Virus-mediated Induction of Interferon A Gene Requires Cooperation between Multiple Binding Factors in the Interferon α Promoter Region*

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Transcriptional activation of interferon A (IFNA) gene in virus-infected cells is controlled by a 35-nucleotide inducible element that is cell type specific. Within this region, two elements, αF1 and IRF-1 binding sites, were shown by mutation analysis to play a crucial role in the expression of inducible element. In this study, we have analyzed the binding of nuclear proteins to the αF1 sequence and have shown that the induction is associated with the formation of a novel complex αF1/B, which contains at least two DNA binding proteins of 68 and 96 kDa. In contrast, no binding of the purified interferon regulatory factor 1 (IRF-1) either to the αF1 or IRF-1 binding sites could be detected in vitro. However, the oligonucleotides corresponding to αF1 or IRF-1 binding sites competed efficiently for the induction of IFNA4 promoter region in a transient transfection assay. We suggest that the induction of IFNA promoter region requires cooperation between αF1 binding proteins and IRF-1. Interestingly, our data also show that the inability of IFNA6 promoter to be expressed in infected L-cells may be a result of a viral-induced repressor, which could act by binding and inactivating αF1 or by competing for the IRF-1 binding site. These results suggest that cell-specific expression of IFNA genes results from corecruitment of trans-acting factors that bind to αF1 and the IRF-1 binding site with the cell-specific virus-induced activator or repressor.

Viral infection of eukaryotic cells leads to a transient expression of a number of cellular genes (1–3). Among these are IFNα, which play an important role in modulating the host immune responses and restriction of viral replication (4–6). Although the expression of IFNs can be subjected to different levels of control, the induction of IFN by virus is mainly regulated at the transcriptional level (7, 8). The transcriptional activation of IFN genes can occur in the absence of cellular protein synthesis (9) suggesting that it is mediated by the preexisting, latent cellular factors that become activated in response to viral infection. The specificity of transcriptional activation of IFN genes is achieved by the interaction of these nuclear factors with various cis-acting elements located within 150 base pairs upstream of the transcription initiation site of both IFNA and IFNb genes. The study of the human IFNB regulatory region revealed that this region consists of three positive regulatory domains (PRD)-I, -II, and -III (10–12). PRD-I and -III can function both as positive and negative regulatory domains, binding at least three nuclear factors; the IRF-1 and -2 can either activate or suppress the transcription, respectively (13–16); PRD-BF1, a zinc finger containing protein, functions as a transcriptional repressor (17, 18). The PRD-II, which contains the xB binding site, interacts with NF-xB proteins and the high mobility group protein 1 (19–22). The synergism between the PRD-I and NF-xB binding sites in the activation of transcription has been shown (22–23). The human IFNA genes display more restricted cell type specific expression and are induced primarily in cells of lymphoid lineage (24, 25). Promoters of IFNA genes contain the virus responsive element or IE with regions similar only to the PRD-I and -III but not to the NF-xB site(s) (24, 25, 27). Very little is known about the factors that bind the virus regulatory element of the human IFNA genes. MacDonald et al. (24) have shown that IEFαα, the factor that mediates the transactivation of the herpes simplex virus-1 (HSV-1) late gene VP-16, also binds to the TA-rich motifs present in the virus responsive element of human IFNA1 gene promoter but does not play a role in the inducibility of this gene.

We have previously shown that, in murine systems, infection of L929 cells with Newcastle disease virus (NDV) activates the expression of murine IFNA4 gene but not the murine IFNA6 gene, while in primary macrophages and in the macrophage-derived cell line, Raw, IFNA6 was induced as well or better than IFNA4 (25, 28–30). The differential expression of these two genes in L-cells is determined by the transcriptional strength of the respective promoter (25); in this region, an enhancer-like, 35-base pairs long sequence (denoted as IE, -109 to -75) was identified and showed to be able to confer viral inducibility to a heterologous minimal promoter (28). This IE motif contains an almost symmetric sequence GATAAGAAAATG (αF1, -103 to -94), which partially overlaps with the putative IRF-1 binding site (Fig. 1A). It has been shown that the integrity of both of these sites is essential for virus-mediated induction (29, 30). The defect in the in-

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1 The abbreviations used are: IFN, interferon; NDV, Newcastle disease virus; IE, inducible element; CAT, chloramphenicol acetyltransferase; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; PRD, positive regulatory domains; CHX, cycloheximide; nt, nucleotide(s).
Role of IRF-1 in Induction of IFNA Genes

The aim of the present study was to analyze and identify trans-activating factors that can specifically bind to the regulatory elements in the murine IFNA4 and -A6 promoters. The critical role of the IRF-1 and IRF-2 in the activation of IFNA genes was suggested by the results of transient transfection assays, overexpression of IRF-1-induced expression of the endogenous IFNA, and increased expression from the transfected IFNA promoter regions (15). Similarly, the cotransfection of IRF-1 expressing plasmid with the IFNA4 and IFNA6 CAT hybrid plasmids to L-cells stimulated expression from both IFNA4 and -A6 promoter regions (30) but did not induce the expression of the endogenous IFNA or B genes in these cells (30).

The aim of the present study was to analyze and identify trans-activating factors that can specifically bind to the regulatory elements in the murine IFNA4 and -A6 promoter regions. A novel induction specific complex (αF1/β) was detected in the nuclear extract from infected L929 cells. By UV cross-linking, we identified that the αF1/β complex is composed of at least two DNA binding proteins of 68 and 96 kDa. Although the multiple repeats of αF1 binding site were not able to confer virus inducibility, they competed effectively for the inducibility of IFNA4 in a transient expression assay. While no binding of purified IRF-1 to the αF1 or to the IE sequences could be detected, oligodeoxynucleotides corresponding to the IRF-1 binding site competed effectively for the induction of IFNA4 promoter region. Interestingly, the lack of inducibility of IFNA6 promoter region in infected L-cells seems to be a consequence of replacement of IRF-1 binding to the IE by a virus-induced suppressor. Neither IRF-1 nor IRF-2 antisense affected virus-mediated inducibility of IFNA4 promoter region. These results suggest that the induction of IFNA genes by virus infections requires cooperation between the αF1 binding proteins and the IRF-1 or a protein(s) binding to the IRF-1 site.

EXPERIMENTAL PROCEDURES

Plasmids and Probes—The antisense constructs, IRF-1AS and IRF-2AS, were made by cloning the XbaI and Xhol cDNA fragments from IRF-1 and IRF-2 expression plasmids in the antisense orientation in the CDM8 vector. PA4 and PA10 plasmids were constructed by insertion of three copies of either αF1 or αF1 Pur1-XhoI fragments in front of the −54αCAT fusion gene. All other plasmids were described previously (28–31). The plasmids used are listed in Fig. 1B. αF1, αF1, and PRD-1 oligomers used as probes for gel shift assay were synthesized with additional 10 nucleotides at the 3′ end, used for primer annealing (Fig. 2D). The probes were made by filling in with 32P-dNTP using Klenow DNA polymerase. IE4, IE6, and CAT probes were made by end-labeling of Pur1-XhoI fragments isolated from plasmids pRB41, pBK123, and pBK98, respectively, as described previously (28–31).

Transfection, Infection, and CAT Assay—Transfection was done by the calcium phosphate precipitation method as described previously (31). Cells were seeded (0.5 × 106 cells/plate) 2 days before transfection. As indicated, 0.5–15 µg of plasmid DNA were used for each transfection. Cells were induced by infection with NDV at multiplicities of 5, 24 h after the transfection. The CAT assay was performed 16 h after the infection, using equal amounts of cell lysate (protein) in a 1-h reaction at 37°C.

Gel Mobility Shift Assay—The nuclear extracts were prepared as described recently (32). The labeled probes (1–10 pg) were incubated with nuclear extract (5–10 µg) in a binding mixture of 100 mM Tris 7.6, 100 mM KCl, 7% glycerol, 5 mM MgCl2, 0.1 mM EDTA, and 8 µg/ml poly(dI)-(dC) for 10 min at 4°C. The protein-DNA complexes were then resolved on nondenaturing 4% polyacrylamide gel. In the competition assay, various amounts of competitors were added into the binding mixture at the time when the binding reached equilibrium (after 15 min of incubation). The mixture containing the competitor was incubated at 4°C for 5 min before the gel electrophoresis.

UV Cross-linking in Solution and in Situ—For the UV cross-linking assay in solution, the binding mixture described above was incubated for 15 min at 4°C, spotted on a parafilm, and UV-irradiated (254 nm) for 5 min at 4°C. The distance from the UV light source was approximately 5 cm. The UV cross-linked product was then resolved on either 10 or 12% SDS-acrylamide gel. In situ UV cross-linking was done as described recently (32). The DNA-protein complex was resolved on a nondenaturing gel and UV-irradiated for 10 min. The protein-DNA complex was eluted from the gel by incubation with 1 M sodium acetate overnight, purified by phenol/chloroform extraction, precipitated with ethanol, and subjected to SDS-PAGE.

Southwestern Blotting Analysis—The method used was based on the procedure used for screening the Agt11 library (33). Briefly, 50–100 µg of nuclear extracts were denatured and separated by SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane, denatured, and renatured; the redenatured filters were hybridized with 32P-labeled probe (106 cpm/ml) for 2 h, washed for 30 min at room temperature, dried, and autoradiographed for 3 days.

In Vitro Translation—Murine IRF-1 mRNA was translated in rabbit reticulocyte lysate according to the directions from the manufacturer (Promega Corp.).

RESULTS

Induction-specific αF1 Binding Activity—Because we have previously shown (30) by mutagenesis analysis that the DNA sequence in the αF1 site is the critical determinant in the differential expression of IFNA4 and IFNA6 genes in infected L-cells, we examined the binding of nuclear proteins from infected cells and controls to DNA probes corresponding to the αF1 sequence. In addition to αF1 probe, the αF1 and PRD-I probes were used for comparison. The binding of IFNA4 promoter region in infected L-cells was found to be due to the replacement of two G residues in A6IE at −103 and −94 by T and A, respectively (Fig. 1). In addition, the mutation at −92 of G residue in A4IE, which resides in the IRF-1 site, could also abolish the activation of IFNA4 promoter (30).

The critical role of the IRF-1 and IRF-2 in the activation of IFNA genes was suggested by the results of transient transfection assays, overexpression of IRF-1-induced expression of the endogenous IFNA, and increased expression from the transfected IFNA promoter regions (15). Similarly, the cotransfection of IRF-1 expressing plasmid with the IFNA4 and IFNA6 CAT hybrid plasmids to L-cells stimulated expression from both IFNA4 and -A6 promoter regions (30) but did not induce the expression of the endogenous IFNA or B genes in these cells (30).

The aim of the present study was to analyze and identify trans-activating factors that can specifically bind to the regulatory elements in the murine IFNA4 and -A6 promoter regions. A novel induction specific complex (αF1/β) was detected in the nuclear extract from infected L929 cells. By UV cross-linking, we identified that the αF1/β complex is composed of at least two DNA binding proteins of 68 and 96 kDa. Although the multiple repeats of αF1 binding site were not able to confer virus inducibility, they competed effectively for the inducibility of IFNA4 in a transient expression assay. While no binding of purified IRF-1 to the αF1 or to the IE sequences could be detected, oligodeoxynucleotides corresponding to the IRF-1 binding site competed effectively for the induction of IFNA4 promoter region. Interestingly, the lack of inducibility of
nuclear and cytoplasmic extracts from uninfected cells to αF1 probe showed the presence of three DNA-protein complexes (A, C, D) on nondenaturing gels (Fig. 2A). From extracts of infected cells, a new band (complex B) could be readily detected as early as 2 h postinfection (Fig. 2A, lane 3) and its intensity increased substantially with time. The formation of complex D shown in Fig. 2 was not consistent in all experiments and therefore its characterization was not further pursued. The formation of complexes (A, B, C) was not a result of nonspecific binding to the primer sequence in the αF1 probe (see Fig. 2D), because the unlabeled primer sequence did not compete the formation of any complexes (data not shown). The appearance of complex B during the course of infection correlated well with the accumulation of IFNA mRNA. The role of complex B formation in the induction of IFNA genes was therefore analyzed in more detail by using the following criteria. It has been shown that the induction of IFNA genes 1) does not require de novo protein synthesis, 2) cannot be mediated by the UV-irradiated, nonreplicative NDV, and 3) is inhibited by the protein kinase C inhibitor, staurosporin (34). The effect of cycloheximide (CHX), staurosporin, and UV-irradiated virus on the formation of complex B is shown in Fig. 2, B and C. The protein synthesis inhibitor, CHX, did not prevent formation of the complex B in infected cells but increased its level, while CHX alone did not induce it. In contrast, formation of this complex was not detected in cells treated with staurosporin and was much reduced in cells induced with UV-NDV. These results support the idea that formation of complex B is related to the transcriptional activation of IFNA genes.

The formation of complex B is not associated with binding of IRF-1 to the αF1 probe. When binding of the purified human IRF-1/ISGF-2 to the αF1 probe was analyzed, no DNA-protein complex could be detected (Fig. 2A, lane 5).

Furthermore, the mobility of the A, B, C, and D complexes was not supershifted in the presence of anti-IRF-1 antibodies (data not shown), indicating that the complexes formed do not contain IRF-1. The formation of complex B, however, was not unique for the αF1 probe but could also be detected with the αF1 probe (Fig. 2A, lanes 11 and 12). The binding of nuclear extracts from infected cells to the PRD-I oligonucleotide showed an entirely different binding pattern with the formation of two specific DNA-protein complexes, which may correspond to the previously described BFc or TH2 and TH1 binding activities in HeLa cells (20, 21). No induction-specific complex corresponding to the previously identified BFi in HeLa cells or to a complex with mobility similar to the αF1/B complex was detected (Fig. 2C).

**Differential Affinity of αF and αF1 Oligonucleotides for αF1 Binding Factor(s)**—We have previously shown that the lack of inducibility of IFNA6 promoter in infected L-cells is a result of the replacement of G residues by T and A at -103 and -94 nt in the αF1 site (30). However, we have not detected any difference in the binding pattern of nuclear proteins to αF or αF1 probes. We, therefore, compared the relative affinity of binding of nuclear proteins with the αF1 and αF1 probes by a competition analysis. As can be seen in Fig. 3, while the formation of complex A could be competed with a 2000-fold molar excess of unlabeled αF1 or αF1, the formation of complex B was effectively competed with a 40-fold molar excess of αF1 oligonucleotide. The amount of αF1 needed for a similar degree of competition was about 10-fold higher (Fig. 3B). Similarly, formation of complex C was competed more effectively by αF1 than by αF1 (Fig. 3C).

These results indicate that the αF1/B binding factor(s) shows higher affinity for the αF1 than for the αF1 oligonucleotides.

The affinity of αF binding activity for the A4 and A6IE and for PRD-I was also examined. All three oligonucleotides

**Fig. 2. Gel shift assay.** A, αF1 and αF1 sequences were used as probes for protein binding. Nuclear and cytoplasmic extracts were prepared from uninfected and NDV-infected L-cells at various times postinfection. In lane 5, binding of purified human IRF-1/ISGF-2 was examined. A, B, C, and D, protein-DNA complexes are indicated by arrows. The little insert on the left shows the shorter exposure of lanes 3, 4, and 5. B, nuclear extracts were prepared either from NDV-infected L-cells treated with CHX (5 µg/ml, 2 h) or staurosporin (STAU.) (80 nm, 4 h) for 30 min before infection or prepared from CHX- (5 µg/ml, 2 h) or IFN- (500 units/ml, 4 h) treated cells. C, comparison of DNA-protein complexes formed with the PRD-I and αF1 probes. D, sequence of the probes used in the gel shift assay. cont., extracts from the uninduced cells.
competed about equally well for the formation of complex A (Fig. 3D). However, formation of complex B was more effectively competed by A4IE than by PRD-I or A6IE (Fig. 3E).

The observation that A4IE appears to compete better than αF1 (Fig. 3, B and E) suggests the presence of cooperative binding between αF1 and another enhancer element (e.g., IRF-1) within the IE. Furthermore, the fact that PRD-I (or AGT-GAA multimers) can compete for the formation of B complex indicates that a factor(s) involved in the formation of this complex can also bind or interact with the PRD-I sequences. None of the complexes could be competed with large amounts of nonspecific competitor, poly(dI)·(dC) (Fig. 3F), suggesting that the formation of complexes A, B, and C is specific. We conclude from these data that the affinity of the α1/β binding protein(s) for αF1 is higher than for αF1 sequences.

Characterization of Proteins Binding to αF1 by UV Cross-linking—The proteins that specifically bind to the αF1 probe were identified by the UV cross-linking analysis. In the initial experiments, we incubated the nuclear extracts with the 5’-bromo-2’-deoxyuridine (BrdUrd) (Boehringer Mannheim) substituted αF1 probe (35). However, the substitution by 5’-BrdUrd in αF1 probe substantially changed the mobility of the formed complexes and the binding pattern on the gel retardation assay (data not shown). Therefore, the uniformly labeled but not the BrdUrd-incorporated αF1 probe was used in all subsequent UV cross-linking experiments. The nuclear extracts from induced cells were incubated with the radioactive αF1 probe, the DNA-protein complexes were separated on nondenaturing gels, and UV was cross-linked in situ, eluted, and subsequently analyzed by SDS-PAGE. The cross-linking of complex A shows the presence of the DNA-protein adduct of about 200, 96, and 68 kDa (Fig. 4A), while both complexes B and C show a strong band of about 96 kDa and a weak band of about 68 kDa. When the cross-linking was done with the extracts from uninfected cells, which can only form the complex C but not complex B, the presence of two bands of 98 and 68 kDa was also detected (data not shown). These results show that the formation of the induction-specific B complex is not due to the binding of a novel, induction-specific protein to the αF1.

The αF1 binding proteins were also identified by the Southwestern analysis (Fig. 4B). Three strong bands representing binding of the αF1 probe to proteins of 96, 68, and 30 kDa were detected in both induced and uninduced cells. The 150- to 200-kDa and the 50-kDa binding proteins shown on this exposure were not detected when the hybridization and washing were done under more stringent conditions (data not shown). The 96- and 68-kDa αF1 binding proteins were identified by both binding of the radioactive αF1 oligonucleotide to the nuclear proteins separated by gel electrophoresis and by the UV cross-linking analysis. The binding of 30-kDa protein to αF1 probe detected by the Southwestern hybridization (Fig. 4B, bottom) showed only a very weak band in situ UV cross-linking. The nuclear extract from human CEM cells (T cell line that is not able to induce IFN after viral infection) was analyzed as a control. In these cells, no binding of the 68- and 30-kDa to αF1 probe was detected. To test whether the binding of αF1 oligonucleotide is specific, nuclear extracts from induced and uninduced L-cells were hybridized with the probe of the 33-nt long oligomer corresponding to the two

![Fig. 3. Quantitation of relative affinity of binding in complexes A, B, and C by competition assay.](image-url)

The probe 32P-labeled oligodeoxyribonucleotide (20 ng) containing the αFl sequence was preincubated with nuclear extracts (10 µg) for 15 min as described under "Experimental Procedures" and followed by addition of indicated competitors. The concentration of these competitors was determined by reading and ethidium bromide staining. After incubation, the DNA-protein binding activity was analyzed by gel shift assay. Each band representing the DNA-protein complex was quantitated by densitometry of the autoradiograph. The given values shown represent the means of two independent experiments.
Role of IRF-1 in Induction of IFNA Genes

FIG. 4. Identification of αF1 binding proteins by UV cross-linking and Southwestern blotting analysis. A, in situ UV cross-linking; 20 μg of nuclear extract from NDV-infected L-cells was used in the binding reaction with uniformly labeled αF1 as described under “Experimental Procedures.” B, Southwestern blotting analysis; 150 μg of nuclear extracts from uninfected and infected L-cells (4 and 8 h) were separated by SDS-PAGE. Nuclear extracts from uninducible CEM cells were used as a control. C, UV cross-linking was done as described in A, using 10 μg of nuclear extract. The UV cross-linked complexes were treated with trypsin (0.5 μg/ml) or V8 (1 μg/ml) for 5 min at room temperature and DNA-protein adducts were separated by SDS-PAGE. In all figures, CONT. represents extracts from the uninduced cell and IND. is the extracts from NDV-treated cells. TYP and V8 represent complexes treated with trypsin or V8, respectively.

NF-κB sites present in HIV-1 LTR (35). In contrast to the αF1 probe, the NF-κB probe did not bind the 96- and 68-kDa proteins but showed a very low level of binding to the protein of 50–55 kDa, which we assume may represent the p50 subunit of NF-κB (data not shown). These results show that the observed binding of αF1 is specific. While the Southwestern blot hybridization shows the appropriate size of the αF1 binding proteins, the mobility of the UV cross-linked DNA-protein complex is expected to be affected by the presence of the 23-base pair long probe. However, the fact that similar mobility of the αF1 binding proteins was detected by both Southwestern hybridization and by the UV cross-linking indicated that the presence of the DNA oligonucleotide in the DNA-protein adduct does not greatly affect the mobility of these complexes.

The UV cross-linking in solution was used to compare the nuclear proteins binding to the α4 and α6F1 probes. To determine whether the DNA-protein complexes detected by the gel shift assay represent binding of different proteins to α4 and α6F1 probe with similar mobility, we digested the DNA-protein UV cross-linked adduct with proteolytic enzymes V8 or trypsin and analyzed the remaining DNA binding polypeptide, αF1, by SDS-PAGE. The results in Fig. 4C show no major difference in the proteolytic pattern between αF1 and α6F1 probes. These data suggest that the formation of induc- tion-specific B complex with α4 and α6F1 probes involves binding of the same proteins.

The Oligonucleotides Corresponding to IRF-1 and αF1 Binding Sites Can Inhibit Inducibility of A4IE—The insertion of multiple copies of AGTGAA hexamer in front of −54αCAT confers virus inducibility (31), while the insertion of three copies of αF1 has no effect.7 The relative contribution of αF1 and IRF-1 binding sites to the inducibility of A4IE was therefore tested by the competition assay. A constant amount of virus-inducible reporter plasmid, A4IE −88αCAT (pBK41), was cotransfected with an increasing amount of the competitor containing either three copies of αF1 sequence in front of the −54 CAT (PA4) or IRF-1 binding site (DS1/2; contains 12 copies of AGTGAA in pSP64 vector). Cotransfections with plasmids containing either the inducible (−109 IFNA4, pBR4) or uninducible (−54A4CAT, pBK29) promoter regions of IFNA4 gene were used for comparison. The results in Fig. 5 show that the transfection with either PA4 or DS1/2 inhibited the inducibility of pBR41. However, while an even 2-fold excess of the PA4 plasmid inhibited the inducibility by 40%, a 20-fold excess of DS1/2 plasmid was required to

7 W.-C. Au, Y. Su, N. B. K. Raj, and P. M. Pitha, unpublished data.

FIG. 5. In vivo competition assay. A reporter plasmid (pBR41) (0.5 μg) was cotransfected with various amounts of the competitor plasmid. These included pRB4, pRB29, pA4, pA10, and DS1/2 (listed in Fig. 1B). The amount of transfected DNA was kept constant. The results represent the mean of three separate experiments; the CAT activity in each experiment was normalized by considering 22% of CAT conversion as zero competition. The standard derivation of each competition result was less than 3%. Symbols representing the inhibitors are as follows: □, pA10, ◆, pRB4, □, pA4, ◇, DS1/2, ▲, pRB29.
achieve a similar degree of inhibition. Surprisingly, a further increase in the amounts of transfected PA4 did not lead to higher inhibition, while the increasing amounts of cotransfected DS1/2 plasmid led to a further decrease in the A4IE expression (55% inhibition). These results indicate that the levels of αF1 binding factors in the induced cells are very low and therefore can be easily competed out by very low amounts of αF1 binding sites. In contrast, the levels of IRF-1 specific factors in the induced cells are relatively high and therefore this factor(s) was not titrated out even with the highest concentration of the competitor. As expected, induction of pBK41 could be most effectively competed by pRB4 that contains the inducible –109 IFNA4 promoter region. The 40% inhibition was achieved with a 2-fold excess of the transfected plasmid and then the inhibition gradually increased with the amounts of plasmid transfected. Thus, the inhibition profile obtained with the pRB4 plasmid had combined characteristics of inhibition achieved by the PA4 and DS1/2 plasmids. Transfection with the pRB29 or plasmid carrying multiple copies of αF1 (pA10) did not inhibit the inducibility of the reporter gene; in contrast, the CAT activity was elevated when increasing amounts of pA10 plasmid were used, suggesting that the NDV-induced repressor can effectively bind the αF1.

Lack of IRF-1 Binding to A4IE—We demonstrated that the IRF-1 binding site plays a role in the induction of IFNA genes by mutagenesis analysis and cotransfection experiments in the absence of detectable binding of IRF-1 to the αF1 sequence. We, therefore, tested whether the IRF-1 binds to the A4IE region and found that, while the purified human IRF-1/ISGF-1 binds effectively to AGTGAA repeats, it was unable to bind A4IE under the same experimental condition (Fig. 6, A and C). However, a weak retarded band was detected when the binding to the A4IE sequence was done in the absence of a nonspecific competitor (data not shown). These results indicate that IRF-1 binds only weakly to the single IRF-1 site present in the A4IE region. To determine whether this inefficient binding was due to the difference in affinity of human IRF-1 and mouse IRF-1 for the IRF-1 site in A4IE, we synthesized the murine IRF-1 in vitro using the rabbit reticulocyte lysate translation system. The translation of the IRF-1 mRNA was confirmed by the presence of a [35S]labeled protein on SDS-acylamide gel, which showed the mobility of IRF-1 (52 kDa) (Fig. 6B). However, when we analyzed the binding of murine IRF-1 to A4IE, we did not observe any difference in the binding pattern between the control and IRF-1 containing reticulocyte lysates, while the rabbit reticulocyte proteins showed high binding affinity for A4IE (data not shown). In contrast, the binding of lysates containing IRF-1 to AGTGAA multimers showed formation of two complexes not present in the control lysates (Fig. 6C). The mobility of one of the IRF-1-specific complexes (faster moving complex) could be supershifted by the anti-IRF-1/ISGF-2 antibodies. The incubation of the purified human IRF-1/ISGF-2 with AGTGAA multimers also led to the formation of two complexes. One of them had mobility similar to that of the newly formed complex with the IRF-1 synthesized in rabbit reticulocyte lysate. We assume that the difference in the mobility of these two complexes is due to the different occupancy of the multiple IRF-1 binding sites in the AGTGAA multimers by IRF-1 or by binding of the homodimers. Addition of anti-IRF-1 antibodies led to the formation of a new, slowly moving complex; the mobility of the supershifted complex was similar to that obtained with in vitro translated murine IRF-1. These data indicate that the binding of both human and murine IRF-1 to the single IRF-1 site in the A4IE probe is much weaker than its binding to the multiple IRF-1 sites present in the AGTGAA multimers.

Differential Effect of IRF-2 Overexpression on NDV and IRF-1-mediated Inducibility of the IFNA-α Promoters—It was shown by the cotransfection studies that, while the IRF-1 can activate a promoter consisting of multiple copies of PRD-1, IRF-2 inhibits the IRF-1-mediated transactivation by competing for the same binding site (13). To further determine the contribution of the IRF-1-mediated activation to the induction of the IFNA promoter region, we examined the effect of IRF-2 on the IRF-1- and NDV-mediated induction of the CAT plasmid containing either −152 IFNA4 (pRB23) or −145 IFNA6 promoter regions. As a control, we used the plasmid containing 12 repeats of AGTGAA inserted in front of −54αCAT. Cotransfection of pRB23 with IRF-1 expressing plasmid led to induction of CAT activity in the transfected cells (Table I). In contrast, cotransfection of this plasmid with IRF-1 and IRF-2 expressing plasmids led to 75% inhibition of IRF-1-induced CAT activity, indicating that the IRF-2 encoded by the transfected IRF-2 expression plasmid competed effectively with IRF-1 for binding to A4IE. As shown previously (29), expression of the pRB23 plasmid was also induced effectively with NDV; however, the overexpression of IRF-2 inhibited only slightly (39%) the virus-mediated induction of pRB23 plasmid. The fact that IRF-2 was able to negate
only partially the virus induction suggests that factor(s) other than IRF-1 is involved in the induction process or that IRF-2 is modified in infected cells and looses its suppressor activity (36). In contrast, the induction of the −145A6CAT plasmid by IRF-1 was much less efficient than induction of pRB23 plasmid (compare 2.4 and 24% conversion, respectively); viral infection and cotransfection with IRF-2 expressing plasmids inhibited effectively the IRF-1-mediated transactivation. This suggests that a factor induced by NDV suppressed the IRF-1-mediated transactivation.

The Effect of IRF-1 and IRF-2 Antisense—To determine the contribution of de novo synthesized IRF-1 and IRF-2 in the virus-mediated induction, we examined the effect of the IRF-1 and IRF-2 antisense on the virus-induced expression of IFNA4 and A6 promoters. The plasmids (IRF-1AS and IRF-2AS) in which the expression of the IRF-1 and IRF-2 antisense was regulated by the cytomegalovirus early promoter were constructed as described under “Experimental Procedures.” The effectiveness of IRF-1AS was first confirmed by its ability to inhibit the IRF-1-mediated activation of pRB23 plasmid (Table I) in transient cotransfection assay. Similarly, the expression of IRF-2AS was demonstrated by its ability to partially reverse the inhibitory effect of IRF-2. However, neither the transfection with IRF-1AS nor with IRF-2AS, at a concentration sufficient to inhibit IRF-1-mediated transactivation, had any significant effect on the NDV-induced expression of pRB23 plasmid. These results suggest that the activation of the IRF-1 in the infected cells occurs mainly at the posttranslational level, and the contribution of de novo synthesized IRF-1 and IRF-2 was minimal within the time interval examined.

**DISCUSSION**

The virus-mediated induction of IFNA and -B gene expression shows a cell type specificity; a question remains as to what determines this specificity? Previous studies have shown that induction of IFNB genes requires the presence of two inducible elements, PRD-I and -II, in the IFNB gene promoter. The activation of transcription was shown to be mediated by the binding of IRF-1, NF-κB, and HMG-1 factors to PRD-I and -II and by their synergetic interaction (19, 21). The role of IRF-1 in the inducibility of IFNA genes has been suggested by transient transfection experiments; however, mutational analysis of the murine IFNA promoter established the importance of a novel domain-aFl, in the inducibility of these genes. The lack of inducibility of IFNA gene promoters could be associated with both the mutation in the IRF-1 site as well as in the aFl sequence (30). In this study, we have shown that the inducibility of IFNA4 gene is associated with the binding of the induction specific complex (aFl/B) to the aFl sequence. The formation of this complex coincides with the expression of IFNA and IFNB genes and likewise does not require de novo protein synthesis. The aFl/B complex is composed of at least two nuclear DNA binding proteins of 68 and 96 kDa. The sizes of these proteins do not correspond to the previously identified IRF-1 (50 kDa), and the mobility of the aFl/B complex could not be supershifted by anti-IRF-1/ISGF-2 antibodies. The binding of these two proteins to aFl does not occur exclusively in the infected cells but can also be detected in uninfected cells where they form a DNA-protein complex with faster mobility (Fig. 7). Thus, virus infection does not result in the induction of a specific aFl binding protein but facilitates either the heterodimer formation between the 68- and 96-kDa aFl binding proteins or the interaction between the aFl binding proteins and another accessory protein. Direct evidence showing that the binding of aFl proteins plays a role in virus induction was demonstrated by the cotransfection of aFl oligonucleotide with the A4IE containing reporter plasmid, which showed that as little as 2-fold excess of aFl multimers was able to decrease virus inducibility. These data strongly suggest that the binding of aFl proteins is one of the essential steps in the induction process.

Our data also showed that neither the purified human IRF-1/ISGF2 nor the in vitro translated murine IRF-1 can bind effectively to the aFl oligomer or to the single IRF-1 site in the A4IE sequence. However, there are at least three indications that the binding of nuclear protein (presumably IRF-1) to the IRF-1 site plays a role in the inducibility of the murine A4IE region. 1) The replacement of the −92 G residue in the IRF-1 site completely abolishes inducibility of A4IE (27). 2) Overexpression of IRF-1 in the transient expression assay activates expression of the A4IE and A6IE. 3) The IRF-1 binding oligomer (PRD-I) inhibits the virus-mediated induction of A4IE or the IFNA4 promoter region. These results indicate that the activation of the IFNA promoter involves the synergistic interaction between IRF-1 and aFl binding proteins; this interaction may stabilize the binding of IRF-1 at the low affinity site. The observation that IRF-1 is not able to activate the expression of the endogenous IFNA and -B genes (29) suggests that IRF-1 alone is not able to induce the expression of IFNA genes but has to interact with factors binding to the site adjacent to the IRF-1 binding site such as aFl in the IFNA and NF-κB in IFNB virus responsive elements (Fig. 7). The interaction between nuclear factors from distinct families plays a role in expression of other genes of the inflammatory response; transactivated transcription of the rat α-1 acid glycoprotein gene involves interaction between NF-IL-6 factor and the glucocorticoid receptor, and the transcription of IL-6 and IL-8 genes requires synergism between NF-IL-6 and NF-κB (37). In the IFN system, the ternary complex between ISGF3α complex and the 48-kDa (ISGF3γ) protein increases the affinity of ISGF3γ for interferon-stimulated response element present in the promoters of IFN-stimulated genes (38, 39). The functional role of the

<table>
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<th>Reporter</th>
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<th>NDV induction</th>
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IRF-1 binding site was demonstrated by inhibition of the IFNA promoter expression by cotransfection with the IRF-1 binding oligonucleotide but not with the IRF-1 antisense. Because the antisense transcripts can affect both mRNA stability and its translatability, our data indicate that the de novo synthesis of IRF-1 is not required for the virus-mediated induction. Presently, however, we cannot completely eliminate the possibility that virus-mediated activation may not require IRF-1, but another virus-activated factor, binding to the IRF-1 site, because the functional redundancy of IRF-1 was recently demonstrated in mice, where the deletion of IRF-1 gene did not abolish the virus-mediated induction of IFN genes.

The GTAAGAAGGT sequence recognized by the αF1 binding proteins contains the GAAANN motif found not only in the PRD-I of the IFNB promoter but also in the interferon-sensitive response element of many IFN stimulated genes. This imperfect inverted motif was also recently identified in the inducible domain of the human IRF-1 promoter. A number of regulatory proteins recognize the GAAANN core sequence. Presently, we cannot eliminate the possibility that the 68-96-kDa αF1 binding proteins are identical to some of the known members of the GAAANN binding proteins, and additional studies to identify these proteins are in progress.

A surprising finding was that, while virus infection enhanced the IRF-1-mediated expression of IFNA4 promoter, it suppressed the IRF-1-mediated expression of the IFNA6 promoter that is not inducible in infected L-cells. The mutations in A41E that abolished its inducibility did not result in the alteration of the binding of αF1-specific proteins to the A4 and A6F1. The abrogation of transcriptional activity of the regulatory domains, but not its binding activity, was also observed with other factors such as the glucocorticoid receptor (44) and the muscle cell-specific transcription factor (45).

The nature of virus-induced suppression is not known, but we assume that the virus-activated repression involves displacement of the bound IRF-1 (Fig. 7). It is unlikely that this suppressor is IRF-2, which is proteolytically cleaved in infected cells with a consequent loss of its inhibitory activity (36). Because the binding of the αF1-specific proteins to the A6F1 sequence is much weaker than to the A4F1 sequence, we speculate that the association of the α1/B complex with the virus-induced repressor may stabilize the binding to the A6F1 site more effectively than the association with the IRF-1. It was recently shown that interaction between the cellular protein, OCT-1, and the viral protein, VP16, leads to corecruitment of these proteins to the new binding site and to the change in specificity of the OCT-1-mediated transcriptional activation (46). By analogy the cell-specific expression of IFNA genes may be a result of formation of a ternary complex between αF1/B and virus-induced activator or suppressor binding to the IRF-1 site as schematically shown in Fig. 7.

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REFERENCES

Role of IRF-1 in Induction of IFNA Genes