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Identification of an inducible factor that binds to a positive regulatory element of the human β-interferon gene

[transcription factors/DNA–protein interactions/virus and poly(I)·poly(C) induction/protein modification]

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ABSTRACT **Human β-interferon gene expression is induced by virus or poly(I)·poly(C). This induction is due at least in part to an increase in the rate of transcription and does not require protein synthesis. A 40-base-pair DNA sequence within the β-interferon promoter, termed the interferon gene regulatory element (IRE), is an inducible enhancer in mouse fibroblasts, and both positive and negative regulatory DNA sequences have been identified within this element. In this paper we identify three factors that bind specifically to two positive regulatory domains within the IRE. Two of these factors are present in nuclear extracts prepared from uninduced and induced cells; one is present only in extracts from induced cells. The functional significance of these binding activities was demonstrated by showing that point mutations within the IRE that decrease human β-interferon gene transcription *in vivo* prevent binding *in vitro*. We propose that induction of the β-interferon gene involves the modification of a protein to a form that binds specifically to a positive regulatory sequence within the IRE.**

Human β-interferon gene transcription is highly inducible by virus or poly(I)·poly(C) (1–5). Analyses of the DNA sequences required for β-interferon gene regulation have identified the interferon gene regulatory element (IRE), an inducible enhancer within the β-interferon promoter (6). Studies of the effects of mutations within the IRE on *in vivo* expression have revealed the presence of both positive and negative regulatory sequences (7–9). These studies suggest that activation of the IRE may involve the removal of a repressor molecule from a negative regulatory domain, NRDI (sequence between positions −63 and −37), and the binding of transcription factors to two positive regulatory domains, PRDI and PRDII (sequence between −77 and −55) (7–9) (Fig. 1). A DNA fragment containing PRDI and PRDII, but lacking NRDI, can function as a constitutive enhancer, but its activity is further increased upon induction (7). In addition, a DNA fragment containing multiple copies of a hexamer sequence motif (AAGTGA) present only in PRDI can function as an inducible regulatory element (10).

The regulatory factors required for β-interferon gene expression must be present in an inactive form before induction, since transcriptional activation occurs in the absence of protein synthesis (11, 12). The increase in activity of PRDI and PRDII after induction could be due to an increase in the activity of the constitutive transcription factors that bind to these positive regulatory sequences or due to the activation of other factors that bind only after induction. To investigate these possibilities we have used gel retardation (13, 14) and DNase protection ("footprinting") (15) assays to identify proteins that bind specifically to the IRE. Two DNA-binding proteins were detected in nuclear extracts from uninduced cells. One of these proteins binds to PRDI, whereas the other binds to PRDII. These two proteins are also detected in extracts from induced cells, but another protein that binds to PRDI is detected as well. These results provide further evidence that induction of the human β-interferon gene involves both constitutive and inducible regulatory factors.

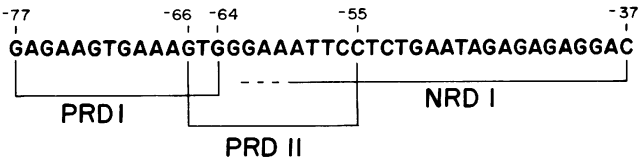

FIG. 1. Organization of regulatory sequences within the IRE. The sequence between positions −37 and −77 of the human β-interferon gene is shown, with the positive (PRDI and PRDII) and negative (NRDI) regulatory domains indicated (9–11). The precise 5′ boundary of NRDI has not been identified.

MATERIALS AND METHODS

Nuclear extracts were prepared by the method of Dignam *et al.* (16) from both uninduced and induced human MG-63 osteosarcoma cells. Cells were induced by treatment with poly(I)·poly(C) (0.1 mg/ml) and cycloheximide (50 μg/ml) for 2 hr followed by an additional 4-hr treatment with cycloheximide alone. Binding-reaction mixtures contained 12 mM Tris (pH 7.9), 60 mM KCl, 2 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM dithiothreitol, 12% (vol/vol) glycerol, and 75 μg of poly(dI)·poly(dC). Ten micrograms of protein was mixed in a 20-μl reaction mixture with 0.1–1 ng of an end-labeled probe comprising nucleotides −77 to −37 of the β-interferon promoter. The binding-reaction mixtures were incubated for 15 min at 30°C and then analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel at 10 V/cm in low-ionic-strength buffer (13, 14, 17). For the competition experiments, nuclear extracts from induced cells were partially purified as a 0.2–0.5 M KCl elution step on a Bio-Rex 70 (Bio-Rad) column in order to reduce nonspecific binding.

In the DNase I footprinting studies, end-labeled probes comprising the IRE were incubated with partially purified extracts from induced MG-63 cells (proteins eluted as a 0.2–0.5 M KCl elution step on a Bio-Rex 70 column) and treated with DNase I (60–125 μg/ml) for 1 min at room temperature. The reaction was stopped by the addition of EDTA to 10 mM, and the unbound probe fragment and the probe–protein complexes were resolved by preparative nondenaturing polyacrylamide gel electrophoresis. The DNA from each band was eluted, denatured, and analyzed on a sequencing gel (15, 18). In the methylation-interference

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Abbreviations: IRE, interferon gene regulatory element; NRDI, negative regulatory domain I; PRDI and PRDII, positive regulatory domains I and II; PRDII-BF, PRDII-binding factor; PRDI-BF_c and PRDI-BF_i, constitutive and inducible PRDI-binding factors.

study, the end-labeled IRE DNA fragment was treated lightly with dimethyl sulfate so that ≈10% of the molecules were methylated. The probe was then incubated with partially purified extracts from induced MG-63 cells. After the unbound probe fragment and complexes were resolved in a nondenaturing polyacrylamide gel, the DNA was eluted from each band and cleaved at the methylated guanosine residues by treatment with piperidine (19).

RESULTS

Gel retardation assays (13, 14) were used to identify factors that bind specifically to the IRE. Two protein–DNA complexes, A and B, were observed when an end-labeled DNA probe containing the IRE was incubated with nuclear extracts prepared from uninduced human MG-63 cells (Fig. 2). When the same probe was incubated with extracts prepared from induced cells, a third complex, C, was observed in addition to A and B (Fig. 2). Competition experiments were performed to determine whether these complexes involve specific interactions with the IRE. As shown in Fig. 3, the addition of competitor DNA containing IRE sequences to the binding-reaction mixture prevented the formation of all three complexes. In contrast, the addition of competitor DNA containing four repeats of the hexamer motif AAGTGA (10) prevented the formation of complexes B and C yet had no effect on complex A. This observation suggests that complexes B and C involve specific binding to PRDI.

The binding site of each of these complexes was localized by DNase I protection (15). The DNA–protein complexes were treated with DNase I before fractionation in nondenaturing polyacrylamide gels (18). DNA from the three complexes was then recovered, denatured, and analyzed by electrophoresis in a sequencing gel. The altered pattern of DNase I cleavage in complex A is included within the region corresponding to the PRDII site (Fig. 4*a*). We will refer to this factor as PRDII-BF (PRDII-binding factor). The altered patterns of DNase I cleavage in complexes B and C are in the region of PRDI and are indistinguishable (Fig. 4*b*). We will refer to the factors that bind to PRDI to form complexes B and C as PRDI-BF_c (constitutive PRDI-binding factor) and PRDI-BF_i (inducible PRDI-binding factor), respectively. Methylation interference (19) was used to identify the guanosine residues involved in essential contacts in complexes B and C. Fig. 5 shows that methylated guanosines at positions −72 and −70 within PRDI block the formation of both complexes.

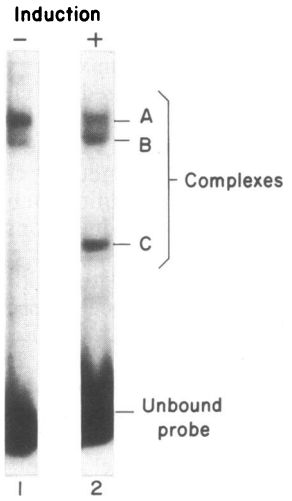

FIG. 2. Identification of cellular factors that bind to the IRE. Shown is an autoradiogram of DNA–protein complexes resolved by gel electrophoresis. An end-labeled DNA fragment containing the IRE was incubated with nuclear extracts prepared from uninduced (lane 1) and induced (lane 2) human MG-63 cells and analyzed by electrophoresis in a nondenaturing polyacrylamide gel (14–16).

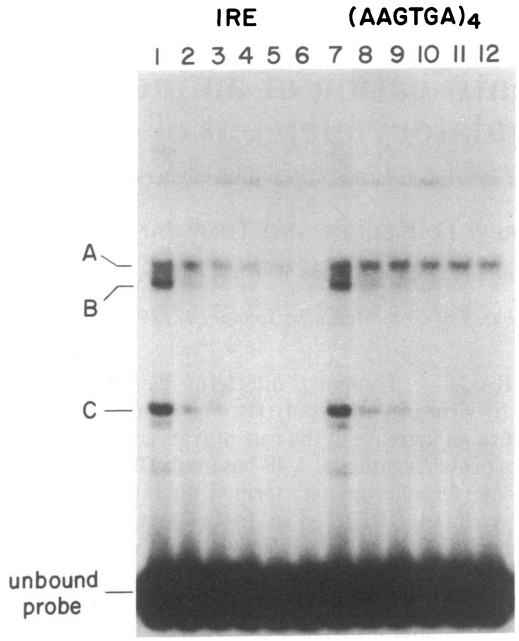

FIG. 3. Competitive binding studies. DNA–protein complexes were formed with extracts prepared from induced MG-63 cells in the presence of competitor IRE (lanes 1–6) or hexamer repeat (lanes 7–12) sequences. Each reaction mixture contained a total of 500 ng of linearized plasmid DNA, of which 0 ng (lanes 1 and 7), 20 ng (lanes 2 and 8), 45 ng (lanes 3 and 9), 100 ng (lanes 4 and 10), 225 ng (lanes 5 and 11), or 500 ng (lanes 6 and 12) contained inserts with specific sequences.

To determine whether these complexes involve specific protein–DNA interactions that are relevant to the function of the IRE, we analyzed the effects of IRE mutations on complex formation. The 5′ boundary of the IRE has been localized between −77 and −73 (20). As shown in Fig. 6*a*, an end-labeled probe lacking sequences between −77 and −73 was unable to form complexes B and C but did form complex A. Thus, a deletion that removes part of PRDI inactivates the IRE and prevents the binding of PRDI-BF_c and PRDI-BF_i to IRE sequences.

Single-base mutations at positions −76, −75, −71, and −65 do not significantly affect the level of β-interferon mRNA *in vivo*, whereas mutations at −70, −68, and −67 markedly decrease the level of transcription after induction (9). When DNA fragments containing these mutations were end-labeled and assayed for complex formation (Fig. 6*b*), mutations at −76, −75, −71, and −65 had little effect. In contrast, mutations at −70, −68, and −67 prevented the formation of complexes B and C but did not affect the formation of complex A. Therefore, point mutations in PRDI that decrease the activity of the IRE after induction *in vivo* correspond precisely to those that block the binding of PRDI-BF_c and PRDI-BF_i to PRDI *in vitro*.

In the region of PRDII, point mutations result in complex phenotypes *in vivo* because they affect overlapping binding sites for a repressor and a transcription factor (9). When IRE mutants in this region were analyzed *in vitro*, mutations between −65 and −57 blocked the formation of complex A. None of the mutations in this region prevented the formation of complexes B and C (Fig. 6*b*). Thus the binding site of PRDII-BF corresponds to the PRDII site defined by expression studies *in vivo* (9).

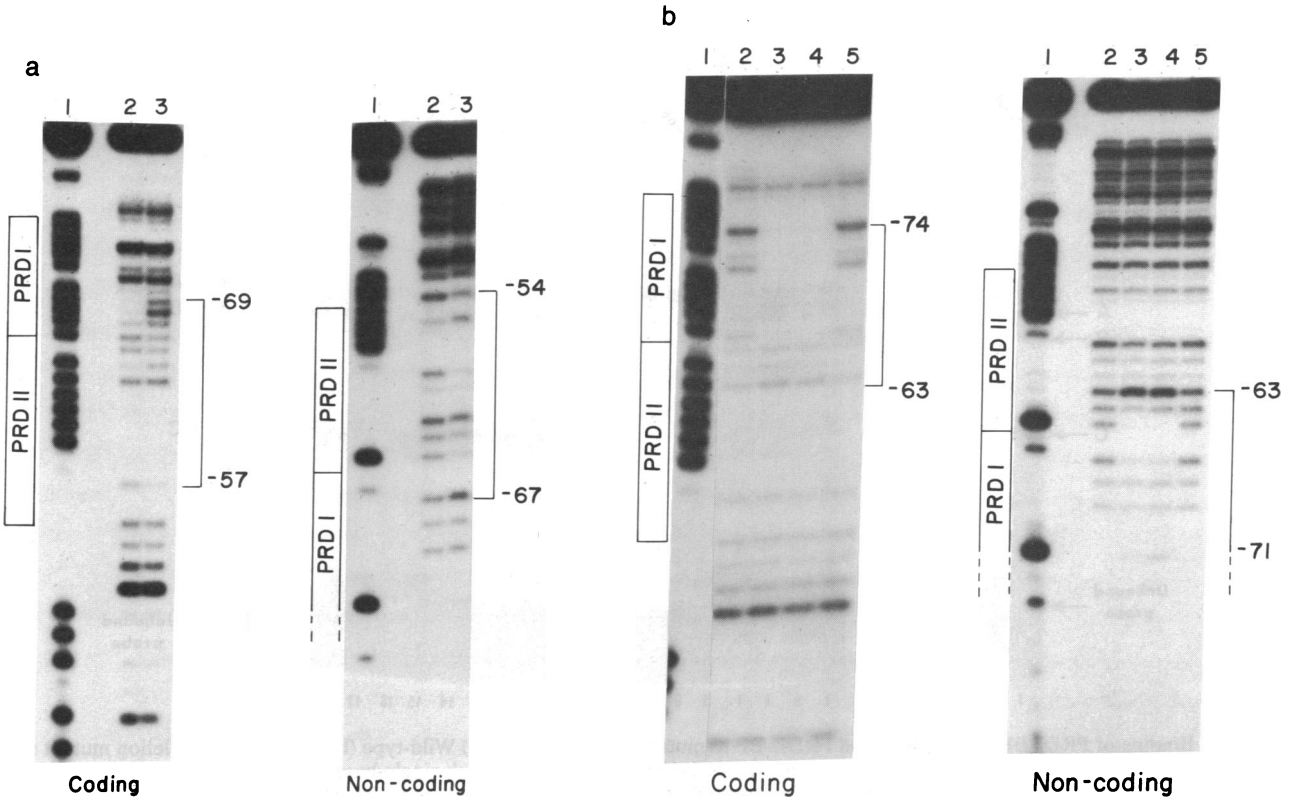

FIG. 4. Footprinting studies of the IRE. (*a*) DNase I cleavage patterns of coding and noncoding strands for complex A. Sequences corresponding to PRDI and PRDII and the region of altered DNase I sensitivity are indicated. Lanes: 1, products of G + A sequencing reactions; 2, unbound probe fragment; 3, complex A. Four sites are protected from DNase I cleavage (positions −65, −60, −57, and −54), and five sites are hypersensitive to cleavage (−69, −68, −67, −64, and −56). (*b*) DNase I cleavage patterns of coding and noncoding strands for complexes B and C. Lanes: 1, products of G + A sequencing reactions; 2 and 5, unbound probe fragment; 3, complex B; 4, complex C. For both complexes, six sites are protected from DNase I cleavage (−74, −72, −71, −67, −66, and −65), and three sites are hypersensitive to cleavage (−71, −64, and −63).

DISCUSSION

We have identified three physically distinct factors that bind specifically to the IRE. One of these factors (PRDII-BF) binds to PRDII and is detected in extracts from both uninduced and induced human MG-63 cells. Multiple copies of a synthetic PRDII sequence can function as a constitutive enhancer whose activity can be greatly increased by virus induction (Chen-Ming Fan and T.M., unpublished data).

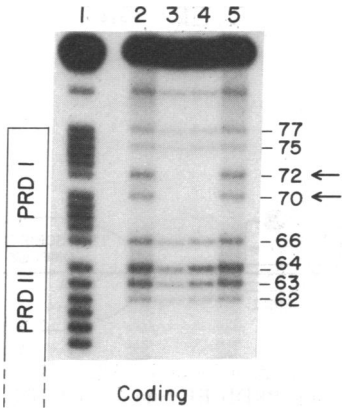

FIG. 5. DNA methylation-interference studies of the IRE. Shown is the pattern of methylation interference of complexes B and C (see *Materials and Methods*). Lanes: 1, products of G + A sequencing reactions; 2 and 5, unbound probe fragment; 3, complex B; 4, complex C. Arrows indicate methylation sites that are underrepresented in the complexes.

However, we have not detected an inducible factor that binds to PRDII. The other two factors bind specifically to PRDI. PRDI-BF_c is detected in extracts from both uninduced and induced human MG-63 cells, whereas PRDI-BF_i is detected exclusively in extracts from induced cells. At present we have no information regarding the relationship, if any, between the two factors that bind to PRDI. The two factors generate the same DNase-protection and methylation-interference patterns, and we have not detected any differences in their affinity for PRDI. The fact that the DNA–protein complex containing the inducible factor migrates more rapidly than the complex containing the constitutive factor suggests that the inducible factor is smaller. If this is the case, the smaller factor could be a proteolytic digestion product of the larger factor or an entirely different protein of lower molecular weight.

Although we have no direct evidence that the binding activities we detect are involved in β-interferon gene regulation *in vivo*, this possibility is strongly suggested by the observation that mutations in PRDI and PRDII that decrease the level of β-interferon transcription *in vivo* prevent the *in vitro* binding of the PRDI-BFs to their respective binding sites. If we assume that these factors do play an important role, the previously reported activities of PRDI and PRDII can be explained in the following manner. A DNA fragment that includes PRDI and PRDII can function as a constitutive enhancer in the absence of NRDI yet still be stimulated by induction (7). Thus, the constitutive activity of this fragment may result from the binding of PRDI-BF_c to PRDI and of PRDII-BF to PRDII (Fig. 7*a*). The increase in transcription after induction would then be due to both the binding of the

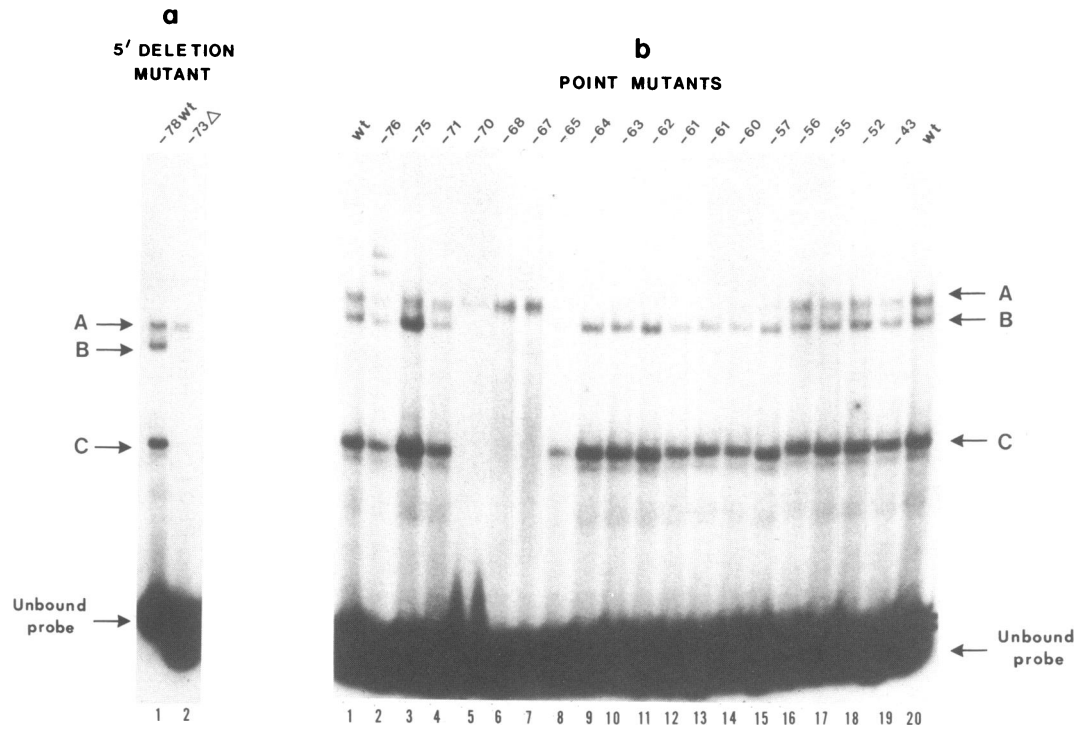

FIG. 6. Binding of PRDI-BF$_c$, PRDI-BF$_i$, and PRDII-BF to mutant IRE templates. (*a*) Wild-type (lane 1) and −73 deletion mutant (lane 2). (*b*) Wild-type (lanes 1 and 20) and single-base mutations (lanes 2–19). The position of each single-base mutation is indicated above each lane. The extra bands in lane 2 may be due to the creation of a new binding site by the point mutation at −76. Mutant IRE templates were end-labeled, incubated with partially purified extracts from induced MG-63 cells, and analyzed as described in *Materials and Methods*.

inducible factor PRDI-BF$_i$ to PRDI and an alteration in the transcriptional activity of PRDII-BF. In the intact IRE the binding of PRDII-BF would be prevented by a repressor bound to NRDI prior to induction (Fig. 7*b*). On the basis of these observations we propose that induction may involve at least three different posttranslational modifications of regulatory factors: (*i*) activation of a preexisting factor to generate the PRDI-BF$_i$ binding activity, (*ii*) alteration of PRDII-BF to generate an enhanced transcriptional activity, and (*iii*) inactivation of the repressor bound to NRDI. This model is consistent with the observed binding activities of PRDI- and PRDII-binding factors and with previously reported *in vivo* DNase-protection experiments (8). However, we have not yet detected an NRDI binding activity *in vitro*.

Posttranslational modification of regulatory factors appears to be a general mechanism for tissue-specific and inducible gene expression (see ref. 21 for review). The modification of a preexisting transcription factor (NF-κB) has been implicated in the B-cell-specific expression of the mouse κ-chain immunoglobulin gene (22). Similarly, the induction of the mouse c-*fos* gene (23), the *Drosophila* heat shock genes (24), and the human metallothionein IIA gene (25) involves an

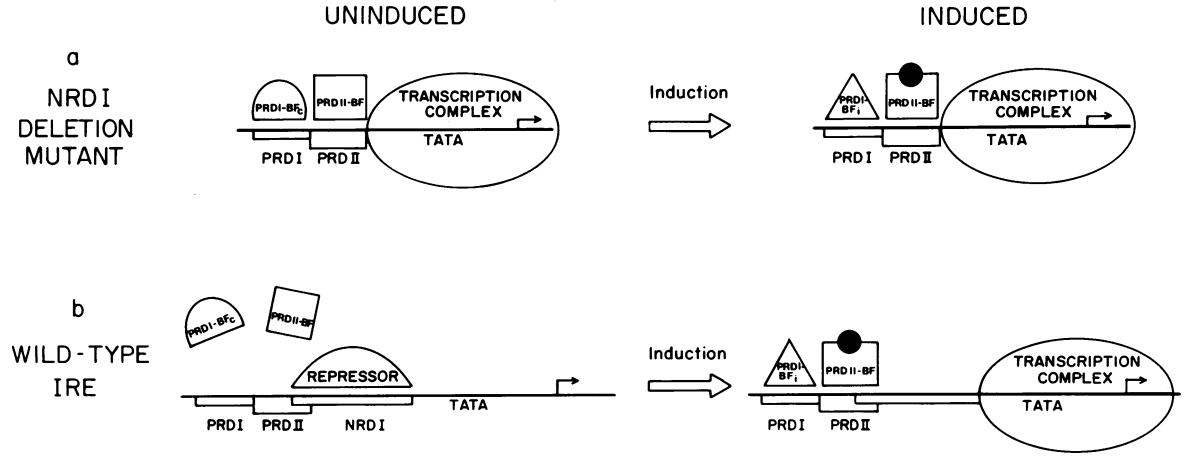

FIG. 7. Model for the interaction of regulatory factors with the positive and negative regulatory domains of the IRE. (*a*) Interaction of factors with NRDI deletion mutants. In the absence of the repressor binding site NRDI, PRDI-BF$_c$ and PRDII-BF bind to the PRDI and PRDII sites, respectively, prior to induction. This interaction results in constitutive transcription. After induction a preexisting protein is modified to form PRDI-BF$_i$, and PRDII-BF is modified to form a more transcriptionally active factor (indicated as a black circle overlapping the PRDII box). Both of these alterations result in a higher level of transcription. (*b*) Interaction of factors with the wild-type IRE. Before induction, repressor binding to NRDI blocks the binding of PRDII-BF to PRDII, thus preventing transcription. After induction the repressor is removed and transcription is activated by the binding of PRDI-BF$_i$ and the modified PRDII-BF to their respective binding sites. Removal of the repressor could be a result either of direct inactivation of the repressor or of an increase in the affinity of the positive transcription factors, leading to the displacement of the repressor.

increase in the binding activity of specific transcription factors that are present prior to induction. Phosphorylation of a specific transcription factor has been implicated in the regulation of a yeast heat shock gene (26). In this case, however, the unmodified transcription factor binds constitutively to DNA, but heat-induced phosphorylation is required for transcriptional activity.

Examination of the nucleotide sequence of the IRE has revealed interesting sequence motifs. For example, similarities have been noted between sequences in PRDI and PRDII and several viral and cellular enhancer motifs, suggesting that the IRE may interact with the same factors that are required for the activity of constitutive enhancers (7). However, we have found that DNA fragments containing these viral enhancer homologues do not compete with the IRE for binding to PRDI-BF_c, PRDI-BF_i, or PRDII-BF (data not shown).

Another notable feature of the IRE sequence is the hexamer sequence motif AAGTGA. As mentioned above, this sequence is found within PRDI. In addition, six imperfect copies of this sequence are present at other sites within the β-interferon promoter (27). Recent studies have shown that four, but not one, two, or three, tandemly repeated copies of the hexamer can confer inducibility on a heterologous promoter (10). We have found that four copies of the hexamer efficiently compete with the IRE for binding to PRDI-BF_c and PRDI-BF_i (Fig. 3), whereas one copy does not (data not shown). The inducibility of four copies of the hexamer could be the result of a specific interaction with PRDI-BF_i. In this regard, we note that tandem repeats of the hexamer sequence fortuitously create the 10-base-pair PRDI-BF_i binding site defined by footprinting studies. Thus, we propose that the 10-base-pair PRDI-BF_i binding site rather than the hexamer is responsible for the positive regulatory activity of the hexamer repeat. This hypothesis could also explain why four but not three copies of the hexamer have regulatory activity. Three copies of the hexamer contain two overlapping PRDI-BF_i binding sites, whereas four copies of the hexamer contain two nonoverlapping sites. It is possible that cooperative interactions between factors bound to these sites are necessary for the observed effects on transcription. Interestingly, an additional binding site for PRDI-BF_c and PRDI-BF_i was found in the β-interferon promoter immediately adjacent to PRDI, at positions -87 to -78 (data not shown). Cooperative interactions between factors bound to adjacent PRDI-BF_i binding sites may be involved in the regulation of the β-interferon promoter as well.

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