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Identification of novel factors that bind to the PRD I region of the human β-interferon promoter

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ABSTRACT

Treatment of cells with virus or synthetic double-stranded RNA (dsRNA) leads to the transient transcriptional activation of the β-interferon gene. Genetic analysis has revealed that the 5′ regulatory sequence responsible for this induction contains multiple positive and negative elements. One of these, Positive Regulatory Domain I (PRD I), has been shown to bind the positively-acting transcription factor IRF-1. In this study we show that this element is inducible under conditions where IRF-1 cannot be detected, suggesting that additional cellular factors are involved in the induction process. To investigate the existence of such factors we have analysed the range and properties of PRD I-binding activities present in HeLa cells. In addition to the repressor protein IRF-2, several novel factors can bind to PRD I in uninduced cells: two of these have properties consistent with a role in negative regulation; levels of two others increase upon priming, and may be alternative candidates for activators. Upon induction we also observe a novel factor whose appearance does not depend upon *de novo* protein synthesis, and which appears to be a truncated form of IRF-2. The potential involvement of these factors in regulating the β-interferon gene is discussed.

INTRODUCTION

The β-interferon gene is transcriptionally activated in many cell types by viral infection or challenge with double-stranded RNA (dsRNA) (see references 1 and 2 for reviews). This induction is transient and can occur in the presence of protein synthesis inhibitors, implying that the requisite transcription factors pre-exist in uninduced cells. The promoter sequences required for induction have been studied by a number of groups (reviewed in 3–5); results indicate that the β-interferon promoter contains multiple independently-inducible elements. Two such elements have received particular attention, namely Positive Regulatory Domains (PRDs) I and II, which reside between −77 and −64 (PRD I) and −64 and −55 (PRD II) (6). A synthetic sequence, the reiterated hexamer AAGTGA, can also act as a virally-inducible element (7). More recent work by Weissmann and co-workers has shown that the region responsible for inducibility within the reiterated AAGTGA shows striking homology to PRD I (8).

The sequence, $(AAGTGA)_4$, when placed between an SV40 enhancer and a TATA box, can also silence expression in uninduced cells (9). Silencing can be relieved by viral induction (9), or by moving $(AAGTGA)_4$ away from the SV40 enhancer (10). In cell lines where the silencing effect is not seen, $(AAGTGA)_4$ stimulates basal level expression from a heterologous promoter (11). These results suggest that PRD I/$(AAGTGA)_4$ can interact with at least two transcription factors (or different forms of the same factor)—one responsible for repressing activity, the second for stimulating transcription. In cell lines which silence efficiently, the repressor might predominate before induction, whereas in cell lines in which $(AAGTGA)_4$ supports basal level transcription, the activator might predominate. Induction could be brought about by a change in the abundance or relative activity of these factors.

Four cDNAs have been isolated which encode proteins that can bind to PRD I/$(AAGTGA)_4$. Taniguchi and co-workers have isolated two cDNA clones, whose products are referred to as IRF-1 and IRF-2 (12,13). Overexpression of IRF-1 activates promoters containing cognate binding sites in transient transfection experiments (10,12), and can activate the endogenous β-interferon promoter to a small degree (14,15). Both of these effects can be blocked by overexpression of IRF-2 (13,15), giving rise to the notion that IRF-1 and IRF-2 are the respective activator and repressor of PRD I/$(AAGTGA)_4$. Since induction of the β-interferon gene can occur in the absence of *de novo* protein synthesis, any involvement of IRF-1 as a direct activator requires the pre-existence of significant levels in uninduced cells. In a study on mouse L929 cells, however, Watanabe et al. (16) could not detect IRF-1 protein in uninduced cells. This was attributed to a low level of specific mRNA (about one transcript per cell) and an unstable protein (a half life of approximately 30 minutes). In a separate study, Pine et al. (17) demonstrated that transcriptional activation of the β-interferon gene could occur

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under conditions in which IRF-1 was not detectable. These results suggest that IRF-1 is unlikely to act as a primary transactivator for induction though PRD I. Since significant amounts of IRF-1 are produced upon viral infection (16), IRF-1 may act to stimulate expression at later times during the induction process.

Keller and Maniatis (18) have obtained a cDNA clone that encodes a PRD I-binding protein (termed PRD I-BF1) that has properties consistent with a role in the post-stimulatory shutdown of the β-interferon gene. The product of a novel cDNA clone (ICSBP-1), isolated by its ability to bind to the Interferon Stimulated Regulatory Element of the MHC class I genes, can also bind to PRD I (19). The role of this factor in the induction process is not clear, as its expression seems restricted to lymphocytes and macrophages. It is not yet understood how the products of these four cDNAs relate to the complexes detected in in vitro binding studies (10, 20–23).

In this study we report that PRD I shares the property of the endogenous β-interferon promoter in responding to induction in the absence of protein synthesis. Uninduced HeLa cells do not contain detectable IRF-1, suggesting that an alternative activator initiates induction in these cells. To investigate the existence of such a factor(s), we have examined the PRD I-binding activities present in HeLa cells under a variety of conditions. At least six distinct PRD I-binding factors are present in crude nuclear extracts prepared from uninduced cells, two of which contain IRF-2. The other two major factors appear to contain novel DNA-binding proteins whose binding activities decrease upon induction, suggesting that they might function as alternative transcriptional repressors. The behaviour of mutant PRD I sites in transfection experiments is consistent with such a role. Two minor factors are also detected in uninduced cells. The levels of these factors increase substantially upon priming—a pre-treatment with type I interferon which greatly enhances the efficiency of induction (24)—suggesting that they may function as activators. Extracts from induced cells contain several factors which react with antiserum specific for IRF-1, in addition to a novel factor which is immunologically related to IRF-2. The latter factor is superinduced if protein synthesis is inhibited. The potential role of these factors in the induction process will be discussed.

MATERIALS AND METHODS

Plasmid Constructions

To construct plasmids for the transient transfection assays, synthetic oligonucleotides containing wild-type or variants of PRD I were inserted into the Bam HI site of pBLCAT2 (25) so that two copies were present in a 5′-3′ orientation adjacent to position −105 of the tk promoter. For the analysis of stable transfectants, the (PRD I)$_2$tk-105CAT cassettes from the above were excised with Hind III and Ssp I and cloned into a vector specifying G418-resistance (pJNT, M. Ellis, unpublished).

cDNAs for the coding regions of IRF-1 and IRF-2 were obtained from HeLa and Jurkat cell RNA respectively by PCR using the oligonucleotides 5′ GCCAACATGCCCATCACTT-GGATGCGCATG 3′ and 5′ GGGGTCGACCTGCTACGGT-GCACAGGGAATGGC 3′ for IRF-1, and 5′ GGCACCATG-CCGGTGGAAAGGATGCGCATG 3′ and 5′ GGGGTCGACG-GCTTAACAGCTCTTGACGCGGGCC 3′ for IRF-2.

The 5′ oligonucleotides contain the Fsp I site present in the IRF-coding region, while the 3′ oligonucleotides contain a synthetic Sal I site engineered just 3′ to the termination codon to facilitate further manipulations (restriction sites are underlined). Each product was completely sequenced, and while IRF-2 showed no differences from published data (26), the product encoding IRF-1 was found to contain several nucleotide mismatches that resulted in three changes from the published sequence (27): Leu34 > Phe, Glu35 > Gln and Ile120 > Thr. The first two of these have also been reported in the sequence of ISGF2 (17), and all three are conserved in the murine IRF-1 sequence (12). We were able to confirm by RNAase mapping that these changes are maintained in HeLa mRNA, and therefore would appear to be allelic differences (data not shown).

To construct plasmids for the production of proteins in reticulocyte lysates (pT7βIRF-1 and pT7βIRF-2), each PCR fragment was cut with Fsp I and Sal I and ligated into pT7βSal (28) digested with Nco I and Sal I. The complementary double-stranded oligonucleotides 5′ CATGCCCATCACTTGGATGC 3′/ 5′ GCATCCAAGTGATGGG 3′ (IRF-1) and 5′ CATGCC-GGTGGAAAGGATGC 3′/ 5′ GCATCCTTTCCACCGG 3′ (IRF-2) were used to join the Nco I and Fsp I sites, and allowed the production of full-length, non-fusion protein products after in vitro transcription and translation reactions. C-terminal deletion mutants were derived from these plasmids by Bal31 nuclease digestion and re-cloning into pT7plink (a gift from R. Treisman), a vector which fuses termination codons in all three frames to the new C-terminus.

To facilitate expression of full-length, non-fusion IRF-2 in a T7 RNA polymerase-bearing strain of *E.coli*, the Fsp I–Bam HI fragment from pT7βIRF-2 was ligated into plasmid pRK171a (a pET vector (29) derivative and a gift from K. Gould) digested with Nde I and Bam HI. The complementary double-stranded oligonucleotides 5′ ATGCCGGTGGAAAGGATGC 3′/ 5′ GC-ATCCTTTCCACCGGC 3′ (IRF-2) were used to join the Nde I and Fsp I sites.

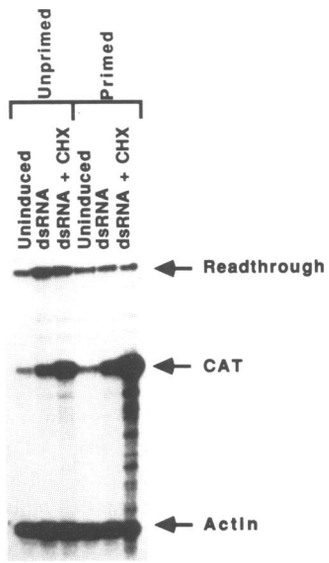

Figure 1. Induction through PRD I does not require protein synthesis. Plasmids containing a CAT gene under the control of a tk promoter with two copies of PRD I upstream were stably introduced into HeLa cells. Pools of G418-resistant transformants were isolated and amplified for expression analysis. Following induction as indicated for each lane, twenty micrograms of total cellular RNA were mapped using RNAase protection probes for both tkCAT and γ-actin and analysed on a 6% polyacrylamide denaturing gel (30). The position of appropriately protected fragments are indicated to the right of the autoradiograph.

Plasmid pT7βPRD I-BF1(Δ276−789), which encodes a truncated form of PRD I-BF1, was constructed by digesting pSP73PRD I-BF1 (a gift from A. Keller) with Hind III, blunting the site by 'filling-in' with Klenow enzyme, and recutting with Eco RI. The resulting fragment was ligated into pT7βSal that had been digested with Acc I, 'filled-in' with Klenow enzyme, and redigested with Eco RI.

Transfection and Expression Analysis

HeLa cells were transfected using standard calcium phosphate co-precipitation protocols (30). For transient analyses, co-transfections included 20μg of test plasmid and 5μg pJATlacZ (which contains sequences of the rat β-actin promoter from −340 to +10 driving transcription of the *E.coli* β-galactosidase gene) as a transfection control. For stable integrants, 20μg of test plasmid was transfected and the drug-resistant colonies pooled. The resulting cell lines were used for further analysis.

HeLa cells were primed and induced as described in Visvanathan and Goodbourn (23). Cellular RNA was isolated as previously described (30) and analysed by SP6 analysis (31). The SP6 probe for tkCAT was a gift from A. Ackrill, and those for γ-actin and β-interferon were as previously described (24, 31). Extracts for the analyses of CAT and β-galactosidase levels were prepared after 40 hours as described (32). CAT activity was assayed according to Sleigh (32) and corrected for variation in transfection efficiencies by normalising to β-galactosidase activity (33).

Preparation of nuclear extracts and gel retardation assays

Nuclear extracts were either prepared by the method of Dignam et al. (34), or by a variation of the method of Schreiber et al. (35) in which 10^6 cells were lysed in Dignam buffer A using 0.1% NP-40. The lowered concentration of detergent minimises effects upon DNA-binding while ensuring efficient lysis of cells. Following the isolation of nuclei by centrifugation, proteins were extracted at 4°C for 60 minutes with Dignam buffer C. Protease inhibitors were added to concentrations as described (23). For gel retardation assay, ten micrograms of a given extract were assayed as in Visvanathan and Goodbourn (23) except that 1.5μg of poly(dI)-(dC) that had been boiled for 10 minutes and quenched on ice replaced the poly(dI-dC).poly(dI-dC). DNA probes are described in the Figure legends and were 3′ end-labelled by 'filling-in'. For experiments using antisera, the nuclear extract was incubated with antiserum in the presence of non-specific carrier DNA at 0°C for 60 minutes before addition of labelled probe.

Expression of recombinant proteins in vitro

Plasmids pT7βIRF-1 and -2 were linearised with Bam HI, pT7βPRD I-BF1(Δ276−789) with Eco RI. Run-off RNA was produced using T7 RNA polymerase (Pharmacia, FPLC-pure) in the presence of m7GpppG as described (36), and synthesis quantitated by ^{3}H-UTP (Amersham) incorporation. Recombinant protein was produced in rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions with 10μg/ml template RNA. Typically 0.5μl of a translation reaction were used in each gel retardation experiment.

Expression and purification of recombinant IRF-2 from *E.coli*

A T7 RNA polymerase-bearing strain (37) carrying pRKIRF-2 was grown to mid-log phase and induced with 100μM IPTG (Sigma) for 4 hours. Cells were harvested by centrifugation and extracts prepared by the method of Harada et al. (15). Recombinant protein was partially purified by fractionating over a Heparin-Agarose column followed by affinity chromatography over DNA-cellulose. The protein was judged to be ~60% pure as judged by SDS PAGE and staining with Coomasssie Blue. Fractions were pooled and acetone precipitated before conjugation with Freund's adjuvant and injection into rabbits to raise antisera (38).

RESULTS

Induction through PRD I does not require protein synthesis

Although the observation that protein synthesis is not required for transcriptional activation of the β-interferon gene (39) would appear to preclude a primary role for IRF-1, it has not been shown that PRD I is itself inducible under these conditions. For example, the inhibition of protein synthesis may lead to a significant loss in labile repressor molecules with the result that the promoter can be efficiently activated by a subset of PRD elements excluding PRD I. Indeed, the binding of NF-κB to PRD II can be activated in the presence of protein synthesis inhibitors (23). Under conditions in which synthesis of labile repressors is not blocked, efficient activation may require PRD I, and IRF-1 might be necessary to stimulate transcription in a protein synthesis-dependent manner.

To investigate induction through PRD I and its dependence upon protein synthesis, two copies of PRD I were cloned directly

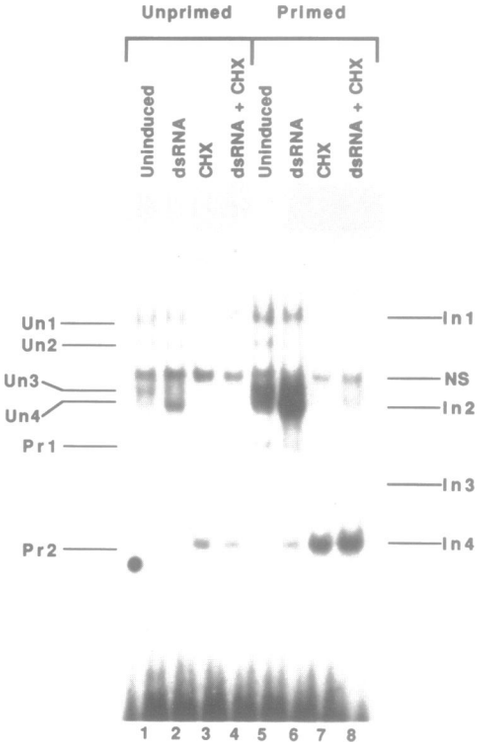

Figure 2. Multiple factors bind to the PRD I region of the β-interferon promoter. Nuclear extracts were prepared from HeLa cells as described in the Materials and Methods, and subjected to gel retardation analysis using a 3′ end-labelled duplex probe of the sequence 5′ GATCCGAGAAGTGAAAGTGAGATC 3′ (−77 is underlined). Complexes specific to uninduced cells are indicated to the left of the autoradiograph, while induced-specific complexes are indicated to the right. The complex In1 in lane 6 has a slightly slower mobility than Un1, whose level has declined in this extract as a result of induction. NS refers to a complex that binds to PRD I in an non-specific fashion as determined by competition assays.

upstream of the HSV thymidine kinase (tk) promoter fused to the CAT gene. This construct was stably introduced into HeLa cells using G418-resistance as a selectable marker. RNAase protection analysis on pools of resistant clones shows that PRD I is capable of conferring dsRNA inducibility upon the tk promoter (Figure 1). This induction is not impaired by addition of cycloheximide (CHX) to a concentration of 50μg/ml. In fact, CHX enhanced the inducibility of dsRNA, while CHX alone was capable of moderate activation. In control experiments, a CAT gene under the control of the tk promoter did not respond to induction (data not shown). We note that, unlike the endogenous gene in these cells (24), induction through PRD I is not dependent on priming, although we routinely see a small stimulation (~2-fold, Figure 1). By analysing the incorporation of radiolabelled amino acids into TCA precipitable material, we found that >99.9% of protein synthesis is inhibited by CHX. These experiments clearly demonstrate that induction of PRD I proceeds using cellular factors that pre-exist in uninduced cells.

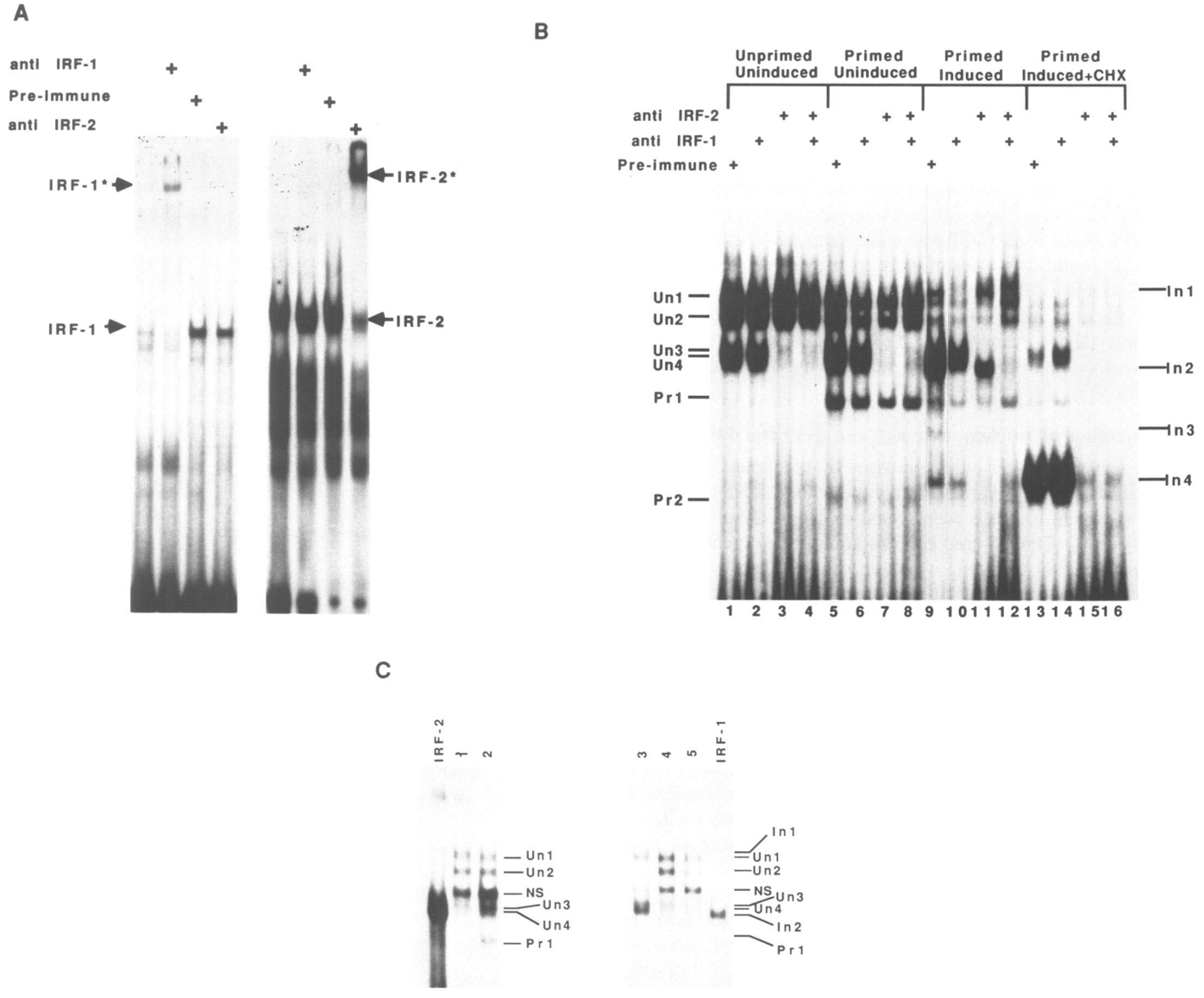

Figure 3. Identification of novel and IRF-containing cellular complexes. (A). Antisera raised against purified IRF-1 or recombinant IRF-2 were incubated with IRF-1 or IRF-2 produced in vitro in a reticulocyte lysate, and the resulting products analysed by gel retardation using the probe described in Figure 2. The position of the native IRF complex is marked, that of the immune complex is asterisked. As a control for non-specific interactions, the gel retardation assay was repeated in the presence of pre-immune serum. The failure of the antisera to cross-react suggests that the antigenic regions of these proteins lie outside of the highly conserved N-terminal DNA-binding domain. Using a set of deletion mutants which produce C-terminally truncated forms of IRF-1 and IRF-2 in reticulocyte lysates we noted that the amounts of antibody required to neutralise the gel retardation complexes increased as larger regions of the C-terminus were removed (data not shown). We presume this reflects a decrease in the number of epitopes present in the shorter DNA-binding proteins. We used this information to establish levels of antisera required to block complex formation in cellular extracts. (B). Nuclear extracts were prepared from HeLa cells as described in the Materials and Methods, and subjected to gel retardation analysis as in Figure 2 in the presence of specific antisera as discussed above. Pre-immune serum was used a negative control for non-specific interactions. Complexes are indicated as in Figure 2. The complexes in lanes 11 and 12 which have a similar mobility to complex In1 are probably due to IRF-2/antibody 'supershifts'. (C). In vitro translated full-length IRF-1 and IRF-2 were compared in a gel retardation assay with extracts from unprimed/uninduced (lanes 1 and 4), primed/uninduced (lanes 2 and 5) and primed/induced (lane 3) HeLa cells using conditions as described in Figure 2.

Multiple factors in HeLa nuclear extracts bind to PRD I

Because induction through PRD I can occur without *de novo* protein synthesis, and IRF-1 mRNA is only detectable in uninduced HeLa cells by PCR (data not shown), either IRF-1 mRNA is very efficiently translated or other activities may be involved in the induction process. To investigate this, we have undertaken a series of gel retardation analyses using an oligonucleotide probe for PRD I. Nuclear extracts from uninduced HeLa cells contain four distinct factors capable of specifically binding to PRD I (complexes Un1−4 in Figure 2, lanes 1 and 5). In addition, a single non-specific complex was detected in these extracts (NS in Figure 2). Upon extended autoradiography we can detect two additional complexes that bind specifically to PRD I in uninduced cells (see Figure 3B, lanes 1−4), whose levels increase upon priming (Figure 2, lanes 1 and 5); we refer to these complexes as Pr1 and Pr2 but note that complex Pr2 is only present at very low levels, even in primed HeLa cells. When nuclear extracts are prepared from cells which have been treated with dsRNA, four further PRD I-specific binding complexes can be detected (In1−4; Figure 2, lanes 2 and 6). Interestingly only one of these complexes (In4) appears in a CHX-resistant manner, whilst the others require *de novo* protein synthesis (Figure 2, lanes 4 and 8). Although the complex In1 has a very similar migration to Un1, it can be distinguished from this complex on the basis of reactivity with IRF-1 specific antiserum and mutational specificity (see below). We note that the complex In3 is present at very low levels in induced cells, and thus its role in the induction process is unclear. In addition to the production of four new activities, induction by dsRNA also leads to a priming-dependent decrease in two of the constitutive complexes, Un1 and Un2 (Figure 2, lanes 2 and 6). This decrease is especially marked in the presence of CHX, under which conditions a decrease is also seen in the complexes Un3 and Un4 (Figure 2, lanes 4 and 8).

Multiple complexes contain IRF-1 and IRF-2

In order to determine which, if any, of these cellular activities contain IRF-1 or IRF-2, we used specific antisera raised against these proteins. An antibody specific for human IRF-1 was obtained from R. Pine (17), while antisera were raised against recombinant IRF-2 overexpressed in bacteria. Since the N-terminal 154 amino acids of IRF-1 and IRF-2 show 62% homology (13), we first analysed the specificity of these antisera against recombinant proteins produced in rabbit reticulocyte lysates. Figure 3A shows that the antibodies specifically interact with their respective antigen.

Of the PRD I-binding complexes detected in extracts from uninduced HeLa cells, only two cross-reacted with an IRF-2 antibody (complexes Un3 and Un4, Figure 3B lane 3 and 7), these being the complexes that co-migrate with those formed by in vitro translated IRF-2 (Figure 3C). As these IRF-2-containing complexes have very similar mobility, and a doublet can be produced from a single transcript in vitro, (Figure 3C) we suggest that Un3 and Un4 are post-translationally modified forms of the same polypeptide. Extracts from uninduced cells were not affected by the addition of IRF-1 antiserum (Figure 3B lane 2). Since the complexes Un1, Un2, Pr 1 and Pr 2 did not react with either antisera (Figure 3B, lanes 4 and 8), they do not appear to be related to either IRF-1 or IRF-2.

Three induced-specific complexes (In1-In3) were affected by IRF-1 antisera (Figure 3B lane 10), one of which (In2) co-migrates with the complex formed by full-length IRF-1 (Figure 3C). The complex In3 is smaller than that formed by full-length IRF-1, and may be a proteolytic breakdown product of IRF-1. The complex In1 is larger than full-length IRF-1, suggesting that it may contain additional protein species. Induction of all three IRF-1-containing activities is absolutely dependent upon protein synthesis (Figure 3B, compare lanes 9 and 13). Addition of IRF-2 antisera to induced extracts revealed that In4 binding is specifically disrupted (Figure 3B, lanes 11 and 15), suggesting that it contains a proteolytic breakdown product of IRF-2, or that it contains a protein that is immunologically related

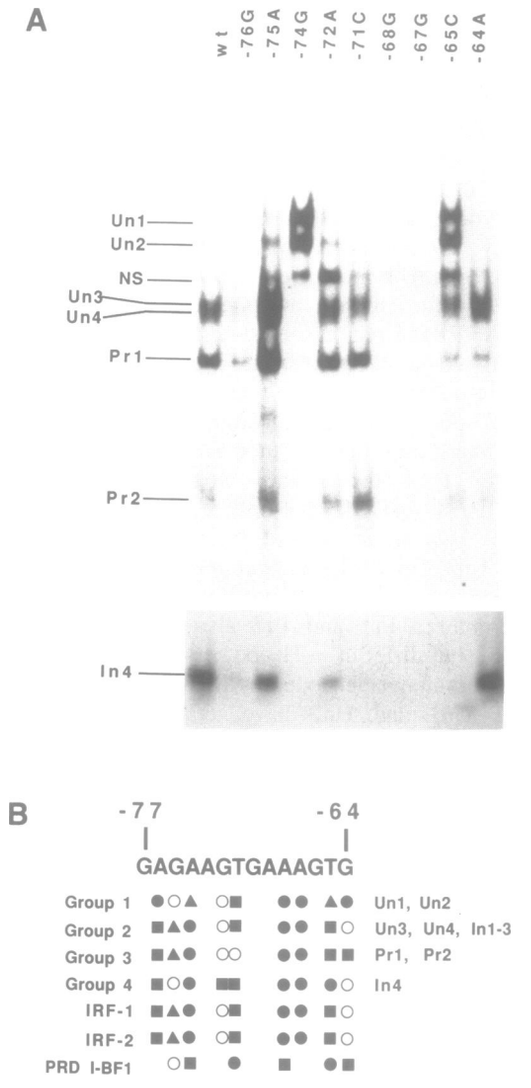

Figure 4. PRD I-binding complexes show discrete binding specificities. (A). A nuclear extract from primed/uninduced HeLa cells was subjected to gel retardation analysis using 3′ end-labelled probes based upon the sequence shown in Figure 2. Specific substitutions were as indicated. The complexes are indicated at the left of the autoradiograph. An insert at the foot of the autoradiograph shows the binding specificity of complex In4, which was established in an experiment using extracts prepared from cells which had been primed and induced in the presence of cycloheximide. These data are representative of a number of experiments conducted with extracts prepared from cells treated in a variety of ways, and also from experiments which analysed in vitro translated IRF-1, IRF-2 and PRD I-BF1. Results are summarised in (B); positions which do not affect binding are indicated by an open circle, those which cause a severe loss of binding a filled circle, those which slightly impair binding a filled square. Those positions which show elevated binding are indicated by a filled triangle.

to IRF-2 but not IRF-1. Western blotting experiments on extracts prepared by directly lysing cells in SDS loading buffer (which should prevent proteolysis during extraction) demonstrate that full-length IRF-2 disappears during induction, and is replaced in a protein synthesis-independent manner by a 29kD immunoreactive product (data not shown). These data support the possibility that In4 contains a truncated form of IRF-2 that is generated during induction. However, we have not determined whether the 29kD product represents the N-terminal fragment of IRF-2 postulated to generate the In4 complex, or whether it comprises a C-terminal fragment of IRF-2 that would be produced by proteolytic cleavage. The latter would seem more likely as we have shown that the majority of the epitopes for this antiserum lie in this C-terminal region (see Figure 3).

PRD I binding activities show distinct point mutation profiles

The observation that a number of complexes did not react with even high concentrations of antisera raised against IRF-1 or IRF-2 suggested that these binding activities may be formed by the products of novel genes. These products might be related to the IRF family as evidenced by similarities in DNA-binding specificity, or might show unrelated specificities. In order to investigate this further we synthesised a series of point mutants spanning the PRD I region, and used them either as probes (a representative data set is shown in Figure 4A) or as competitor DNAs (data not shown) in gel retardation assays. Analysis of the binding specificities of the cellular activities showed them to fall into four groups (data summarised in Figure 4B). As might be expected from their highly homologous DNA-binding domains, IRF-1 and IRF-2 produced in vitro have identical specificities, which are shared by the IRF-immunoreactive complexes Un3, Un4, In1, In2, and In3. The complex In4 shows minor differences, notably at positions −75, −72, −71 and −65. Complexes Pr1 and Pr2 also show similar binding specificities, but differ at −71 and −64, suggesting that these cellular factors may be encoded by IRF-like genes. In contrast, complexes Un1 and Un2 have totally dissimilar binding specificities to the IRF group, and thus may be the products of unrelated genes. In addition, we have determined that the cellular factors present in complexes Un1 and Un2 appear to be able to bind to other sequences within the β-interferon promoter (manuscript in preparation).

It is possible that some of the unassigned cellular complexes contain PRD I-BF1 (18) or ICSBP-1 (19). Since antibodies were not available against PRD I-BF1, we have expressed a partial product in reticulocyte lysates, and examined its ability to bind to PRD I and point mutant variants. Our results indicate that PRD I-BF1 shows a specificity distinct from any observed for the cellular complexes detected in these experiments (Figure 4B), and are in agreement with results recently published by Keller and Maniatis (40). Furthermore, the product of even this partial cDNA forms a gel retardation complex that migrates more slowly than any of the cellular complexes described here (data not shown). We have been unable to detect ICSBP-1 mRNA by PCR in our HeLa cells. Thus we believe that the unassigned cellular complexes do not contain either PRD I-BF1 or ICSBP-1.

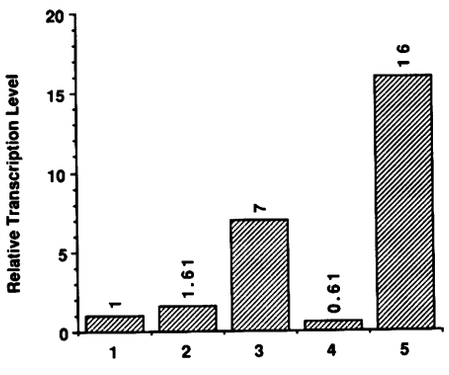

Figure 5. Point mutations in PRD I affect expression from a heterologous promoter. Plasmids were transiently transfected into HeLa cells and extracts prepared from uninduced cells as described in the Materials and Methods. The graph shows expression of CAT (corrected for variations in transfection efficiency by normalising to β-galactosidase levels) from: Lane 1, tk-105CAT; Lane 2, two copies of PRD I (−77/−64) inserted upstream from tk-105CAT; Lane 3, two copies of PRD I (−77/−64), containing a G to A change at −75, inserted upstream from tk-105CAT; Lane 4, two copies of PRD I (−77/−64), containing an A to G change at −74, inserted upstream from tk-105CAT; Lane 5, two copies of PRD I (−77/−64), containing a G to A change at −64, inserted upstream from tk-105CAT.

Complexes Un1 and Un2 may function as novel transcriptional repressors

Since the binding of cellular complexes could be distinguished by their point-mutational specificities, we chose to analyse the genetic properties of some PRD I variants. To do this, two copies of each binding site were cloned upstream of the tk promoter and CAT reporter gene. We elected to use the full-length tk promoter as it has a constitutive activity which would enable us to analyse both positive and negative effects of upstream sequences. Analysis of CAT protein levels in transfected cells shows that two copies of PRD I slightly activate a heterologous promoter in uninduced cells (Figure 5, lanes 1 and 2). We have shown above that these cells contain factors which form complexes Un1-Un4, and also contain low levels of factors which form complexes Pr1 and Pr2. A mutant that shows enhanced binding of complexes Un1 and Un2 (−74G), but which does not appear to bind to any other complexes, lowers expression about 3-fold relative to wild-type PRD I (Figure 5, lanes 2 and 4). Since the level of expression from this construct is lower than that from the tk promoter alone (Figure 5, lane 1), it appears that the consequence of Un1 and Un2 binding is net repression of transcription. Consistent with this, a mutant that shows no detectable binding of Un1 and Un2 while retaining wild-type affinities for other complexes (−64A) has a substantially elevated level of expression (Figure 5, lane 5). This result clearly indicates that uninduced cells contain activities that can stimulate expression from PRD I. A mutant at −75, which does not affect binding by Un1 and Un2, but which has an increased affinity for all the other PRD I-binding complexes present in uninduced cells also shows elevated levels of CAT activity (Figure 5, lane 3), suggesting that the expression from the heterologous promoter in these experiments is determined by the opposing effects of Un1/Un2 and activities which function to stimulate expression.

DISCUSSION

In this study we show that the PRD I region of the β-interferon promoter acts in an analogous manner to the related sequence $(AAGTGA)_4$ in that it can convey inducibility by dsRNA upon a heterologous promoter. We also show that this induction can occur in the absence of protein synthesis, suggesting that components required pre-exist in uninduced cells. We have

detected a number of complexes using extracts from HeLa cells and summarise their properties in Table 1.

It has been suggested by others (12,13,15) that induction through PRD I utilises the positively-acting transcription factor IRF-1. However, IRF-1 was not detected in uninduced L929 (16) or HeLa cells (17). In the HeLa cell line used in our experiments, IRF-1 mRNA was undetectable by RNAase mapping in uninduced cells. However, we were able to detect IRF-1 mRNA by PCR (data not shown), suggesting that it is a very rare message. In addition, IRF-1 has a rapid turnover (30 minutes−ref. 16) and it may be the combination of these two phenomena that accounts for the inability to detect any IRF-1 protein by gel retardation assay (Figure 3B) or by Western Blotting (data not shown) in uninduced cells. Failure to detect IRF-1 is maintained using various extraction procedures (13,15,34,35) and is not affected by the presence of phosphatase inhibitors (16), or substantial amounts of protease inhibitors (see Materials and Methods). Upon treatment with dsRNA, IRF-1 becomes easily detectable, although this is absolutely dependent upon *de novo* protein synthesis.

As induction can occur in the absence of protein synthesis, any relevant positive factor must pre-exist at levels sufficient to activate transcription. Models involving IRF-1 in such a role must take account of the vanishingly small levels of this protein under such circumstances. Since activation of the β-interferon gene is restricted to a small fraction of the total cells in a population (24,41) it is possible that IRF-1 shows a similar distribution. In such a model, the IRF-1 concentration could be relatively high in responsive cells, yet appear undetectable in an extract from a population of cells which is predominantly uninducible.

An alternative hypothesis is that a factor other than IRF-1 is involved in the primary induction of PRD I. A positive role for IRF-1 in induction is not excluded by such a model—IRF-1 produced in a protein synthesis-dependent manner might be required to boost expression in a 'second phase' event, while not itself being the primary activator. Such a role has already been proposed for IRF-1 (ISGF2) in the induction of genes responsive to interferons (17). The primary activation of these genes appears to utilise the factor ISGF3, whose DNA-binding activity is induced in a protein synthesis-independent manner (42). Could ISGF3 be activated by dsRNA and be the primary activator of PRD I? Despite the similarity between PRD I and the canonical ISRE sequence (5), we have been unable to detect ISGF3 binding to the PRD I probe used in these experiments (manuscript in preparation), and thus do not believe this factor to be involved.

The complex In4 is produced in a protein synthesis-independent manner in response to induction, and is therefore a candidate for the primary activator. We note that there is a close correlation between the levels of In4 and the magnitude of induction. Since this complex is immunologically related to IRF-2 rather than IRF-1, and because the DNA-binding domains of these two molecules reside in the N-terminal portions of these molecules, we consider it highly probable that In4 represents a C-terminally truncated form of IRF-2. The generation of the In4 complex is unlikely to be occurring during extraction since formation is not inhibited by a range of protease inhibitors, including leupeptin added to 100μg/ml. Western blotting experiments demonstrate that full length IRF-2 disappears during the induction process and is replaced in a protein synthesis-independent manner by an IRF-2 immunoreactive product of 29kD. The generation of this product is not an artefact of proteolysis during extraction. Taken together, these results suggest that In4 is generated by an induction-specific proteolytic cleavage of IRF-2. A positive role for In4 seems difficult to reconcile with the proposed repressor function of IRF-2 (13,15). However, it may be that IRF-2 can function as a repressor in uninduced cells, but becomes an activator following an induction-specific processing event. Alternatively, IRF-2 may be a transactivator whose ability to stimulate transcription is inhibited by C-terminal sequences that leave its ability to bind to DNA unimpaired. In co-transfection studies, such a molecule could down-regulate IRF-1 transactivation in uninduced cells (13,15) by competing for cognate binding sites.

Finally, induction might be brought about by loss of repressor function followed by binding of a constitutive factor. We have detected two novel complexes (Un1 and Un2) which have properties of putative repressors: the binding activities of these factors decrease upon induction, and a high affinity binding site for these complexes down-regulates a heterologous promoter. Furthermore, a variant of PRD I which shows decreased binding of Un1 and Un2 significantly activates transcription from a tk promoter, suggesting that uninduced cells do contain a constitutive activity. The identity of this activity is not clear. In addition to Un1 and Un2, uninduced cells contain IRF-2 and low levels of complexes Pr1 and Pr2. Mutant PRD I binding sites that support constitutive activity retain binding of IRF-2, which indicates that

Table 1. Properties of PRD I-binding complexes.

Complex	Unprimed Uninduced	Primed Uninduced	Unprimed Induced	Primed Induced	IRF-1 Reactive	IRF-2 Reactive	Protein Synthesis Required?	DNA binding class
Un1	+++	+++	++	+	−	−	N/A	1
Un2	+++	+++	++	+	−	−	N/A	1
Un3	+++	+++	++	++	−	+	N/A	2
Un4	+++	+++	++	++	−	+	N/A	2
Pr1	(+)	++	(+)	++	−	−	N/A	3
Pr2	(+)	++	(+)	++	−	−	N/A	3
In1	−	−	++	+++	+	−	YES	2
In2	−	−	++	+++	+	−	YES	2
In3	−	−	++	+++	+	−	YES	2
In4	−	−	+	+++	−	+	NO	4

The relative abundance of specific complexes in different extracts is indicated on the following scale +++ > ++ > + > (+) > −. IRF-1 or IRF-2 reactive complexes are indicated as + or − depending on reactivity with specific antisera. Protein synthesis requirement refers to the ability or otherwise of CHX to block the appearance of complexes during induction by dsRNA; N/A is used to indicate that complexes Un1−4 and Pr1 and Pr2 pre-exist in uninduced cells. Binding class indicates the binding specificity as determined by point mutations in PRD I as indicated in Figure 4B.

IRF-2 does not function as an efficient repressor in these cells. Assuming that IRF-2 does not act as an activator in uninduced cells, the only other complexes which we can detect are Pr1 and Pr2. The low levels of Pr1 and Pr2 may therefore be sufficient to stimulate transcription. Although the levels of Pr1 and Pr2 are significantly increased by priming, it should be noted that induction through PRD I is not greatly enhanced by this treatment.

The induction through PRD I is clearly a complex process which will require molecular cloning and analysis of these newly-identified components and the construction of cell lines which are altered in their responsiveness to dsRNA to further elucidate the molecular mechanisms involved.

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