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Viral Induction of the Human Beta Interferon Promoter: Modulation of Transcription by NF-κB/*rel* Proteins and Interferon Regulatory Factors

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Multiple regulatory domains within the −100 region of the beta interferon (IFN-β) promoter control the inducible response of the IFN gene to virus infection. In this study, we demonstrate that the formation of NF-κB-specific complexes on the positive regulatory domain II (PRDII) precedes the onset of detectable IFN-β transcription in Sendai virus-infected cells. By using NF-κB subunit-specific antibodies, a temporal shift in the composition of NF-κB subunits in association with the PRDII domain is detected as a function of time after virus infection. Furthermore, a virus-induced degradation of IκBα (MAD3) protein is observed between 2 and 8 h after infection; at later times, de novo synthesis of IκBα restores IκBα to levels found in uninduced cells and correlates with the down regulation of IFN-β transcription. In cotransfection experiments using various NF-κB subunit expression plasmids and two copies of PRDII/NF-κB linked to a chloramphenicol acetyltransferase reporter gene, we demonstrate that expression of p65, c-Rel, or p50 or combinations of p50-p65 and p65–c-Rel differentially stimulated PRDII-dependent transcription. Coexpression of IκBα completely abrogated p65-, c-Rel-, or p65-p50-induced gene activity. When the entire IFN-β promoter (−281 to +19) was used in coexpression studies, synergistic stimulation of IFN-β promoter activity was obtained when NF-κB subunits were coexpressed together with the IFN regulatory factor 1 (IRF-1) transcription factor. Overexpression of either IκB or the IRF-2 repressor was able to abrogate inducibility of the IFN-β promoter. Thus, multiple regulatory events—including differential activation of DNA-binding NF-κB heterodimers, degradation of IκBα, synergistic interaction between IRF-1 and NF-κB, and decreased repression by IκB and IRF-2—are all required for the transcriptional activation of the IFN-β promoter.

The type 1 interferon (IFN) genes (IFN-α and IFN-β) have served as an important paradigm to examine the mechanisms of virus-inducible gene expression. The DNA sequences that regulate IFN-β gene transcription are located immediately upstream of the intronless structural gene and consist of multiple, overlapping positive and negative regulatory domains essential for virus-induced activation and/or silencing of the promoter (reviewed in references 44 and 45). Four positive regulatory domains (PRDs) contribute to transcriptional activation of IFN-β (14, 16, 20, 23, 24, 31, 39, 49, 75). PRDI (−77 to −64) and PRDIII (−94 to −78) contain permutations of a hexameric motif, 5′-AAGTGA-3′ (20), and participate in virus-induced activation of the IFN promoter in synergy with another domain called PRDII (16, 39) (discussed below). PRDIV has been shown to interact with ATF-2 and octamer binding proteins (14, 25). The induction of the IFN-β promoter likewise requires the chromosome-associated high-mobility-group proteins I/Y (HMG I/Y) which interact with AT-rich nucleotides within PRDII and PRDIV (15). The HMG I/Y proteins appear to facilitate the binding of NF-κB and ATF-2 by bending DNA. Recently, an 11-bp element that acts as a negative regulatory element of the PRDII domain has been described (54); the silencing activity of the negative regulatory element is overcome in virus-infected cells by the cooperative activity of PRDI and PRDII. The capacity of the negative regulatory element to silence an NF-κB element complements the results of an earlier study describing the regulatory effect of the negative regulatory domain (23).

When multiple copies of PRDI, PRDIII, or the hexamer sequence are placed adjacent to a reporter gene, a virus-inducible promoter element is generated (16, 20). PRDI, PRDIII, and multimers of AAGTGA constitute binding sites for the IFN regulatory factors IRF-1 and IRF-2 (18–20, 26, 28, 49, 57, 58). Expression of mouse and human IRF-1 genes was shown to increase transcription from reporter genes under the control of IRF-binding sites (18, 19, 26, 39, 44, 49, 73); activation of transcription was abrogated with concomitant expression of IRF-2 (26–28). Recently, stable cell lines that expressed either sense or antisense IRF-1 cDNA were selected; antisense expression of IRF-1 abolished IFN-β induction by virus or double-stranded RNA, whereas cells overexpressing IRF-1 produced higher induced levels of IFN-β compared with that in control cells (58). In addition to IRF-2, a second repressor of the IFN-β promoter—PRDI-BF1—that binds to the PRDI domain was cloned (34). PRDI-BF1 cDNA is unrelated to IRF proteins and encodes an 88-kDa zinc finger protein that appears to be involved in postinduction repression of the promoter (34).

The second positive regulatory domain of IFN-β, PRDII (−64 to −55), is a decamer sequence (5′-GGGAAATTCC-3′) that serves as a recognition site for the NF-κB/*rel* family of transcription factors (10, 17, 31, 41, 72). Base mutations or deletions which alter the PRDII domain cause a loss of virus inducibility, suggesting a critical role for this element in virus

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induction (23, 24, 71). The NF-κB/*rel* family of transcription factors binds to a decameric recognition sequence (consensus 5′-GGGRNNYYCC-3′) and participates in the activation of numerous genes involved in immune regulatory functions including the acute-phase proteins, cytokines, cell surface receptors involved in immune recognition, and enhancer domains of several viruses (reviewed in references 2, 3, and 42).

Studies by Baeuerle and Baltimore demonstrated that a complex of three NF-κB subunits existed in the cytoplasm of most cells: a DNA-binding 48- to 55-kDa protein (p50), a DNA-binding 65- to 68-kDa protein (p65), and a non-DNA-binding regulatory subunit termed IκB that interacted specifically with p65 (1, 2, 76). IκB appears to be responsible for the cytoplasmic localization of the inactive NF-κB complex (5, 30, 32, 43, 67). Molecular cloning of the NFKB1 (p50) and RelA (p65) genes of NF-κB revealed that the DNA-binding, amino-terminal portion of these proteins shared strong homology with the c-*rel* proto-oncogene and with the *Drosophilia* morphogen *dorsal* (8, 22, 36, 53, 62, 69). The NF-κB family now consists of multiple NF-κB/*rel* peptides ranging in molecular mass from ~105 to 49 kDa (6, 42, 48, 51, 63–65).

In addition to the DNA-binding forms of NF-κB, three distinct forms of the regulatory IκB proteins have also been identified (reviewed in reference 3); all possess multiple ankyrin repeat motifs that may play an important role in protein-protein dimerization and cytoplasmic anchoring of NF-κB: (i) IκBα (MAD3 or pp40), cloned as an immediate-early response gene in phorbol ester-induced macrophages (29); (ii) *bcl3*, identified initially as a gene translocated in B-cell lymphoma (35, 52, 55, 74); and (iii) IκBγ, a unique 70-kDa gene product encoded by the carboxy-terminal 607 amino acids of the p105 gene (32). The cytoplasmic localization of both NF-κB/*rel* subunits is mediated via the masking by IκB of a nuclear localization sequence in the *rel* homology domains of these subunits (5, 30, 35). The ankyrin-containing precursors of NFKB1 (p105) and NFKB2 (p100) also participate in the cytoplasmic localization of DNA subunits (42, 43, 47, 59). Phosphorylation and rapid degradation of IκBα are the first detectable changes in NF-κB–IκB complexes after induction; loss of IκBα results in the translocation of NF-κB to the nucleus, where p65 has been shown to stimulate IκBα transcription de novo by an autoregulatory mechanism (3, 4, 7, 21, 67, 70).

In this study, we demonstrate that the formation of the PRDII-specific complexes preceded the onset of detectable IFN-β transcription in Sendai virus-infected cells and, using NF-κB subunit-specific antibodies, that a temporal shift in the composition of NF-κB subunits in association with the PRDII domain was detected as a function of time after virus infection. Virus-induced degradation of IκBα also correlated with NF-κB and IFN-β gene transcription. The activation of the IFN-β promoter was examined in a coexpression system with eukaryotic vectors expressing different NF-κB subunits and the IRF-1 and IRF-2 transcription factors. Synergistic stimulation of the IFN-β promoter was obtained when NF-κB subunits were coexpressed together with IRF-1 protein; in contrast, overexpression of IκB or IRF-2 decreased the inducibility of the IFN-β promoter.

MATERIALS AND METHODS

Cell culture and transfection. Cells were grown in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% calf serum, glutamine, and antibiotics. Subconfluent human 293 cells were transfected with 5 μg of CsCl-purified chloramphenicol acetyltransferase (CAT) reporter plasmid by the calcium phosphate coprecipitation method. All cotransfections contained equivalent amounts of DNA; in those assays in which less reporter or expression plasmid was used, additional pUC8 DNA was added. In some experiments, cells were infected with Sendai virus (500 hemagglutinating units/ml for 90 min). Protein extracts were obtained 16 to 20 h postinfection by freeze-thaw, and CAT assays were performed as previously described (38, 39). For individual transfections, 100 μg of total protein extract was assayed for 2 to 4 h at 37°C (described for each experiment). All transfections were performed three to six times.

Plasmid construction and oligonucleotide synthesis. Plasmids SV_2CAT, SV_1CAT, P2(1)-CAT, and SVoβ-CAT have all been previously described (38, 39) and are derivatives of pSV_2CAT. The NF-κB expression plasmids were produced by subcloning different NF-κB genes into the SVK_3 vector: (i) p50, a 1,381-bp *Eco*RI-*Rsa*I fragment from KBF-1 (36) subcloned into the *Eco*RI-*Sma*I site of SVK3; (ii) p65Δ, a 2,572-bp *Xba*I-*Xho*I fragment from plasmid BL-SK (61) subcloned into the *Bam*HI-*Xho*I site of SVK3; (iii) c-*rel*, the 2,340-bp *Eco*RI fragment of c-*rel* cDNA (8) cloned into the SVK3 *Eco*RI site; (iv) IκB, a 1,190-bp *Eco*RI fragment (29) from pGEX-2T subcloned into the *Eco*RI site of SVK3. The CMIN-p65 vector (61) was used to express the p65 subunit.

RT-PCR analysis of IFN-β mRNA. Total RNA isolated from Sendai virus-infected cells (9) was analyzed by a quantitative reverse transcriptase (RT)-PCR assay with IFN-β, SV_2CAT, and glyceraldehyde phosphate dehydrogenase primers and procedures previously described (12, 13).

Electrophoretic mobility shift analysis. Nuclear extracts (2.5 μg) were preincubated with the nonspecific DNA competitor poly(dI-dC) (5 μg; Pharmacia) for 10 min on ice in a total volume of 15 to 20 μl of whole-cell extract buffer containing 0.1% Nonidet P-40 (31). Binding activity was assessed with 0.2 ng of probe end labelled with ^{32}P corresponding to the PRDII domain of the IFN-β promoter (P2, 5′-GGGAAATTCCGGG AAATTCC-3′), which was incubated with the extract for 30 min at room temperature. Protein-DNA complexes were then separated on a 5% native polyacrylamide gel (60:1 cross-link) in Tris-glycine (25 mM Tris, 195 mM glycine [pH 8.8]). In competition analysis, a 200-fold molar excess of unlabelled oligonucleotide was incubated with the nuclear extract for 10 min on ice prior to the addition of the probe. The sequences of oligonucleotides used in competition experiments are as follows: mutant NF-κB sites 5′-actAAATTCCactAAATTCC-3′ (Gm) and 5′-GGGAcgTTCCGGGAcgTTCC-3′ (Am). To examine the individual proteins present in the complex, polyclonal subunit-specific antisera against p50/p105 (AR27, N terminus specific), p65 (AR28, C terminus specific), c-*rel* (AR22, C terminus specific), and p52/p100 (AR43, N terminus specific) were used in combination with the mobility shift assay. Antisera (1 μl) were incubated with whole-cell extracts (2.5 μg) in the presence or absence of specific competitor peptide (1 μg, final amount) for 20 min at room temperature prior to the addition of poly(dI-dC) and radiolabelled probe as described for mobility shift analysis.

Western blot analysis of IκBα. Cytoplasmic proteins (15 μg) were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell), and incubated with rabbit IκBα antiserum at a dilution of 1:1,000 in 5% milk-PBS overnight. After four washes with PBS for 10 min, membranes were reacted with peroxidase-conjugated anti-rabbit immunoglobulin antibody (Amersham) and visualized with the enhanced chemiluminescence detection system (Amersham Corp.).

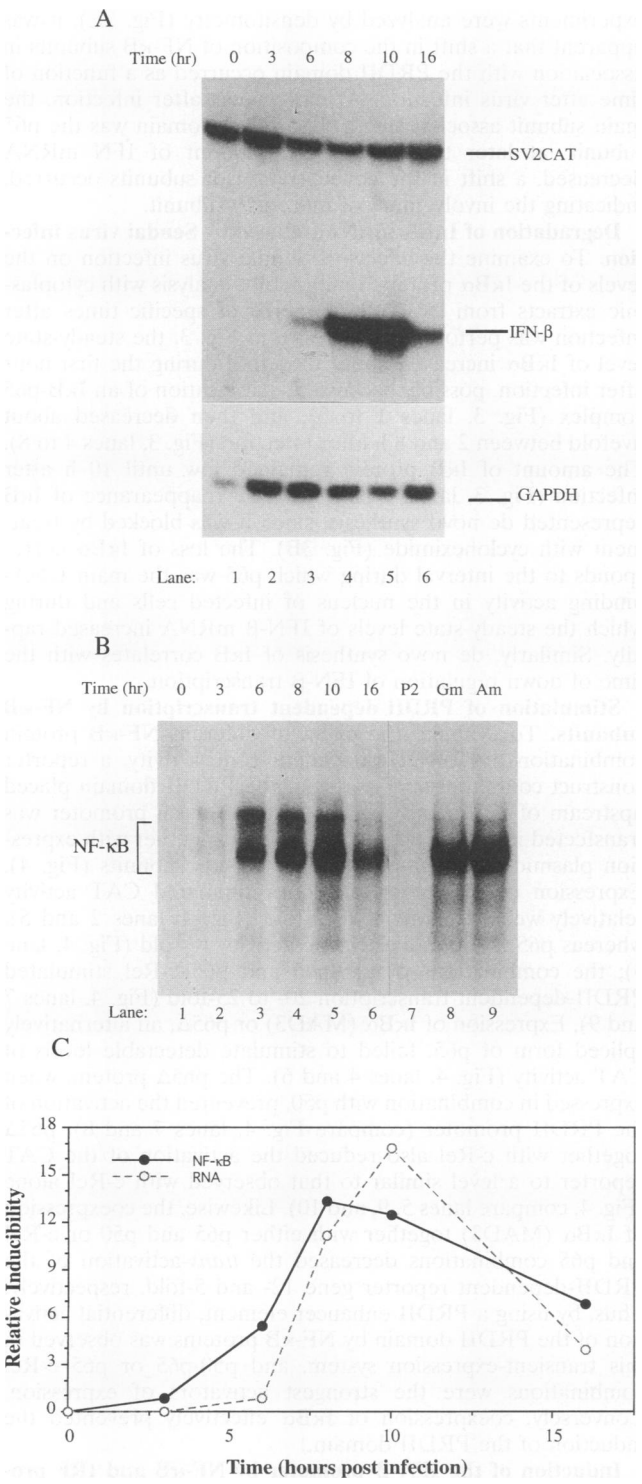

FIG. 1. Induction of NF-κB binding activity and IFN-β mRNA by Sendai virus infection. (A) Total RNA (9) was isolated from 293 cells infected with Sendai virus (500 hemagglutinating units/ml) for various times (indicated above the lanes). Quantitative RT-PCR amplification was performed with 2 μg of RNA, using primers specific for IFN-β, glyceraldehyde phosphate dehydrogenase (GAPDH), or SV_2CAT, essentially as previously described (15, 16). (B) At the same times (lanes 1 to 6), whole cell extracts were prepared and 10 μg of protein was analyzed for NF-κB-specific binding complexes, using the P2 probe (39); a 200-fold excess of competitor oligonucleotide (indicated above the lanes) was included in reaction mixtures containing whole-cell extracts from the 10-h sample (lanes 7 to 9). (C) The relative intensities of the PCR products and the protein-DNA complexes were scanned by laser densitometry and plotted as a function of time.

RESULTS

PRDII-specific protein-DNA complex formation in virus-infected cells. The Sendai virus-inducible expression of IFN-β was examined with a quantitative RT-PCR assay for IFN-β mRNA (12, 13). As shown in Fig. 1A, the induction of IFN-β mRNA was detected in human renal carcinoma (293) cells between 3 and 6 h after virus infection and peaked at about 10 h postinfection; no IFN-β RNA was detectable at either 0 or 3 h after infection, using the RT-PCR assay, thus verifying that induction of IFN-β expression is a predominantly transcriptional event. To examine the potential changes in NF-κB proteins that may occur during virus infection, the protein-DNA complexes formed with the PRDII probe were assessed by mobility shift analysis (Fig. 1B). No PRDII-specific protein-DNA complex was formed by using protein extracts from unstimulated cells. At 3 h after Sendai virus infection, a small amount of NF-κB–PRDII complex was detected, while at 6 h and at time points thereafter two prominent NF-κB–PRDII-specific complexes were generated. These complexes represent different homo- and heterodimeric p50 and p65 NF-κB complexes (see below). The specificity of these complexes for the PRDII probe was confirmed by competition analysis with an excess of the wild-type PRDII sequence (5′-GGGAAATTCC-3′) or oligonucleotides mutated in the first three guanine residues (GGG→ACT) or the two adenine residues at positions 5 and 6 (AA→CG). As illustrated in Fig. 1C, this result demonstrated that formation of the PRDII-specific complex preceded the onset of detectable IFN-β transcription in Sendai virus-infected cells.

Subunit composition of NF-κB–PRDII complexes. To investigate the subunit composition of the protein-DNA complexes formed with the PRDII probe, mobility shift analyses with antibodies specific to p65, p105/p50, p100/p52, and c-Rel were performed (Fig. 2). In each case, the antibody was titrated to ensure that an excess of antibody was used (data not shown); also, an excess of peptide used to generate the monospecific antibody was used in competition to verify the specificity of the shifted complex. At 3 h after infection, antibody specific for p65 resulted in a prominent shift-up of the two PRDII complexes (Fig. 2A, lane 4), indicating that p65 was the main subunit in association with the PRDII domain at 3 h. One of the bands that shifted with the anti-p65 antibody appeared to be nonspecific, since it did not compete in the presence of an excess of p65 peptide (Fig. 2A, lane 5). In addition, antibody to p50 produced two specific shifted complexes (Fig. 2A, lanes 2 and 3) that appear to represent p50 homodimers and p50-p65 heterodimers. Antibody to c-Rel also produced a shifted complex, indicating the involvement of some c-Rel in complex formation (Fig. 2A, lanes 6 and 7). No specific interaction with the p52 subunit was observed (Fig. 2A, lanes 8 and 9). At 6 h after infection, a similar profile of protein-DNA complexes was observed (Fig. 2B). By 10 h after infection (Fig. 2C), corresponding to the peak of IFN-β mRNA production, a shift in the relative abundance of p50 and p65 was observed by using the shifted shift assay, indicating that p50 was now an abundant NF-κB subunit in association with the PRDII probe (Fig. 2C and D, lanes 2 and 4). Also at both 10 or 16 h after infection, the amount of c-Rel protein associated with PRDII decreased (Fig. 2C and D, lanes 6). When the results of the mobility shift

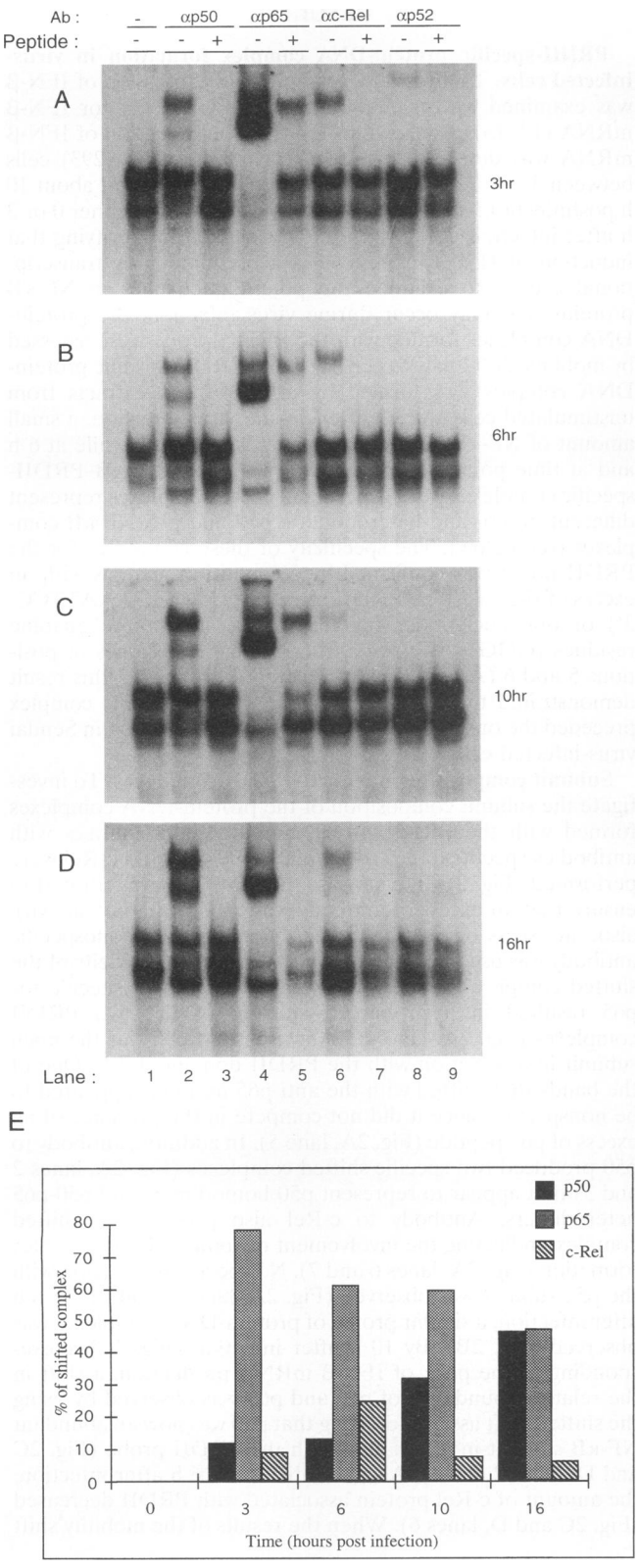

FIG. 2. Temporal shift in NF-κB protein-DNA complexes during viral infection. Nuclear extracts (2.5 μg) from 293 cells infected with Sendai virus for 3 h (A), 6 h (B), 10 h (C), or 16 h (D) were preincubated with NF-κB subunit-specific antisera (indicated above each lane) in the presence of 5 μg of poly(dI-dC) for 20 min prior to the addition of radiolabelled P2 probe. Specificity of antigen-antibody (Ab) association was confirmed by competition with an excess (1 μg) of antibody-specific peptide (lanes marked +). The intensity of the shifted protein-DNA complexes was evaluated by laser densitometry and expressed as a percent of the shifted complexes (E).

experiments were analyzed by densitometry (Fig. 2E), it was apparent that a shift in the composition of NF-κB subunits in association with the PRDII domain occurred as a function of time after virus infection. At early times after infection, the main subunit associated with the PRDII domain was the p65 subunit; at later times when the amount of IFN mRNA decreased, a shift in the concentration of subunits occurred, indicating the involvement of more p50 subunit.

Degradation of IκBα mRNA induced by Sendai virus infection. To examine the effects of Sendai virus infection on the levels of the IκBα protein, immunoblot analysis with cytoplasmic extracts from 293 cells obtained at specific times after infection was performed. As shown in Fig. 3, the steady-state level of IκBα increased about threefold during the first hour after infection, possibly because of stabilization of an IκB-p65 complex (Fig. 3, lanes 1 to 3), and then decreased about fivefold between 2 and 8 h after infection (Fig. 3, lanes 4 to 8). The amount of IκB protein remained low until 10 h after infection (Fig. 3, lanes 9 and 10). The reappearance of IκB represented de novo synthesis, since it was blocked by treatment with cycloheximide (Fig. 3B). The loss of IκBα corresponds to the interval during which p65 was the main DNA-binding activity in the nucleus of infected cells and during which the steady-state levels of IFN-β mRNA increased rapidly. Similarly, de novo synthesis of IκB correlates with the time of down regulation of IFN-β transcription.

Stimulation of PRDII-dependent transcription by NF-κB subunits. To evaluate the effect of different NF-κB protein combinations on PRDII-dependent gene activity, a reporter construct containing two copies of the PRDII domain placed upstream of the inactive simian virus 40 basal promoter was transfected into 293 cells either alone or together with expression plasmids producing individual NF-κB subunits (Fig. 4). Expression of p50 or c-Rel alone stimulated CAT activity relatively weakly, about 4- to 5-fold (Fig. 4, lanes 2 and 5), whereas p65 alone induced gene activity 18-fold (Fig. 4, lane 3); the combinations of p50-p65 and p65–c-Rel stimulated PRDII-dependent transcription 20- to 25-fold (Fig. 4, lanes 7 and 9). Expression of IκBα (MAD3) or p65Δ, an alternatively spliced form of p65, failed to stimulate detectable levels of CAT activity (Fig. 4, lanes 4 and 6). The p65Δ protein, when expressed in combination with p50, prevented the activation of the PRDII promoter (compare Fig. 4, lanes 7 and 8); p65Δ together with c-Rel also reduced the activation of the CAT reporter to a level similar to that observed with c-Rel alone (Fig. 4, compare lanes 5, 9, and 10). Likewise, the coexpression of IκBα (MAD3) together with either p65 and p50 or c-Rel and p65 combinations decreased the *trans*-activation of the PRDII-dependent reporter gene 12- and 5-fold, respectively. Thus, by using a PRDII enhancer element, differential activation of the PRDII domain by NF-κB proteins was observed in this transient-expression system, and p50-p65 or p65–c-Rel combinations were the strongest activators of expression. Conversely, coexpression of IκBα effectively prevented the induction of the PRDII domain.

Induction of the IFN-β promoter by NF-κB and IRF proteins. To examine the effects of different NF-κB proteins on the induction of the PRDII domain in the context of the natural IFN-β promoter, coexpression studies were performed

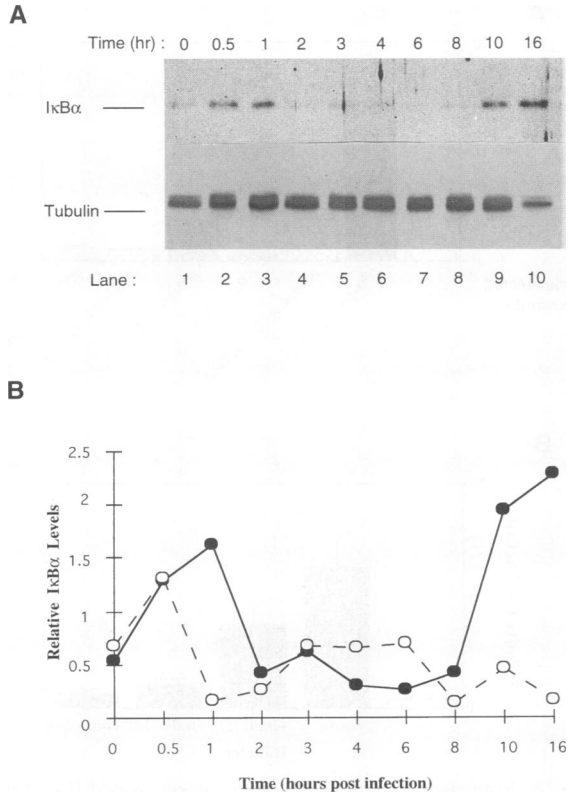

FIG. 3. Sendai virus-induced degradation of IκBα. 293 cells were induced with Sendai (500 hemagglutinating units/ml) in the presence or absence of cycloheximide (50 μg/ml). At the indicated times, cells were collected for the preparation of subcellular extracts. (A) Immunoblotting analysis of IκBα. Cytoplasmic extracts (15 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with antiserum to IκBα and an enhanced chemiluminescence assay (Amersham). Tubulin levels were similarly detected as a control by using 5 μg of cytoplasmic extract. (B) Relative levels of IκBα in the presence (○) or absence (●) of cycloheximide were evaluated by laser densitometry and expressed relative to the levels of tubulin.

with a CAT reporter gene driven by the entire IFN-β promoter from −281 to +19. Surprisingly, expression of individual NF-κB proteins—p65, p50, or c-Rel—failed to stimulate the intact IFN-β promoter (Fig. 5A), even though these proteins were able to differentially activate the PRDII element in isolation (Fig. 4). Similarly, coexpression of IRF-1 increased IFN-β activity only about twofold. The combination p50 and p65 likewise did not activate the IFN-β promoter. However, as shown in Fig. 5B, combinations of NF-κB proteins and IRF-1 were capable of stimulating the IFN-β transcription unit; coexpression of p65, p50, and IRF-1 stimulated IFN-β activity about 15-fold. Surprisingly, the combination of c-Rel and IRF-1 was also an activating combination in these experiments; this result was unexpected, since c-Rel binding activity was only weakly detected in extracts from infected cells (Fig. 2). On the basis of these assays, NF-κB proteins or IRF-1 alone was not sufficient to stimulate the IFN-β promoter; synergistic interactions between IRF-1 and NF-κB proteins appear to be essential for IFN-β *trans*-activation.

Inhibition of IFN-β promoter activity by IRF-2 and IκB. Next, the expression of proteins implicated as repressors of either PRDI- and PRDIII- or PRDII-dependent gene activity were examined to assess their potential to inhibit activation of the IFN-β promoter by Sendai virus infection or by subunit coexpression. As shown in Fig. 6A, when IRF-2 was substituted for IRF-1, either with c-Rel or p50 plus p65, the IFN-β promoter was not activated. Similarly, the expression of IκBα along with c-Rel plus IRF-1 or p50 plus IRF-1 inhibited the activation of the IFN-β promoter. By using the synergistic combination of p50, p65, and IRF-1, it was possible to demonstrate that coexpression of IRF-2 also blocked the *trans*-activation of the IFN-β promoter (Fig. 6A); IRF-2 also blocked Sendai virus-mediated induction of the IFN-β promoter (41a). The inhibition of Sendai virus-induced activation of the IFN-β promoter was also examined by overexpressing the IκBα protein. As shown in Fig. 6B, cotransfection of increasing amounts of the IκBα expression plasmid decreased the stimulation of the IFN-β promoter in a concentration-dependent manner. Thus, overexpression of either IRF-2 or IκBα proteins was sufficient to inhibit the induction of the IFN-β promoter in a *trans*-dominant negative manner. On the basis of the ability of IκBα to complex with p65 (1, 2, 7, 60, 70), it appears that overexpression of IκB prevents association of

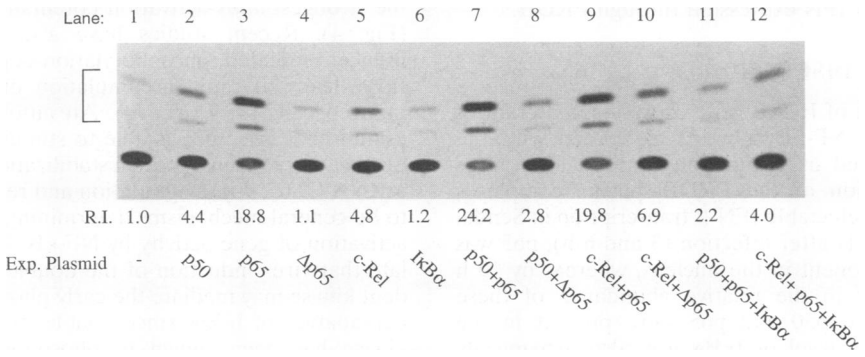

FIG. 4. Activation of the IFN-β PRDII domain by distinct NF-κB subunits. The PRDII-CAT reporter plasmid (5 μg) was transfected into 293 cells by the calcium phosphate method together with different SVK3-derived plasmids expressing the IκB, NFKB1 (p50), ΔRelA (p65Δ), or c-*rel* proteins; the RelA (p65) subunit was expressed from the CMIN-p65 vector (50); expression vectors were used at 5 μg with the exception of CMIN-p65, which was used at 2 μg. Cultures were harvested at 48 h after transfection and assayed for CAT activity (50 to 100 μg of protein for 4 h). The relative inducibility of the reporter plasmid (R.I.) is shown below the lanes and represents the activity of the reporter in the presence of an expression construct(s) divided by the activity of the reporter alone. The bracket shows the position of the acetylated forms of [^{14}C]chloramphenicol.

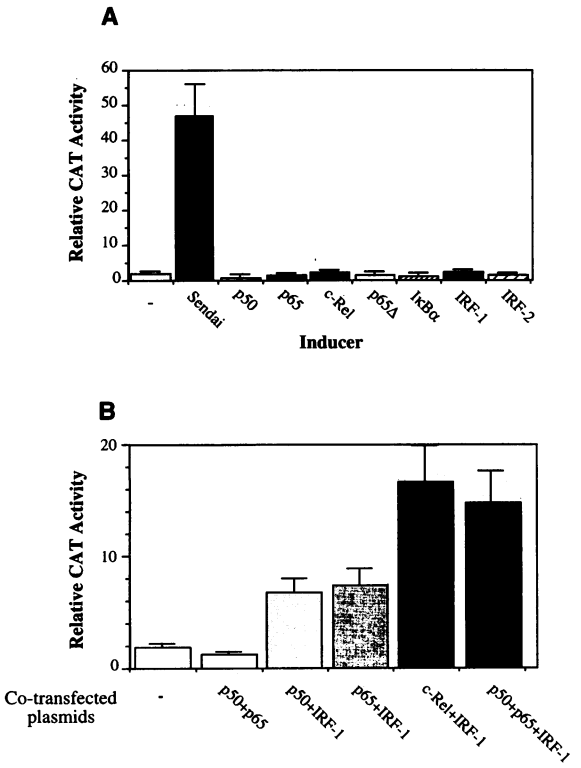

FIG. 5. Synergistic activation of the IFN-β promoter by NF-κB and IRF-1. (A) Human renal carcinoma (293) cells were transfected with a reporter plasmid (5 μg) containing the IFN-β promoter from −281 to +19 linked to the CAT gene (31, 39). Cotransfections were performed with individual expression plasmids (5 μg each) as indicated below the bar graph. Sendai virus infection of transfected 293 cells was performed as described previously (39). Lysates were prepared and analyzed at 48 h after transfection. (B) Cotransfections were performed with combinations of different expression plasmids (5 μg each, indicated below the bar graph).

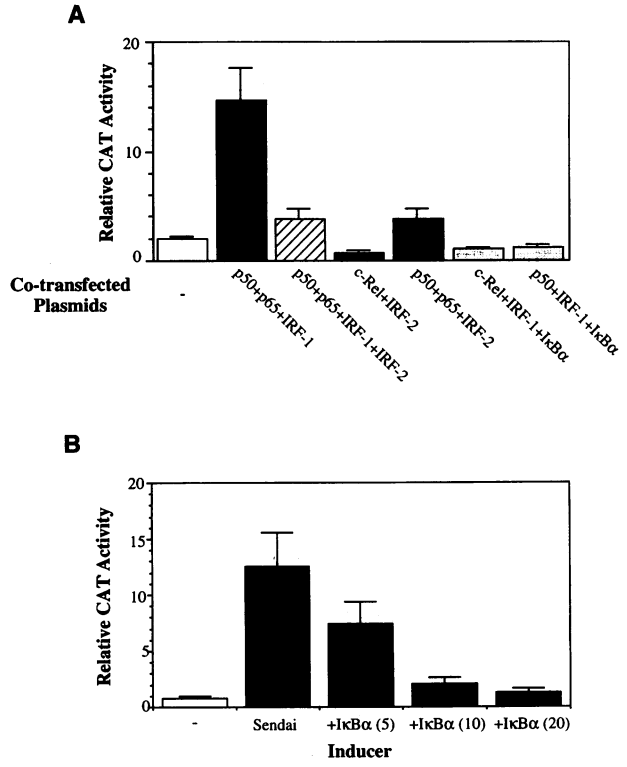

FIG. 6. Repression of virus-induced or *trans*-activated IFN-β promoter activity by overexpression of IRF-2 or IκBα. (A) 293 cells were transfected with the IFN-β–CAT reporter plasmid (5 μg) and different NF-κB and IRF combinations (indicated below the bar graph); some cells were infected with Sendai virus (500 hemagglutinating units/ml for 90 min) at 24 h after transfection; all lysates were analyzed for CAT activity at 48 h. (B) 293 cells were transfected with the IFN-β–CAT reporter plasmid (5 μg) and the IκBα expression plasmid at 5, 10, or 20 μg. At 24 h after transfection, cells were infected with Sendai virus, and lysates were prepared and analyzed for CAT activity at 48 h.

p65 with the PRDII domain. By analogy with the turnover of IκBα in virus-infected cells, IκBα degradation permits release of cytoplasmic NF-κB complexes, while resynthesis of IκB by 10 h after infection contributes to the sequestration of NF-κB and down regulation of IFN expression through PRDII.

DISCUSSION

During the induction of IFN-β gene expression, alterations in the composition of NF-κB subunits associated with the PRDII domain occurred as a function of time after virus infection. The formation of the PRDII-specific complexes preceded the onset of detectable IFN-β transcription in Sendai virus-infected cells. Early after infection (3 and 6 h), p65 was the main NF-κB component in the nucleus, whereas by 10 h after infection, a shift in the relative abundance of these subunits occurred; both p50 and p65 were present in the specific complexes. The level of IκBα was also dynamically altered by virus infection; by 2 h after infection, the amount of IκBα decreased about fivefold relative to cytoplasmic extracts from control cells. At 10 h after infection, de novo synthesis of IκBα restored the levels of this inhibitory protein. These results are consistent with a Sendai virus-induced degradation of IκBα. Previous studies with Sendai virus-infected cells demonstrated that de novo-synthesized IκB can be chased into an immunoprecipitable complex with p65 (60). Thus, de novo synthesis of IκB may contribute to the postinduction shutoff of transcription by sequestering p65-p50, which clearly possesses the strongest *trans*-activation potential for the PRDII domain (Fig. 4). Recent studies have also demonstrated that an inducer-mediated, phosphorylation-dependent degradation of IκBα leads to rapid accumulation of p65 in the nuclei of induced cells (4, 7, 67, 70). An autoregulatory loop is thus established, since p65 is able to stimulate IκBα levels by two distinct mechanisms: protein stabilization and induction of IκB mRNA (7, 67, 70). Degradation and resynthesis of IκB appear to be general mechanisms determining the rapid but transient activation of gene activity by NF-κB. It is attractive to speculate that virus induction of the double-stranded RNA-dependent kinase may mediate the early phosphorylation-dependent degradation of IκBα, since double-stranded RNA-dependent kinase has been shown to phosphorylate IκB and induce NF-κB binding activity (37). Surprisingly, IκB levels were decreased from 2 to 8 h after infection, a relatively long interval compared with those for other inducers such as tumor necrosis factor or phorbol myristate acetate that promote phosphorylation and degradation of IκB. The heterogeneous nature of viral infection or sustained activation of a virus-induced kinase may contribute to the longer period of IκB

decay than previously described for inducers such as cytokine or phorbol esters (4, 7, 52, 67, 70).

Concomitant with the modulation of protein-DNA interactions at the PRDII element shown in this study, numerous changes also occur within the PRDI and PRDIII domains (11, 19, 23, 26, 28, 33, 49, 56–58). Earlier models of IFN transcriptional control proposed that in uninduced cells IRF-2 binding caused silencing of the IFN-β promoter, whereas following viral infection, transcriptional induction and synthesis of IRF-1 displaced IRF-2, leading to gene activation (19, 23, 26, 28). On the basis of several observations—(i) induction of IFN gene expression may involve the inducible processing of the IRF-2 protein (11, 33, 56), (ii) IFN-β induction correlates with NF-κB binding to PRDII (31, 41, 72), and (iii) PRDII activation requires the simultaneous interaction of HMG I/Y with PRDII and NF-κB (71)—it is apparent that efficient IFN-β gene activation by IRF proteins requires synergistic interaction with NF-κB subunits and possibly with additional IRF-associated proteins (73). Also, although IRF-1 is phosphorylated in vivo, it has not been possible to correlate posttranslational changes in phosphorylation with alterations in IRF-1 activity.

On the basis of cotransfection experiments using various NF-κB subunit expression plasmids and two copies of PRDII–NF-κB linked to a CAT reporter gene, we showed that expression of p65, c-Rel, or p50 or combinations of p50-p65 and p65–c-rel differentially stimulated NF-κB-dependent transcription. The results of the coexpression assays are consistent with the idea that p65 homodimers or p65-p50 heterodimers stimulate transcription from PRDII at early times following virus infection (66). At later times the expression of PRDII is down regulated by the appearance of p50 homodimers that only weakly affect PRDII and the resynthesis of IκBα which restores the cytoplasmic pool of latent NF-κB by sequestering p65-p50. When the entire IFN-β promoter (−281 to +19) was used in coexpression studies, the combination of p50 and p65 only weakly increased IFN gene activity. Synergistic stimulation of IFN-β promoter activity was obtained when NF-κB subunits were coexpressed together with the IRF-1 transcription factor, which has been shown to bind to adjacent PRDI (−79 to −64) and PRDIII (−94 to −78) domains. This result is consistent with previous studies demonstrating that efficient virus induction of IFN-β required the synergistic activities of the different PRD domains (16, 24, 39).

Two recent studies are directly relevant to the synergistic activation of the IFN-β promoter. Matsuyama et al. demonstrated that ablation of IRF-1 by homologous recombination decreased the double-stranded RNA-induced activation of the IFN-β promoter but did not affect virus-induced stimulation of IFN-α or IFN-β mRNA production (46). This result suggests that IRF-1-independent pathways that are important in virus-mediated induction of IFN-α and IFN-β also exist (46). The IRF-2 gene was also ablated by homologous recombination, and functional knock-out of this gene resulted in a three- to fourfold increase in the steady-state levels of IFN-α and IFN-β mRNA, reflecting a role for IRF-2 in the posttranscriptional shutoff of IFN gene transcription (46). The studies of Du et al. provide important new insights into the synergistic activation of the IFN-β promoter (15). These experiments demonstrated that HMG I/Y was also required for the activity of PRDIV; in this case, HMG I/Y stimulated the binding of ATF-2 and the assembly of inducible complexes on PRDIV containing ATF-2 and c-*jun*. ATF-2, in turn, was shown to interact with PRDII-bound NF-κB. It is proposed that HMG I/Y plays an important role in establishing transcriptional synergy between PRDII and PRDIV (15). The HMG I/Y-stimulated interactions between PRDII and PRDIV may represent the IRF-1-independent pathways suggested by Matsuyama et al. (46). Consistent with the idea of synergistic activation of IFN-β by IRF-1 (acting at PRDI and PRDIII) and NF-κB (acting at PRDII), overexpression of either IκBα or IRF-2 was able to repress inducibility of the IFN-β promoter (Fig. 6). Together with the present studies describing synergistic stimulation by PRDI, PRDIII, and PR DII, these studies indicate that the IFN-β promoter contains several interactive domains that contribute to synergistic transcriptional activation.

Given the pleiotropic role of NF-κB in the transcriptional control of immunoregulatory and inflammatory genes and the broad range of activators capable of stimulating NF-κB binding activity (3, 42), additional regulatory mechanisms, active at the level of individual promoters, represent a necessary evolutionary requirement to maintain the specificity of gene activation. With regard to the IFN-β promoter, several important mechanisms that ensure the specificity of IFN-β induction are operative. (i) Functional synergism between NF-κB, IRF-1, and ATF-2 proteins ensures that three independent signalling pathways converge at the level of the IFN-β promoter. To date it has not been possible to demonstrate physical association between NF-κB and IRF proteins (41a), suggesting perhaps the involvement of other associated proteins. Nonetheless, physical and functional interactions between the *rel* domain of NF-κB and the leucine zipper domain of C/EBP proteins and ATF-2 have been demonstrated recently (15, 40, 68); functional cross-talk between transcription factors may be a general mechanism that increases transcriptional specificity, as well as complexity, among eukaryotic promoters. (ii) Virus-induced degradation of IκBα (60) coupled with a p65-dependent synthesis of IκBα provides an additional autoregulatory mechanism that ultimately limits IFN activation via cytoplasmic sequestration of NF-κB (7, 67, 70). (iii) Temporal variations in the nuclear appearance of different NF-κB proteins, together with their differential ability to *trans*-activate the PRDII domain, also modulate the level of PRDII-dependent activation. (iv) The interaction of NF-κB and HMG I/Y on the PRDII element provides a unique IFN-β-specific mechanism that increases the affinity of NF-κB for the PRDII domain (71). Thus, multiple regulatory events—including differential activation of DNA-binding NF-κB heterodimers, virus-induced degradation of IκBα, synergistic interaction between IRF-1 and NF-κB, and decreased repression by IκB and IRF-2—are all required for the transcriptional activation of the IFN-β promoter.

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