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Double-stranded RNA activates binding of NF-κB to an inducible element in the human β-interferon promoter

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The human β-interferon promoter contains at least two positive acting domains (PRD I and PRD II). PRD I has been previously shown to stimulate basal transcription and to respond to induction by double-stranded RNA (dsRNA). Here we show that PRD II functions independently as a constitutive element that also responds to induction. A cellular factor that specifically binds to PRD II has been identified, and the levels of this factor increase markedly in extracts from cells treated with dsRNA. The inducible factor has a binding specificity that is indistinguishable from the transcription factor NF-κB. As has been shown for NF-κB, the PRD II-specific factor can be activated in uninduced extracts by treatment with detergent, suggesting that the inactive state is due to association with an inhibitory factor. Induction by dsRNA therefore provides a novel means for the post-translational activation of NF-κB. Potential binding sites for NF-κB are present in the 5′ flanking regions of a number of genes involved in the immune response, several of which are inducible by dsRNA. These findings demonstrate a role for NF-κB in the physiological activation of genes in non-lymphoid cells.

Key words: cytokines/HIV/point mutations/transcription factor/viral infection

Introduction

Many cell lines respond to viral infection or treatment with double-stranded RNA (dsRNA) by increasing the expression of a number of genes. The best characterized of these genes are the type I interferons (IFNs), which function to limit the further replication of virus, and which may also have a role in regulating cell growth. In fibroblastoid cells, dsRNA predominantly induces β-, rather than α-IFN. The increase in expression is a result of transcriptional activation, a process which does not appear to require protein synthesis indicating that the cellular factors which are involved in this process pre-exist in an inactive form in uninduced cells (for reviews see Stewart, 1979; Lengyel, 1982; Taniguchi, 1988).

Detailed genetic analysis has revealed that the human β-IFN gene is regulated by *cis*-acting sequence domains that are positioned 5′ to the mRNA startpoint (Zinn *et al.*, 1983; Fujita *et al.*, 1985; Goodbourn *et al.*, 1985, 1986). In mouse C127 cells, a short region of DNA (between −77 and −36) is necessary and sufficient to confer the induction response on a heterologous promoter element (Goodbourn *et al.*, 1985). This region contains at least two positive regulatory domains (PRDs) which are prevented from functioning in uninduced cells by an overlapping negative regulatory element (Goodbourn *et al.*, 1986).

PRD I, defined genetically with outer boundaries of −77 and −64 (GAGAAGTGAAAGTG), functions as a weakly constitutive transcription element (Goodbourn *et al.*, 1986). In addition, PRD I, or a PRD I-like sequence created by multimerization of a hexamer (AAGTGA), confers an increased level of transcription following induction (Goodbourn *et al.*, 1985; Fujita *et al.*, 1987). Several lines of evidence suggest that PRD II, defined genetically with outer boundaries of −66 and −55 (GTGGGAAATTCC; Goodbourn and Maniatis, 1988) may have similar properties. Firstly, an internal deletion in the β-IFN promoter ('ID−55/−40' in Goodbourn *et al.*, 1986) which partially inactivates the negative regulatory region but retains PRD I and PRD II has a higher basal level and greater inducibility than a comparable construction which lacks PRD II. Secondly, analysis of single base substitutions throughout the β-IFN promoter indicates that mutations in PRD II cause a dramatic loss of inducibility (Goodbourn and Maniatis, 1988). Thirdly, internal deletions that remove only PRD II from the intact promoter respond poorly to induction (Goodbourn and Maniatis, 1988, and our unpublished observations). PRD II may therefore have similar properties to PRD I, with optimal expression requiring synergy between the two elements. Alternatively, PRD II may lack such properties on its own, but may accentuate the properties of PRD I.

In keeping with the complex organization of the β-IFN promoter, a number of cellular factors which can bind this region have been identified. Keller and Maniatis (1988) demonstrated that uninduced MG63 cells (a human osteosarcoma line) contain factors that can bind to PRD I (PRDI-BFc), and to PRD II (PRD II-BF). Upon induction, an additional PRD I-binding factor can be detected (PRD I- BFi) in MG63 nuclear extracts, although no inducible PRD II-binding factor was observed. A factor present in mouse L929 cells which binds to the synthetic PRD I-like sequence $(AAGTGA)_4$ has recently been identified (Fujita *et al.*, 1988) and a cDNA clone has been isolated that appears to encode a protein capable of binding to this sequence and to the natural β-IFN promoter (Miyamoto *et al.*, 1988).

In this paper we demonstrate that following induction by dsRNA there is an increase in expression from promoters containing PRD II as their only β-IFN component. A cellular factor has been identified which can bind this region of the β-IFN promoter in extracts from induced cells. This factor appears to be indistinguishable from the transcription factor NF-κB previously identified in mature B cells (Sen and Baltimore, 1986a). The binding activity of NF-κB, and stimulation of expression of promoters containing NF-κB binding sites has been shown to be activated by lipopolysaccharide treatment of pre-B cells or by TPA treatment of

a variety of cell lines (Sen and Baltimore, 1986b; Lenardo *et al.*, 1987; Nabel and Baltimore, 1987; Nelsen *et al.*, 1988; Pierce *et al.*, 1988). Binding activity can also be liberated by treatment of cellular extracts with detergents, prompting the suggestion that NF-$\varkappa$B is associated with an inhibitory factor in uninduced cells (Baeuerle and Baltimore, 1988a). The results presented in this paper suggest that the binding activity of NF-$\varkappa$B can also be released from the inhibitor-associated form by treatment of cells with dsRNA.

Results

PRD II is an independently inducible genetic element

To investigate the properties of PRD II, we examined the expression in mouse C127 cells of 5′ deletion mutants which were derived from ID −55/−40 (described above). The results shown in Figure 1A demonstrate that inactivation of PRD I did not substantially lower basal expression (Figure 1A, lanes 1−5), although inducibility was reduced (Figure 1A, lanes 10−14). A plasmid that retains only sequences between −64 and −55 still had a substantially higher basal level than a β-IFN mutant lacking any sequences upstream from −40 whereas further 5′ deletion to −57 abolished this (Figure 1A, lanes 6−8). In contrast to the −40 deletion mutant which was only ~2-fold inducible, the plasmid which contained the −64/−55 region was inducible ~5-fold (Figure 1A, lanes 15−18). Thus, when positioned upstream from a β-IFN gene with a 5′ deletion to −40, the PRD II element could stimulate basal expression and increase inducibility.

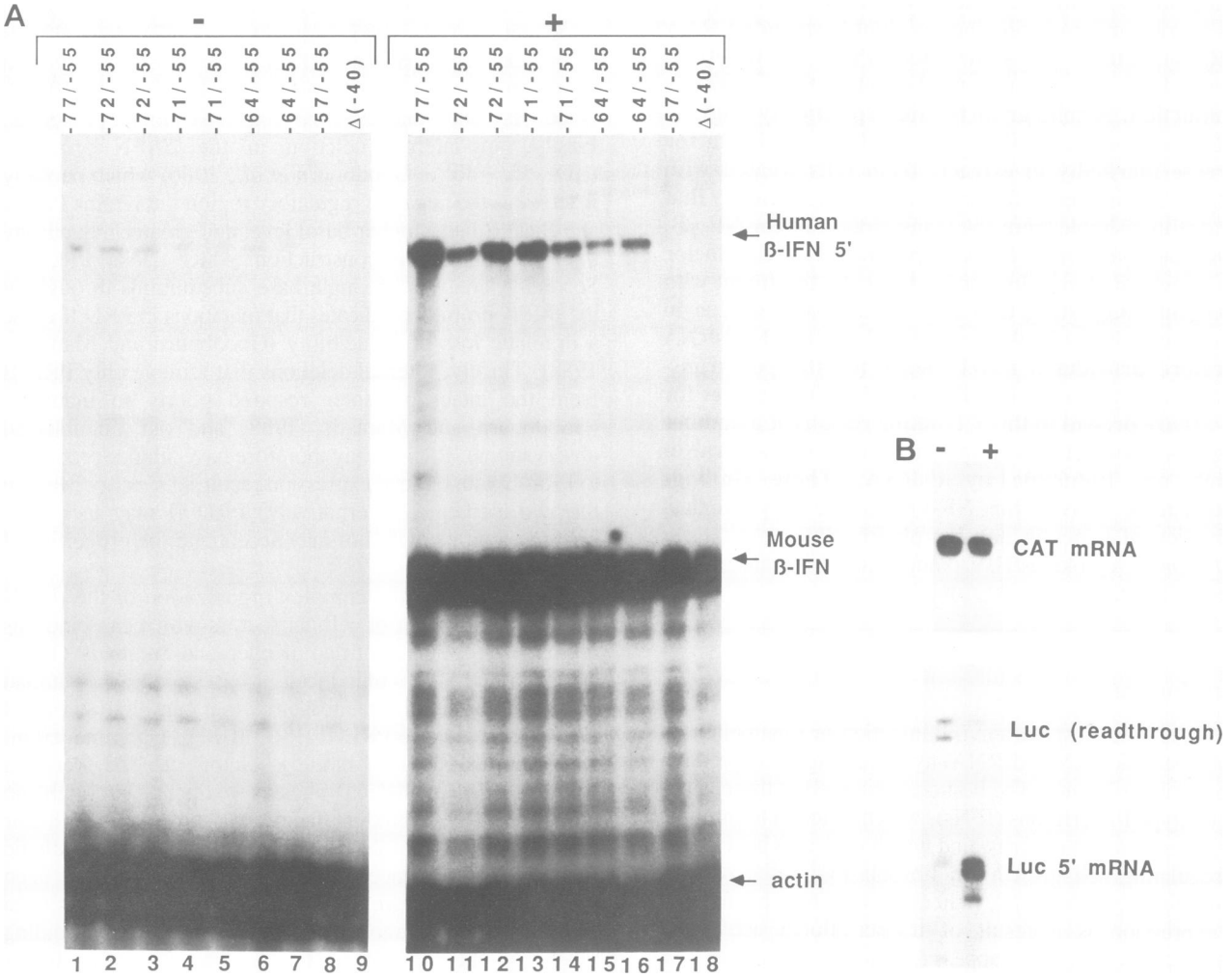

Fig. 1. Analysis of the effects of PRD II on basal expression and inducibility. **(A)** The plasmid ID−55/−40 (Goodbourn *et al.*, 1986) and 5′ deletion derivatives were cloned into the vector pBPV-BV1 (Zinn *et al.*, 1983), and introduced into mouse C127 cells as described in the Materials and methods. 20 μg of total cellular RNA from uninduced cells (minus lanes) or poly(rI)−poly(rC)-induced cells (plus lanes) was analysed for the presence of human and mouse β-IFN transcripts, and mouse γ-actin transcripts, using the quantitative RNase mapping procedure of Zinn *et al.* (1983). The position of protected RNA is indicated to the right of the panel. The human β-IFN 5′ and mouse β-IFN probes are described in Goodbourn *et al.* (1985), and the γ-actin probe is described in Enoch *et al.* (1986). The specific deletions are indicated above each lane using nomenclature as in the following example: The plasmid marked '−72/−55' has β-IFN sequences from between −72 and −55, which are fused upstream of the −40 position using a *Bam*HI linker. The Δ(−40) plasmid has no β-IFN sequences upstream from −40. **(B)** The plasmid (PRD II) << tkΔ(−39)Luc, which contains two copies of PRD II (see Materials and methods and Table I), was co-transfected into HeLa cells with a plasmid containing the CAT reporter gene under the control of the SV40 enhancer. Transiently transfected cells were induced as described in Materials and methods. 5 μg of total cellular RNA from induced (+ lane) or uninduced (− lane) cells was analysed for the presence of luciferase or CAT transcripts, which are indicated on the right (CAT and Luc 5′). The luciferase probe spans the mRNA cap site, and the protected material is correctly initiated; the CAT probe protects an 'internal' region of CAT mRNA.

To determine whether PRD II retains these properties when examined independently from any other β-IFN sequences, we inserted PRD II oligonucleotides (−66/−55 sequences) in a position 5′ to a heterologous transcription unit consisting of a herpes simplex virus (HSV) thymidine kinase (tk) TATA box fused to the firefly luciferase gene. These constructions were co-transfected into a variety of mammalian cell lines with a reference plasmid which contains the chloramphenicol acetyl transferase gene (CAT) under the control of the SV40 enhancer. Extracts of transfected cells were assayed for luciferase activity, and the value obtained was corrected for variations in transfection efficiency using the CAT activity value. The results of this analysis are presented in Table I.

Prior to induction, expression from the plasmid lacking upstream PRD II sequences [tkΔ(−39)Luc] was very low in C127, L929 or HeLa cells, and was undetectable in MG63 cells. Following induction, a decrease in expression was often observed. Single copies of PRD II placed in either orientation 5′ to the tk TATA box [PRD II > and PRD II < tkΔ(−39)Luc], stimulated basal expression in both C127 and HeLa cells, although a net decrease was still observed upon induction. In L929 cells the stimulation of basal levels was much smaller than in C127 or HeLa cells. When the effect of two or more copies of PRD II was examined [PRD II < < and (PRD II)$_5$ tkΔ(−39)Luc], further increases in basal expression were observed, and luciferase signals could be detected in uninduced MG63 cells. Expression was now inducible in all four cell lines. Increasing the copy number of PRD II from two to five caused a further increase in basal expression and inducibility in C127 cells, although the other lines did not show this effect. To test the specificity of the PRD II effects, we investigated the expression in L929 cells of a plasmid containing a −63 G to A change in each of two copies of PRD II; this change results in severely diminished binding of an inducible PRD II-specific binding factor (see below). A small basal stimulation was observed, but no inducibility resulted (Table I).

While the inducibility of constructions with PRD II multimers may appear small, a plasmid which contains the luciferase gene under the control of a β-IFN promoter including sequences between −104 and +72 [IFΔ(−104)Luc in Table I] shows only a limited inducibility (6- to 12-fold) in all cell lines. Since this promoter sustains >50-fold inducibility at the mRNA level (Zinn *et al.*, 1983), the luciferase assay appears to result in the level of induction being underestimated. When HeLa cells were transfected with the plasmid containing two copies of PRD II, an increase of at least 20-fold in the luciferase mRNA level was obtained (Figure 1B), in contrast to the 2.8-fold inducibility in luciferase activity (Table I). These data clearly demonstrate that PRD II can independently confer dramatic inducibility upon a heterologous promoter.

Cellular factors that bind to PRD II

To identify cellular factors that could mediate inducibility through PRD II we used gel-retardation assays. We initially investigated binding to a β-IFN probe spanning the −77/−12 region (−77/−12 probe). Three complexes, C1, C2 and C3, which were weakly visible in extracts from uninduced cells, were detected at significantly higher levels in extracts prepared from induced HeLa and MG63 cells (Figure 2A). Extracts from HeLa cells which had been primed (Enoch *et al.*, 1986) contained increased levels of all three complexes following induction (Figure 2A). In addition, a number of complexes were present in extracts from both uninduced and induced cells; these are currently under investigation. The binding specificity of the inducible complexes was examined by competition with a variety of oligonucleotides, and by using radiolabelled −77/−12 β-IFN probes bearing single base changes (Figure 2B). The inducible complexes C1 and C2 were competed for specifically by the −66/−55 oligonucleotide used in the *in vivo* experiments described above (Table I), and were highly sensitive to mutations at −64, −63, −62, −56 and −55. Mutations in the centre of the binding site affected complexes C1 and C2 slightly differently, but none of these changes

Table I PRD II elements increase basal expression and confer inducibility upon a heterologous transcription unit.

	Cell line	Uninduced	Induced	Induction ratio
tkΔ(−39)Luc	C127	1.0	0.7	0.7
	L929	1.0	1.0	1.0
	MG63	UD	UD	UD
	HeLa	1.0	0.6	0.6
(PRD II) > tkΔ(−39)Luc	C127	2.4	1.4	0.6
	L929	1.1	0.6	0.6
	MG63	UD	UD	UD
	HeLa	2.7	1.4	0.5
(PRD II) < tkΔ(−39)Luc	C127	2.4	1.0	0.4
	L929	1.6	1.5	0.9
	MG63	UD	UD	UD
	HeLa	2.5	1.4	0.6
(PRD II) << tkΔ(−39)Luc	C127	9.3	44.7	4.8
	L929	3.6	11.0	3.0
	MG63	1.0	19.0	19.0
	HeLa	4.5	12.7	2.8
(PRD II) << tkΔ(−39)Luc (−63 mutant)	L929	2.1	1.6	0.8
(PRD II)$_5$tkΔ(−39)Luc	C127	22.1	164.5	7.4
	L929	3.1	11.7	3.7
	MG63	0.9	15.0	15.9
	HeLa	5.8	12.7	2.2
IFΔ(−104)Luc	C127	399	3509	8.8
	L929	14.9	108.4	7.4
	MG63	8.0	47.0	5.9
	HeLa	4.5	50.5	11.2

The tkΔ(−39)luciferase construction (see Materials and methods) and derivatives containing PRD II were co-transfected into cell lines with a plasmid containing the CAT reporter gene under the control of the SV40 enhancer. Cellular extracts were assayed for both CAT and luciferase activity, and expression of luciferase normalised using the CAT value. Identical results were obtained for HeLa cells transfected using either calcium phosphate or DEAE−dextran transfection protocols. For C127, L929 and HeLa cells, a value of 1.0 is assigned to the expression levels of the tkΔ(−39)Luc plasmid in uninduced cells, and expression levels of other plasmids in uninduced or induced cells are given relative to this. Since we cannot detect any luciferase expression in MG63 cells until two copies of PRD II are inserted upstream from tkΔ(−39), a value of 1.0 has been assigned to the level of expression of this plasmid in uninduced cells. The PRD II complement of each construction is indicated by arrows; for example (PRD II) << tkΔ(−39)Luc has two copies of PRD II in the opposite direction from the orientation in the β-IFN promoter.

had a major effect on complex formation (Figure 2B). Thus the binding specificity of complexes C1 and C2 was very similar.

The third inducible complex, C3 (Figure 2A), showed reduced binding to the −77/−12 probes containing mutations in the PRD I region (−71, −70, −68, −67 and −65; data not shown). The sensitivity of this factor to point mutations between −77 and −55 was identical to that of the recently reported inducible factor PRD I-BFi (Keller and Maniatis, 1988). In addition, C3 and PRD I-BFi complexes have similar mobilities, and are competed by the PRD I-like oligonucleotide $(AAGTGA)_4$. We therefore believe complex C3 to contain PRD I-BFi.

The ability of PRD II oligonucleotides to compete for complexes formed on the −77/−12 probe suggested that PRD II could function as an independent binding site. To confirm this we used the 12 bp PRD II (−66/−55) oligonucleotide as a probe. The −66/−55 probe revealed the presence of two highly inducible complexes (the major species, A1, and the minor species, A2, in Figure 3, lane 2) which can be specifically competed for by the PRD II oligonucleotide (Figure 3, lanes 3−5). The complexes could be observed in extracts from uninduced cells, but at much reduced levels (Figure 3, lane 1). The PRD II probe also detected a complex of faster mobility (complex B in Figure 3) that was present in approximately equal amounts in extracts from both uninduced and induced cells. Complex B is also specific to PRD II, since it can be prevented from forming by an excess of homologous competitor oligonucleotide (Figure 3, lanes 3−5).

To determine whether the inducible factors detected by the −66/−55 probe (complexes A1 and A2) correspond to either of the −77/−12 complexes (C1 and C2), and to investigate the binding specificity of complex B, we evaluated the ability of mutant PRD II oligonucleotides to compete for complex formation. Examples of these experiments are shown in Figure 3 (lanes 9−14). A point mutation at −61 (A → G) is capable of competing efficiently for complexes A1 and A2 at a lower molar excess than the wild-type competitor (Figure 3, lanes 9−11 and 3−5 respectively), indicating that this mutant has an increased affinity for factors present in these complexes. In contrast, an oligonucleotide bearing a change at −63 (G → A) competes less efficiently than the wild-type oligonucleotide (Figure 3, lanes 12−14

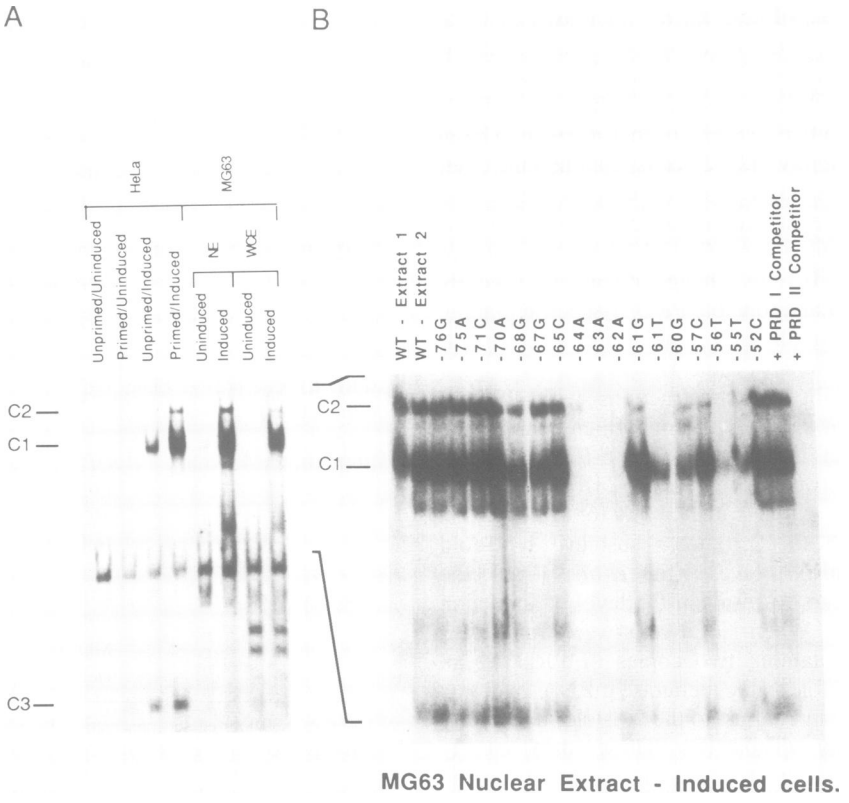

Fig. 2. Detection and characterization of the sequence requirements of dsRNA inducible factors which bind to the 5′ flanking region of the β-IFN gene. (**A**) Nuclear (NE) and whole-cell extracts (WCE) were prepared from uninduced and induced cells as described in Materials and methods. Binding reactions were performed using an end-labelled probe from the −77/−12 region of the β-IFN promoter. The extract examined in each lane is indicated. The three inducible complexes are indicated (C1, C2 and C3). (**B**) Nuclear extracts from induced MG63 cells were analysed for the ability to form complexes with end-labelled probes which contain single base substitutions in the −77/−12 β-IFN region. The gel shown in this panel has been run for longer than the gel shown in (A). MG63 nuclear extract (extract 2) was incubated with wild-type (indicated by WT) or a series of point mutant −77/−12 probes with the nucleotide that has been substituted as indicated. Competition experiments were performed using wild-type −77/−12 probe and the oligonucleotides $(AAGTGA)_4$ (+ PRD I competitor) and −66/−55 (+ PRD II competitor) respectively. Complexes C1 and C2 are indicated. Extract 1 was prepared as described in Materials and methods, whereas extract 2 lacked protease inhibitors other than PMSF and benzamidine in the lysis buffer; the minor complexes that have a faster mobility than complex C2 are probably proteolytic breakdown products.

and 3–5 respectively), indicating that this mutation decreases the affinity for factors in complexes A1 and A2. We have also analysed the ability of mutant PRD II oligonucleotides to compete for complexes C1 and C2. Table II summarizes the results of these competition experiments. All four complexes show an identical DNA binding specificity as determined by their affinities for competitor mutant PRD II oligonucleotides. These results demonstrate that complexes C1, C2, A1 and A2 either contain a common factor or contain factors that cannot be distinguished on the basis of binding specificity. We will subsequently refer to this factor as PRD II-BF II [after Keller and Maniatis (1988) who have previously detected a distinct PRD II binding factor, PRD II-BF].

Although the competition data demonstrate that the binding specificities of complexes C1, C2, A1 and A2 cannot be distinguished, the pattern obtained is slightly different from that derived for either C1 or C2 when the −77/−12 fragment is used as a probe (Figure 2). These differences are confined to the central nucleotides (−61 to −57), and the effects of these mutants on complex formation are less severe than the effects of mutations at outer positions (−64, −63, −62, −56, −55). The discrepancies between the competitor and the probe data could be due to the presence of flanking sequences in the −77/−12 probe which could subtly alter the binding specificity by modifying the DNA structure, or by facilitating the interaction with additional factors.

Complex B was severely affected by mutations at −65, −64, −62 and −61, and slightly affected by mutations at −60, −59 and −57. Therefore complex B and PRD II-BF II have partially overlapping but distinct binding sites.

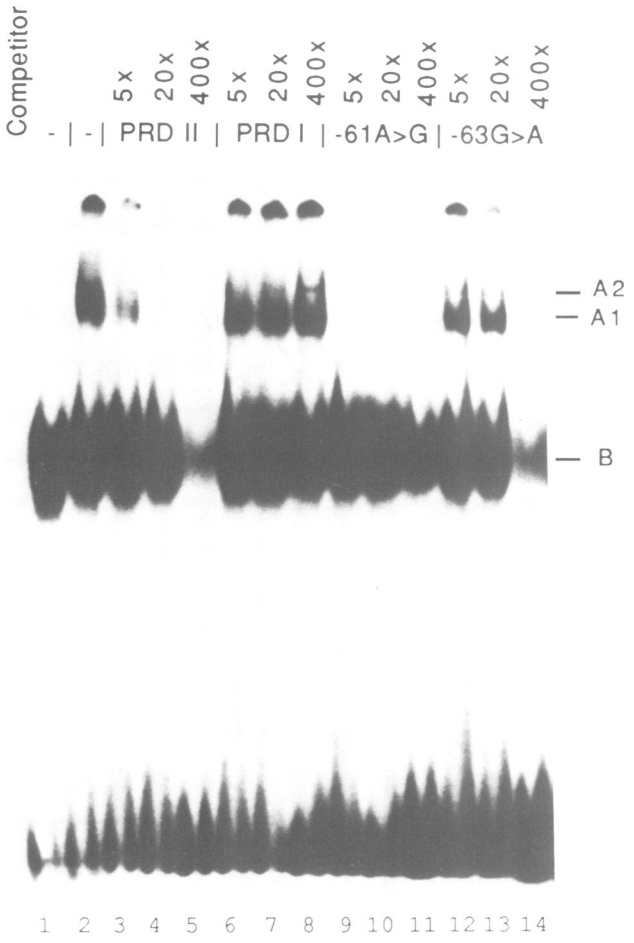

Fig. 3. Detection and characterization of the sequence requirements of constitutive and dsRNA inducible factors which bind to a PRD II (−66/−55) probe. Nuclear extracts from uninduced (**lane 1**) and induced (**lanes 2–14**) MG63 cells were incubated with end-labelled −66/−55 (GTGGGAAATTCC) oligonucleotide probe (all lanes), in the absence of competitor (lanes 1 and 2), or in the presence of the following oligonucleotide competitors at three different molar excesses: **lanes 3–5**, wild-type −66/−55 PRD II; **lanes 6–8**, $(AAGTGA)_4$ (PRD I-like); **lanes 9–11**, −66/−55 PRD II with −61 A → G mutation; **lanes 12–14**, −66/−55 PRD II with −63 G → A mutation. For each set of competition experiments the three molar ratios were 5-, 20- and 400-fold respectivley (left to right). The inducible complexes, indicated as A1 and A2, can be detected in uninduced cells (lane 1) after autoradiography for longer periods (data not shown). Inspection of lower autoradiographic exposures reveals no change in abundance of a third PRD II specific complex (B) upon induction.

PRD II-BFII is indistinguishable from NF-κB

The analysis of the effects of single base substitutions on binding to the PRD II oligonucleotide has allowed us to determine which nucleotides are of key importance in binding to PRD II-BF II. The data demonstrate that the nucleotides between −64 and −55 (GGGAAATTCC) delineate the binding site, and the G-C base-pairs are crucial for binding. This sequence has an 8/10 homology to the binding site for the transcription factor NF-κB in the mouse immunoglobulin (Ig) *kappa* gene enhancer (Table III). The two mismatches correspond to −60 A → C and −59 A → T changes in PRD II. Our studies on the sensitivity of PRD II-BF II to

Table II. Competition of PRD II-specific complexes by wild-type and mutant PRD II oligonucleotides

	A1, A2, C1, C2	B
Wild-type	+++	+++
−66 G → A	+++	+++
−65 T → G	+++	(+)
−65 T → C	+++	+
−64 G → C	+	(+)
−64 G → T	+(+)	(+)
−64 G → A	+	(+)
−63 G → A	(+)	+++
−62 G → A	+	+
−61 A → T	++	+
−61 A → G	++++	+
−60 A → G	++(+)	++
−59 A → G	++	++(+)
−59 A → T	+++	+++
−58 T → G	++	+++
−57 T → C	++	++(+)
−56 C → T	(+)	+++
−55 C → T	+	+++

The ability of wild-type and mutant −66/−55 oligonucleotides to compete for complexes A1, A2, C1, C2 and B in extracts from induced MG63 cells was determined using 20- and 400-fold molar excess relative to probe. The mutants used are indicated by referring to the position of the nucleotide relative to the β-IFN cap site, the wild-type nucleotide at that position and the relevant substitution. The efficiency of competition shown by the wild-type or the mutant −66/−55 oligonucleotide is indicated on the following scale: +++ > ++ > +, with intermediate values given in parenthesis (+). Since no differences were detected for complexes A1, A2, C1 and C2, these complexes are tabulated in one column. All mutant oligonucleotides were better competitors than a non-specific $(AAGTGA)_4$ (PRD I-like) oligonucleotide at high molar excess.

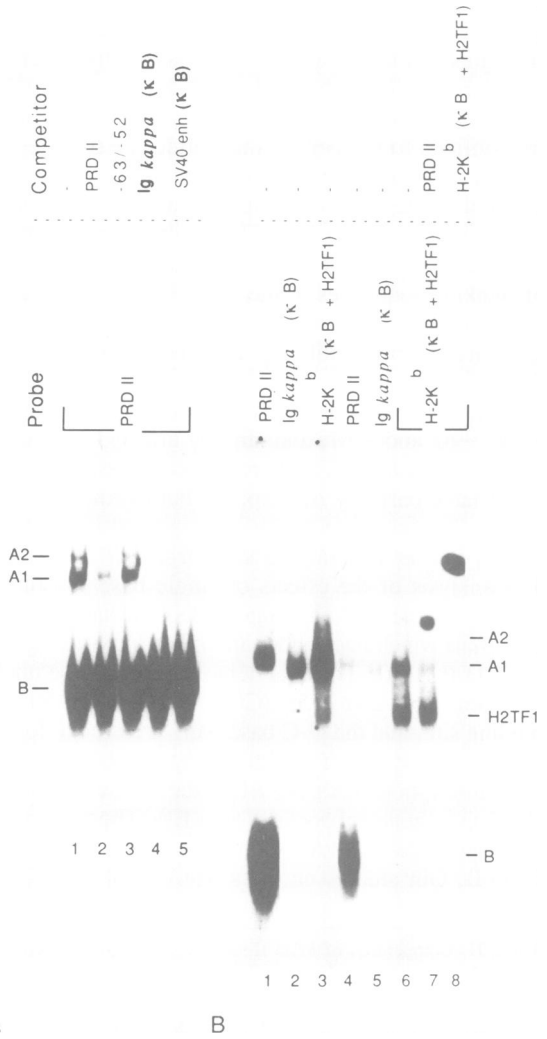

Fig. 4. PRD II-BF II recognises an NF-$\varkappa$B binding site, and NF-$\varkappa$B probes detect dsRNA-inducible factors that are similar to those formed by PRD II-BF II. (**A**) Nuclear extracts from induced MG63 cells were incubated with an end-labelled −66/−55 (GTGGGAAATTCC) oligonucleotide probe in the absence of competitor (**lane 1**), or in the presence of the following oligonucleotide competitors: −66/−55 (PRD II) (**lane 2**); −63/−52 β-IFN sequences (**lane 3**); immunoglobulin enhancer NF-$\varkappa$B site, Ig *kappa* ($\varkappa$B), (**lane 4**); and SV40 enhancer sequences containing an NF-$\varkappa$B site, SV40 enh ($\varkappa$B) (**lane 5**). The sequences of each competitor are as follows: −63/−52 β-IFN, GGAAATTCCTCT; Ig *kappa* ($\varkappa$B), TGGGGACTTTCCGAG; SV40 enh $\varkappa$B, CGGGACTTTCCACACCCTA. All oligonucleotides are flanked by a 5′ overhang end of GATC. (**B**) Nuclear extracts were incubated with end-labelled probes as follows: **lanes 1−3** show nuclear extracts from induced MG63 cells incubated with PRD II probe (**lane 1**), Ig *kappa* ($\varkappa$B) probe (**lane 2**), and H-2K^b ($\varkappa$B + H2TF1) probe (**lane 3**) respectively; **lanes 4−8** show nuclear extracts from uninduced HeLa cells incubated with PRD II probe (**lane 4**), Ig *kappa* ($\varkappa$B) probe (**lane 5**) and H-2K^b ($\varkappa$B + H2TF1) probe (**lanes 6−8**) respectively. Experiments with extracts from uninduced MG63 cells show equivalent results except the levels of the PRD II-BF II factor are much lower. The probes were made by filling in the competitor oligonucleotides referred to above. The H-2K^b ($\varkappa$B + H2TF1) probe comprises nucleotides −170 to −159 from the promoter region of the mouse MHC gene, and has the sequence TGGGGATTCCCCAG. This region has been shown to bind both NF-$\varkappa$B and the factor H2TF1 (Baldwin and Sharp, 1988). The complex formed with the H-2K^b probe was examined for specificity by competition with PRD II (**lane 7**) or with the Ig *kappa* NF-$\varkappa$B site (**lane 8**). This gel represents a longer electrophoresis run than the gel shown in (A). A slightly lower mobility was obtained for the complex detected by the PRD II probe on prolonged electrophoresis, possibly due to the effects of small sequence differences on the conformation of the protein−DNA complexes.

point mutations indicated no effect on binding of the −59 A → T change and a relatively minor decrease for a −60 A → G change. We therefore examined the ability of an oligonucleotide, Ig *kappa* ($\varkappa$B), which contains the NF-$\varkappa$B binding site previously identified in the Ig *kappa* enhancer (Sen and Baltimore, 1986a), to compete for PRD II-BF II. This oligonucleotide competed efficiently at low molar excess (10-fold) for PRD II-BF II complexes A1 and A2 (Figure 4A, lanes 1 and 4), as well as complexes C1 and C2 (data not shown), and was in fact a slightly more efficient competitor than PRD II (Figure 4A, lanes 2 and 4); at the same molar excess, an oligonucleotide with an incomplete PRD II site (−63/−52) did not compete (Figure 4A, lane 3). An oligonucleotide containing a previously identified NF-$\varkappa$B binding site in the SV40 enhancer was also found to compete for PRD II-BF II (Figure 4A, lane 5).

The ability of the inducible factor PRD II-BF II to recognize specifically NF-$\varkappa$B binding sites raised the possibility that PRD II-BF II was similar, if not identical, to NF-$\varkappa$B. This was investigated by using oligonucleotide probes containing PRD II, the Ig *kappa* ($\varkappa$B) NF-$\varkappa$B site,

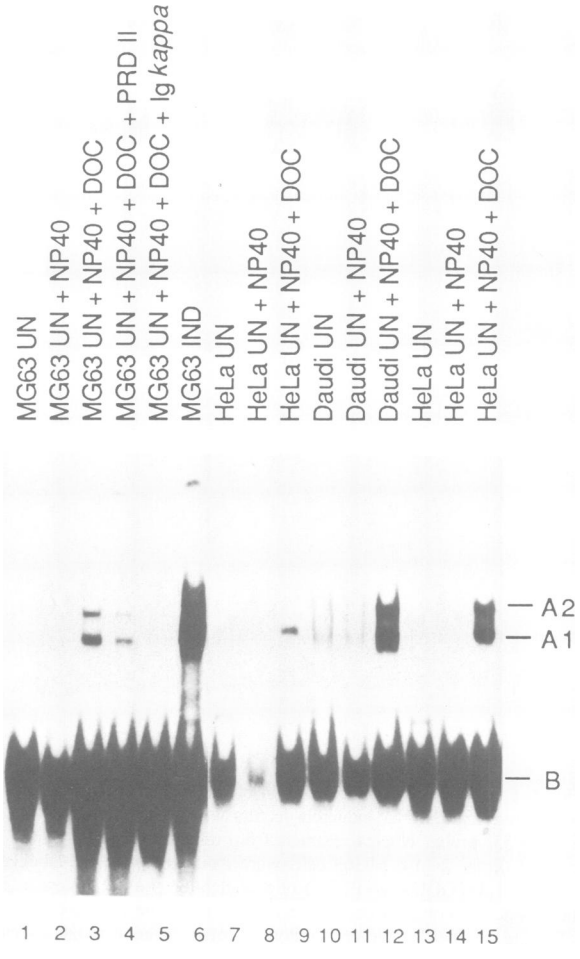

Fig. 5. Activation of PRD II-BF II DNA binding activity by addition of sodium desoxycholate to nuclear and cytosolic extracts. The cellular extracts indicated above each lane were examined by gel-retardation analysis with −66/−55 (PRD II) oligonucleotide probe in the presence or absence of NP-40 and sodium desoxycholate (at a final concentration of 0.2%). PRD II (**lane 4**) and NF-$\varkappa$B (**lane 5**) oligonucleotide competitors, used at a 10-fold molar excess, demonstrate the specificity of complex formation.

Table III. NF-κB binding sites

(A1)		**(B1)**	
PRD II	(T) GGGAAATTCC	−61 A → G	(T) GGGGAATTCC
−65 T → C	(C) GGGAAATTCC	H-2K^b	(G) GGGGAATCCC
−65 T → G	(G) GGGAAATTCC		
−60 A → G	(T) GGGAGATTCC		
−59 A → T	(T) GGGAATTTCC		
IgκB-m	(G) GGGACTTTCC		
IL-2Rα	(G) GGGAGATTCC		
(A2)		**(B2)**	
Factor B	(G) GGGAAATTCC	IgκB-h	(G) GGGGATTTCC
IP-10	(A) GGGAAATTCC	CMV	(G) GGGGATTTCC
IL-6	(T) GGGATTTTCC	TNF-β	(G) GGGGCTTCCC
TNF-α	(T) GGGTTTCTCC		
JE	(G) GGGTGTTTCC		

The identified binding sites for NF-κB, and potential sites identified on the basis of binding site requirements determined in this manuscript are listed. (**A1**) The sequence of the wild-type binding site determined for PRD II-BF II (−64/−55) is compared to those of point mutant PRD II sites which show similar binding affinity and to the NF-κB binding sites in the mouse Ig κ gene enhancer (Ig κB-m) (Sen and Baltimore, 1986a) and interleukin-2 receptor α enhancer (IL-2Rα) (Leung and Nabel, 1987). (**A2**) Putative NF-κB binding sites which are identical to PRD II, or differ in positions which do not severely impair binding. The TNF-α and JE sites are likely to be relatively weak due to the presence of a T at the position −61 in PRD II which reduces but does not abolish binding (see Figure 2B). The putative NF-κB sites are located as follows: factor B (−456/−465, Wu *et al.*, 1987); IP-10 (−1958/−1967, Luster and Ravetch, 1987): IL-6 (−135/−126, Yasukawa *et al.*, 1987); TNF-α (−100/−91, Nedwin *et al.*, 1985) and JE (−145/−154, Rollins *et al.*, 1988). (**B1**) The PRD II oligonucleotide with a −61 A → G mutation (−61 A → G) and the NF-κB site in the H-2K^b class I MHC enhancer (H-2K^b) (−170/−161, Baldwin and Sharp, 1988; Israel *et al.*, 1987) which both have increased affinity for PRD II-BF II relative to wild-type PRD II and Ig κB sites and listed separately. (**B2**) Proposed NF-κB binding sites which also contain a change equivalent to −61G. The putative NF-κB sites are located as follows: Ig human κ gene enhancer (Ig κB-h) (Emorine *et al.*, 1983); cytomegalovirus enhancer (CMV) (Boshart *et al.*, 1985), and TNF-β (−100/−91, Nedwin *et al.*, 1985).

or the previously identified NF-κB binding site in the 5′ flanking region of the MHC class I H-2K^b gene (Baldwin and Sharp, 1988). All three probes produced one major dsRNA inducible complex with a similar mobility; complex B was not detected by the Ig *kappa* (κB) and H-2K^b (κB + H2TF1) probes (Figure 4B, lanes 1−6), consistent with its identified binding specificity (Table II). Since NF-κB is formally defined as a B-cell-specific transcription factor (Sen and Baltimore, 1986a) and has been purified from Namalwa cells (Kawakami *et al.*, 1988), we examined extracts from these cells for the ability to form complexes with the oligonucleotide probes. One major complex was formed with the Ig *kappa* (κB) probe in uninduced cells, and the amount of this complex increased in extracts from induced cells (data not shown). The mobility of the Namalwa NF-κB complex was similar to the PRD II-BF II complexes from the other cell extracts. A second B-cell line (Daudi cells, which express κ immunoglobulin chains) also gave a single specific complex of the expected mobility with PRD II probe, and this was competed for by the NF-κB oligonucleotide (see Figure 5). We therefore cannot distinguish the binding properties of PRD II-BF II from those of NF-κB.

The H-2K^b NF-κB site appeared to have the highest affinity for the inducible factor in MG63 extracts: increased levels were detected by the H-2K^b probe (Figure 4B, lanes 3 and 6), and competition by unlabelled H-2K^b oligonucleotide was more efficient than that by PRD II (Figure 4B, lanes 7 and 8). The increased affinity is probably due to the presence of a G at the position corresponding to −61 in PRD II, since introduction of this change in PRD II resulted in increased affinity for PRD II-BF II (Figures 2 and 3, and Tables II and III). The H-2K^b probe was also expected to detect the ubiquitous H2TF1 transcription factor, which has been shown to require additional nucleotides flanking the NF-κB binding site, and which are present in the H-2K^b oligonucleotide but not in the PRD II or Ig *kappa* (κB) oligonucleotides (Table III). An additional complex was indeed seen with the H-2K^b probe in MG63 and HeLa nuclear extracts (Figure 4B, lanes 3−7). The PRD II oligonucleotide was unable to compete with H-2K^b probe for this complex (Figure 4B, lane 7) whereas the H-2K^b oligonucleotide competed efficiently (Figure 4B, lane 8).

Activation of PRD II-BF II by addition of deoxycholate to cellular extracts

It has recently been reported that NF-κB binding activity can be activated in cytosolic extracts from pre-B cells by the addition of a mixture of the detergents NP-40 and sodium deoxycholate (Baeuerle and Baltimore, 1988a). To investigate further the similarity between PRD II-BF II and NF-κB we attempted to determine if the induction of PRD II-BF II binding activity by dsRNA *in vivo* could be obtained *in vitro* by the addition of detergents to extracts from uninduced cells. MG63 extracts were treated with NP-40 alone, or NP-40 and sodium deoxycholate (DOC) and analysed in a gel retardation assay with the −66/−55 PRD II probe (Figure 5). Addition of NP-40 alone had little effect other than a slight decrease in the level of complex B (Figure 5, lane 2). Addition of NP-40 and DOC resulted in the production of complexes that were indistinguishable from those formed by PRD II-BF II (Figure 5, lanes 3−6). An analysis of the point mutation specificity profile also failed to distinguish the complexes from those formed by PRD II-BF II (data not shown).

As we have noted above, nuclear extracts from uninduced HeLa cells contain a low level of PRD II-BF II binding activity (see Figure 3). The level of PRD II-BF II is only marginally increased in these extracts by detergent treatment (Figure 5, lanes 7−9). In contrast, an equal amount of HeLa whole cell extract contains much lower levels of PRD II-BF II (Figure 5, lanes 13 and 7 respectively) but the PRD II-BF II binding activity can be highly activated by detergent (Figure 5, lane 15). These results suggest that most of the

inhibitor-free NF-κB activity in HeLa cells resides in the nucleus. As would be expected from a B cell line constitutively expressing the Ig *kappa* gene, whole-cell extracts from Daudi cells contained detectable levels of PRD II-BF II; these levels could also be increased following detergent treatment (Figure 5, lanes 10–12).

Discussion

The experiments reported here indicate that the PRD II element is an independent target for cellular factors which promote basal expression, and which increase in activity upon induction. We have shown that an element with a 5′ boundary of −64 is inducible (Figure 1), and coupled with the observation that mutations at nucleotides −55 and −56 have a major effect on induction (Goodbourn and Maniatis, 1988) the limits of PRD II can be narrowed to between −64 and −55. The binding site of the inducible factor PRD II-BF II is exactly coincident with these nucleotides, unlike the sites for PRD II-BF of Keller and Maniatis (1988) and the factor which forms complex B, neither of which increase in binding activity in extracts from induced cells. Based upon the binding site requirements and the inducibility of PRD II-BF II binding activity, it is highly probable that PRD II-BF II plays a role in the induction of the β-IFN gene.

In addition to the response to induction, the experiments described in this paper demonstrate that PRD II stimulates basal expression when analysed in isolation from other β-IFN sequences, or when fused to β-IFN position −40. It is likely that the low level of PRD II-BF II detected in nuclear extracts from uninduced cells is responsible for this basal expression. In this regard it is interesting that a cellular factor, EBP-1, which has similar binding requirements to PRD II-BF II, has been purified from uninduced HeLa cells (L.Clarke and R.Hay, personal communication). In contrast to the basal expression demonstrated by the plasmids analysed here, the endogenous β-IFN gene does not express detectable mRNA levels in any cell line we have examined, including Namalwa cells which have a significant level of PRD II-BF II in uninduced cells. These observations indicate that a dominant repression mechanism prevents PRD II-BF II from activating the β-IFN promoter. Since a number of agents which have not been reported to be β-IFN inducers can activate the binding activity of PRD II-BF II/NF-κB (see below) the repression mechanism would prevent β-IFN induction in cells under inappropriate conditions. A negative regulatory element, which is disrupted in the experiments reported here, physically overlaps PRD II (Goodbourn *et al.*, 1986; Goodbourn and Maniatis, 1988) and would prevent binding of PRD II-BF II to the β-IFN promoter in uninduced cells. Both PRD II-BF and complex B are detected in uninduced cells and have binding sites which partially overlap PRD II. It is possible that these factors may be involved in negative regulation of the β-IFN promoter.

The finding that not only PRD I, but also PRD II is independently inducible has interesting implications for the regulation of β-IFN gene activity. For maximal expression, both domains are required, and it seems reasonable to assume that both NF-κB and the inducible factor that binds to PRD I act in synergy. PRD II also seems to act in synergy with β-IFN sequences which are located downstream of −40, since single copies of PRD II respond to induction in this context in contrast to their behaviour when part of a heterologous promoter. Since our experiments demonstrate that PRD II activates transcription and augments the response to induction when a negative regulatory region is partially inactivated by deletion, the endogenous β-IFN gene may be induced through PRD II alone following physiological derepression. Such a stimulation may allow a mechanism whereby low levels of β-IFN are produced in the absence of stimulation by PRD I binding factors. Several groups have noted the similarity between PRD I and a consensus element found in all IFN-inducible promoters (see for example Hug *et al.*, 1988; Porter *et al.*, 1988; Wathelet *et al.*, 1988). It is therefore possible that IFN production achieved by mechanisms that do not involve PRD I could activate transcription factors that bind to PRD I, thus achieving further stimulation of β-IFN synthesis. The fact that β-IFN mRNA induction can occur in the presence of protein synthesis inhibitors would suggest that this cannot be obligatory for β-IFN induction, but it is possible that such a positive autoregulation might be important in generating the high levels of β-IFN achieved during induction.

The cellular factor PRD II-BF II appears to have many properties in common with NF-κB. The complexes detected by PRD II can be specifically competed for by NF-κB binding sites and vice versa. Point mutations that severely impair the binding of PRD II-BF II to PRD II map to positions conserved between natural NF-κB binding sites identified to date, and changes in PRD II which are equivalent to variations between these sites have little effect on the binding of PRD II-BF II (see Table III). Probes containing known sites for NF-κB detect dsRNA inducible complexes that have very similar mobilities to those detected by PRD II probes in B cells as well as non-lymphoid cells. Complexes which can bind to PRD II and NF-κB probes can be activated in extracts from uninduced cells by treatment with detergent, and these complexes appear to be indistinguishable from those detected in extracts from dsRNA induced cells. In addition to these properties, relatively low concentrations of poly(dI)–poly(dC) prevent detection of PRD II-BF II and NF-κB complexes (unpublished observations). Thus, by all available criteria PRD II-BF II is NF-κB.

In our experiments NF-κB binding activity can be induced by detergent in nuclear extracts. This result would appear to be in contrast to the findings of Baeuerle and Baltimore (1988a), who detected activation in cytoplasmic, but not nuclear extracts. A number of explanations could account for this discrepancy. Firstly, the relatively crude separation procedures commonly used to prepare nuclear and cytoplasmic extracts may give some cross-contamination. Secondly, Baeuerle and Baltimore (1988a) assayed significantly less protein from the nuclear fraction than the cytoplasmic fraction (1.35 and 9 μg respectively for HeLa cells)—all of our experiments have been performed with 10 μg of nuclear extract. When we compared 10 μg of cytoplasmic and nuclear protein from HeLa cells, we found that NP-40 + DOC could activate a higher level of binding activity in the cytoplasmic fraction (data not shown). Thirdly, in contrast to their results with HeLa cells, Baeuerle and Baltimore (1988a) were able to activate low levels of NF-κB in nuclear extracts from 70Z/3 pre-B cells using 4.4 μg protein. This is consistent with our data, which indicates differences between cell lines in the level of inactive NF-κB in nuclear extracts (Figure 5).

The results of Baeuerle and Baltimore (1988a,b) suggest that NF-κB is associated with an inhibitory factor in situations in which the binding activity is not required. Since NF-κB can be produced in a DNA binding form by treatment of cells with phorbol esters, it has been suggested that the NF-κB–inhibitor complex is disrupted by phosphorylation in response to activated protein kinase C. The liberated NF-κB would then be free to migrate into the nucleus. Since we have demonstrated that NF-κB-like activity can be activated by dsRNA, it seems pertinent to ask whether a similar mechanism of activation could be involved. The observation that β-IFN mRNA synthesis can be induced by dsRNA in the presence of protein synthesis inhibitors suggests that the NF-κB activation step is direct. Double-stranded RNA is not known to be a protein kinase C activator, but it has been suggested that dsRNA could activate the β-IFN gene via the DI-kinase (Zinn *et al.*, 1988; Marcus and Sekellick, 1988). It is therefore possible that the NF-κB–inhibitor complex might be the target for more than one kinase pathway, allowing it to respond to diverse cellular signals.

The activation of NF-κB in response to dsRNA may be important for the activation of the human immunodeficiency virus type I (HIV-I). A number of recent reports have demonstrated that NF-κB is capable of binding to, and activating expression from, the HIV-I enhancer (Kaufmann *et al.*, 1987; Nabel and Baltimore, 1987). It has been previously proposed that bacterial infections could promote activation of the HIV-I enhancer in T lymphocytes by the production of lipopolysaccharide and activation of NF-κB (Nabel and Baltimore, 1987). Activation of NF-κB by dsRNA (a replicative intermediate in many viral infections) could provide an alternative mechanism whereby opportunistic infections could activate HIV.

Potential NF-κB binding sites are present in the 5′ flanking regions of a number of other genes which have been reported to be induced by dsRNA, namely IL-6 (Kohase *et al.*, 1987; Seghal *et al.*, 1987), JE (Rollings *et al.*, 1988) and TNF-α and TNF-β (Wong and Goeddel, 1986)—see Table III. The α-interferon genes, which are not inducible by dsRNA (Kelly and Pitha, 1985) do not contain an NF-κB site in their promoter regions. It is therefore possible that direct activation of NF-κB can be responsible for the induction of these genes by dsRNA. The possibility that induction of NF-κB by viral infection or activation of T cells results in stimulation of IL-6 and TNF-β is particularly intriguing in view of their recently reported properties (Billiau, 1986; Gauldie *et al.*, 1987; Seghal *et al.*, 1987; Tosato *et al.*, 1988), and suggests that this transcription factor has a key role in the regulation of many genes which participate in the immune response. This regulation appears to be governed by a complex network of interactions since many of these genes appear to be capable of regulating the expression of themselves and each other (Kronke *et al.*, 1988; Niitsu *et al.*, 1988; Taniguchi, 1988).

Materials and methods

Cell lines

C127, L929 and MG63 cell lines were obtained from the American Type Culture Collection (ATCC). C127 cells (ATCC CRL 1616) are a non-transformed clonal line from a mouse mammary tumour; L929 cells (ATCC CCL 1) are a non-transformed clonal line from mouse connective tissue; MG63 cells (ATCC CRL 1427) are a line derived from a human osteosarcoma. HeLa cells were obtained from E.Laufer, Imperial Cancer Research Laboratories, London.

Plasmid constructions and transfections

The 5′ deletion derivatives of the BPV-based plasmid, ID −55/−40 (Goodbourn *et al.*, 1986), were constructed by linearizing with *Bgl*II to generate a 5′ end at −77, followed by limited digestion with T4 DNA polymerase in the absence of nucleotides; single-stranded DNA was removed with Mung Bean nuclease, and *Bgl*II linkers were ligated to the new 5′ ends. These plasmids were introduced into mouse C127 cells by calcium phosphate co-precipitation, and pools of transformed foci isolated. Pools of foci were maintained for at least 35 days before inducing and harvesting RNA. The tk–luciferase plasmid consists of HSV tk promoter sequences from between −39 and −15 fused to −17 of the firefly luciferase cassette of De Wet *et al.* (1987); the tk fragment is bounded by a *Bam*HI site at the 5′ end, and a dimeric SV40 terminator element is positioned upstream from this site. The *Bam*HI site was used to insert PRD II oligonucleotides adjacent to the tk −39 position. The tk–luciferase plasmids, together with a pSV2CAT (Gorman *et al.*, 1982) derivative, were transiently introduced into C127, L929 and HeLa cells by DEAE–dextran precipitation, and into HeLa and MG63 cells by calcium phosphate co-precipitation.

Induction protocols and expression analysis

C127 cells and L929 cells were induced using poly(rI)–poly(rC) as previously described (Zinn *et al.*, 1983). For transient analysis, cells were induced or mock-induced 42–45 h after transfection, and extracts were made 8 h after induction. HeLa and MG63 cells were induced by incubating for 2 h in serum-free medium containing 100 μg/ml Poly(rI)–poly(rC) and 50 μg/ml cycloheximide; the medium was then replaced with medium containing only cycloheximide for a further hour and then again replaced with medium containing 10% serum for 5 h, followed by extract preparation. HeLa cells were primed ~15 h before induction (Enoch *et al.*, 1986) by the addition of 500 U/ml of Wellferon (a mixture of α- and β-IFN). Cellular RNA was prepared and analysed as previously described (Zinn *et al.*, 1983). The SP6 probes specific for CAT and luciferase mRNAs were a gift of E.Laufer; other probes have been previously described as indicated. CAT activity was assayed using the procedure of Sleigh (1986), and luciferase activity was assayed using an LKB 1251 luminometer using the buffer of Nguyen *et al.* (1988).

Cellular extracts and gel-retardation assays

HeLa and MG63 cells were induced as described above, except that the cycloheximide chase was extended to 2 h, at which point cells were harvested for extract preparation. Nuclear extracts and S100 fractions were prepared by the method of Dignam *et al.* (1983), and whole cell extracts were prepared according to Manley *et al.* (1980). Protease inhibitors were added to all buffers at the following final concentrations: pepstatin (5 μg/ml), aprotinin (5 μg/ml), leupeptin (30 μg/ml), benzamidine (1 mM) and PMSF (0.5 mM). Whole cell extracts from HeLa and Daudi cells prepared by the procedure of Zimarino and Wu (1987) were gifts from T.Dale and A.Imam respectively. Ten micrograms of a given extract was assayed in a total volume of 20 μl binding buffer containing 20 mM Tris (pH 8.0), 60 mM KCl 2 mM $MgCl_2$, 0.3 mM dithiothreitol (DTT), 12% glycerol, 1.5 μg poly(dI–dC)·poly(dI–dC) (Pharmacia), and 2–5 × 10^4 c.p.m. of end-labelled probe (added last). The reaction mixture was incubated for 15 min at 30°C, loaded onto a pre-electrophoresed native 5% polyacrylamide (containing 0.17% bisacrylamide) gel and electrophoresed at 14 V/cm. The gel was dried onto DE52 paper (Whatman) and subjected to autoradiography at −70°C. DNA probes are described in the appropriate figure legends and were 3′ end-labelled by 'filling-in'.

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