating interactions among these factors and permitting their alignment on the same face of the DNA helix to promote cooperative transcription (53, 55, 61). Enhanceosome formation has not been well documented.

FIGURE 9. IKKe and TBK1 differentially affect IRF7-mediated activation of IFN-A4. Relative transcription levels of plIF4J and plIF11J promoters (A) were determined in the presence of 25 ng of plasmid expressing IRF-7A alone or together with 75 ng of pcDNA3-IKK or TBK1. Ind., inducibility. B, the relative transcription levels of plasmids plIF4T-L and plIF11T-L were determined in the presence of 100 ng of MyD88-expressing plasmid, 25 ng of IRF-7A-expressing plasmid, or both. C, stimulatory effect of IKKe or TBK1 on plIF4J, plIF4J[Brev], and plIF4J[Crev] was determined in the presence of increasing amounts of IKKe- or TBK1-expressing plasmids together with 25 ng of pcDNA3-IRF-7A. D, Western blotting was performed as described in the legend to Fig. 8, using anti-FLAG or anti-phosphoserine 477/479 IRF-7 antibodies in nuclear or cytoplasmic extracts from 293T cells transfected with pcDNA3 (lanes 1 and 6), pcDNA3-FLAGIKKe (lanes 2 and 7), or pcDNA3-FLAGTBK1 (lanes 4 and 9) alone or together with pcDNA3-IRF-7A (lanes 3, 5, 8, and 10). The phosphorylated form of IRF-7 is indicated by an asterisk.
for IFN-A gene promoters, although IRF-3 and IRF-7 have been suggested to bind to human IFN-A1 promoter as heterodimers (62). In the case of the IFN-A4 promoter, virus-induced transcription is dependent solely on the capacity of IRF-3 or IRF-7 homodimers to bind on the IRF elements of the VRE-A4. The factors facilitating this enhanceosome or “irfosome” formation are unknown, although IRF-3 itself may play this role. Our data suggest that IRF-3 homodimers bound to the B, C, and D elements adopt precisely defined orientations with respect to each other on the DNA helix. Accordingly, nucleotide substitutions disrupting specifically the C element (the M78 construct) or the C and D elements (IFN-A11 gene promoter and the M78/57 construct) led to a proportional decrease in IRF-3 and CBP recruitment, affected synergistic transcription, and altered cooperativity between IRF-7 and IRF-3. This particular organization of the IFN-A4 promoter accounts also for its differential responsiveness to IKKe or TBK1 compared with IFN-A11. IRF-3-mediated induction of IFN-A4 was activated at low concentrations of IKKe but required high levels of TBK1 expression. This distinct stimulatory effect of IKKe and TBK1 on transcription may be related to subcellular localization: IKKe was localized predominantly to the nuclear compartment, whereas TBK1 was only detected in the cytoplasm. Since IRF-3 constitutively shuttles in and out of the nucleus (41, 56), IKKe may promote phosphorylation of nuclear pools of this factor, leading to rapid and efficient IRF-3-mediated transcription. In contrast, TBK1 phosphorylates only the cytoplasmic pool of IRF-3, thereby stimulating a relatively delayed transcription that initiates following IRF-3 translocation to the nucleus. The IFN-A11 gene promoter, on the other hand, lacks the irfosome required for IRF-3-mediated transcription, thereby losing its ability to respond to IKKα stimulated by IKKe.

Differential IFN-A Gene Regulation by IKKe and TBK1

The IFN-A11 gene promoter, which is unresponsive to IRF-3, was activated at low concentrations of IKKe but required high levels of TBK1 expression. The distinct differential responsiveness to IKKα and IKKe in lymphoid cells or mature dendritic cells may thus reduce the differential expression levels of IFN-A gene family members. Since the expressions of IRF-7 and IKKe are both virus-inducible (11, 49, 51), the differential expression of IFN-A may be premed in the early phase of virus infection but may be balanced in fibroblasts or epithelial cells during the delayed phases. The “gradient” effect of IRF-3-mediated transcription that favors differential IFN-A gene expression might constitute a regulatory mechanism allowing the return to homeostasis in different cells, in comparison with particular subsets of dendritic cells expressing both IRF-7 and IKKe and acting as high IFN-A producers.

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FEBRUARY 24, 2006•VOLUME 281•NUMBER 8
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