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Inducible Processing of Interferon Regulatory Factor-2

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PRDI-BFc and PRDI-BFi are proteins that bind specifically to a regulatory element required for virus induction of the human beta interferon (IFN-β) gene. PRDI-BFc is a constitutive binding activity, while the PRDI-BFi binding activity is observed only after cells are treated with inducers such as virus or poly(I) · poly(C) plus cycloheximide or in some cells by cycloheximide alone. In this paper we report that PRDI-BFc is interferon regulatory factor-2 (IRF-2), a known transcriptional repressor. In addition, we find that PRDI-BFi is a truncated form of IRF-2, lacking approximately 185 C-terminal amino acids. Thus, PRDI-BFi appears to be generated by inducible proteolysis. Although the affinity of PRDI-BFc/IRF-2 for the IFN-β promoter does not appear to be affected by the removal of C-terminal amino acids, the ability of PRDI-BFi to function as a repressor in cotransfection experiments is significantly less than that of intact IRF-2. Studies have shown that IRF-2 can block the activity of the transcriptional activator IRF-1, which also binds specifically to the IFN-β gene promoter. Thus, the inducible proteolysis of IRF-2 may be involved in the regulation of the IFN-β gene or of other genes in which the ratio of IRF-1 to IRF-2 can affect the level of transcription.

The human beta interferon (IFN-β) gene is highly inducible by virus or synthetic double-stranded (ds) RNA [poly(I) · poly(C)] (for a review, see references 5 and 50). Prior to induction the gene is inactive, but within 90 min after virus infection, IFN-β mRNA can be detected. The level of IFN-β mRNA continues to increase and reaches a level of approximately 2,000 molecules per cell between 6 and 12 h postinduction. Subsequently, the level of IFN-β mRNA rapidly decreases, returning to nearly preinduction levels 20 to 24 h after infection (34, 42). The increase in IFN-β mRNA after induction is a result of transcriptional activation of the gene, and this activation does not require protein synthesis in most cell types (47, 48, 55). In contrast, the postinduction decrease in IFN-β mRNA levels, which is due to a combination of transcriptional repression and rapid turnover of the mRNA, requires ongoing protein synthesis (47, 48, 55). These observations suggest that virus infection leads to the activation of preexisting transcription factors and/or inactivation of repressors. Postinduction repression, on the other hand, appears to involve repressors that are synthesized during virus infection. There is some evidence that these repressors are encoded by genes that are themselves inducible by virus infection (21, 27, 56).

The regulatory sequences required for this transient induction are located within an approximately 200-bp region immediately upstream from the transcription start site of the gene (for a review, see reference 34). Detailed studies of this region have revealed a complex array of positive and negative regulatory sequences. With respect to positive control, four distinct sequence elements, designated PRDI through PRDIV (positive regulatory domains I to IV), are required for maximal levels of induction (7, 9, 19, 29, 34, 59). In the intact promoter these elements interact synergistically to activate the gene. However, two or more copies of any of these elements or one copy each of PRDI and PRDII are capable of conferring virus induction on a heterologous promoter (7, 9, 29). PRDI contains two copies of the hexamer sequence AAGTG(A/G), and a synthetic 6-bp (AAGTGA) oligomer can also function as a virus-inducible enhanson (9, 14, 17, 28, 29, 32, 38, 41). Analysis of promoters containing four copies of the PRDI hexamer or two copies of PRDII show that these elements are sufficient not only for virus induction but also for postinduction repression (56). Thus, these elements can function as either positive or negative regulatory sequences and must therefore be the targets of transcriptional activators and repressors.

Considerable effort has been made to identify transcriptional regulatory proteins that bind specifically to the IFN-β promoter (for a review, see reference 34). In particular, a number of proteins that bind specifically to the PRDI and PRDII elements have been identified. The proteins designated interferon regulatory factor-1 (IRF-1) and IRF-2 are homologous proteins which bind specifically to both PRDI and another element of the IFN-β promoter called PRDIII (16, 21, 36). A number of observations suggest that IRF-1 and possibly IRF-2 are involved in regulating IFN-β gene expression. Cotransfection studies have shown that IRF-1 can act as a transcriptional activator on promoters containing multiple copies of PRDI hexamer (21, 29, 32, 38), while IRF-2 can function as a repressor of IRF-1-activated gene expression (21, 22). High levels of IRF-1 expression in mouse L929 and monkey COS cells can also activate the endogenous IFN-α and IFN-β genes (12, 21, 22). Finally, stable transfectants constitutively expressing antisense IRF-1 mRNA produced little or no IFN-β mRNA and protein after induction with poly(I) · poly(C) or Newcastle disease virus (43). IRF-2 may be involved in the pre- and/or postinduction repression of the IFN-β gene. Another protein, designated PRDI-BF1, may also be a postinduction repressor. In contrast to IRF-2, PRDI-BF1 is able to block virus induction of the IFN-β promoter (27).

The only inducible, cycloheximide (CHX)-insensitive PRDI-binding activity thus far identified is a protein designated PRDI-BFi (i stands for inducible). This protein and a constitutive PRDI-binding protein designated PRDI-BFc (c stands for constitutive) were first detected in extracts from human MG63 cells (26). PRDI-BFc was found to be present before and after induction, while PRDI-BFi is present only after the cells are treated with virus or poly(I) · poly(C) and

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CHX. The DNA sequence requirements for PRDI-specific binding were identical for PRDI-BFi, PRDI-BFc, IRF-1, and IRF-2 (21, 26, 36). In addition, the DNase I footprints for these two proteins were indistinguishable from each other and from those of IRF-1 and IRF-2.

In this paper we investigate the relationship between these four proteins and examine the possible role of PRDI-BFi and PRDI-BFc in the transcription of the IFN-β gene. We purified both of these proteins from CHX-treated HeLa cells and showed that PRDI-BFc is identical to IRF-2. In addition, we found that PRDI-BFi is a C-terminal truncated form of IRF-2. Furthermore, we found that IRF-2 repressed the IRF-1-dependent activation of the human IFN-β promoter, while a truncated form of IRF-2, having properties similar to those of PRDI-BFi, only weakly repressed IRF-1-stimulated gene expression. These observations suggest that inducers of IFN-β decrease the negative regulatory activity of IRF-2 by proteolytic processing.

MATERIALS AND METHODS

Cell culture and inductions. Human HeLa, human MG63, and simian COS cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Mouse L929 cells were maintained in minimum essential medium plus 5% fetal bovine serum. HeLa S_3 cells (6) were grown in RPMI 1640 supplemented with 5% horse serum. HeLa S_3 cells were grown in spinner flasks and maintained at 3×10^5 to 6×10^5 cells per ml.

For virus induction, cells were grown to confluence and treated with Sendai virus (SPAFAS, Inc.) as described previously (18). Cells were induced with poly(I) · poly(C) plus CHX as described by Keller and Maniatis (26) except that for L929 and COS cells the poly(I) · poly(C) was added with 0.5 mg of DEAE-dextran per ml. In these experiments, control samples received 0.5 mg of DEAE-dextran per ml in serum-free medium alone. Induction of HeLa S_3 cells (6×10^5 to 8×10^5 cells per ml) was with 200 μg of CHX per ml for 6 h. The CHX was added directly to the spent medium of the cultures.

Extract preparation and electrophoretic mobility shift assay (EMSA). Whole-cell extracts were prepared by the method of Fujita et al. (16) with some modification. Briefly, the cells were washed with phosphate-buffered saline (PBS), harvested, and collected by centrifugation. The cell pellet was frozen at −80°C and thawed by the addition of 3 volumes of lysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 0.2 mM EDTA, 0.2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.5 mM spermidine, 0.15 mM spermine, 1.0 mM dithiothreitol [DTT], 10% glycerol, 0.5 mM phenylmalanine sulfonyl fluoride [PMSF], 2 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 500 μM L-1-tosylamide-2-phenylalanine chloromethyl ketone, 25 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 0.5 mM benzamidine, 5 μg of aprotinin per ml, 10 mM sodium molybdate, 2.0 mM sodium PP_i, 2.0 mM sodium orthovanadate). In some cases the leupeptin concentration was increased to 100 μg/ml, with no apparent effect on EMSA binding patterns. After thawing, 2 M KCl was added (one cell-pack volume) and mixed at 4°C for 30 min. The samples were centrifuged at 80,000 × *g* for 60 min at 4°C. The supernatant was collected and diluted with lysis buffer to give a final KCl concentration of 0.15 M. The sample was centrifuged at 13,000 × *g* to remove any precipitate. The extracts were aliquoted and stored at −80°C. Protein concentration was measured by using the Bradford reagent (Bio-Rad).

Nuclear extracts were made from CHX-treated HeLa S_3 cells as previously described (1, 6) except that the final dialysis was against buffer A (20 mM Tris [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) plus 0.2 M KCl. Protein concentrations of the nuclear extracts were typically 8 to 16 mg/ml.

PRDI-BFc and PRDI-BFi activity was determined by using the EMSA (11). The binding reaction mixtures contained 20 mM HEPES or Tris (pH 7.5), 20 to 100 mM KCl (final), 0.5 mM DTT, 0.1 mM EGTA, 1.0 mM $MgCl_2$, 5% glycerol, and 2 μg of poly(dI-dC) · poly(dI-dC). Extracts were mixed in a 10- or 20-μl reaction mixture with 0.2 to 0.8 ng of end-labeled PRDI hexamer [$(AAGTGA)_4$] or the interferon gene regulatory element (IRE; −77 to −37) (18) probe at 0°C for 30 min. The reaction was analyzed by electrophoresis in a nondenaturing 4.5% polyacrylamide gel at 4°C in 25 mM Tris–192 mM glycine–1 mM EDTA. For antibody (Ab) inhibition experiments, the extracts were preincubated with the appropriate antiserum (anti-human IRF-1 serum kindly provided by R. Pine and J. E. Darnell [40] and anti-murine IRF-2 serum kindly provided by H. Harada and T. Taniguchi [22]) for 30 min at 0°C before addition of radiolabeled probe for another 30 min.

Purification of PRDI-BFc and PRDI-BFi. All chromatographic separations were performed at 4°C. The column fractions were assayed for PRDI-BFc and PRDI-BFi activity by EMSA with the PRDI hexamer as a probe. Nuclear extract from 40 liters of HeLa S_3 cells was applied to a DE-52 (Whatman) column (10 mg of protein per ml of packed-bed volume) equilibrated in buffer A–0.2 M KCl. The flowthrough was collected, and the fractions were pooled. The active flowthrough fractions were then applied to a heparin Sepharose (Pharmacia) column (16 mg of protein per ml of bed volume) equilibrated in buffer A–0.2 M KCl. After the column was washed with starting buffer, proteins were step eluted with buffer A–0.32 M KCl and buffer A–0.6 M KCl. PRDI-BFc and PRDI-BFi eluted at 0.6 M KCl. The active fractions were pooled and frozen at −80°C. The heparin Sepharose active pool was adjusted with buffer A (without KCl) to 0.3 M KCl. The sample was centrifuged, and the supernatant (20 mg of protein) was incubated with 100 μg of poly(dI-dC) · poly(dI-dC) per ml for 15 min at 0°C. The sample was then loaded onto a 2-ml specific PRDI hexamer oligonucleotide-Sepharose column equilibrated with buffer A, 0.3 M KCl, and 0.5% Nonidet P-40. The specific oligonucleotide DNA affinity column was prepared by annealing oligonucleotides 5′ GATCCAAGTGAAAGT GAAAGTGAAAGTGA 3′ and 5′ GATCTCACTTTCACTT TCACTTTCACTTG 3′, ligating the ds oligonucleotide and coupling it to cyanogen bromide-activated Sepharose CL-2B (Pharmacia) as described by Kadonaga and Tjian (25). The flowthrough was collected and immediately reloaded onto the column. After the column was washed with starting buffer, PRDI-BFc and PRDI-BFi were eluted with buffer A–0.6 M KCl. The column fractions were frozen at −80°C until assay. The active fractions were then pooled, diluted with buffer A (without KCl) to 0.3 M KCl, and centrifuged to remove any precipitate. Poly(dI-dC) · poly(dI-dC) (50 μg/ml) was added to the supernatant, and the sample was reloaded onto the recycled 2-ml specific oligonucleotide affinity column. The DNA affinity column was recycled by extensive washing with buffer A, 2.0 M KCl, and 1 mM EDTA and reequilibration with buffer A–0.3 M KCl. Chromatography was as described above. Active fractions were collected in

siliconized tubes, pooled, and passed through the DNA affinity column for a third time. All fractions were frozen at −80°C, and activity was tested by EMSA. Purified PRDI-BFc and PRDI-BFi gave EMSA DNA binding profiles identical to those of PRDI-BFc and PRDI-BFi from crude nuclear extracts. In addition, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis and silver staining of gels were performed after the second and third PRDI hexamer column steps to analyze the purification.

Photoaffinity cross-linking and renaturation of PRDI-BFc and PRDI-BFi. UV cross-linking was done as previously described (56). Briefly, the PRDI-BFc and PRDI-BFi protein-DNA complexes from CHX-induced HeLa cells were resolved by EMSA by using a 5-bromodeoxyuridine-substituted PRDI hexamer as probe. The complexes were UV cross-linked in the gel, excised, eluted, and separated by SDS–12% PAGE. The samples were not DNase I treated prior to SDS-PAGE.

Renaturation of PRDI-BFc and PRDI-BFi was done essentially as described by Baeuerle and Baltimore (2). CHX-induced HeLa cell nuclear extract or purified PRDI-BFc and PRDI-BFi was separated by SDS–12% PAGE. Molecular size fractions were cut from the gel, and the proteins were eluted from the gel, precipitated with acetone, and denatured with 8 M urea. The proteins were then renatured in 20 mM Tris (pH 7.5), 10 mM KCl, 1.0 mM DTT, 20 μM PMSF, and 0.2 mM EDTA. These fractions were analyzed by EMSA with the PRDI hexamer as a probe.

Plasmid construction. Full-length human IRF-2 cDNA (a gift from H. Harada and T. Taniguchi [24]) was cloned into the mammalian expression vectors pcDNA1 (Invitrogen) and pXM (27). pXM has the adenovirus major late promoter at the site of cDNA insertion and the simian virus 40 replication origin (58). For the peptide-tagging experiments, the first 7 amino acids of human IRF-2 were removed by fusing an 11-amino-acid *myc* tag (a *Hin*dIII-*Eco*RI fragment, made blunt by filling in with Klenow [10, 37]) in frame to the N terminus of IRF-2 at the *Fsp*I site (*Fsp*I-*Xba*I fragment, +118 to +1358). This construct was then cloned into the pcDNA1 mammalian expression plasmid.

pcDNA1 and pXM human IRF-2 C-terminal deletion constructs were generated by using the convenient restriction sites *Ava*II (+575) and *Bst*EII (+710) within the IRF-2 cDNA. These IRF-2 deletion constructs were subcloned to put them in frame with a stop codon. The IRF-2/*Ava*II construct contains amino acids 1 to 160 [IRF2(Δ160)], and the IRF-2/*Bst*EII construct encodes amino acids 1 to 206 [IRF2(Δ206)]. The additional C-terminal amino acids following the IRF2(Δ160) and IRF2(Δ206) deletions that differ from IRF-2 are as follows: IRF2(Δ160), Arg; and IRF2(Δ206), Ile and His. The pcDNA1 deletion constructs were expressed and translated in vitro by using a kit from Promega. The *myc* tag was also fused to the IRF2(Δ160) and IRF2(Δ206) constructs at the N-terminal *Fsp*I site (+118). These were then cloned into the pcDNA1 vector as described above.

Transfection analysis. COS and HeLa cells were seeded in 10-cm plates 1 day prior to transfection and were approximately 50% confluent at the time of transfection. COS cells were transfected by using the standard DEAE-dextran method (10, 46). Typical COS cell transfections included pXM vector (as control), pXM human IRF-2, or the pXM IRF-2 deletion constructs (3 μg of DNA). Forty-eight hours after transfection, the cells were induced with poly(I) · poly(C) plus CHX for 6 h as described above (26). Whole-cell extracts were prepared and analyzed by EMSA.

HeLa cells were transfected with calcium phosphate by using the method of Chen and Okayama (4). Sixteen hours after transfection, the cells were shocked with 15% glycerol in PBS (1 min, 37°C), washed with PBS, and refed with fresh medium. In one set of experiments, HeLa cells were transfected with pCMVβ-LacZ (33) transfection control (5 μg) and 25 μg of pcDNA *myc* tag-IRF-2 or pcDNA1 vector. Forty-eight hours after transfection, the cells were induced with poly(I) · poly(C) plus CHX (26). Whole-cell extracts were prepared and separated (5 μg) by SDS–12% PAGE. The proteins were then transferred to nitrocellulose and incubated with anti-*myc* tag monoclonal Ab 9E10 (10). The secondary Ab was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad). The proteins were visualized by color developing with NBT and BCIP (Promega). β-Galactosidase activity in the extracts was measured as described previously (9).

For cotransfection analysis, HeLa cells were transfected with 5 μg of −104 human IFN-β chloramphenicol acetyltransferase (CAT) reporter construct (27), 5 μg of pCMVβ-LacZ transfection control, and 20 μg of cDNA expression plasmids. Forty hours after transfection, extracts were prepared for CAT assays as described previously (20). CAT assays were normalized to β-galactosidase activities (9). For RNase protection assays, HeLa cells were transfected with 5 μg of −104 human IFN-β CAT reporter and 20 μg of expression plasmids as indicated. The πSV40 α-globin plasmid (0.5 μg) was also transfected as an internal transfection control (9). RNA was isolated 16 or 24 h after transfection by the guanidium thiocyanate procedure (46). RNase protection experiments were carried out as previously described (9, 46) by using 50 μg of total RNA. The probe used to detect correctly initiated CAT mRNA was psp73 −40 IFN-β CAT/*Pvu*II, which protects a 170-bp fragment. The probe used to detect γ-actin mRNA was psp64 γ-actin/*Hin*fI (8), which protects approximately 140 nucleotides. The probe for 5′ α-globin mRNA protects approximately 135 nucleotides.

RESULTS

Purification and characterization of PRDI-BFc and PRDI-BFi. PRDI-BFi binding activity was originally identified in poly(I) · poly(C) plus CHX-treated MG63 cells (26). Subsequently, we detected PRDI-BFi in a variety of other cell types induced by dsRNA and CHX (i.e., L929, COS, and HeLa cells) and this binding activity was also detected in Sendai virus-infected cells (39a). However, induction of PRDI-BFi binding activity is weaker in virus-treated cells compared with poly(I) · poly(C) plus CHX-induced cells (data not shown).

While examining the effect of different agents on PRDI-BFc and PRDI-BFi binding activity, we found that CHX alone could induce PRDI-BFi in HeLa cells. In the absence of CHX only PRDI-BFc is detectable by EMSA with PRDI hexamer as a probe (Fig. 1, lane 1). In contrast, both PRDI-BFc and PRDI-BFi are present after induction with CHX (Fig. 1, lane 2). The induction of PRDI-BFi is slightly less than that seen with poly(I) · poly(C) plus CHX (data not shown). In addition there is less PRDI-BFc present after CHX induction in this experiment (Fig. 1), suggesting a reciprocal relationship in the amounts of the two proteins. However, in other experiments there was only a slight decrease in PRDI-BFc binding activity after induction. This variability in the amount of PRDI-BFc present after induction was also seen in MG63 and L929 cells (data not shown).

To determine the molecular masses of PRDI-BFc and

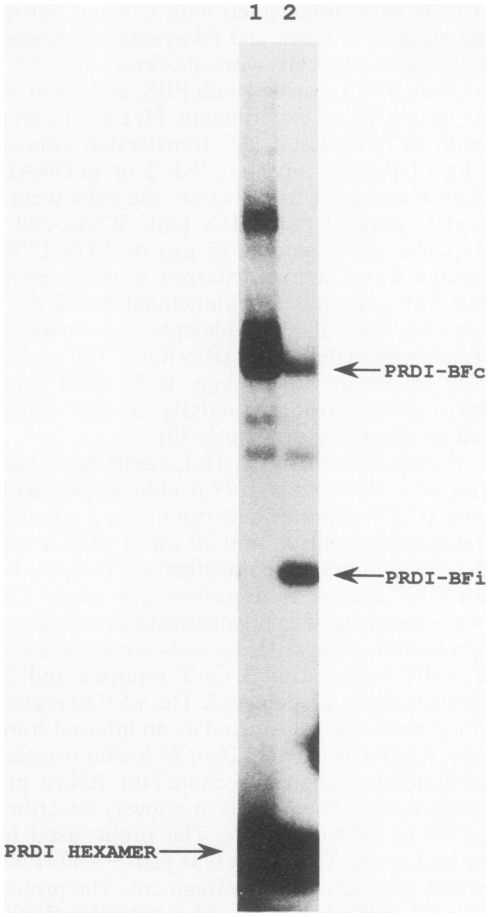


FIG. 1. PRDI-BFi binding activity is inducible by CHX in HeLa cells. HeLa S_3 cells were untreated (lane 1) or treated (lane 2) with 200 μg of CHX per ml for 6 h. Nuclear extracts (2 μg) were prepared and analyzed by EMSA with PRDI hexamer probe as described in Materials and Methods. Arrows indicate PRDI-BFc and PRDI-BFi protein-DNA complexes. The hexamer probe is functionally equivalent to two PRDI sites, and the more slowly migrating PRDI-DNA binding complexes in lane 1 are probably due to the simultaneous binding of proteins to each of the two PRDI sites.

PRDI-BFi, CHX-treated HeLa cell nuclear extract was separated on an SDS-polyacrylamide gel, and different size fractions were excised. The proteins were eluted and renatured. PRDI hexamer DNA binding activity corresponding to PRDI-BFc was identified in the size fractions corresponding to approximately 60 to 50 kDa and 50 to 45 kDa (Fig. 2A). PRDI-BFi binding activity was renatured from a molecular size fraction of approximately 28 to 25 kDa (Fig. 2A). To further characterize PRDI-BFc and PRDI-BFi, these factors were purified from CHX-treated HeLa S_3 cells as described in Materials and Methods. SDS-PAGE analysis and silver staining of an active fraction from the third hexamer DNA affinity column step revealed three major bands, corresponding to molecular masses of >100, 50, and 24 kDa and a minor doublet of around 40 kDa (data not shown). The purification of a 50- and a 24-kDa protein correlates well with the presence of PRDI-BFc and PRDI-BFi binding activity in molecular size fractions of approximately 50 and 28 to 25 kDa, respectively (Fig. 2A). PRDI binding activity was also detected in size fractions of 45 to 39 kDa (Fig. 2A) and probably corresponds to the doublet at 40 kDa seen with silver staining (not shown). These proteins, which are not consistently observed in different preparations, may be breakdown products of PRDI-BFc. A denaturation and renaturation experiment was also performed with highly purified PRDI-BFc and PRDI-BFi from the third hexamer column step, with essentially identical results.

In another experiment PRDI-BFc and PRDI-BFi were UV cross-linked to a labeled bromodeoxyuridine-substituted PRDI hexamer probe, and the cross-linked products were analyzed by SDS-PAGE (Fig. 2B). The molecular masses of PRDI-BFc and PRDI-BFi were determined by subtracting the molecular mass (14 kDa) of the DNA adduct. The small amounts of labeled PRDI-BFc in the PRDI-BFi lane and PRDI-BFi in the PRDI-BFc lane (Fig. 2B) are probably due to slight contamination when the proteins were resolved by EMSA and excised. The results from the photoaffinity cross-linking and denaturation and renaturation experiments indicated that the molecular masses of PRDI-BFc and PRDI-BFi are 50 and 24 to 27 kDa, respectively.

The amino acid composition of purified PRDI-BFc was determined after SDS-PAGE and transfer to a polyvinylidene difluoride membrane. Comparison of the amino acid composition of PRDI-BFc with that of IRF-1 and IRF-2 is consistent with the possibility that PRDI-BFc is a member of the IRF family of proteins (data not shown). However, on the basis of this information alone, we could not determine whether PRDI-BFc is IRF-1 or IRF-2.

Abs against IRF-2 recognize PRDI-BFc and PRDI-BFi. To determine whether PRDI-BFc and PRDI-BFi are indeed related to IRF-1 and IRF-2, we examined the effect of Abs against IRF-1 and IRF-2 on the formation of specific PRDI-BFc- and PRDI-BFi-DNA complexes in an EMSA. Abs against IRF-1 (40) had no effect on PRDI-BFc- and PRDI-BFi-DNA complex formation (data not shown). However, anti-IRF-2 Abs blocked the binding of both PRDI-BFc and PRDI-BFi to the IRE in an EMSA (Fig. 3). Whole-cell extracts from mouse L929 cells were used because the anti-IRF-2 serum was generated against murine IRF-2 (22). In this experiment, the binding of PRDI-BFc from uninduced (lanes 4 and 5) and poly(I) · poly(C)–CHX-induced (lanes 9 and 10) cells was inhibited by anti-IRF-2 serum. The formation of PRDI-BFi DNA binding complex from poly(I) · poly(C)–CHX-treated cells (lanes 9 and 10) also interacted with anti-IRF-2 Ab. Preimmune serum had no effect on the mobility of any of the complexes (Fig. 3, lanes 2, 3, 7, and 8). It should be noted that other protein-DNA complexes are detected in L929 cell extracts when the IRE is used as probe, and these correspond to factors that bind to PRDII (13, 30). The specific binding of PRDI-BFc and PRDI-BFi to the IRE can be competed with an excess of unlabeled IRE or PRDI DNA fragments (26, 39a).

We also investigated the effect of anti-IRF-2 serum on highly purified PRDI-BFc and PRDI-BFi from CHX-induced HeLa cells. In this experiment the antiserum specifically blocked the binding of PRDI-BFc and PRDI-BFi to the IRE (data not shown). Furthermore, the IRF-2 antiserum detected a 50-kDa protein in a Western immunoblot analysis by using an active fraction from the final PRDI-BFc and PRDI-BFi purification step (not shown). Even though this antiserum specifically recognized PRDI-BFi in an EMSA (Fig. 3), it did not recognize a 24- to 27-kDa protein in a Western blot. These results show that PRDI-BFc, PRDI-BFi, and IRF-2 are related to each other. PRDI-BFc appears to be full-length IRF-2 from the amino acid analysis data, the EMSA profile (Fig. 1) (22), the DNA footprinting and methylation interference patterns (21, 26, 36), the IRF-2 Ab inhibition experi-

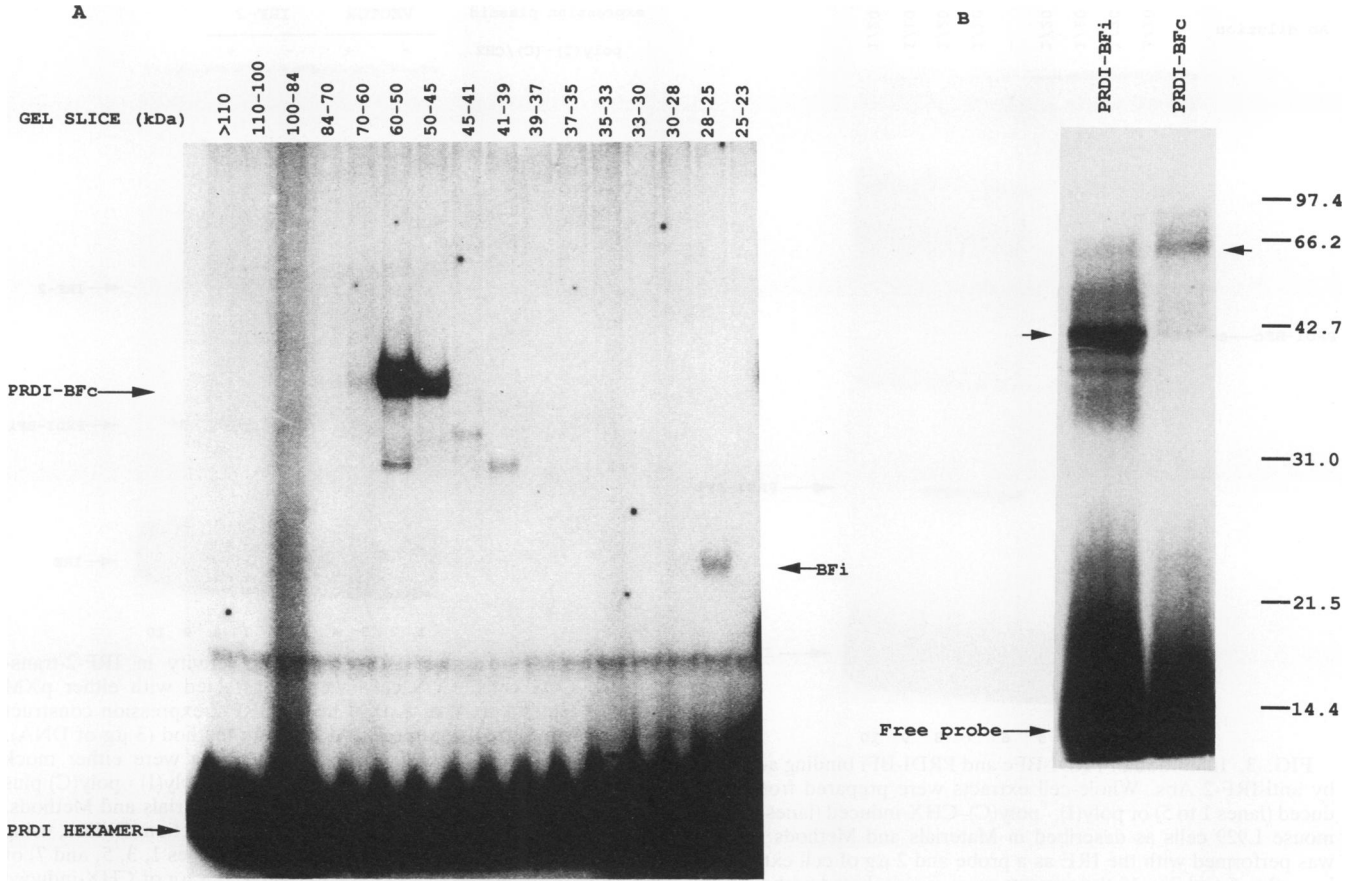


FIG. 2. Estimation of the molecular masses of PRDI-BFc and PRDI-BFi DNA binding activities. (A) CHX-induced HeLa S_3 cell nuclear extract (200 μg) was separated by SDS–12% PAGE under reducing conditions. Proteins from different molecular mass regions of the gel were eluted and renatured as described in Materials and Methods. PRDI hexamer binding activity was detected by EMSA with 10 μl of the renatured fractions. Molecular mass fractions and PRDI-BFc/PRDI-BFi binding activities are indicated. (B) A preparative EMSA was performed with 60 μg of CHX-induced HeLa cell nuclear extract and an internally labeled 5-bromodeoxyuridine-substituted PRDI hexamer oligonucleotide. The protein-DNA complexes were UV cross-linked in the gel, excised, eluted, and resolved by SDS–12% PAGE. The excised PRDI-BFi and PRDI-BFc complexes are specified above each lane, and the labeled cross-linked proteins are indicated by arrows. The free probe is also shown. Molecular mass markers (in kilodaltons) are indicated.

ments, the Western blot analysis, and the molecular mass (50 kDa) of purified PRDI-BFc. On the other hand, PRDI-BFi may be a truncated form of PRDI-BFc/IRF-2 and this could be why antibodies against IRF-2 preferentially bound to PRDI-BFc/IRF-2 compared with PRDI-BFi in the Western blot. A number of epitopes recognized by the polyclonal antiserum may be missing in PRDI-BFi. Alternatively, the anti-IRF-2 antiserum may not recognize denatured PRDI-BFi in a Western blot, in contrast to native PRDI-BFi in an EMSA (Fig. 3).

PRDI-BFi is a truncated form of IRF-2. To further investigate the relationship between PRDI-BFi and PRDI-BFc/IRF-2, we examined levels of PRDI-BFi in cells in which IRF-2 is overproduced. To do this, COS cells were transfected with a pXM human IRF-2 expression construct or pXM vector as a control, and 48 h after transfection the cells were induced with poly(I) · poly(C) plus CHX. In the control cells transfected with vector alone, there was a little PRDI-BFi activity before induction (Fig. 4; lanes 1, 2) compared with after poly(I) · poly(C)-CHX treatment (lanes 3 and 4) determined by an EMSA with the IRE as a probe. In the IRF-2-transfected cells, both IRF-2 and PRDI-BFi binding were detected before induction (lanes 5 and 6). Moreover, there was much more PRDI-BFi formed after induction (lanes 7 and 8). The amount of IRF-2 present after induction was decreased, and this was probably due to both the formation of PRDI-BFi from full-length IRF-2 and the effect of CHX on protein synthesis. Importantly, the PRDI-BFi activity generated after poly(I) · poly(C)-CHX treatment of the IRF-2-transfected cells comigrated with PRDI-BFi from HeLa cell nuclear extract in the EMSA (Fig. 4, lane 9). As a control, a pXM IRF-1 expression plasmid was transfected into COS cells, and after poly(I) · poly(C)-CHX induction there was no increase over the endogenous level in the amount of PRDI-BFi binding activity (data not shown). These observations provide further evidence that PRDI-BFi is a truncated form of IRF-2.

The DNA binding domain of IRF-2 is located in the N-terminal half of the protein (21). Therefore, PRDI-BFi must be a C-terminal truncated form of IRF-2. To address this question a peptide-tagging experiment (10, 37) was carried out by using an N-terminal *myc* tag-IRF-2 fusion construct. HeLa cells transfected with this construct produced a protein with a molecular mass of approximately 50 kDa detected by an anti-*myc* monoclonal Ab (10) on a Western blot (Fig. 5, lane 3). This is identical to the

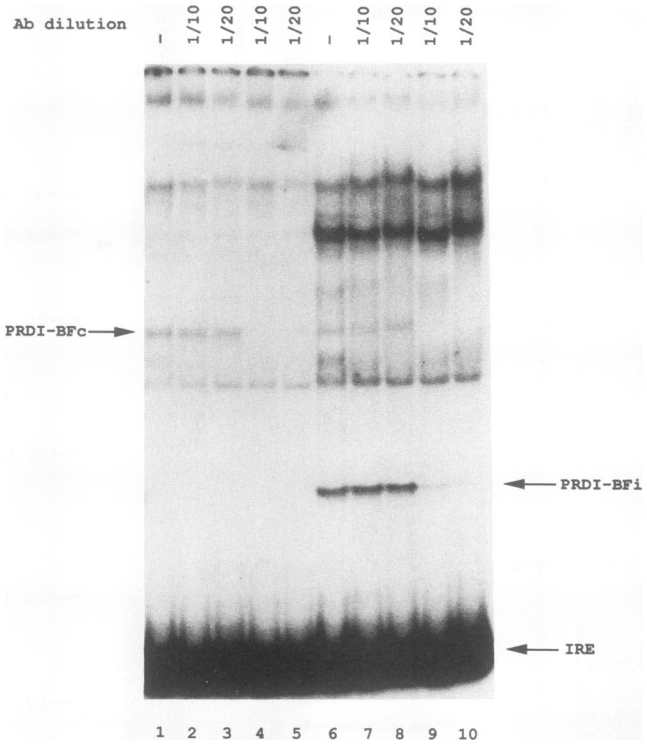


FIG. 3. Inhibition of PRDI-BFc and PRDI-BFi binding activities by anti-IRF-2 Abs. Whole-cell extracts were prepared from uninduced (lanes 1 to 5) or poly(I) · poly(C)–CHX-induced (lanes 6 to 10) mouse L929 cells as described in Materials and Methods. EMSA was performed with the IRE as a probe and 2 μg of cell extract. In lanes 2 to 5 and 7 to 10 the extracts were preincubated with the sera for 30 min at 0°C before addition of labeled probe. Lanes 2, 3, 7, and 8 received a dilution of preimmune serum, while lanes 4, 5, 9, and 10 received an appropriate dilution of IRF-2 antiserum as indicated. Samples from lanes 1 and 6 were untreated. PRDI-BFc and PRDI-BFi DNA binding activity is indicated. PRDI-BFc binding activity is much weaker on the IRE compared with on the hexamer (Fig. 1), because both PRDI-BFc and PRDI-BFi appear to have a higher affinity for the hexamer probe.

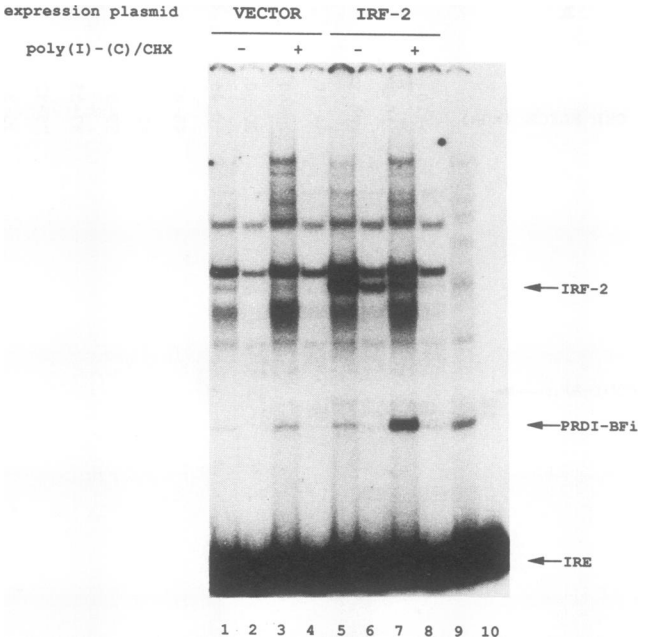


FIG. 4. Increased PRDI-BFi binding activity in IRF-2-transfected COS cells. COS cells were transfected with either pXM vector (lanes 1 to 4) or a pXM human IRF-2 expression construct (lanes 5 to 8) by using the DEAE-dextran method (3 μg of DNA). Forty-eight hours after transfection, the cells were either mock induced (lanes 1, 2, 5, and 6) or induced with poly(I) · poly(C) plus CHX (lanes 3, 4, 7, and 8) as described in Materials and Methods. Whole-cell extracts were prepared 6 h after induction. EMSA, using the IRE as probe, was performed with 3 μg (lanes 1, 3, 5, and 7) or 1 μg (2, 4, 6, and 8) of COS cell extract and 8 μg of CHX-induced HeLa cell nuclear extract (lane 9). Lane 10 is free IRE probe. IRF-2 and PRDI-BFi are indicated by arrows.

molecular mass of PRDI-BFc. Treatment of these transfected cells with poly(I) · poly(C) and CHX resulted in a decrease in the amount of full-length *myc*-IRF-2 and the formation of a 25-kDa protein (Fig. 5, lane 4). This is similar to the molecular mass of purified PRDI-BFi. Extracts from HeLa cells transfected with vector alone did not have any detectable 50- or 25-kDa proteins before (lane 1) or after (lane 2) induction. The endogenous *myc* protein was detectable (62 kDa) in the extracts from untreated cells (lanes 1 and 3), while poly(I) · poly(C)-CHX treatment inhibited the synthesis of the endogenous *myc* protein (lanes 2 and 4). When a similar tagging experiment was performed in COS cells, the 25-kDa protein was present before and after induction (data not shown). This result correlates well with the fact that PRDI-BFi is detected before poly(I) · poly(C)-CHX induction of COS cells (Fig. 4, lane 1), while no PRDI-BFi activity is detected in HeLa cells before induction (Fig. 1, lane 1). We conclude that since the *myc* tag of the fusion protein, as well as the DNA binding domain of IRF-2 (21), is at the N-terminal half of the protein, the formation of PRDI-BFi must be due to a C-terminal truncation of preexisting IRF-2.

We believe this proteolytic cleavage to be specific and not the result of nonspecific proteolysis during extract preparation, because the extracts were made in the presence of a large excess of many different protease inhibitors (see Materials and Methods) and PRDI-BFi was not detected in extracts from most uninduced cells. Furthermore, the 25-kDa *myc*-IRF-2 fusion protein (see above) was detected in transfected COS cells after the cells were lysed with hot (100°C) SDS-lysis buffer and immediately boiled for 10 min (data not shown). Thus, the formation of PRDI-BFi occurs inside the cell.

Transcriptional repression by IRF-2 and IRF-2(Δ160). To examine the effect of this C-terminal truncation on IRF-2 function, we constructed C-terminal deletion expression plasmids as described in Materials and Methods. IRF2(Δ160) and IRF2(Δ206) code for amino acids 1 to 160 and 1 to 206, respectively. SDS-PAGE analysis of in vitro-translated IRF2(Δ160) and IRF2(Δ206) yielded proteins with molecular masses of approximately 25 and 33 kDa, respectively (data not shown). COS cells were transfected with pXM IRF2(Δ160) or pXM IRF2(Δ206), and whole-cell extracts were analyzed by EMSA (IRE probe) for the expressed proteins (Fig. 6). The COS cells were also transfected with pXM vector (negative control) and pXM IRF-2 (positive control) (Fig. 6, lanes 1 and 2). Expression of IRF-2(Δ160) produced a protein with a mobility in the EMSA (lane 3) that is similar to those of PRDI-BFi from COS cells (lane 2) and HeLa cells (lane 5). A very small difference was observed in the mobility of PRDI-BFi-IRE and IRF2(Δ160)-IRE complexes. The relative affinity of IRF2(Δ160) and IRF-2 for the

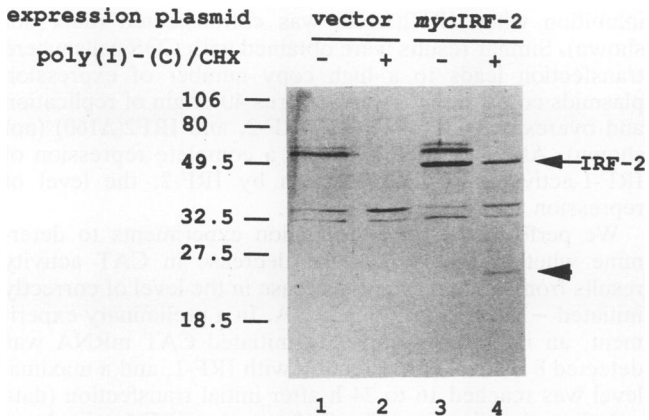


FIG. 5. Western analysis of *myc*-tagged IRF-2 from transfected HeLa cells. HeLa cells were transfected with 25 μg of pcDNA1 vector (lanes 1 and 2) or pcDNA *myc*-human IRF-2 fusion plasmid (lanes 3 and 4) by using the calcium phosphate method. In the fusion construct, the first 7 amino acids of IRF-2 were replaced with an 11-amino-acid peptide tag derived from c-*myc* (10, 37). pCMVβ-LacZ (5 μg) was also transfected as an internal control. Forty-eight hours after transfection, the cells were mock induced (lanes 1 and 3) or induced with poly(I) · poly(C)-CHX (lanes 2 and 4) for 6 h as described in Materials and Methods. Whole-cell extracts (5 μg) were separated by SDS–12% PAGE. The *myc*-IRF-2 proteins were detected by Western analysis (see Materials and Methods) using anti-*myc* monoclonal Ab 9E10. Arrow indicates position of IRF-2. Arrowhead indicates truncated form of IRF-2. Molecular mass standards (in kilodaltons) are also shown.

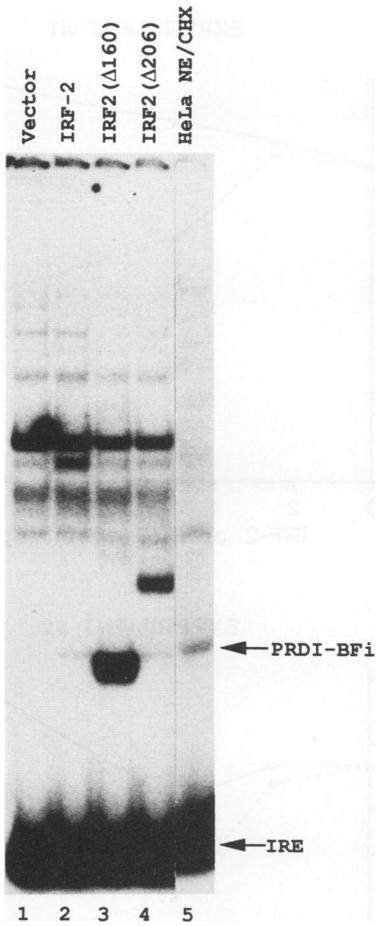
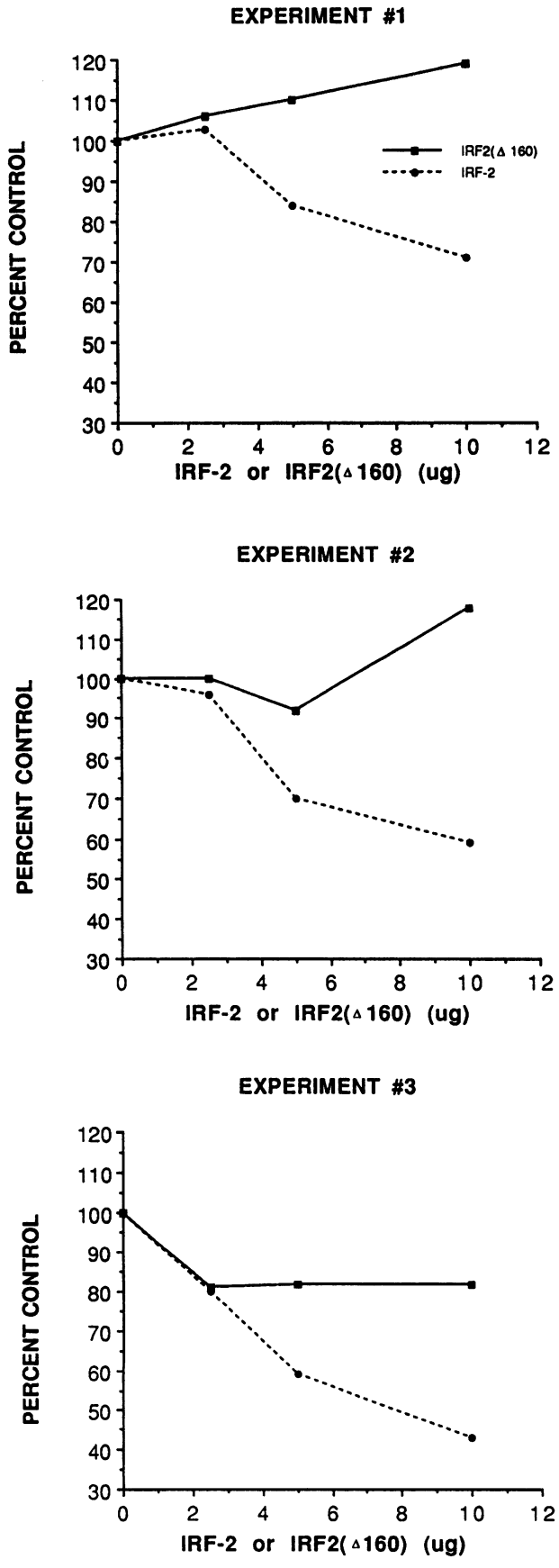


FIG. 6. EMSA of IRF-2 deletion (Δ) proteins produced in COS cells. COS cells were transfected (Fig. 4) with 3 μg of the following plasmids: pXM vector (lane 1), pXM human IRF-2 (lane 2), pXM IRF2(Δ160) (lane 3), and pXM IRF2(Δ206) (lane 4). Whole-cell extracts were prepared 48 h after transfection as described in Materials and Methods. EMSA was performed with 2 μg of lysate and IRE as a probe. Lane 5 is CHX-induced HeLa cell nuclear extract (12 μg). The position of PRDI-BFi is indicated.

IRE in an EMSA appears to be similar on the basis of competitions with increasing concentrations of unlabeled IRE and PRDI hexamer oligonucleotides (data not shown). IRF2(Δ206) has a mobility in the EMSA that is intermediate between full-length IRF-2 and IRF2(Δ160) (compare lanes 2 to 4). Interestingly, overexpression of IRF2(Δ206) led to a slight increase in the amount of PRDI-BFi activity (lane 4), suggesting that IRF2(Δ206) can be clipped in COS cells to yield PRDI-BFi. In addition, identical DNA binding profiles were observed when in vitro-translated IRF-2, IRF2(Δ160), and IRF2(Δ206) were used in the EMSA (data not shown). In a control experiment, COS cells were also transfected with N-terminal *myc* tag-IRF2(Δ160) or -IRF2(Δ206) plasmids, and the expressed proteins were analyzed by immunofluorescence with an anti-*myc* monoclonal Ab. Both deletion constructs expressed proteins that localized strictly to the nucleus (data not shown).

Because IRF2(Δ160) is similar to PRDI-BFi (molecular mass, EMSA mobility, and nuclear localization), this deletion construct was used to examine the effect of truncated IRF-2 on the transcription of the human IFN-β gene promoter. Cotransfection experiments were carried out with HeLa cells by using IRF-2 expression constructs and the CAT gene under the control of the human IFN-β gene promoter (−104 to +20) (27). In the first set of experiments, expression of full-length IRF-2 and IRF2(Δ160) had no effect on the basal and virus-induced levels of CAT expression (not shown).

We then studied the effect of IRF-2 and IRF2(Δ160) on IRF-1-activated gene expression. IRF-2 has been shown to repress IRF-1 activation of a reporter gene under the control of multiple copies of the PRDI hexamer (21). IRF-1 strongly activates this reporter construct (21, 32). Interestingly, murine IRF-1 has a much weaker stimulatory effect on the human IFN-β gene promoter driving the CAT gene (21, 32). Figure 7 shows three separate experiments in which IRF-1 stimulates −104 IFN-β CAT gene expression. In experiment 1, human IRF-1 (5 μg) was used (3.1% chloramphenicol acetylation). In experiments 2 and 3, 3 μg of human IRF-1 was cotransfected (2.7 and 3.4% chloramphenicol acetylation, respectively). The percent chloramphenicol acetylations for vector controls were 0.2, 0.17, and 0.34% for experiments 1, 2, and 3, respectively. Cotransfection of IRF-1 with increasing amounts of human IRF-2 led to a decrease in the amount of CAT expression (Fig. 7; expressed as percent control IRF-1-stimulated CAT activity). However, when increasing amounts of IRF2(Δ160) were cotransfected with IRF-1, there was either a slight enhancement (Fig. 7, experiments 1 and 2) or a weak inhibition (Fig. 7, experiment 3) of CAT activity. In another experiment HeLa cells were cotransfected with maximal amounts of human IRF-1 (10 μg) and human IRF-2 (10 μg) or IRF2(Δ160) (10 μg). In this experiment there was a 44% inhibition of IRF-1-activated −104 IFN-β CAT gene expression when full-length IRF-2 was cotransfected compared with a 20%



inhibition when IRF2(Δ160) was cotransfected (data not shown). Similar results were obtained with COS cells where transfection leads to a high copy number of expression plasmids containing the simian virus 40 origin of replication and overexpression of IRF-1, IRF-2, and IRF2(Δ160) (not shown). Although there was not a complete repression of IRF-1-activated CAT expression by IRF-2, the level of repression seen was reproducible.

We performed RNase protection experiments to determine whether the increase or decrease in CAT activity results from an increase or decrease in the level of correctly initiated −104 IFN-β CAT mRNA. In a preliminary experiment, an increase in correctly initiated CAT mRNA was detected 8 h after cotransfection with IRF-1, and a maximal level was reached 16 to 24 h after initial transfection (data not shown). Likewise, Fig. 8 shows that IRF-1 stimulated the expression of correctly initiated CAT mRNA 24 h after transfection (lane 2, stimulation index [SI] of 3.75 over vector control). In contrast, there was very little detectable CAT mRNA from cells cotransfected with −104 IFN-β CAT and vector (lane 1), IRF-2 (lane 3), or IRF2(Δ160) (lane 4). When IRF-2 was cotransfected with IRF-1 there was a lower level of CAT mRNA observed (lane 5, SI of 3.3, 12% inhibition) compared with IRF-1 plus pXM vector (lane 2). Importantly, IRF2(Δ160) expression did not appear to inhibit the increase in CAT mRNA but rather enhanced IRF-1-activated CAT gene expression (140% enhancement) at this time (lane 6). CAT mRNA levels were measured by densitometry scanning of the autoradiographs and corrected for the amount of RNA loaded per lane (γ-actin mRNA) and the transfection efficiency of each sample (α-globin mRNA). Densitometric analysis of a similar RNase protection experiment, performed with RNA made 16 h after transfection, showed a 90 and a 0% inhibition of IRF-1-activated CAT mRNA expression by IRF-2 and IRF2(Δ160), respectively (data not shown). There was no enhancement of IRF-1-stimulated CAT mRNA expression by IRF2(Δ160) in this experiment. The results from these RNase protection experiments are similar to those seen when CAT enzyme activity

FIG. 7. Inhibition of IRF-1 activation of the human IFN-β promoter by IRF-2 and IRF2(Δ160). Three separate experiments performed on different days are shown. For each experiment, HeLa cells were cotransfected with 5 μg of −104-+20 human IFN-βCAT reporter, 20 μg of test expression plasmids, and 5 μg of pCMVβ-LacZ reference plasmid. The test plasmids were as follows: IRF-1+IRF-2/IRF2Δ (2.5 μg) is 2.5 μg of pXM human IRF-2 or pXM IRF2(Δ160), 7.5 μg of pXM, and 10 μg of IRF-1/pcDNA1; IRF-1+IRF-2/IRF2Δ (5 μg) is 5 μg of pXM IRF-2 or pXM IRF2(Δ160), 5 μg of pXM, and 10 μg of IRF-1/pcDNA1; IRF-1+IRF-2/IRF2Δ (10 μg) is 10 μg of pXM IRF-2 or pXM IRF2(Δ160) and 10 μg of IRF-1/pcDNA1. IRF-1/pcDNA1 is 5 μg of pcDNA human IRF-1 plus 5 μg of pcDNA1 (experiment 1) or 3 μg of pcDNA IRF-1 plus 7 μg of pcDNA1 (experiments 2 and 3). For 100% control values the IRF-1/pcDNA1 plasmids were cotransfected with 10 μg of pXM in the absence of IRF-2/IRF2(Δ160). Cotransfection of full-length pXM human IRF-2 (10 μg) or pXM IRF2(Δ160) (10 μg) with 10 μg of pcDNA1 had no effect on −104IFN-βCAT reporter when transfected in the absence of IRF-1. Extracts for CAT assays were prepared 40 h after transfection, and CAT assays were normalized to β-galactosidase activities from the reference plasmid. The data from the three experiments are expressed as percent control IRF-1-stimulated CAT activity versus increasing amounts of cotransfected pXM IRF-2 or pXM IRF2(Δ160). The stimulation indices for IRF-1-activated −104IFN-βCAT expression (100% control) are 15.5, 15.9, and 21.6 for experiments 1, 2, and 3, respectively.

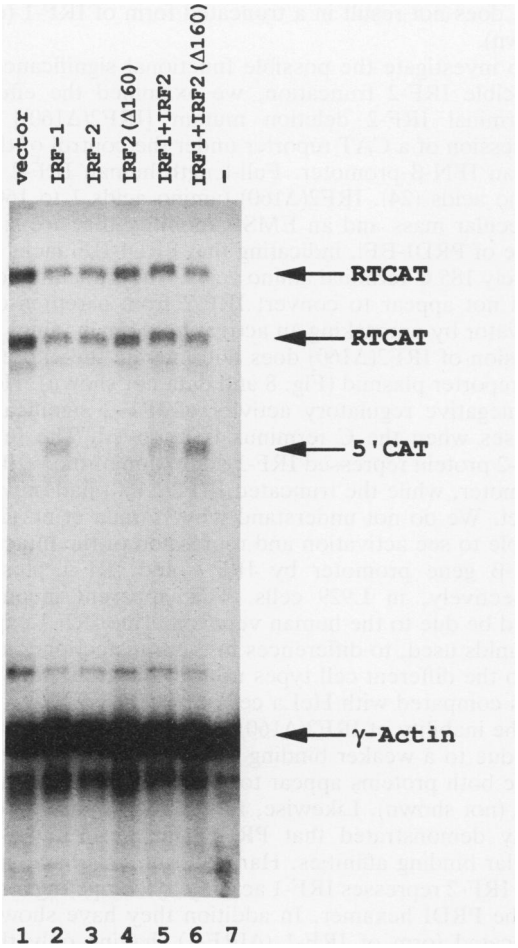


FIG. 8. RNase protection analysis of RNA from HeLa cells cotransfected with −104IFN-βCAT, IRF-1, and IRF-2. HeLa cells were cotransfected with 5 μg of −104–+20 IFN-βCAT reporter, 0.5 μg of πSV40 α-globin reference plasmid, and 20 μg of cDNA expression plasmids. The test plasmids were as follows: 10 μg of pXM and 10 μg of pcDNA1 (lane 1), 10 μg of pcDNA human IRF-1 and 10 μg of pXM (lane 2), 10 μg of pXM human IRF-2 and 10 μg of pcDNA1 (lane 3), 10 μg of pXM human IRF2(Δ160) and 10 μg of pcDNA1 (lane 4), 10 μg of pcDNA IRF-1 and 10 μg of pXM IRF-2 (lane 5), and 10 μg of pcDNA IRF-1 and 10 μg of pXM IRF2(Δ160) (lane 6). Total RNA was prepared 24 h after transfection, and 50 μg of RNA was analyzed with probes specific for IFN-βCAT and γ-actin mRNAs by the RNase mapping procedure. In separate samples the α-globin mRNA was mapped to determine transfection efficiencies for each sample. Lane 7 is RNA from untransfected HeLa cells. Correctly initiated CAT mRNA (5'CAT) is indicated. γ-Actin-protected fragments are also indicated. The bands corresponding to readthrough CAT mRNA (RTCAT) are above the correctly initiated CAT mRNA.

is measured (Fig. 7). Overall, the results show that full-length IRF-2 repressed activation of the human IFN-β promoter by IRF-1 and that a truncated form of IRF-2 [IRF2(Δ160)], which has properties similar to those of PRDI-BFi, had a slight effect on IRF-1 activation of the IFN-β promoter.

DISCUSSION

We have shown that two previously identified PRDI-binding factors, PRDI-BFc and PRDI-BFi, correspond to intact and truncated forms, respectively, of the transcriptional repressor IRF-2. PRDI-BFc was originally identified as a constitutive binding factor in human MG63 cells, while IRF-2 was identified in a mouse L929 cDNA library by virtue of its ability to cross-hybridize with an IRF-1 cDNA clone (21, 26). IRF-1 is a transcriptional activator which may be necessary, but is not sufficient, for induction of the IFN-β gene by virus and dsRNA (15, 21, 29, 43). IRF-1 is a potent activator of promoters containing multiple copies of the PRDI hexamer but is much less active with reporters containing the intact IFN-β promoter (21, 32, 39b). This difference is most likely due to the combinatorial nature of the mechanism of IFN-β gene activation (34). For example, both IRF-1 and NF-κB (which bind to PRDI/PRDIII and PRDII, respectively) are presumably required for induction (13, 23, 29, 30, 43, 51, 52).

The mechanism by which IRF-1 activates transcription is not known. Virus, poly(I) · poly(C) and IFNs can induce IRF-1 mRNA and protein (15, 21, 40, 52), but the increase in IRF-1 binding activity is blocked by cotreatment of the cells with CHX (39c, 40). This observation suggested that IRF-1 may not be necessary for IFN-β gene expression because induction of the gene does not require protein synthesis (47, 48, 55). However, a small amount of IRF-1 is present in the cells before induction and virus could stimulate the post-translational modification of IRF-1 (52). This modification may be required for transcriptional activation but is not essential for binding to the IFN-β promoter (34, 39b). Alternatively, the low level of IRF-1 activity present in cells before induction may not stimulate the IFN-β gene because repressors prevent IRF-1 binding to the *cis*-acting regulatory regions prior to induction. Along these lines, IFN can stimulate IRF-1 expression but does not appear to activate IFN-β gene transcription (15). IFN may fail to activate some other protein(s) involved in IFN-β gene expression or fail to stimulate a posttranslational modification of IRF-1 that is necessary for transcriptional activation. The C-terminal half of IRF-1 contains a transcriptional activation domain (12, 21). Within the C terminus there is a serine- and threonine-rich region (36) which could be phosphorylated to modulate the transcriptional activity of IRF-1. In fact, Pine et al. (40) have shown that three different phosphorylated isoforms of IRF-1 exist in HeLa cells.

In contrast to IRF-1, IRF-2 has been shown to act as a transcriptional repressor. IRF-2 represses IRF-1 activation of PRDI hexamers, and in EC cells, IRF-2 represses activation of the IFN-α/β promoters by IRF-1 (21, 22). IRF-2 has also been shown to repress the transcriptional activity of the simian virus 40 enhancer (21) when multiple PRDI hexamer sites are inserted between the enhancer and the TATA box (17, 21, 27, 28). However, we have not been able to inhibit virus induction of the IFN-β promoter by overexpression of IRF-2 in HeLa cells (data not shown).

As described above, the C-terminal half of IRF-1 contains a transcriptional activation domain (12, 21). If this C terminus is removed and replaced with the C terminus of IRF-2, the resulting fusion protein no longer activates transcription (21). However, this chimeric protein can repress IRF-1 activation of the PRDI hexamer in cotransfection analysis. This experiment suggests that the C-terminal half of IRF-2 either is inert or has a transcriptional repression domain. Interestingly, this is the portion of IRF-2 that is absent in PRDI-BFi.

As with PRDI-BFc, PRDI-BFi was originally identified in human MG63 cell extracts. However, PRDI-BFi was observed only after virus or poly(I) · poly(C)-CHX induction,

and the appearance of the PRDI-BFi binding activity did not require protein synthesis (26). As mentioned above, induction of the IFN-β gene also does not require ongoing protein synthesis in most cell types (47, 48, 55). Thus, PRDI-BFi was a candidate for a virus-inducible transcriptional activator of the IFN-β gene. PRDI-BFc and PRDI-BFi gave identical DNase protection and methylation interference patterns, and PRDI-BFi appeared to be smaller than PRDI-BFc (26). On the basis of these observations, Keller and Maniatis proposed that PRDI-BFi is a proteolytic digestion product of PRDI-BFc or an entirely different protein. Subsequently, Xanthoudakis et al. (57) also suggested that PRDI-BFi is generated by proteolytic cleavage of PRDI-BFc. In this paper we provide strong evidence that PRDI-BFi is indeed a truncated form of PRDI-BFc/IRF-2, generated by an inducible proteolytic cleavage.

First, we observed that PRDI-BFi activity can be induced by poly(I) · poly(C) plus CHX, by CHX alone, and by Sendai virus in different cell lines. All of these agents have been shown to activate transcription of both the endogenous and transfected IFN-β genes (5, 8, 35, 50). The kinetics of virus induction of PRDI-BFi is much slower and quantitatively weaker than that observed with poly(I) · poly(C)-CHX. PRDI-BFi can be detected 2 h after CHX treatment compared with 8 h after infection with virus (data not shown). The kinetics of virus-induced PRDI-BFi activity should, in fact, be slower because of the time it takes virus to enter the cell, uncoat, and form dsRNA during infection.

We showed by many different criteria (e.g., Ab studies, molecular mass) that PRDI-BFc is IRF-2 and that PRDI-BFi represents a truncated form of IRF-2. Overexpression of IRF-2 in COS cells, followed by induction with poly(I) · poly(C)-CHX, resulted in a much higher level of PRDI-BFi compared with COS cells that were transfected with the vector lacking IRF-2 cDNA. The results observed with the IRF-2-transfected COS cells and the *myc*–IRF-2-transfected HeLa cells also revealed that the formation of PRDI-BFi is due to a C-terminal truncation of IRF-2. PRDI-BFi is formed from preexisting IRF-2, since it appears when protein synthesis is inhibited by CHX. All of these results confirmed earlier speculation (26, 57) that PRDI-BFi is a smaller-molecular-weight form of PRDI-BFc that is generated by a proteolytic cleavage event. As discussed above, we believe this proteolytic cleavage to be specific and not an artifact generated during the preparation of extracts. After this paper was submitted, Whiteside et al. (54) reported the identification of a truncated form of IRF-2. After lysing the cells directly in SDS buffer, they observed that this truncated IRF-2 was present only after the cells were induced with dsRNA-CHX.

Virus-induced proteolysis of a transcriptional regulatory protein is not unprecedented. Infection of T cells with the human immunodeficiency virus leads to the formation of the p50 subunit of the NF-κB transcriptional activator. In this case, a virus-encoded protease can process the p105 precursor of p50 to generate a 45-kDa protein (45). This 45-kDa protein still interacts with the p65 subunit, and the entire NF-κB complex formed binds specifically to DNA.

Virus infection may be similar to CHX and inhibit host cell protein synthesis. By blocking protein synthesis, the level of specific protease inhibitors may decrease, leading to an increase in protease activity. This could be one way PRDI-BFi is generated. Alternatively, virus infection could specifically activate a particular protease responsible for generating PRDI-BFi. A protease does appear to be specific for IRF-2, since overexpression of IRF-1, followed by induction, does not result in a truncated form of IRF-1 (data not shown).

To investigate the possible functional significance of the inducible IRF-2 truncation, we examined the effect of a C-terminal IRF-2 deletion mutant [IRF2(Δ160)] on the expression of a CAT reporter under the control of the −104 human IFN-β promoter. Full-length human IRF-2 has 349 amino acids (24). IRF2(Δ160) (amino acids 1 to 160) has a molecular mass and an EMSA mobility that are similar to those of PRDI-BFi, indicating that PRDI-BFi lacks approximately 185 C-terminal amino acids. The truncation of IRF-2 does not appear to convert IRF-2 from a repressor to an activator by unmasking an activation domain, since overexpression of IRF2(Δ160) does not activate the expression of the reporter plasmid (Fig. 8 and data not shown). However, the negative regulatory activity of IRF-2 significantly decreases when the C terminus is removed. The full-length IRF-2 protein repressed IRF-1 activation of the −104 IFN-β promoter, while the truncated IRF2(Δ160) had only a weak effect. We do not understand why Harada et al. (21) were unable to see activation and repression of the intact human IFN-β gene promoter by IRF-1 and IRF-1 plus IRF-2, respectively, in L929 cells. This apparent inconsistency could be due to the human versus murine IRF-1 expression plasmids used, to differences in the transfection conditions, or to the different cell types used in the experiments (L929 cells compared with HeLa cells).

The inability of IRF2(Δ160) to repress IRF-1 activation is not due to a weaker binding affinity compared with IRF-2, since both proteins appear to have a similar affinity for the IRE (not shown). Likewise, Keller and Maniatis (26) previously demonstrated that PRDI-BFc and PRDI-BFi have similar binding affinities. Harada et al. (21) have suggested that IRF-2 represses IRF-1 activity by competing for binding to the PRDI hexamer. In addition they have shown that a truncated form of IRF-1 (ΔIRF-1), having only the DNA binding domain, can repress transcriptional activation by full-length IRF-1 presumably by a similar competition for binding. By implication, a truncated form of IRF-2 should also repress IRF-1 activation. However, Harada et al. (21) did not examine a truncated form of IRF-2. In addition, the PRDI hexamer was used in their cotransfection experiments, while we used the intact IFN-β promoter. The ΔIRF-1 truncation may out-compete full-length IRF-1 for binding to the hexamer, and at present the binding affinity of ΔIRF-1 for the hexamer is not known. Assuming that both IRF-2 and IRF2(Δ160) bind to the IFN-β promoter with equal affinities in vivo, our results suggest that the C-terminal half of IRF-2, which is absent in PRDI-BFi/IRF2(Δ160), is required for repression. Furthermore, we do not believe that the slight enhancement of IRF-1-activated IFN-βCAT gene expression by IRF2(Δ160) is significant (Fig. 7, experiments 1 and 2, and Fig. 8), because it was not reproducible. In other experiments (Fig. 7, experiment 3, and data not shown) IRF2(Δ160) either had no effect or slightly repressed IRF-1-stimulated CAT expression.

The poly(I) · poly(C)-CHX or virus-induced cleavage of IRF-2 is reminiscent of *recA*-induced proteolysis of *lexA*, λ*c*I, or phage P22 repressors (for a review, see references 39 and 53). Agents that damage DNA or inhibit DNA synthesis induce the SOS response in *Escherichia coli*, and this response is triggered by proteolytic inactivation of *lexA* or λ repressors by *recA* (39). There are many different mechanisms of transcriptional repression (for a review, see references 31 and 44). Competition for binding to *cis*-acting elements is one mechanism. For example, IRF-2 appears to

repress IRF-1 activity by competing for binding to the PRDI hexamer (21). Repression could also occur by a silencing mechanism whereby the binding of transcriptional repressors to DNA disrupts the assembly of a transcription initiation complex. This mechanism was first observed in the yeast silent mating type locus (3). Finally, repressors can form nonproductive complexes with transcriptional activators to inhibit transcriptional activation and/or DNA binding.

IFN-β gene expression is turned off in the absence of virus or dsRNA, but after induction, a rapid and transient increase in IFN-β mRNA is detected. IFN-β mRNA peaks 6 to 12 h after induction and then decreases rapidly (34, 42). Therefore, repression of IFN-β gene expression is seen before and after induction. IRF-2 could be a preinduction repressor of the IFN-β gene. It is present at high levels before induction, and the half-life of IRF-2 is more than 8 h, compared with that of IRF-1, which is approximately 30 min (52). Interestingly, CHX alone can stimulate the expression of a reporter gene under the control of multiple PRDI hexamer sites (56a) and CHX alone can induce PRDI-BFi. In addition, CHX can induce the endogenous IFN-β gene in MG63 cells (8). However, CHX cannot induce endogenous human IFN-β gene expression in HeLa cells, even though it is capable of inducing PRDI-BFi (Fig. 1) and activating NF-κB (49). Other signals or factors must therefore be required for induction of the gene in these cells. Nevertheless, poly(I) · poly(C)-CHX or virus treatment of cells could result in the inactivation of the repression activity of IRF-2 by proteolysis, and this could be one of the mechanisms leading to induction of the IFN-β gene.

Alternatively, IRF-2 could be a postinduction repressor of IFN-β. Virus as well as IFN itself can induce the expression of IRF-2 mRNA, leading to an increase in the amount of available IRF-2 protein (21). Virus and IFN could also stimulate the posttranslational modification of IRF-2, enhancing its repression activity. CHX has been shown to block transcriptional repression of the IFN-β gene, resulting in the maintenance of transcription and superinduction of IFN-β mRNA (47, 48, 55). Inactivation of the repression activity of IRF-2 by CHX and virus could be responsible for this superinduction. In either case, our results suggest that IRF-2 is a repressor that is inactivated through a proteolytic cleavage event by inducers of IFN-β gene expression. Considering the opposing activities of IRF-1 and IRF-2, an alteration of the ratio of the activities of these two proteins by virus- or poly(I) · poly(C)–CHX-induced proteolysis of IRF-2 may be part of the mechanism involved in the activation of the IFN-β gene.

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