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Postinduction repression of the β-interferon gene is mediated through two positive regulatory domains

(positive regulatory domain I/positive regulatory domain II/virus induction/interferon induction/repressor)

LISA-ANNE WHITTEMORE AND TOM MANIATIS

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138

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ABSTRACT **Virus induction of the human β-interferon (β-IFN) gene results in an increase in the rate of β-IFN mRNA synthesis, followed by a rapid postinduction decrease. In this paper, we show that two β-IFN promoter elements, positive regulatory domains I and II (PRDI and PRDII), which are required for virus induction of the β-IFN gene are also required for the postinduction turnoff. Although protein synthesis is not necessary for activation, it is necessary for repression of these promoter elements. Examination of nuclear extracts from cells infected with virus reveals the presence of virus-inducible, cycloheximide-sensitive, DNA-binding activities that interact specifically with PRDI or PRDII. We propose that the postinduction repression of β-IFN gene transcription involves virus-inducible repressors that either bind directly to the positive regulatory elements of the β-IFN promoter or inactivate the positive regulatory factors bound to PRDI and PRDII.**

β-Interferon (β-IFN) gene expression is highly inducible by virus or double-stranded RNA (for review, see ref. 1). Prior to induction the gene is repressed, but virus infection results in a transient, high-level accumulation of β-IFN mRNA. Previous studies demonstrated that the increase in β-IFN mRNA levels after induction is due to an increase in the rate of transcription (2, 3). We have recently shown that the postinduction decrease in β-IFN mRNA levels is due to a combination of transcription repression and rapid mRNA turnover (4). In addition, we demonstrated that this transcription repression requires protein synthesis (4). These observations suggest that the postinduction decrease in β-IFN gene transcription requires a virus-inducible repressor and that the positive regulatory proteins are stable, since high rates of transcription continue in the absence of protein synthesis.

Analysis of the DNA sequence requirements for virus induction of the β-IFN gene has revealed a complex regulatory element consisting of overlapping positive and negative regulatory domains (5, 6). The arrangement of these domains is diagrammed in Fig. 1. Although the sequence requirements for maximum levels of induction are cell-type specific (7–9), the region between −37 and −77 base pairs upstream from the start point of transcription is both necessary and sufficient for high levels of induction in mouse C127 cells (10). This region, which has been designated the IRE (IFN gene regulatory element), is a virus-inducible transcription enhancer (10). We have recently demonstrated that the IRE is sufficient for postinduction repression of β-IFN gene transcription (4).

The IRE consists of two positive regulatory domains (PRDI and PRDII) and one negative regulatory domain (NRDI) (5). Although a single copy of PRDI or PRDII is not sufficient to activate the β-IFN gene, one copy of both elements, or multiple copies of either, functions as a virus-inducible enhancer (5, 11–13). In this paper, we show that postinduction repression of the β-IFN gene is regulated through both PRDI and PRDII and that NRDI is not necessary for this regulation. Thus, the same transcription elements are used for both virus induction and postinduction repression. Additional studies show that both PRDI- and PRDII-dependent repression requires protein synthesis, suggesting that virus-inducible repressors act on these sequences. Further evidence for this possibility is provided by the detection of virus-inducible, cycloheximide (CHX)-sensitive factors that bind to PRDI or PRDII.

MATERIALS AND METHODS

Cell Culture and Transfection. Mouse C127, human MG63, or HeLa cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS). C127 cells were seeded 1 day prior to transfection and were 30–50% confluent at the time of transfection. Cells were transfected and transformants were selected as described (5).

Virus and IFN Induction. Cells were induced with Sendai virus (Spafas) as described (5). Virus treatment was for 4 hr in serum-free DMEM, after which the cells were rinsed in phosphate-buffered saline and incubated in DMEM/2% FCS. Superinduction was carried out in the same manner as virus induction, except that 50 μg of CHX per ml was included in the induction medium as well as the DMEM/2% FCS. Induction with IFN was carried out by adding 1000 units of mouse β-IFN per ml (Lee Biomolecular Laboratories, San Diego, CA) to each plate of cells.

RNA Analysis. RNA was isolated by the guanidinium thiocyanate procedure (14). Total cellular RNA (10 μg) was assayed by RNase protection as described (9).

Plasmid Constructions. The NRDI deletion mutant, −55ID, and its isogenic wild-type counterpart, −41ID, are described in Goodbourn *et al.* (15) as pBVIFΔ(−77)ID−55/−40 and pBVIFΔ(−77)ID−41/−40, respectively. The test genes $(PRDI)_4$ and $(PRDII)_4$ have two copies of the PRDIX2 or PRDIIX2 oligonucleotides (Fig. 1*B*) adjacent to a −40 β-IFN gene and inserted at the *Bam*HI site of BPV-BV1.

Probes for RNase protection are described elsewhere. pSP65'IF (9) was used as a human β-IFN probe, pSP6mif (10) was used as a mouse β-IFN probe, and pSP6γ-actin (16) was used as a γ-actin probe.

DNA-Binding Experiments. Low salt (200 mM KCl) nuclear extracts were prepared from C127, HeLa, or MG63 cells at appropriate times after virus, virus plus CHX, or β-IFN induction. Preparation of nuclear extracts and binding assays were done as described by Levy *et al.* (17). The concentrations of protein for each extract preparation were approxi-

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Abbreviations: CHX, cycloheximide; IFN, interferon; IRE, IFN gene regulatory element; IRF, IFN regulatory factor; ISGF, IFN-stimulated gene factor; ISRE, IFN-stimulated regulatory element; NRD, negative regulatory domain; PRD, positive regulatory domain; SRE, serum response element.

FIG. 1. (*A*) Human β-IFN gene regulatory sequences. The three boxes represent the regulatory domains PRDI, PRDII, and NRDI. (*B*) Sequence of the synthetic oligonucleotides used for expression and binding studies. Adapted from Fan and Maniatis. Reprinted by permission of Oxford University Press from figure 1 in ref. 11 (copyright 1989).

mately equal so that equal volumes of extract and equal concentrations of protein were used in each binding assay. Probes for the binding assay were end-labeled oligonucleotides, PRDIX2 and PRDIIX2 (Fig. 1*B*). These oligonucleotides were ligated into high copy number multimers and used as competitors in the binding reaction. UV crosslinking was done as described by Chodosh *et al.* (18) except that binding reactions were run on a native gel, crosslinked in the gel, and eluted before DNase treatment and running on an SDS/polyacrylamide gel.

RESULTS

In previous studies, mutations in the NRDI element were shown to increase both the basal and induced levels of β-IFN mRNA accumulation (5, 15), but the kinetics of induction were not examined. To determine whether NRDI is required for postinduction repression of the β-IFN gene, we compared the kinetics of mRNA accumulation from the wild-type β-IFN gene and an NRDI promoter deletion mutant designated −55ID (15). Both the wild-type and mutant genes were introduced into mouse C127 cells by using a bovine papilloma virus (BPV) vector, and stable transformants were selected, pooled, and induced with Sendai virus. The level of accurately initiated β-IFN mRNA was assayed by RNase protection. We confirmed that the −55ID deletion leads to an increase in both the basal (Fig. 2*B*) and induced (Fig. 2*A*) levels of β-IFN mRNA accumulation compared with the wild-type promoter. However, the NRDI deletion did not alter the kinetics of induction, indicating that NRDI is not required for postinduction repression of the β-IFN gene. Moreover, since we have previously shown that the IRE is sufficient for postinduction repression of transcription (4), these results indicate that PRDI and PRDII are also sufficient for postinduction repression.

To elucidate the roles of PRDI and PRDII in postinduction repression, we analyzed the expression of β-IFN genes containing multiple copies of PRDI or PRDII inserted upstream from the TATA box of the β-IFN promoter (Fig. 1*B*). We find that both $(PRDI)_4$ and $(PRDII)_4$ are turned off with kinetics similar to the wild-type (Fig. 2*A*) and endogenous mouse β-IFN genes (Fig. 3), indicating that multiple copies of either element are sufficient for postinduction repression. $(PRDII)_4$ is a more potent transcription activator than $(PRDI)_4$ (11), as indicated by a higher basal and induced level of β-IFN mRNA (Fig. 3). However, in both cases the level of

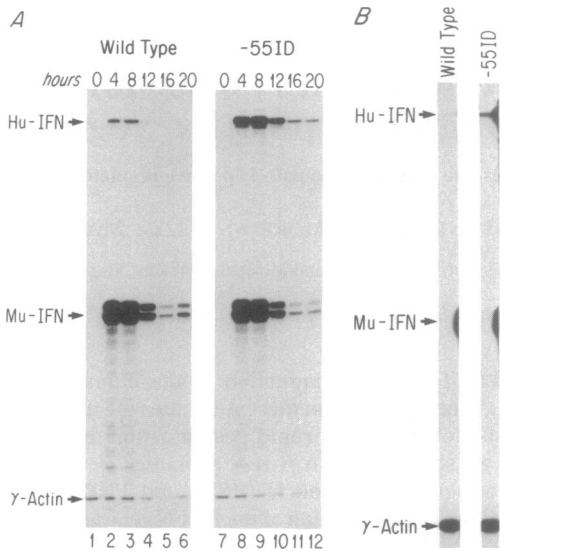

FIG. 2. Time course of virus induction for a NRDI deletion mutant (−55ID) and its isogenic wild type (−41ID). Pools of transfected C127 cells were induced with Sendai virus and RNA was isolated at the indicated times postinduction. RNase protection analysis was performed with a probe for the 5′ end of human β-IFN mRNA (Hu-IFN), a probe for an internal portion of mouse β-IFN mRNA (Mu-IFN), and a probe for an internal portion of mouse γ-actin mRNA (γ-Actin). (*A*) Exposure (3.5 hr) of the entire autoradiogram showing the induction and postinduction repression of human and mouse β-IFN mRNAs. (*B*) Exposure (2.5 days) of lanes 1 and 7 of *A* to show the relative basal levels of transcription of the wild-type and −55ID genes.

β-IFN mRNA decreases significantly by 16 hr after induction.

We previously demonstrated that protein synthesis is required for the postinduction repression of β-IFN gene transcription (4). These experiments involved the endogenous β-IFN gene or transfected genes containing the entire IRE. To determine whether repression of PRDI and/or PRDII also requires protein synthesis, we examined the effect of CHX on the kinetics of virus induction for cells transformed with the $(PRDI)_4$ or $(PRDII)_4$ vectors. When pools of foci were induced in the presence of virus and CHX, the steady-state level of mRNA remained high 16 hr after induction for the

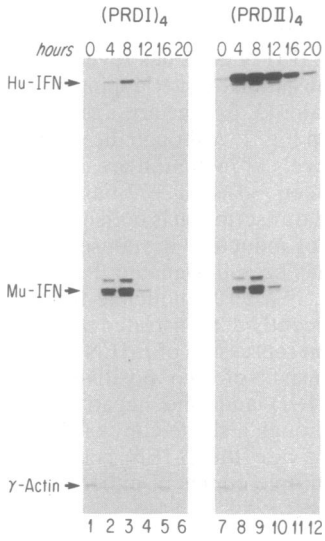

FIG. 3. Time course of virus induction for $(PRDI)_4$ and $(PRDII)_4$. Probes for RNase protection are described in Fig. 1.

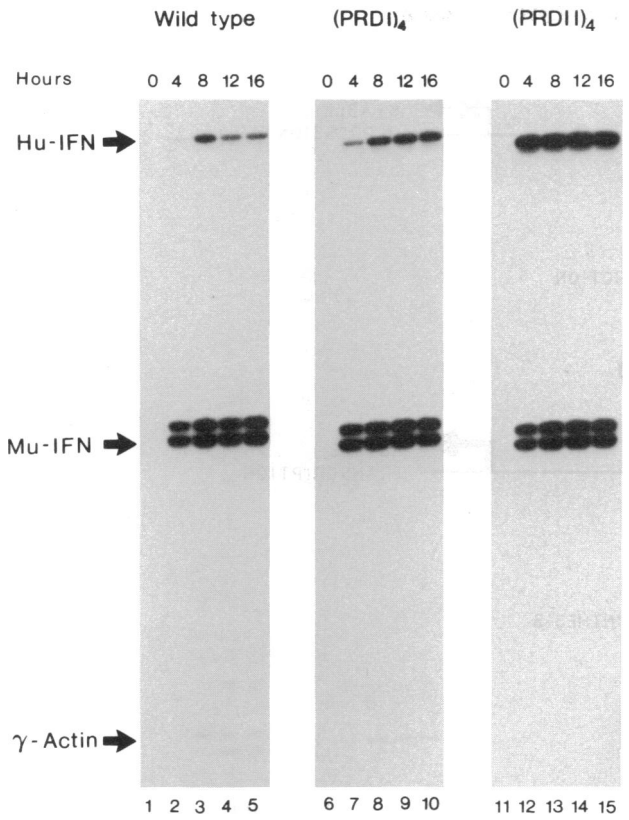

FIG. 4. Time course of virus plus CHX induction for wild type (−41ID), $(PRDI)_4$, and $(PRDII)_4$. Probes for RNase protection are described in Fig. 1.

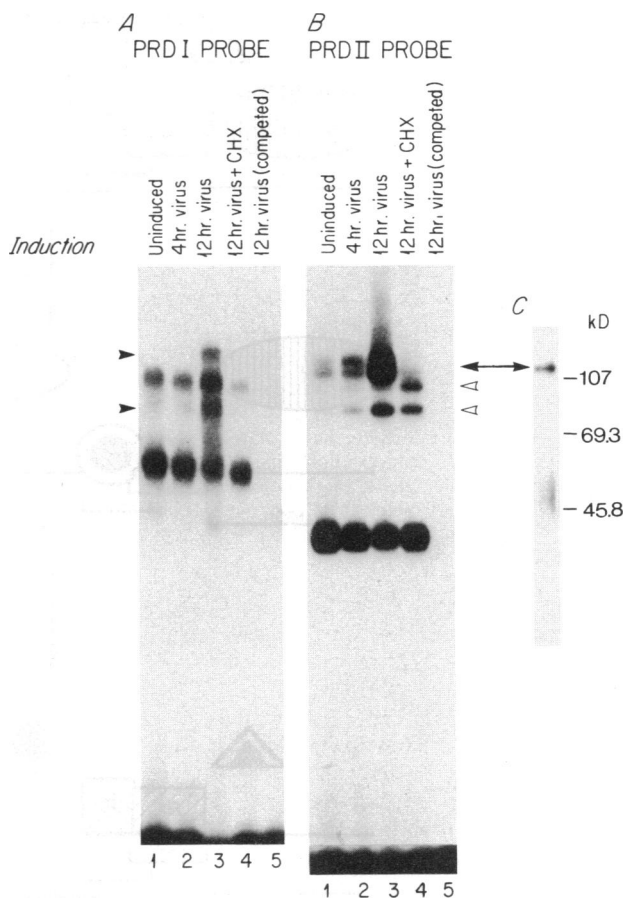

FIG. 5. Gel-shift analysis of C127 cell nuclear extracts using PRDI (*A*) and PRDII (*B*) DNA probes. Cells were mock induced (lane 1), induced for 4 hr with virus (lane 2), induced for 12 hr with virus (lanes 3 and 5), or induced for 12 hr with virus plus CHX (lanes 4). The binding in lane 5 is blocked by competition with 50 ng of ligated PRDI oligonucleotide (*A*) and 50 ng of PRDII oligonucleotide (*B*). The PRDI complexes are not blocked by competition with PRDII oligonucleotide and the PRDII complexes are not blocked by competition with PRDI oligonucleotide (data not shown). Solid arrowheads indicate complexes that are virus inducible and CHX sensitive and open arrowheads indicate NF-κB complexes. (*C*) SDS/polyacrylamide gel showing the size of the CHX-sensitive PRDII-binding complex (*B*) after UV crosslinking and gel isolation. The positions of molecular mass standards (BRL) are shown.

endogenous mouse β-IFN gene as well as the transfected wild type, $(PRDI)_4$, and $(PRDII)_4$ (Fig. 4). Nuclear transcriptions indicate that superinduction by CHX is primarily at the level of transcription for both PRDI and PRDII (ref. 4; unpublished data). Thus, the postinduction repression of both PRDI and PRDII requires protein synthesis, suggesting that one or more virus-inducible repressors may be involved in turning off transcription mediated by these elements.

To test the possibility that virus-inducible repressors bind to PRDI and PRDII, we carried out electrophoretic gel-shift experiments with nuclear extracts prepared from uninduced and virus-induced C127 cells in the presence or absence of CHX. As shown in Fig. 5, virus-inducible, CHX-sensitive factors are detected with both the PRDI and PRDII DNA probes. Although these factors are present at low levels in uninduced cells, their levels increase significantly at a time after induction when transcription of the β-IFN gene is decreasing (8–12 hr postinduction). In the case of PRDI, we detect a pair of shifts that are virus inducible and CHX sensitive (Fig. 5*A*). These complexes are also induced by β-IFN and γ-IFN (data not shown), which suggests that activation may result from virus induction of IFN, followed by IFN induction of the binding factors.

A PRDII-specific factor that is virus inducible and CHX sensitive is also detected (Fig. 5*B*). This factor has a molecular mass of 110–130 kDa as determined by UV crosslinking (Fig. 5*C*). We have also identified a complex that, based on its mobility and competition analysis, corresponds to NF-κB. This complex is virus inducible and remains at high levels 12 hr after induction and in the presence of CHX (Fig. 5*B*).

We find similar PRDI and PRDII binding factors in extracts made from HeLa and MG63 cells, although the mobilities of the complexes differ from the mobilities of the complexes formed by the factors in C127 cell extracts. All of the factors described above bind to a single copy of the IRE, indicating that they bind to PRDI and PRDII in the context of the β-IFN promoter.

DISCUSSION

We have identified the regulatory sequences required for postinduction repression of β-IFN gene expression. Analysis of the kinetics of β-IFN mRNA accumulation and decay after virus induction of wild-type and mutant promoters reveals that the NRDI element is not necessary for postinduction repression. However, one copy each of PRDI and PRDII, or multiple copies of either element, are both necessary and sufficient for virus induction and postinduction repression. Thus, PRDI and PRDII appear to interact with positive regulatory factors immediately after induction and with negative regulatory factors at later times after induction.

To our knowledge, the only other positive regulatory element that has been demonstrated to have a role in postinduction repression is the serum response element (SRE) of the *fos* gene. The *fos* gene is autoregulated (19–21) and the fos protein trans-represses the *fos* gene through the SRE (22–25). However, the fos protein does not bind to the SRE *in vitro*

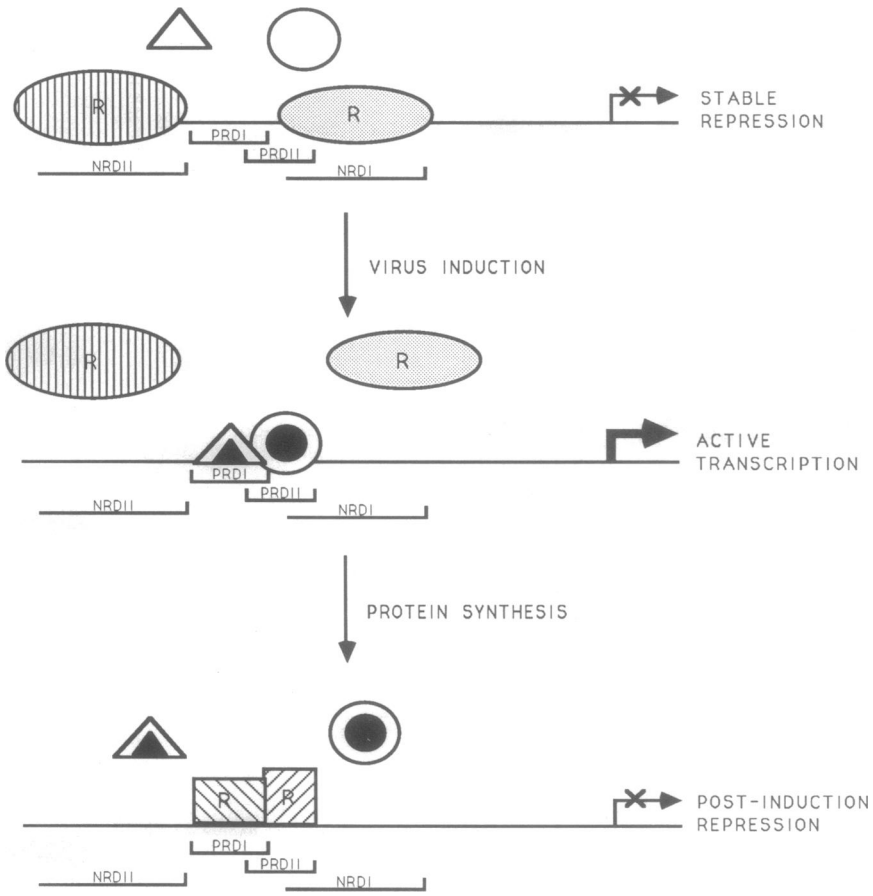

FIG. 6. Model for negative control of β-IFN gene expression showing stable repression and postinduction repression. Virus induction leads to the displacement or inactivation of negative regulatory factors, the activation and binding of positive regulatory factors, and the synthesis and binding of postinduction repressors at later times after induction. Represssors are indicated by the letter R.

(25) and mutations in the DNA-binding region do not affect the protein's ability to trans-repress (24). This indicates that the fos protein represses the SRE indirectly.

Although virus induction of the β-IFN gene occurs in the absence of protein synthesis (for review, see ref. 1), we find that postinduction repression of both PRDI and PRDII requires protein synthesis. This suggests that the positive regulatory proteins are stable and that there are virus-inducible repressor proteins. These proteins could either bind to PRDI or PRDII and block transcription or inactivate the positive regulatory factors that act on PRDI and PRDII. The detection of virus-inducible, CHX-sensitive factors that bind specifically to PRDI or PRDII provides evidence for the first possibility.

PRDI differs by a single base from the IFN-stimulated regulatory element (ISRE), which is found in many IFN-inducible genes (17, 26–34). Studies of the ISRE have revealed many parallels to PRDI (11, 35, 36). Treatment of cells in culture with α-IFN leads to the rapid, protein synthesis-independent activation of a factor (IFN-stimulated gene factor; ISGF3) that binds to the ISRE and activates transcription (37). This is followed by the activation of a second factor (ISGF2), which also binds to the ISRE but requires protein synthesis for its activation (17, 37). A γ-IFN-inducible and CHX-sensitive factor that binds to both the IFN response sequence of H-2K^b and the IRE of β-IFN has also been identified (IBP-1) (38) and, based on its molecular mass (38, 39), may be the same factor as ISGF2. Since ISGF2 and IBP-1 bind to the IRE, and the CHX-sensitive PRDI-binding factor that we observe is inducible by virus, β-IFN and γ-IFN (Fig. 5A; data not shown), this factor may be the murine analogue of ISGF2 and/or IBP-1. Interestingly, the cDNA clone for ISGF2 is the same as the human cDNA clone for IFN regulatory factor 1 (IRF-1) (37). Although overexpression of IRF-1 in monkey COS cells leads to the expression of α- and β-IFN (40), more recent studies suggest that IRF-1/ISGF2 may not play a major role in virus induction of the IFN genes (37).

The fact that the PRDI-binding factor is induced late after virus treatment and is sensitive to CHX suggests that it may be a postinduction repressor. Since this factor is also IFN inducible, its activation may be indirect and repression of β-IFN gene transcription may involve a feedback loop in which virus induces the β-IFN gene that induces both antiviral genes and postinduction repressors. However, since pretreatment of cells with IFN enhances virus induction of the IFN gene (priming; for review, see ref. 1), the IFN-inducible factor could only repress transcription when synthesized after the peak of induction. Further functional analysis is necessary to elucidate the role of this factor in virus and IFN induction.

Two candidates for postinduction repressors have been identified by cDNA expression cloning, IRF-2 (41) and PRDI-BF1 (A. Keller and T.M., unpublished data). Both of these factors act as transcription repressors in cotransfection experiments. IRF-2, which was cloned from mouse L929 cells, is inducible by virus and β-IFN (41). PRDI-BF1, which was cloned from human MG63 cells, is also inducible by virus but is not inducible by β-IFN (A. Keller and T.M., unpublished data). The level of PRDI-BF1 mRNA reaches a plateau late after virus induction, consistent with a role of postinduction repression. Additional studies will be required to determine whether one or both of these factors is involved in β-IFN gene regulation.

PRDII is a virus-inducible element that binds a number of factors, including PRDII-BF (6), PRDII-BF1 (42), NF-κB (13, 43–45), and EBP-1 (46). Thus far, NF-κB is the only PRDII-binding factor that has been implicated in virus induction of the β-IFN gene (for review, see ref. 47). In this paper, we show that PRDII also plays a role in the postinduction repression of the β-IFN gene. In addition, we find that a virus-inducible, CHX-sensitive factor with a molecular mass of 110–130 kDa binds to PRDII (Fig. 5*B*). Based on its molecular mass and binding properties, this factor may be the same as H2TF, a 120-kDa factor (A. S. Baldwin and P. A. Sharp, personal communication) that binds the major histocompatibility complex class I *H-2K*^{b} gene promoter (48). Further studies will be required to determine whether these proteins are the same, and whether they are involved in regulation of the β-IFN gene.

Based on previous studies and the results presented here, we propose that there are two distinct mechanisms for negative control of β-IFN gene expression, stable repression, and postinduction repression (Fig. 6). According to this model, the gene is maintained in a repressed state as a result of repressor proteins bound to the NRDI and NRDII elements. This proposal is based on the observation that mutations in NRDI and NRDII increase the basal level of transcription (5, 9, 15) and on the results of genomic footprinting experiments that indicate that a protein binds to both NRDI and NRDII before induction (49). Virus induction leads to the inactivation or displacement of the repressors bound to NRDI and NRDII and activation of preexisting positive regulatory factors that bind to PRDI and PRDII (6, 49). These positive regulatory factors are stable and in the absence of protein synthesis continue to activate transcription at late times after induction. Consistent with this model is our observation that NF-κB, a probable positive activator of PRDII, is present at late times after induction when transcription of the β-IFN gene is decreasing. In addition, we and others (50) find that NF-κB is induced in the presence of CHX. According to the model, virus induction also leads to the activation of postinduction repressors that bind to PRDI and PRDII and displace the positive regulatory factors. The binding of postinduction repressors is sensitive to CHX, indicating that protein synthesis is required for their activation. An alternative model is that the postinduction repressors inactivate positive regulatory factors that are bound to PRDI and PRDII. To distinguish between these models, the virus-inducible, CHX-sensitive factors that we detect by gel-shift analysis must be demonstrated by functional analysis to be repressors. As discussed above, viral activation of PRDI-binding repressors may be indirect in that these factors could be activated by virally induced IFN. Therefore, postinduction repression appears to involve at least two CHX-sensitive pathways. One is mediated through PRDI and causes the repression of IFN and possibly IFN-inducible genes, while the other is mediated through PRDII.

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