Identification and characterization of a novel repressor of β -interferon gene expression

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We have identified and characterized a novel repressor of human β -interferon (β -IFN) gene expression. This protein, designated PRDI-BF1, binds specifically to the PRDI element of the β -IFN gene promoter and is distinct from previously reported proteins that bind to this sequence. PRDI-BF1 is an 88-kD protein containing five zinc-finger motifs. Cotransfection experiments in cultured mammalian cells revealed that PRDI-BF1 is a potent repressor of PRDI-dependent transcription. PRDI-BF1 blocks virus induction of the intact β -IFN gene promoter and of synthetic promoters containing multiple PRDI sites. PRDI-BF1 can also block the SV40 enhancer when PRDI sites are located between the enhancer and the promoter. This repression is highly dependent on the location of the PRDI sites, however, indicating that PRDI-BF1 cannot act at a distance. On the basis of the properties of PRDI-BF1 and the observation that PRDI-BF1 mRNA accumulation is virus inducible, we propose that PRDI-BF1 may act as a postinduction repressor of the β -IFN gene by displacing positive regulatory proteins from the PRDI site of the promoter.

[Key Words: Negative control of gene expression; zinc-finger protein; virus induction]

Received December 27, 1990; revised version accepted February 20, 1991.

Transcription of the human β -interferon (β -IFN) gene is highly inducible by virus (for review, see DeMaeyer and DeMaeyer-Guignard 1988). β -IFN mRNA can be detected within 90 min after virus infection, peaks at ~6–12 hr after infection, and rapidly decreases. The rate of RNA transcription follows the same time course, indicating that the β -IFN gene is turned on and off at the level of transcription (Whittemore and Maniatis 1990a). New protein synthesis is not required for induction but is required for postinduction repression (Sehgal et al. 1977; Sehgal and Tamm 1979; Whittemore and Maniatis 1990a).

The DNA sequences required for this transient induction are located within the β -IFN gene promoter, between 37 and 104 bp upstream from the start site of transcription (for review, see Maniatis et al. 1991). This region contains both positive and negative regulatory sequences, including an element designated positive regulatory domain 1 (PRDI) (Goodbourn and Maniatis 1988; Keller and Maniatis 1988). PRDI differs by only one nucleotide from the interferon-stimulated response element (ISRE), an element required for induction of a number of genes by IFN (Levy et al. 1988; Fan and Maniatis 1989). PRDI is necessary for the induction of the β -IFN gene and is sufficient when multimerized to confer both virus (Fujita et al. 1987; Kuhl et al. 1987; Fan and Maniatis 1989) and IFN (Fan and Maniatis 1989; MacDonald et al. 1990) inducibility to a heterologous promoter. Virus induction of synthetic promoters containing multi-

ple PRDI sites occurs with the same kinetics as the intact β -IFN gene promoter, and the postinduction turnoff also requires protein synthesis (Whittemore and Maniatis 1990b). Thus, PRDI can function as both a positive and negative regulatory element at different times after induction. These observations suggest that PRDI-dependent virus induction requires the activation of a preexisting transcription factor, whereas postinduction repression requires the synthesis of a repressor (Whittemore and Maniatis 1990b).

Further evidence that PRDI can act as a negative regulatory element is provided by the observation that multiple copies of PRDI can reduce the activity of the SV40 enhancer in some cell types when inserted between the enhancer and the TATA box (Kuhl et al. 1987; Fujita et al. 1988; Fan and Maniatis 1989). This blocking activity is relieved upon virus induction. Thus, PRDI may be involved in both pre- and postinduction repression of the β -IFN gene.

Several proteins that bind specifically to PRDI have been identified. Interestingly, all of these factors also bind to the ISRE. PRDI-BF_c and PRDI-BF_i were identified as binding activities in extracts from human cells induced with double-stranded RNA (Keller and Maniatis 1988). PRDI-BF_c is present before and after virus induction. On the other hand, PRDI-BF_i is present only after cells are treated with virus or poly(I-C) and cycloheximide. Recently, the genes encoding two PRDI-binding factors, IRF-1 (Miyamoto et al. 1988; Pine et al. 1990) and

IRF-2 (Harada et al. 1989), have been cloned. These two factors are related to each other, although they have different activities *in vivo* when transfected into tissue culture cells. IRF-1 can act as a transcriptional activator (Fujita et al. 1989; Harada et al. 1989, 1990), whereas IRF-2 can function as a repressor (Harada et al. 1989, 1990). A number of observations show that IRF-1 and IRF-2 can influence β -IFN gene expression (Harada et al. 1990). However, neither protein has been shown to play a direct role in virus induction or postinduction repression. An additional PRDI-binding protein, ICSBP, has been cloned and exhibits similarity with the amino-terminal regions of both IRF-1 and IRF-2 (Driggers et al. 1990).

In an effort to isolate and characterize proteins involved in the PRDI-dependent induction and repression of the β -IFN gene promoter we screened bacteriophage λ expression cDNA libraries for clones encoding factors that bind specifically to PRDI. In this discussion we report the characterization of a new PRDI-binding protein, designated PRDI-BF1. This protein contains a DNA-binding domain comprised of five zinc fingers. We show that PRDI-BF1 binds specifically to PRDI, and can block virus induction of reporter genes containing the intact β -IFN gene promoter or multiple copies of PRDI. Interestingly, unlike previously described PRDI-binding proteins, which bind to both PRDI and the ISRE with comparable affinities, PRDI-BF1 preferentially binds to PRDI. Evidence that PRDI-BF1 may be involved in the postinduction repression of the β -IFN gene is provided by the observation that the PRDI-BF1 gene is virus inducible.

We find that PRDI-BF1 can block the SV40 enhancer when bound to PRDI sites located between the enhancer, and a promoter. PRDI-BF1 repression, however, is highly dependent on the relative positions of the PRDI site, the enhancer and the promoter. Thus, PRDI-BF1 appears to act by interfering with the function of transcription factors that interact with PRDI or sites immediately adjacent to PRDI.

Results

PRDI-BF1 is an 88-kD protein containing five zinc fingers

A cDNA clone encoding a protein that binds specifically to the PRDI site of the β -IFN gene promoter was isolated by screening a human λ gt11 cDNA expression library with a probe containing multiple PRDI sites (Singh et al. 1988; Vinson et al. 1988). This protein, designated PRDI-BF1 (positive regulatory domain 1-binding factor 1), was expressed in bacteria, and the cell extracts were used for DNA footprinting studies (Galas and Schmitz 1978). As shown in Figure 1A, the bacterially produced protein protects a 19-bp region of the β -IFN gene promoter centered on the PRDI regulatory element. Methylation interference studies (Staudt et al. 1986) show that three guanine residues are contact points for the protein-DNA complex (Fig. 1B). Methylation at positions -72,

Figure 1. PRDI-BF1 binds specifically to the PRDI element of the β -IFN gene promoter. (A) DNase I protection patterns of noncoding and coding strands of the β -IFN gene promoter. Lanes: (G + A and T + C) Maxam and Gilbert sequencing ladders cleaved after G + A and T + C, respectively; (- and +) DNase I treatment of β -IFN gene promoter fragment incubated in the absence and presence of PRDI-BF1, respectively. (B) DNA methylation-interference study of PRDI-BF1 binding to the β -IFN gene promoter sequence. Lanes: (G + A) Maxam and Gilbert sequencing ladder cleaved after G + A; (-) free complex; (+) bound complex. (C) Summary of binding studies. The β -IFN gene promoter sequence of PRDI is shown. The horizontal lines above and below the sequence indicate the regions protected from DNase I cleavage. The arrows represent the guanine residues which, when methylated, interfere with the binding of PRDI-BF1.

-70, and -66 disrupted the binding of PRDI-BF1. This methylation interference pattern contrasts with that reported for other proteins that bind to the PRDI motif, including PRDI-BF_c and PRDI-BF_i (Keller and Maniatis 1988) and IRF-1 and IRF-2 (Harada et al. 1989). The complexes between these factors and DNA were strongly disrupted by methylation at positions -72 and -70 but only weakly disrupted by methylation at -66 (Fig. 1C).

The original cDNA clone used for the binding studies encodes a partial PRDI-BF1 protein. A full-length cDNA clone of PRDI-BF1 was therefore isolated from an oligo-

primed human cDNA library. The DNA sequence of this cDNA clone contains an open reading frame of 789 amino acids, predicting a molecular mass of 88 kD. The sequence encodes five zinc fingers of the TFIIIA type near the carboxyl terminus (Fig. 2). This type of finger motif is an independently folding zinc-containing protein domain with the consensus Tyr/Phe-X-Cys-X_{2/4}-Cys-X₃-Phe-X₅-Leu-X₂-His-X₃-His-X₅ (for review, see Evans and Hollenberg 1988; Berg 1990). All five zinc fingers of PRDI-BF1 are common variants of the consensus. In each case, the cysteines are separated by only two amino acids, and each finger is joined to the next through a Thr-Gly-Glu-Lys-Pro consensus linker. The fifth finger contains a cysteine instead of the second histidine, and these residues are separated by four, rather than three, amino acids.

A basic region of 24 amino acids (charge +8) is located immediately amino terminus to the set of five zinc-finger motifs. Immediately carboxy-terminal to these motifs is a predicted acidic α -helical region of 82 amino acids with a net charge of -11. An additional acidic region is located within a predicted α -helix in the amino-terminal 44 amino acids (net charge of -10). Similar regions in other proteins have been shown to function as transcriptional activation domains (Hope and Struhl 1986; Ma and Ptashne 1987a). Finally, PRDI-BF1 contains a serine- and threonine-rich region of 40 amino acids located between amino acid residues 285 and 324 (Kadonaga et al. 1987). Computer searches of the EMBL and NBRF data banks did not reveal similarities to any other protein sequence outside of the zinc-finger domain.

The PRDI-BF1 gene is virus inducible

Transcription of the PRDI-BF1 gene was examined in RNA blotting experiments. As shown in Figure 3A, two RNA bands were detected in RNA prepared from the human osteosarcoma cell line U2OS. The major and minor bands correspond to RNAs of 5700 and 3500 nucleotides, respectively. The full-length cDNA clone used in the studies described below corresponds to the shorter transcript. Both RNAs are derived from the same gene, as genomic blotting experiments revealed only one gene corresponding to PRDI-BF1 (data not shown). At present, we do not know whether the two RNA species result from differential polyadenylation or splicing, or whether the two RNAs encode different proteins. A number of independent cDNA clones, however, were isolated which all contain the same open reading frame.

PRDI-BF1 mRNA is expressed at a low level in human MG63 cells but increases significantly upon virus induction (Fig. 3B). As demonstrated previously (Zinn et al.

1983), β -IFN mRNA is not detected before induction but is present in high levels by 4 hr postinduction. β -IFN mRNA levels peak between 8 and 12 hr postinduction and then rapidly decrease. In contrast, PRDI-BF1 mRNA levels continue to increase over the entire 24-hr induction period. Thus, the highest levels of PRDI-BF1 mRNA are observed during the postinduction turn off of the β -IFN gene. Significantly, neither the β -IFN nor the PRDI-BF1 gene is inducible by IFN (data not shown). Thus, the induction of both genes must be a direct response to virus infection, rather than a response to IFN induced by virus.

PRDI-BF1 is a transcriptional repressor

The function of PRDI-BF1 was studied by conducting cotransfection experiments in human HeLa cells. The PRDI-BF1 full-length cDNA (corresponding to the 3500-nucleotide mRNA) was cloned into a mammalian expression vector (pXM; Yang et al. 1986) and then cotransfected with reporter plasmids containing various promoters driving the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene. As a control, the mammalian expression vector lacking the PRDI-BF1 cDNA insert was cotransfected with the reporter genes. As shown in Figure 4A, expression of PRDI-BF1 results in a dramatic decrease in expression of the -104 β -IFN gene promoter before and after virus induction. A reporter construct containing four copies of the PRDI motif upstream of the β -IFN gene TATA box was similarly repressed by PRDI-BF1 (Fig. 4B). The expression of PRDI-BF1 decreased both the basal- and virus-induced levels of (PRDI)₄ CAT activity.

Multiple copies of the PRDI element are not only virus inducible but they also respond to IFN induction (Fan and Maniatis 1989). Therefore, we carried out experiments to determine whether PRDI-BF1 is capable of blocking PRDI-dependent IFN induction. As shown in Figure 4B, expression of PRDI-BF1 completely blocks IFN induction of the reporter gene containing four copies of PRDI. Thus, PRDI-BF1 can repress both virus and IFN induction of promoters containing PRDI elements.

To determine whether the negative regulatory activity of PRDI-BF1 is specific to the PRDI element, we carried out cotransfection experiments with a reporter gene containing four copies of the PRDII element of the β -IFN gene promoter. PRDII is virus inducible (Fan and Maniatis 1989) but does not bind to PRDI-BF1 (data not shown). As shown in Figure 4C, expression of the (PRDII)₄ CAT reporter is not repressed by PRDI-BF1 before or after virus induction. In fact, a slight increase in CAT activity is consistently observed. We conclude that PRDI-BF1 repression is specific for the PRDI element.

Figure 2. PRDI-BF1 cDNA encodes an 88-kD zinc-finger protein. Nucleotide sequence of the PRDI-BF1 cDNA. The amino acid residues comprising a 2367-bp open reading frame are indicated by one-letter abbreviations. The five zinc fingers are indicated by arrows below the sequence. The basic, acidic, and serine/threonine-rich regions are indicated by +, -, and *, respectively.

Figure 3. The accumulation of PRDI-BF1 mRNA is virus inducible. (A) Northern blot of poly(A)⁺ RNA isolated from the human osteosarcoma cell line U2OS. Arrows indicate the size of the observed messages in kb. (B) Time course of virus induction in MG63 cells. RNA was prepared from cells treated with virus for the times indicated and analyzed with SP6 probes for β-IFN, PRDI-BF1, and γ-actin mRNAs by using the RNase mapping procedure (Zinn et al. 1983). The specific activities of the β-IFN and γ-actin probes were 100 times lower than that of the PRDI-BF1 probe. The PRDI-BF1 probe does not distinguish between the two transcripts observed on the Northern blot. Lanes: (1) 0-hr virus; (2) 2-hr virus; (3) 4-hr virus; (4) 8-hr virus; (5) 12-hr virus; (6) 16-hr virus; (7) 20-hr virus; (8) 24-hr virus; (9) 24 hr, no virus.

Differential affinity of PRDI-BF1 for PRDI and the ISRE correlates with in vivo activity of PRDI-BF1

As mentioned above, the methylation interference pattern of PRDI-BF1 binding to the β-IFN gene promoter differs from that of other proteins that bind to PRDI. Methylation of the guanine residue at –66 of the β-IFN gene promoter prevents binding of PRDI-BF1 but does not affect the binding of other proteins, such as IRF-1 and IRF-2. Significantly, the guanine at this position is the only difference between PRDI and the ISRE. In contrast to PRDI, the ISRE contains a cytosine at this position. The methylation interference data therefore predict that PRDI-BF1 will not bind specifically to the ISRE. The H-2K^b gene promoter contains one copy of the ISRE, which is identical to PRDI over a 14-nucleotide stretch except that it has a C at the equivalent of position –66 in the β-IFN gene promoter (Kimura et al. 1986). As predicted, in vitro binding studies demonstrate that the bacterially produced partial PRDI-BF1 protein has a much higher affinity for the β-IFN gene promoter than for the H-2K^b gene promoter (Fig. 5A). In contrast, IRF-1 binds to both promoters with equal affinity, consistent with the observation that IRF-1 binding to the PRDI element in the β-IFN gene promoter is not affected by methyl-

ation of the guanine residue at position –66. Because the partial PRDI-BF1 protein used for these studies contains all five zinc fingers, which we have shown to be sufficient for PRDI binding (A.D. Keller and T. Maniatis, in prep.), its binding behavior likely reflects that of the full-length protein.

To show that the difference in binding affinity of PRDI-BF1 to the β-IFN and H-2K^b gene promoters is due to the G/C difference at the equivalent of position –66 in the β-IFN gene promoter, isogenic motifs differing at only that position were synthesized and tested for binding to PRDI-BF1. As shown in Figure 5B, PRDI-BF1 shows a dramatic preference for PRDI over the ISRE, whereas IRF-1 binds both equally well. These observations were further supported by competition experiments shown in Figure 5C. PRDI is much more effective than the ISRE at competing with the PRDI-BF1/PRDI complex, whereas both are equally effective at competing with the IRF-1/PRDI complex.

To determine whether these in vitro DNA-binding studies can be correlated with the in vivo activity of PRDI-BF1, we conducted cotransfection experiments with reporter genes containing promoters with ISRE elements. Expression of PRDI-BF1 leads to only a slight decrease in the activity of the H-2K^b gene promoter (Fig. 6A) or of a promoter containing four copies of the ISRE (Fig. 6B) after induction with either virus or IFN. This effect was much smaller than that observed with reporters containing the PRDI element (cf. Fig. 4A). Therefore, the full-length PRDI-BF1 protein represses promoters containing the PRDI motif much more than promoters containing the ISRE, in agreement with the in vitro binding studies with the partial protein (Fig. 5).

Mechanism of PRDI-BF1 repression

PRDI-BF1 could act as a repressor by preventing the binding of positive regulatory proteins to PRDI, by actively interfering with the assembly of a transcription initiation complex, or both. To determine whether PRDI-BF1 can interfere with the activity of an adjacent enhancer element, we constructed a reporter gene in which PRDI or ISRE sites were inserted between the SV40 enhancer and the TATA box. A similar construct was used previously to show that PRDI sites can block the SV40 enhancer in mouse L929 cells, and the block is removed upon virus induction (Kuhl et al. 1987). We find, however, that the activity of the SV40 enhancer is not diminished by the presence of PRDI sites when the gene is transfected into human HeLa cells (Table 1). Thus, the negative regulatory factors present in mouse L929 cells may not be present in HeLa cells. In any case, the lack of repression in HeLa cells provided the opportunity to determine whether PRDI-BF1 can block the activity of the SV40 enhancer.

A reporter gene containing four copies of PRDI is expressed at low levels when transfected into HeLa cells. However, a reporter gene, containing the SV40 enhancer inserted 40 nucleotides upstream from the PRDI sites is expressed at 50-fold higher levels (Table 1). Remarkably,

Figure 4. PRDI-BF1 is a negative regulator of β -IFN gene expression. Histogram showing the CAT activity produced in HeLa cells transfected with a reporter gene cotransfected with the expression vector lacking (solid bar) or containing (hatched bar) the PRDI-BF1 cDNA insert. CAT activities are normalized to the activity of a cotransfected tkHGH gene. (A) Reporter gene containing the -104 β -IFN gene promoter fused to the CAT gene. (Control) Uninduced cells; (virus) virus-treated cells. (B) Reporter gene containing four copies of the PRDI element and the β -IFN gene TATA box fused to the CAT gene. (Control) Uninduced cells; (virus) virus-treated cells; (IFN) interferon-treated cells. (C) Reporter gene containing four copies of the PRDI element and the β -IFN gene TATA box fused to the CAT gene. (Control) Uninduced cells; (virus) virus-treated cells.

the activity of this reporter is decreased nearly 500-fold when PRDI-BF1 is expressed in the same cells. This effect requires PRDI-BF1 binding, as no decrease in CAT activity is observed with reporter genes containing the SV40 enhancer, but missing the PRDI-binding sites (Table 1).

Additional evidence that PRDI-BF1 repression is PRDI specific is provided by an analysis of the effects of PRDI-BF1 on a reporter gene containing the SV40 enhancer and four copies of the ISRE element. As shown in Table 1, a somewhat higher basal level of CAT activity is observed with the SV40-(ISRE)₄ CAT reporter compared with the SV40-(PRDI)₄ CAT reporter. Only a sixfold decrease in CAT activity is observed, however, with the SV40-(ISRE)₄ CAT reporter in the presence of PRDI-BF1 (Table 1). Thus, as with the reporter genes lacking the SV40 enhancer, the activity of PRDI-BF1 *in vivo* can be correlated with differences in the binding affinity of PRDI-BF1 to the PRDI and ISRE motifs *in vitro*.

These experiments show that PRDI-BF1 can block the transcriptional activity of the SV40 enhancer when bound to sites located between the enhancer and the promoter. We considered three possible mechanisms for this blocking activity. First, PRDI-BF1 could act as a silencer (Brand et al. 1985), a factor that can repress transcription irrespective of its location relative to the enhancer and promoter. Second, PRDI-BF1 could physically block interactions between the enhancer and the promoter. Third, PRDI-BF1 may interfere with binding of TFIID or other general transcription factors to the TATA box, which is located only 15 bp away in the constructs examined above.

To determine whether PRDI-BF1 can act as a silencer

we introduced four copies of PRDI 50 bp upstream from the SV40 enhancer. We found that PRDI-BF1 decreased the activity of this reporter by only twofold (Table 1). Thus, PRDI-BF1 does not appear to act as a silencer. The second possibility was tested by placing the SV40 enhancer and four copies of PRDI immediately upstream from the -128 β -globin gene promoter (Fan and Maniatis 1989). In this case, the PRDI sites are located nearly 100 bp upstream from the TATA box. Surprisingly, we found that PRDI-BF1 decreased the activity of this reporter by only twofold. This observation rules out the possibility that PRDI-BF1 blocks enhancer-promoter interactions but is consistent with the possibility that PRDI-BF1 directly interferes with the function of transcription factors that interact with the TATA box. Thus, PRDI-BF1 may repress transcription by preventing transcription factors from binding to PRDI or by interfering with the function of transcription factors that interact with sites immediately adjacent to PRDI.

Discussion

PRDI-BF1 is a candidate for a postinduction repressor of the β -IFN gene

PRDI-BF1 is a negative regulatory protein that binds specifically to the PRDI site of the human β -IFN gene promoter. Although a role for PRDI-BF1 in β -IFN gene regulation has not been demonstrated directly, a number of observations suggest that this protein is a PRDI-dependent postinduction repressor. Cotransfection experiments show that PRDI-BF1 is a potent repressor of the

Figure 5. Comparison of the affinities of PRDI-BF1 partial protein and IRF-1 to the PRDI and ISRE elements. (A) DNA complexes were formed between a β -IFN gene promoter DNA fragment (lanes 1, 2, 5, and 6) or an H-2K^b gene promoter DNA fragment (lanes 3, 4, 7, and 8) and varying amounts of PRDI-BF1 or IRF-1 protein produced in bacteria. Two complexes are observed in the presence of IRF-1. The faster migrating complex is due to an IRF-1 breakdown product. No additions (lanes 1 and 3); 50 ng of PRDI-BF1 (lanes 2 and 4); 10 ng of IRF-1 (lanes 5 and 7); 50 ng of IRF-1 (lanes 6 and 8). (B) DNA complexes were formed between a PRDI oligonucleotide (lanes 1, 2, 5, and 6) or an ISRE oligonucleotide (lanes 3, 4, 7, and 8) and varying amounts of PRDI-BF1 or IRF-1 protein produced in bacteria. No additions (lanes 1 and 3); 50 ng of PRDI-BF1 (lanes 2 and 4); 10 ng of IRF-1 (lanes 5 and 7); 50 ng of IRF-1 (lanes 6 and 8). (C) Competition binding study. Complexes between PRDI-BF1 and PRDI were competed with increasing amounts of PRDI competitor (lanes 1–4) or ISRE competitor (lanes 5–8). Complexes between IRF-1 and PRDI were competed with increasing amounts of PRDI competitor (lanes 9–12); ISRE competitor (lanes 13–16); or PRDII competitor (lanes 17–20). (Lanes 1, 5, 9, 13, and 17) No competitor; (lanes 2, 6, 10, 14, and 18) 1 ng of competitor; (lanes 3, 7, 11, 15, and 19) 10 ng of competitor; (lanes 4, 8, 12, 16, and 20) 100 ng competitor.

Figure 6. The effect of PRDI-BF1 on the activity of promoters containing the ISRE element. Histogram showing the CAT activity produced in HeLa cells transfected with a reporter gene cotransfected with the expression vector lacking (solid bar) or containing (hatched bar) the PRDI-BF1 cDNA insert. CAT activities are normalized to the activity of a cotransfected tkHGH gene. (A) Reporter gene containing the -365 H-2K^b gene promoter fused to the CAT gene. (Control) Uninduced cells; (IFN) interferon-treated cells. (B) Reporter gene containing four copies of the ISRE element and the β -IFN gene TATA box fused to the CAT gene. (Control) Uninduced cells; (virus) virus-treated cells; (IFN) interferon-treated cells.

intact β -IFN gene promoter and of synthetic promoters containing multiple PRDI sites before and after virus induction. In addition, the PRDI-BF1 gene is inducible by virus, and maximal levels of PRDI-BF1 mRNA are observed at late times after virus induction. Thus, if levels

of PRDI-BF1 protein correspond to mRNA levels, the timing of PRDI-BF1 expression correlates well with the timing of postinduction repression.

Evidence for a PRDI-dependent postinduction repressor was provided by the demonstration that protein syn-

Table 1. The ability of PRDI-BF1 to block the activity of the SV40 enhancer depends on the relative positions of the enhancer, promoter, and PRDI sites

Reporter construct	Normalized CAT activity		Fold repression (X)
	vector	PRDI-BF1	
	52	83	0.6
	2.2	<0.1	>22
	142	0.3	474
	33	14	2
	127	64	2
	22	4.1	5
	298	49.2	6

The levels of CAT activity resulting from cotransfection of reporters into HeLa cells with an expression vector or expression vector producing PRDI-BF1 are listed. The reporters are diagrammed, indicating the relative positions of the SV40 enhancer, the TATA box, and the PRDI or ISRE sites. CAT activities shown are normalized to the activity of a cotransfected tkHGH gene.

thesis is required for postinduction turn off of promoters containing multiple PRDI sites (Whittemore and Maniatis 1990b). In addition, several virus-inducible, cycloheximide-sensitive PRDI-binding activities were detected in gel-shift experiments (Whittemore and Maniatis 1990b). At present, we do not know whether any of these binding activities correspond to PRDI-BF1. The two most prominent binding activities were also found to be IFN inducible and therefore do not correspond to PRDI-BF1. One of these IFN-inducible activities, IRF-2, is also a candidate for a postinduction repressor (Harada et al. 1989). Experiments to determine whether IRF-2 is capable of blocking virus induction of PRDI-containing promoters, however, have not been reported.

Differential regulation of virus and IFN induction

Although both PRDI-BF1 and IRF-2 bind specifically to PRDI, the two proteins display important differences in their sequence requirements for binding. The guanine residue at position -66 in the β -IFN gene promoter is critical for efficient PRDI-BF1 binding (Fig. 1) but is not required for IRF-2 binding. For this reason, PRDI-BF1 binds to PRDI 100 times more tightly than to the ISRE, but IRF-1 binds equally well to both. This difference in binding affinity of PRDI-BF1 to PRDI and the ISRE in vitro is reflected in vivo, where PRDI-BF1 represses promoters containing the PRDI motif nearly 100 times more than promoters containing the ISRE. Thus, PRDI-BF1 appears to be a PRDI-specific repressor, whereas IRF-2 has the potential to repress the activities of both PRDI and the ISRE. It may be relevant in this regard that the PRDI-BF1 gene is inducible only by virus, whereas the IRF-2 gene is inducible by both virus and IFN (Harada et al. 1989).

Significantly, two closely related transcriptional regulatory pathways, virus and IFN induction, require very similar regulatory elements—PRDI and the ISRE. Virus-infected cells produce and secrete IFN. This IFN binds to specific cell-surface receptors on neighboring cells, inducing the transcription of a large set of genes involved in the antiviral response. IFN also induces genes that enhance virus induction of the β -IFN gene, a phenomenon known as priming (DeMaeyer and DeMaeyer-Guignard 1988). The similarity between PRDI and the ISRE may allow communication between the virus and IFN induction pathways, yet still allow the pathways to be independently regulated. For example, in cells that are initially infected with virus, the β -IFN gene must be repressed at late times in induction to achieve a local response. It may be advantageous, however, to maintain the antiviral response in these cells by not simultaneously repressing the IFN response genes. PRDI-BF1 would be able to achieve such differential repression.

In uninfected cells, IFN-induced factors, such as IRF-1 and IRF-2, bind to both PRDI and the ISRE. IFN induction could therefore influence directly the activity of the β -IFN gene promoter upon subsequent virus induction. For example, factors such as IRF-1 and IRF-2 could be involved in priming. In contrast, ISGF3, which is

thought to be responsible for much of the IFN-induced activity on the ISRE, binds to the ISRE and not to PRDI (Kessler et al. 1988). Thus, regulatory interactions between the virus and IFN-inducible pathways could be achieved through factors, such as IRF-1 and IRF-2, but the pathways could be independently regulated through factors such as ISGF3 and PRDI-BF1.

Mechanism of repression by PRDI-BF1

Three different mechanisms have been proposed for negative control of gene transcription in eukaryotes (for review, see Levine and Manley 1989; Renkawitz 1990). The simplest mechanism is competition between repressors and activators for common or overlapping DNA-binding sites. This mechanism has been suggested for the stable repression of the β -IFN gene (Goodbourn et al. 1986), for IRF-2 repression of multimerized PRDI (Harada et al. 1989), for negative control by hormone receptors (Sakai et al. 1988; Drouin et al. 1989), and for *Drosophila* homeo box proteins (Jaynes and O'Farrell 1988; Han et al. 1989).

A second mechanism is silencing, where repressor binding blocks transcription irrespective of the location of the operator relative to the enhancer and the promoter. This mechanism has been proposed to explain silencer activity at the mating-type locus in yeast (Brand et al. 1985), the silencing of the SV40 enhancer by multimerized PRDI in mammalian cells (Kuhl et al. 1987), and the negative regulatory activity of the *Drosophila* zinc-finger protein *Krüppel* (Licht et al. 1990). In the case of *Krüppel*, a region of the protein that does not include the zinc fingers is both necessary and sufficient to confer repression activity to another DNA-binding protein (Licht et al. 1990).

A third mechanism for negative control of gene transcription is the neutralization of activators. In this case, a repressor interacts directly with a positive activator to shield either its DNA-binding domain or its transcriptional activation domain. The former mechanism has been proposed for T-antigen repression of AP2 in mammalian cells (Mitchell et al. 1987), whereas the latter has been proposed to explain GAL80 repression of GAL4 in yeast (Ma and Ptashne 1987b). A somewhat different mechanism is provided by the transcription factor NF- κ B, which is found in the cytoplasm bound to an inhibitor I κ B. This inhibitor prevents the transport of NF- κ B to the nucleus (Baeuerle and Baltimore 1988a,b; Lenardo and Baltimore 1989).

The third mechanism clearly does not apply to PRDI-BF1, as the protein must act by directly binding DNA. The PRDI site is required for PRDI-BF1 repression, and several mutants of PRDI-BF1 that do not bind to PRDI in vitro do not repress PRDI promoter constructs in vivo (data not shown). Two experiments show that PRDI-BF1 does not act as a silencer. First, only a twofold decrease in the activity of the SV40 enhancer is observed if multiple copies of PRDI are placed upstream from the enhancer. Second, the ability of PRDI-BF1 to block the SV40 enhancer in constructs containing PRDI sites be-

tween the enhancer and the promoter depends on the distance between the PRDI sites and the TATA box. Complete repression is observed when PRDI sites are located 15 bp upstream from the TATA box, but this effect is significantly diminished when the PRDI sites are located 100 bp from the TATA box. In the former case, PRDI-BF1 may interfere with the formation of a transcription complex on the TATA box, rather than directly blocking the activity of the SV40 enhancer. This observation also brings into question the interpretation of previous β -IFN gene-silencing experiments (Kuhl et al. 1987). Because the PRDI sites in these studies were located only 26 bp upstream from the TATA box, the "silencing" observed may be due to interference with the promoter rather than the upstream enhancer.

On the basis of these observations we propose that PRDI-BF1 acts as a postinduction repressor of the β -IFN gene by preventing interactions between PRDI and positive regulatory proteins. This mechanism may be characteristic of regulatory regions involved in both positive and negative control, whereas silencers may act at sites that are used exclusively for negative control. Stable repression of the β -IFN gene prior to virus induction may also involve direct competition between positive and negative regulatory factors. In this case, a negative regulatory sequence designated NRDI, overlaps with PRDII, a positive regulatory element (Goodbourn et al. 1986; Goodbourn and Maniatis 1988). A repressor bound to NRDI before induction may prevent the binding of positive regulatory proteins to PRDII. Significantly, NRDI can also repress an immediately adjacent heterologous promoter, but repression is dramatically diminished when the distance between NRDI and the promoter is increased by only a few base pairs (Goodbourn et al. 1986). Thus, competition between positive and negative regulatory proteins for interactions with a common binding site may be a general mechanism for negative control in higher eukaryotes.

Materials and methods

Library screening and oligonucleotide synthesis

The PRDI-BF1 clone was isolated from a λ gt11 expression library by using ligated PRDI oligonucleotides as probe (Singh et al. 1988; Vinson et al. 1988). The initial partial cDNA clone was isolated from a library prepared from U2OS poly(A)⁺ RNA (gift of J. Wozney). The full-length clones were isolated from oligo-primed libraries prepared from MG63 RNA (Maniatis et al. 1982). The human IRF-1 cDNA clone was similarly isolated from an oligo-primed MG63 library (M. Tian and J. Jing, unpubl.).

The PRDI and ISRE oligonucleotides were synthesized on an Applied Biosystems 391 DNA Synthesizer. The H-2K^b clone used for gel shifts contained sequences between nucleotides -153 and -134 of the H-2K^b gene promoter (gift of P. Sharp).

Bacterial protein production and DNase I footprinting

PRDI-BF1 was produced as a partial peptide by cloning the originally isolated partial cDNA insert (initiating at bp 1613) into the phage T7 expression vector (Studier and Moffatt 1986). Full-

length IRF-1 protein was expressed in the same vector (gift of V. Palombella). Protein was purified from inclusion bodies by the method of Gaul et al. (1987). Protein was dialyzed against buffer containing 50 mM Tris (pH 7.9), 0.5 M NaCl, 10% glycerol, and 1 mM PMSF. This resulted in protein of >50% purity as judged by SDS-PAGE and staining with Coomassie brilliant blue.

In the DNase I footprinting studies, 0.1–1 ng of end-labeled probe comprising sequences between -104 and -10 of the β -IFN gene promoter was incubated with 40–100 ng of the bacterially produced protein in a 15- μ l reaction for 20 min at room temperature. Binding reactions contained 13 mM Tris (pH 7.9), 60 mM KCl, 12.5 mM NaCl, 12% glycerol, and 75 μ g/ml of poly[d(I-C)]. DNase I (0.3 ng) in 35 μ l of 5 mM MgCl₂, 5 mM CaCl₂ solution was then added for 1 min at room temperature. The reaction was terminated by extraction with phenol. The digested DNA was precipitated with ethanol, resuspended in denaturing formamide mix, and fractionated on a denaturing polyacrylamide gel.

Gel shifts and methylation interference

Bacterially produced PRDI-BF1 or IRF-1 was incubated with end-labeled probes for 20 min at room temperature under the same buffer conditions used for footprinting. The mixtures were then loaded onto a low ionic strength 4.5% nondenaturing polyacrylamide gel and electrophoresed at 10 V/cm at room temperature with circulation of buffer (Strauss and Varshavsky 1984). In the methylation interference study, the end-labeled DNA fragment was treated with DMS before incubation with PRDI-BF1. Binding reactions were then loaded onto a nondenaturing polyacrylamide gel to resolve the bound complex from free probe. Bands were cut out of the gel, and the DNA was eluted from each band in TE, phenol-extracted, and ethanol-precipitated. The DNA was then cleaved at the methylated guanine residues by treatment with piperidine and resolved on a sequencing gel (Staudt et al. 1986).

RNA analysis

RNA was isolated by the guanidinium thiocyanate procedure (Chirgwin et al. 1979). The Northern blot was prepared and probed according to Maniatis et al. (1982). The probe used for hybridization to the blot contained PRDI-BF1 sequences between bp 1613 and 3280. RNase mapping experiments were carried out as described previously using 15 μ g of RNA (Zinn et al. 1983). The probe used to detect β -IFN mRNA 5' ends was pSP65'IF, which protects 277 nucleotides (Zinn et al. 1983). The probe used to detect γ -actin mRNA was pSP64 Hu γ -actin, which protects 143 nucleotides (Enoch et al. 1986). The probe for PRDI-BF1 extends from the *Pst*I site at bp 2080 to the *Sma*I site at bp 2273, protecting 193 nucleotides of mRNA.

Virus and interferon inductions

Cells were treated with Sendai virus (SPAFAS) as described (Goodbourn and Maniatis 1988). Interferon inductions were performed by treatment with 200 U/ml of recombinant human β -IFN (Lee Biomolecular) for 18 hr in serum containing medium.

Mammalian cell transfections

HeLa cells were transfected with calcium phosphate according to the method of Chen and Okayama (1988). Typical transfections included 15 μ g of expression plasmid, 5 μ g of reporter construct, and 5 μ g of tkHGH transfection control. The expres-

sion plasmid pXM includes the adenovirus major late promoter at the site of cDNA insertion (Yang et al. 1986). At 24 hr post-transfection, the cells were split onto separate plates for control and induced samples. At 48 hr post-transfection, the cells were induced with either virus or IFN for 14 hr. Cells were harvested by scraping, and extracts were prepared for CAT assays according to Gorman et al. (1982). The chloramphenicol products were resolved on TLC and cut out for quantitation by liquid scintillation counting. Human growth hormone assays were performed for a transfection control by radioimmunoassay by using the Allegro kit (Nichols Institute Diagnostics). All transfections were performed in duplicate. Transfection efficiencies were found to vary by no more than twofold between samples.

Acknowledgments

We thank Alan Michelson, Vito Palombella, and Lisa Whittemore for critical review of the manuscript. This work was supported by a Merck Predoctoral Fellowship granted to A.K. and U.S. Public Health Service grant AI-20642 to T.M. from the National Institutes of Health.

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Identification and characterization of a novel repressor of beta-interferon gene expression.

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Genes Dev. 1991, **5**:

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