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An IL-2 response element in the human IL-2 receptor α chain promoter is a composite element that binds Stat5, Elf-1, HMG-I(Y) and a GATA family protein

Susan John, Christiane M.Robbins[1] and Warren J.Leonard[2]

Laboratory of Molecular Immunology, National Heart, Lung and Blood Institute, Bldg 10, Rm 7N244 and [1]Genome Technology Branch, National Center for Genome Research, Bethesda, MD 20892-1674, USA

[2]Corresponding author

Expression of the human interleukin-2 (IL-2) receptor α chain gene is potently upregulated by its own ligand, IL-2. In this study, we characterize an essential upstream IL-2 response element that contains both consensus and non-consensus GAS motifs, two putative Ets binding sites (EBS), one of which overlaps the consensus GAS motif, and a GATA motif, which overlaps the non-consensus GAS motif. We demonstrate that although the individual components of this element do not respond to IL-2, together they form a composite element capable of conferring IL-2 responsiveness to a heterologous promoter. Multiple factors including Stat5, Elf-1, HMG-I(Y) and GATA family proteins bind to the IL-2 response element and mutation of any one of these binding sites diminishes the activity of this element. An unidentified Ets family protein binds to the EBS overlapping the consensus GAS motif and appears to negatively regulate the human IL-2Rα promoter. Thus, IL-2-induced IL-2Rα promoter activity requires a complex upstream element, which appears to contain binding sites for both positive and negative regulatory factors.

Keywords: Elf-1/GATA/HMG-I(Y)/IL-2 receptor/STAT

Introduction

There are three classes of interleukin-2 (IL-2) receptor proteins. High affinity receptors (K_d ~10^{-11} M) contain the α, β and γ chains; intermediate affinity IL-2Rs (K_d ~10^{-9} M) contain the β and γ chains; and low affinity receptors (K_d ~10^{-8} M) contain only the α chain (Leonard *et al.*, 1994; Sugamura *et al.*, 1995; Taniguchi, 1995). The intermediate and high affinity forms are capable of transducing mitogenic signals in response to IL-2, whereas low affinity receptors are not, indicating the importance of the β and γ chains (reviewed in Leonard *et al.*, 1994; Sugamura *et al.*, 1995; Taniguchi, 1995). Although IL-2Rα is not required for IL-2-induced signaling, its essential role is underscored by the observation that mice lacking expression of this gene develop severe autoimmunity and die at a young age (Willerford *et al.*, 1995). The importance of IL-2Rα is presumably based on the need to achieve high affinity receptors in order to bind the relatively low concentrations of IL-2 that are physiologically produced.

The IL-2Rα gene is potently induced by antigens and mitogens, the transactivator protein of HTLV-I and certain cytokines, including IL-2 (Depper *et al.*, 1985; Inoue *et al.*, 1986; Cross *et al.*, 1987; Maruyama *et al.*, 1987; Siegel *et al.*, 1987; Siekevitz *et al.*, 1987). The IL-2Rα promoter contains two important positive regulatory regions, PRRI (–276 to –244) (Bohnlein *et al.*, 1988; Leung *et al.*, 1988; Ballard *et al.*, 1989; Cross *et al.*, 1989; Lowenthal *et al.*, 1989; Lin *et al.*, 1990; Roman *et al.*, 1990; Toledano *et al.*, 1990; Algarte *et al.*, 1995; John *et al.*, 1995) and PRRII (–137 to –64) (John *et al.*, 1995), that together are required for mitogenic stimulation of the IL-2Rα gene (John *et al.*, 1995). PRRI binds to NF-κB1, c-Rel and serum response factor (SRF), while PRRII binds the lymphoid/myeloid-specific Ets family protein, Elf-1, and the high mobility group proteins, HMG-I (Y) (John *et al.*, 1995). Intramolecular interactions between proteins that bind to PRRI and PRRII appear to result in a highly-ordered stereospecific complex that regulates the transcriptional activity of the IL-2Rα promoter upon mitogenic stimulation (John *et al.*, 1995).

Although IL-2 potently induces IL-2Rα, in transfected YT cells, it did not induce IL-2Rα–CAT reporter constructs spanning PRRI and PRRII. In the murine IL-2Rα chain gene, an IL-2 response element is located ~1.3 kb upstream of the transcription start site (Sperisen *et al.*, 1995). We now characterize a similar IL-2 response element (denoted PRRIII) in the human IL-2Rα promoter, located ~3.7 kb upstream of the initiation of transcription. We demonstrate that PRRIII is a complex element capable of conferring IL-2 inducibility to a minimal heterologous promoter. It contains both consensus and non-consensus GAS motifs and two distinct Ets binding sites (EBSs), one of which overlaps the consensus GAS motif, and an important GATA motif, which overlaps the non-consensus GAS motif. By performing binding studies and mutational analysis, we clarify how the components of PRRIII cooperate to form a composite unit that regulates IL-2-induced IL-2Rα promoter activity.

Results

Identification of a sequence in the human IL-2Rα promoter similar to the murine IL-2 response element

We have demonstrated that two positive regulatory elements, PRRI and PRRII, are essential for the mitogen-induced activation of the human IL-2Rα promoter (John *et al.*, 1995). However, a reporter construct containing PRRI and PRRII (–472 to +109 IL-2Rα–CAT) was not induced by IL-2 (our unpublished observations). In the murine IL-2Rα promoter, an IL-2 response element was identified between nucleotides –1376 and –1304 relative to the transcription start site (Sperisen *et al.*, 1995). Analysis of the human IL-2Rα promoter revealed that a

similar sequence was located between nucleotides –3780 and –3703 (PRRIII, Figure 1A). Analogous to the murine sequence, this human sequence contains a consensus GAS motif (GAS-c), a non-consensus GAS motif (GAS-n), a putative GATA motif and an 'Ets' motif (EBS), (Figure 1B). Unlike the murine consensus GAS motif sequence (TTCTGAGAA), the human sequence contains an overlapping GGAA Ets motif (TTCTAGGAA, Figure 1B). To determine if PRRIII was sufficient to confer IL-2 inducibility to a heterologous promoter, the full-length PRRIII oligonucleotide (–3780 to –3703) and each of its individual sub-elements were inserted upstream of a minimal TK promoter in pBLCAT2 and the resulting constructs were transfected into YT natural killer-like cells (Figure 1C). Only the full-length PRRIII oligonucleotide conferred maximal IL-2 inducibility to the TK promoter, suggesting that PRRIII functions as a composite element.

Signal transducer and activator of transcription (STAT), Ets and GATA family protein recognition sites are contacted in PRRIII

To characterize the range of proteins that bind to PRRIII, we performed electrophoretic mobility shift assays (EMSA) with a PRRIII oligonucleotide and nuclear extracts prepared from PBL that were preactivated with phytohemagglutinin (PHA), rested, and then either left untreated or stimulated with IL-2 (Figure 2A). We used preactivated PBL rather than freshly isolated PBL for this study in order to ensure that the cells were primed to be maximally responsive to IL-2 treatment. EMSAs with the full-length PRRIII probe (–3780 to –3703) revealed the presence of three major complexes (C1, C2 and C3) in uninduced extracts (Figure 2A, lane 1). Complex C3 appears to be a degradation product of C2 (see below). Upon stimulation with IL-2, a new complex (C4) was formed (lane 2). Treatment of cells with cycloheximide did not prevent formation of complex C4, indicating that its formation did not depend on new protein synthesis (data not shown), a finding consistent with our ability to detect complex C4 within 30 min of IL-2 stimulation. Formation of complexes C1–C4 was specific as demonstrated by the ability of unlabeled excess PRRIII oligonucleotide to compete the binding activities (data not shown). Similar results were obtained with nuclear extracts from YT cells that were not stimulated or stimulated with IL-2 (data not shown).

To identify the nucleotide sequences contacted by complexes C1, C2 and C4, we performed methylation interference analyses using the full-length PRRIII oligonucleotide and nuclear extracts from preactivated PBL that were not induced or induced with IL-2 (Figure 2B and summarized in Figure 2C). In complex C1, factor(s) strongly contacted the Gs at –3765, –3764, –3725 and –3724 (Figure 2B, lanes 1–4). The fact that both pairs of Gs are contained within Ets binding sites suggested that C1 contains Ets family proteins. Protein(s) in complex C2 also contacted these same nucleotides albeit somewhat less strongly than those in C1 (lanes 5–8 and 9–12), suggesting that C2 may also contains Ets family proteins. The IL-2-inducible complex (C4) strongly contacted G nucleotides at –3768, –3765, –3764, –3761 and –3748 within the two GAS motifs, the G nucleotide at –3746 of the putative GATA motif and the G at –3739, immediately following the putative GATA motif; in addition, the Gs of the Ets motif located at nucleotides –3725 and –3724 were also weakly protected (lanes 13–16). These results suggest that Ets family proteins in preactivated PBL bind to PRRIII and that upon stimulation of these cells with IL-2, a new complex is formed that may contain STAT and GATA family proteins in addition to Ets family proteins.

EMSAs were performed with wild type and mutant PRRIII oligonucleotides (Figure 3A) to determine how each binding site in PRRIII contributed to the formation of complexes C1, C2, C3 and C4. Binding to the wild type probe is shown in Figure 3B (lanes 1 and 2). Mutation of the consensus GAS motif, so that the overlapping Ets motif was left intact (lanes 3 and 4; mutant M1.0) resulted in a loss of C4. Interestingly, this mutant exhibited an increase in C1 and also formed a new complex with both uninduced and IL-2-induced extracts. Selective mutation of the overlapping Ets motif (leaving intact the TTCXXX-GAA consensus GAS motif) resulted in a loss of C1, a decrease in C2 and C3 and a marked decrease in C4 (lanes 5 and 6; mutant M1.1), although a low level of C4 was still visible on a longer exposure (data not shown). Simultaneous mutation of both the consensus GAS motif and the overlapping EBS (lanes 7 and 8; mutant M1.2) resulted in a total loss of C1 and C4 and a decrease in C2 and C3. Mutation of the non-consensus GAS motif and the 'T' in the associated GATA motif (lanes 9 and 10; mutant M2.1) or of only the non-consensus GAS motif (from TTCTGATAA to TTGTGATAA, data not shown), eliminated only complex C4, while mutation of only the 'T' in the GATA motif still allowed C4 formation albeit at a lower level (lanes 11 and 12; mutant M2.2). Mutation of the EBS at nucleotides –3724 and –3725 (lanes 13 and 14; mutant M3) resulted in a loss of C1, and a decrease in the intensity of C2 and C3 and a more modest decrease in C4. These results suggest that this downstream Ets motif is required for the formation of C1. Furthermore, they suggest that the formation of C4 is dependent on multiple sites. Mutation of both GAS motifs resulted in a loss of C1 and C4 (lanes 15 and 16; mutant M5). As expected, mutation of all three protein binding sites (lanes 17 and 18; mutant M6), resulted in the loss of C1, C2 (C3) and C4. These data (summarized in Figure 3C) suggest that the formation of C4 is dependent on the integrity of the consensus and non-consensus GAS motifs, with their overlapping EBS and GATA motifs respectively, and to a lesser extent on the downstream EBS. In contrast, formation of complex C1 depends on the presence of intact Ets binding sites.

Elf-1, Stat5 and a GATA protein can bind to PRRIII

Next we sought to determine the identity of the proteins contained within each complex. Since IL-2 potently activates both Stat5A and Stat5B (Lin *et al.*, 1996), we used specific antisera in antibody supershift experiments and found that both Stat5A and Stat5B antibodies affected complex C4, suggesting that both Stat5A and Stat5B were present in this complex (Figure 4A). In contrast, antibodies to Stat3 and Stat1 had no effect (data not shown).

The methylation interference analysis suggested that C1, C2 and C4 contained Ets family proteins. Since a low affinity Elf-1 site in PRRII has been shown to be vital for IL-2Rα transcription (John *et al.*, 1995, 1996), we

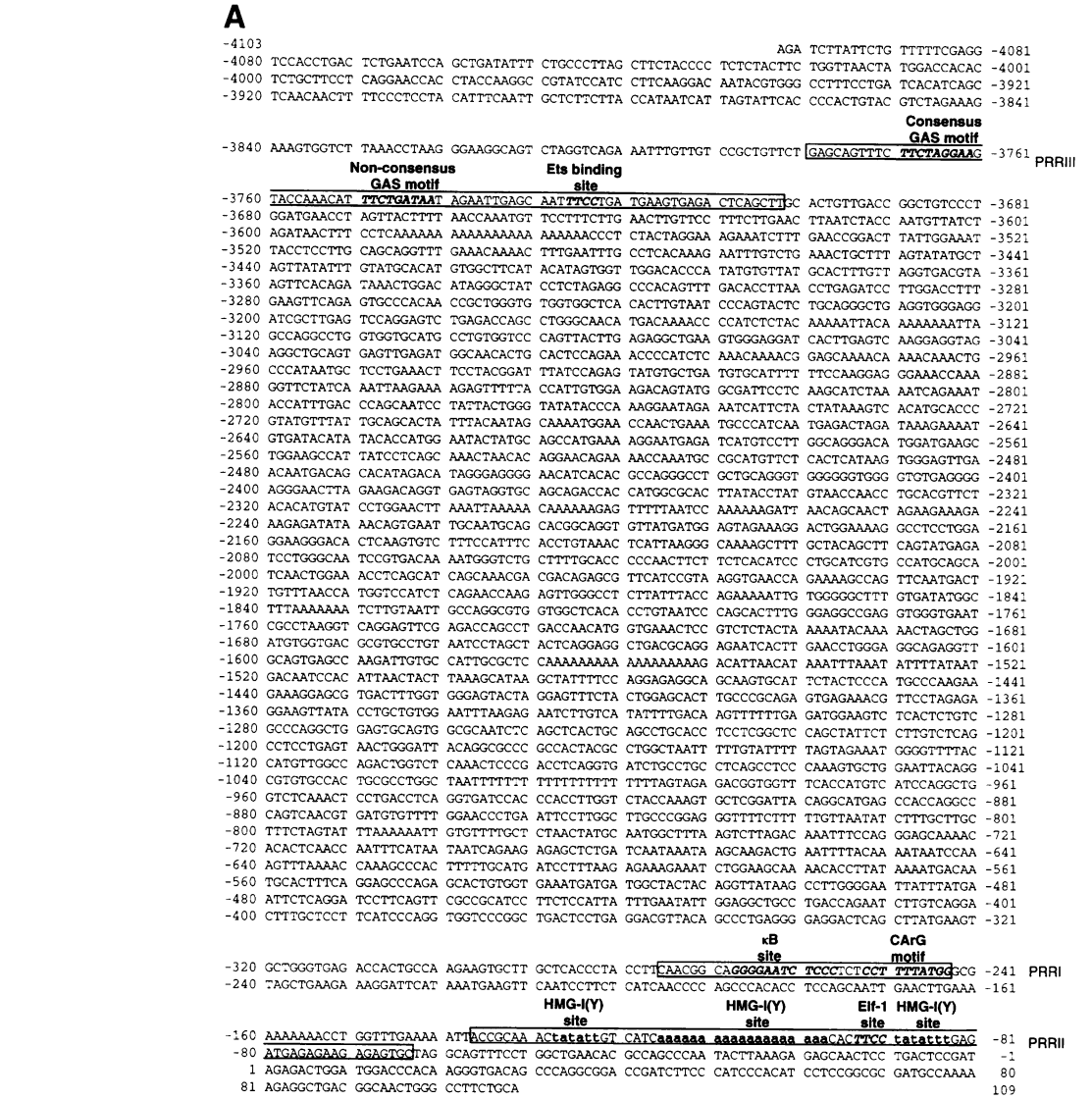

Fig. 1. (**A**) The –4103 to +109 sequence from the IL-2Rα gene (GenBank accession number U57613). Boxed are PRRI (–276 to –244, containing an NF-κB site and a CArG motif capable of binding SRF), PRRII [–137 to –64, containing Elf-1 and HMG-I(Y) binding sites] and PRRIII (–3780 to –3703, containing a consensus GAS motif, a non-consensus GAS motif and an Ets binding site). (**B**) Schematic representation of PRRIII highlighting the consensus GAS motif with the overlapping EBS (GAS-c/EBS), the non-consensus GAS motif with its overlapping GATA motif (GAS-n/GATA) and the separated EBS. (**C**) IL-2-induced activities in transfected YT cells of the full-length PRRIII, GAS-c (–3780 to –3756), GAS-n (–3757 to –3737) and EBS (–3734 to –3703) oligonucleotides cloned upstream of the TK promoter in pBLCAT2. The activity of pBLCAT2 without treatment was assigned a value of 1, and the activities of the other constructs are expressed as fold increase over this activity. The means ± SEM for three independent experiments are expressed relative to this activity.

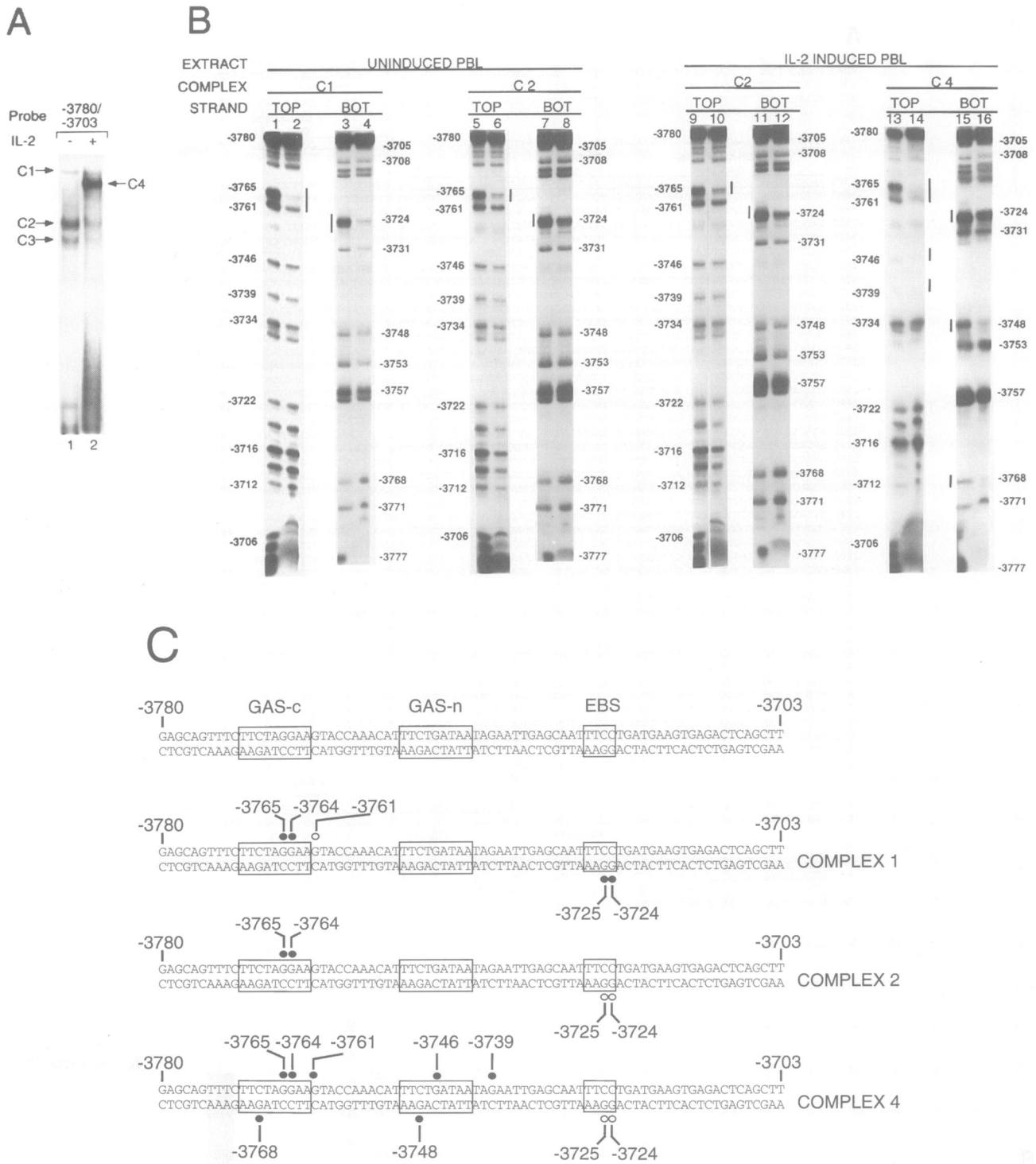

Fig. 2. Characterization of PRRIII binding proteins. (**A**) EMSAs using full-length PRRIII, –3780 to –3703, and nuclear extracts from uninduced (lane 1) or IL-2-induced (lane 2) preactivated PBL. Four major DNA binding complexes, C1–C4, that are reproducibly seen are indicated. (**B**) Methylation interference analysis of C1, C2 and C4. Shown are piperidine-mediated cleavage of free probe (lanes 1, 3, 5, 7, 9, 11, 13 and 15) or of PRRIII bound to protein (lanes 2, 4, 6, 8, 10, 12, 14 and 16). TOP and BOT refer to the sense (top) and antisense (bottom) strands, respectively. The apparent protections of the bands at the bottom of lanes 2, 6, 10 and 14 are artefactual since those nucleotides correspond to artificial overhangs rather than PRRIII sequences. (**C**) Summary of methylation interference analysis. Filled circles represent strong protection and open circles indicate weak protection.

investigated whether Elf-1 could also bind to the putative EBSs in PRRIII. *In vitro* translated Elf-1 protein (Figure 4B, lane 2), but not control lysate (lane 3), generated two complexes that co-migrated with the C2 and C3 complexes formed with nuclear extracts from PBL (lane 1). C3 is therefore likely to be a degradation product of Elf-1, the existence of which was previously suggested (John *et al.*, 1995). Elf-1 binds to both the –3734 to –3703 and –3780 to –3756 oligonucleotides (lanes 5 and 7), each of which contains an EBS, but not to an oligonucleotide containing

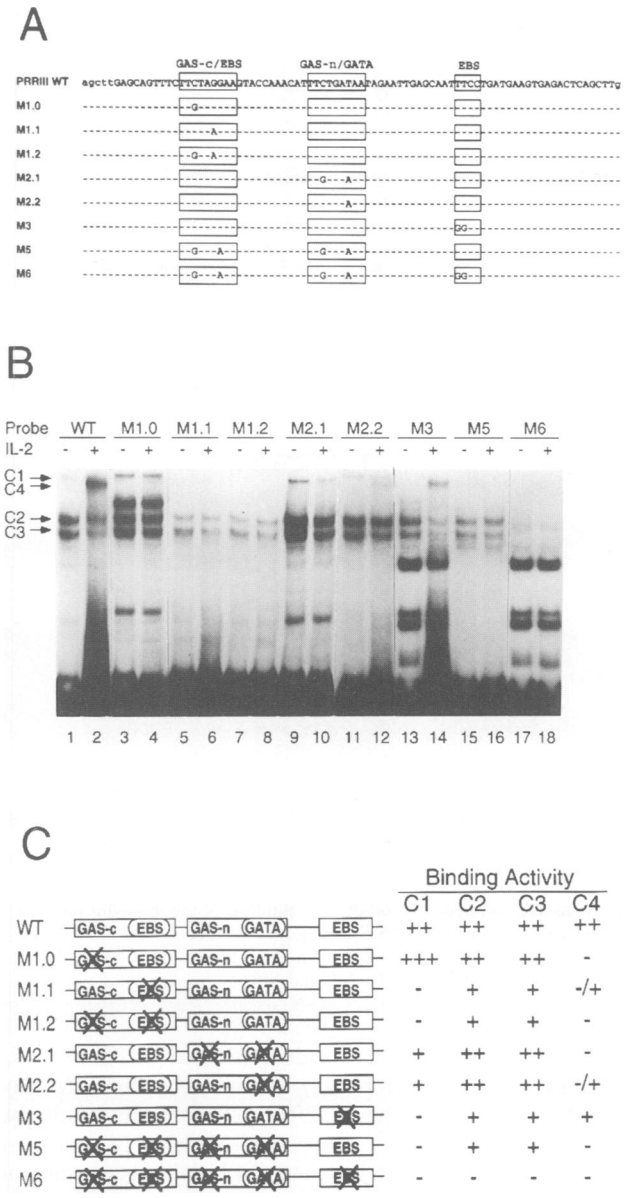

Fig. 3. EMSAs using wild type and mutant PRRIII oligonucleotides. (**A**) Sequence of the wild type (WT) PRRIII oligonucleotide. The positions of the GAS-c/EBS, GAS-n/GATA and EBS are indicated. For the mutant oligonucleotides, only the mutations are shown; hyphens indicate sequence identity with the wild type sequence. (**B**) EMSAs using wild type and mutant PRRIII oligonucleotides: wild type PRRIII (lanes 1 and 2); mutant M1.0, mutation of consensus GAS motif leaving intact the EBS (lanes 3 and 4); mutant M1.1, mutation of the overlapping EBS in the consensus GAS motif (lanes 5 and 6); mutant M1.2, mutation of the overlapping EBS and the consensus GAS motif (lanes 7 and 8); mutant M2.1, mutation of the non-consensus GAS and GATA motifs, (lanes 9 and 10); mutant M2.2, mutation of the GATA motif, which overlaps the non-consensus GAS motif (lanes 11 and 12); mutant M3, mutation of the downstream EBS between –3705 and –3702 (lanes 13 and 14); mutant M5, mutation of both GAS motifs (lanes 15 and 16); mutant M6, mutation of all protein binding sites (lanes 17 and 18). In some lanes, including 3, 4, 9, 10, 13, 14, 17 and 18, new binding activities are seen. Some of these binding activities are also seen as faint bands at the same positions with the wild type PRRIII probe. Although the origin and significance of the new bands are unclear, they may represent the enhanced abilities of other factors that can potentially bind to PRRIII when the binding of the major complexes, C1–C4 are inhibited as a result of the mutations. Alternatively, they could represent new binding sites created by the mutations. (**C**) Summary of binding activities.

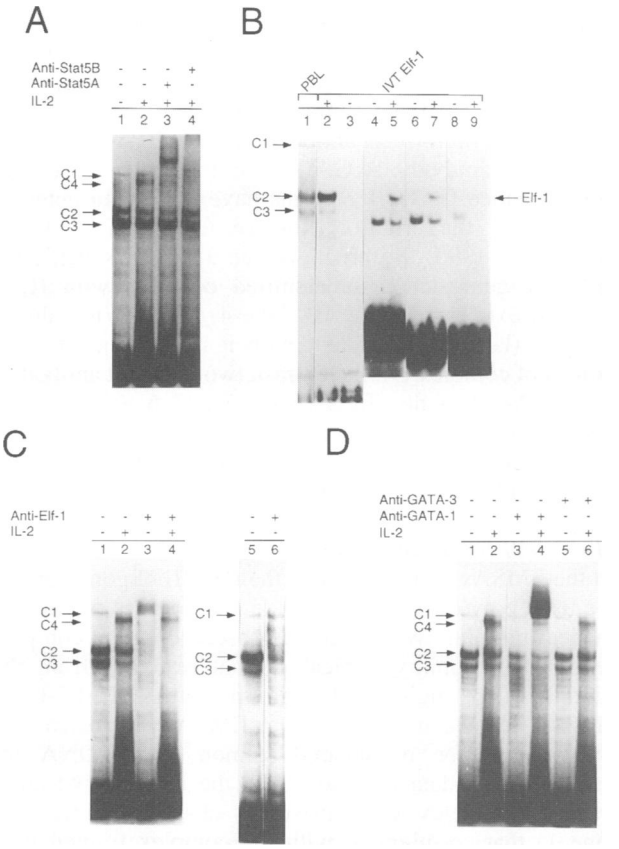

Fig. 4. Identification of proteins contained in complexes C1, C2 (C3) and C4. (**A**) C4 contains Stat5. Stat5A and Stat5B specific antisera supershift C4 but not C1, C2 or C3 (lanes 3 and 4 versus 2). (**B**) *In vitro* translated Elf-1 binds to PRRIII. Lane 1 shows binding of uninduced preactivated PBL nuclear extract to PRRIII. Binding of unprogrammed lysates (lanes 3, 4, 6 and 8) or Elf-1 programmed lysates (lane 2, 5, 7 and 9) to PRRIII (lanes 2 and 3), –3734 to –3703 (lanes 4 and 5), –3780 to –3756 (lanes 6 and 7) and –3757 to –3737 (lanes 8 and 9) oligonucleotides. (**C**) C2 and C4 contain Elf-1. Lanes 1 and 2 show binding of proteins in preactivated PBL nuclear extracts to PRRIII in the absence of any antibody added. An anti-Elf-1 antibody supershifts C2 and C3 (lanes 3 and 4) and decreases the intensity of C4 (lane 4). Complex C1 seen with uninduced preactivated PBL extracts (lane 5), is not affected by preincubation with an anti-Elf-1 antibody (lane 6). (**D**) GATA proteins are present in C4. EMSAs were performed with nuclear extracts from preactivated PBL in the absence (lanes 1 and 2) or presence of 1 μl of anti-GATA-1 (lanes 3 and 4) or GATA-3 (lanes 5 and 6) specific antibodies.

the non-consensus GAS motif (–3757 to –3737, lane 9). The presence of Elf-1 in C2 and C3 was confirmed by the ability of an antibody specific for Elf-1 to supershift C2 and C3 (Figure 4C, lane 3 versus 1). Since the supershifted complexes in lane 3 superimposed on complex C1, it was unclear if C1 was also affected by the Elf-1 antibody. However, when the same antibody was preincubated with uninduced nuclear extracts prior to addition of probe, it primarily diminished Elf-1 complexes (C2 and C3), showing that complex C1 was not affected and therefore does not appear to contain Elf-1 (lane 6 versus 5). The identity of the protein(s) contained in C1 are not yet known. The anti-Elf-1 antibody also modestly decreased the intensity of C4 (lane 4 versus 2), consistent with the possibility that C4 might contain Elf-1 as well as Stat5.

As indicated in Figure 1B, a putative GATA binding

motif overlaps the non-consensus GAS motif. GATA-3 has previously been shown to be a lineage restricted factor that regulates the expression of T cell receptor genes (Ho *et al.*, 1991). To determine if GATA-3 could bind to PRRIII, we performed antibody supershift analysis with a GATA-3 antibody. A GATA-1 antibody was used as a control, since GATA-1 proteins have only been detected in cells of the erythroid lineage (reviewed in Weiss and Orkin, 1995). Surprisingly, the antibody specific for GATA-1 generated a supershifted complex with IL-2-induced extracts (Figure 4D, lane 4), but not uninduced extracts (lane 3), consistent with it recognizing a component of complex C4. In contrast, two different antibodies to GATA-3 had no effect (lanes 5 and 6 and data not shown). These results suggest that upon IL-2 stimulation, GATA-1 or a closely related protein binds to PRRIII as part of a complex that also contains Stat5 and Elf-1 proteins.

HMG-I(Y) binds to PRRIII

In the EMSAs performed with the PRRIII oligonucleotide we used poly(dI-dC) to eliminate non-specific binding of nuclear proteins. We sometimes observed a faint complex with rapid mobility, typical of that resulting from the binding of the high mobility group proteins, HMG-I and HMG-Y (John *et al.*, 1995). When EMSAs were performed in the presence of sheared salmon sperm DNA [or poly(dG–dC), data not shown] as the non-specific competitor, a complex was consistently observed (Figure 5B, lane 1) that co-migrated with the complex formed with purified recombinant HMG-I (lane 2). EMSAs performed with a series of mutant PRRIII oligonucleotides (see Figure 5A for sequences) revealed that this binding activity was decreased with oligonucleotides which contain mutations in the Elf-1 binding site located between –3725 and –3724 (M3 and M6, Figure 5B, lanes 9 and 12 versus 3). However, the residual HMG-I binding seen with mutant M3 was abrogated when the A-T rich sequence within the GATA motif was simultaneously mutated (mutant M8, lane 14), while mutation of the GATA motif alone had a minimal effect (mutant M7, lane 13). Thus, HMG-I(Y) binds to two different sequences in PRRIII, overlapping the Elf-1 binding site and the GATA motif. Furthermore, it appears that the major HMG-I binding site overlaps the Elf-1 motif since mutation of this site caused the greatest decrease in HMG-I binding activity.

The coordinated activity of each of the binding sites in PRRIII is required for the IL-2 inducibility of IL-2Rα promoter

The above studies indicate that the human IL-2Rα IL-2 response element is a complex structure containing two GAS motifs, a GATA motif, HMG-I(Y) binding sites and two EBSs. To clarify the functional significance and relative contributions of each of these sites, we performed site directed mutagenesis analysis of the –4103 to +109 IL-2Rα–CAT construct (mutations are shown in Figure 3A) and assayed for IL-2 responsiveness of mutant constructs in YT cells (Figure 6). The wild type construct consistently showed a 3- to 4-fold IL-2 inducibility in YT cells (Figure 6, WT). Mutation of the overlapping EBS in the consensus GAS motif (mutant M1.1), which abrogates C1 binding activity but importantly still retains weak C4 binding activity, significantly increased basal activity,

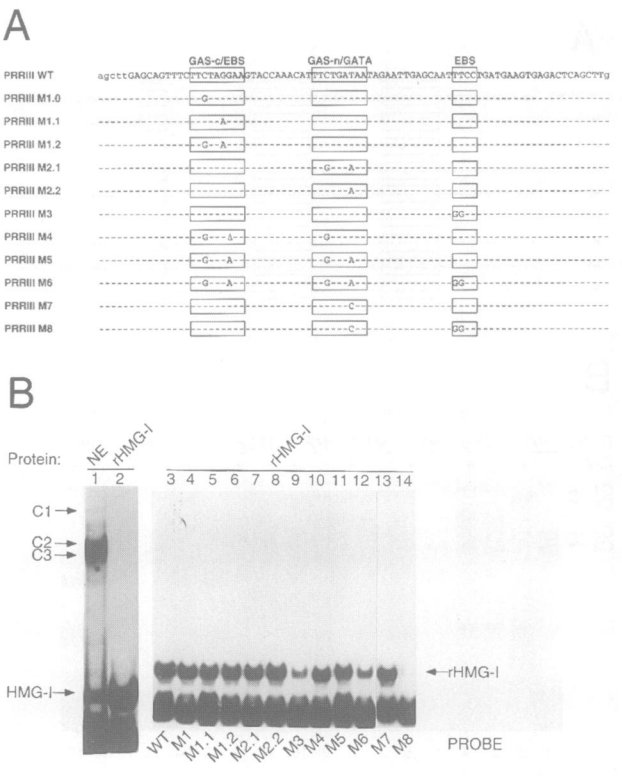

Fig. 5. HMG-I(Y) binds to PRRIII. (**A**) Nucleotide sequences of PRRIII oligonucleotides used for EMSAs in (B). (**B**) EMSAs showing the binding of nuclear extracts from preactivated PBL not stimulated with IL-2 (lane 1) or recombinant purified HMG-I (lane 2) to the wild type PRRIII oligonucleotide in the presence of sheared salmon sperm DNA as the non-specific competitor. Binding of recombinant purified HMG-I to WT or mutant PRRIII oligonucleotides; lane 3 shows binding to WT, lanes 4–14 are binding to mutants M1.0, M1.1, M1.2, M2, M2.1, M3, M4, M5, M6, M7 and M8.

thus decreasing the IL-2 inducibility of this construct. This suggested that protein(s) which bind to this site may negatively regulate the IL-2Rα promoter. Interestingly, mutation of this same consensus GAS motif in a fashion that affected both the STAT and Ets binding sites (mutant M1.2), which shows no detectable C1 or C4 binding activity, restored basal activity to that of the wild type construct; in addition, it exhibited a modest but reproducible decrease in IL-2-induced promoter activity relative to that of the WT construct. Thus, the difference in the levels of IL-2-induced transcriptional activity observed with mutants M1.1 and M1.2 might result from the ability of M1.1, but not M1.2, to form the inducible complex C4.

Remarkably, mutant M2.1, which affected only the non-consensus GAS motif and overlapping GATA motif, displayed a profound reduction in both basal and IL-2 inducible activity, suggesting that these motifs are essential to the activation of the IL-2Rα promoter. In contrast, in mutant M2.2, which affects the GATA motif, although there was a decrease in both basal and IL-2-induced levels of activity; some IL-2-induced activity was still seen, indicating the critical role of the non-consensus GAS motif. Interestingly, mutation of the downstream EBS (mutant M3), which affects Elf-1 and HMG-I(Y) binding, showed a greater decrease in IL-2 inducibility than mutants M1.1, M1.2 and M2.2, all of which displayed greatly diminished or a total loss of STAT protein binding activity.

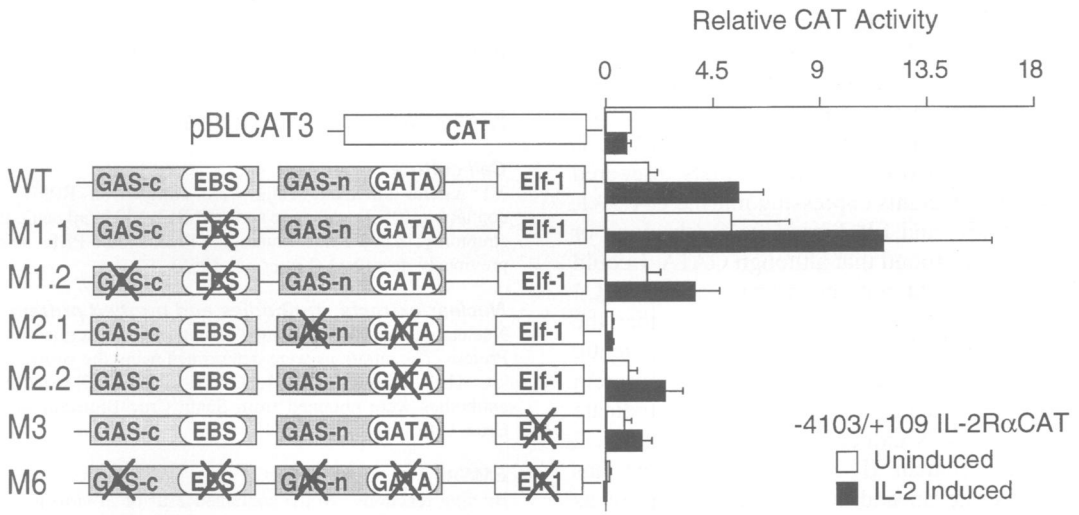

Fig. 6. All components of PRRIII are required for maximal IL-2 responsiveness. Mutations in PRRIII were incorporated into –4103 to +109 IL-2Rα–CAT reporter constructs and transfected into YT cells, followed by no stimulation or stimulation with IL-2. Mutations are designated as in Figure 3A. The activity of pBLCAT3 in cells not stimulated with IL-2 was assigned a value of 1, and the activities of the other constructs are expressed as fold increase over this activity. The means ± SEM for seven independent experiments are shown.

This result suggests that binding of Elf-1 and HMG-I(Y) are essential for IL-2-induced activation of PRRIII. Finally, simultaneous mutation of all the components of PRRIII (mutant M6), abrogated IL-2Rα promoter activity. These results indicate that functional cooperativity between Stat5, GATA proteins, Elf-1 and HMG-I are required for maximal IL-2-induced IL-2Rα promoter activity.

Discussion

IL-2Rα transcription is tightly regulated in a lineage- and activation-dependent manner. IL-2 itself is an activator of IL-2Rα gene expression, thereby modulating the number of high affinity IL-2 receptors on the surface of activated T cells. IL-2Rα is therefore perhaps one of the most important genes activated by IL-2 making it essential to understand the molecular basis underlying IL-2-induced IL-2Rα regulation. Since IL-2 activates the STAT proteins Stat5 and Stat3 (Nielsen *et al.*, 1994; Fujii *et al.*, 1995; Gaffen *et al.*, 1995; Gilmour *et al.*, 1995; Hou *et al.*, 1995; Lin *et al.*, 1995; Wakao *et al.*, 1995) it was likely that STAT proteins would play a role in regulating IL-2-induced IL-2Rα gene expression. In this study, we report a detailed analysis of the human IL-2 response element, PRRIII, which is essential for IL-2-induced IL-2Rα promoter activity. Interestingly, PRRIII is a complex composite unit that is composed of binding sites not only for the IL-2-inducible STAT proteins, Stat5A and Stat5B, but also for the lymphoid/myeloid-specific Ets family protein, Elf-1, the high mobility group protein, HMG-I(Y) and a GATA-1-like protein.

The combinatorial nature of PRRIII is reflected in the finding that mutations in the binding sites for any one of these proteins decreased the IL-2 inducibility of reporter constructs in transfected cells. This feature of PRRIII is reminiscent of the positive regulatory domains of the β-IFN promoter where multiple factors act in concert to regulate the viral inducibility of that promoter (Thanos and Maniatis, 1992, 1995; Du *et al.*, 1993). We therefore hypothesize that extensive protein–protein interactions exist between the various factors that bind to PRRIII and that IL-2 stimulation of the IL-2Rα promoter induces the formation of a highly ordered 'enhanceosome' structure (Thanos and Maniatis, 1995). Based on its ability to interact with TFIIB (our unpublished observations), Elf-1 may be required to relay activation-induced signals to the basal transcription machinery, whereas Stat5 binding to PRRIII may represent the nuclear sequela of IL-2-mediated activation of the Jak–STAT pathway. In our transfection studies performed with mutant IL-2Rα constructs, we found that mutation of the downstream Elf-1 binding site (mutant M3) resulted in a decrease in IL-2 inducibility even though Stat5 binding activity was clearly detectable with this mutant. This suggests that both Elf-1 and Stat5 binding to PRRIII are important for IL-2-mediated IL-2Rα induction.

Several elegant studies have now shown that HMG-I(Y) proteins can promote selective binding of transcription factors and induce bends in DNA thereby facilitating enhanceosome formation and permitting cross-talk between spatially separated transcription factors (Thanos and Maniatis, 1992, 1995; Du *et al.*, 1993; Falvo *et al.*, 1995). We have demonstrated previously that Elf-1 and HMG-I can synergistically activate PRRII (John *et al.*, 1995). The Elf-1 site in PRRIII is also juxtaposed to HMG-I binding sites, suggesting that these proteins may also functionally interact to regulate the activity of PRRIII. In addition, we also detected a second lower affinity binding site for HMG-I, which overlaps the GATA motif, a situation that is analogous to that found in the γ-globin promoter, where HMG-I binding to GATA motifs may function to form transcriptionally active multiprotein complexes (Magis and Martin, 1995). Moreover, similar to its role in regulating PRRII activity, HMG-I(Y) may also function to modulate the architecture of PRRIII, thereby facilitating functional interactions between key regulatory proteins such as Elf-1, Stat5 and GATA proteins in the generation of IL-2-induced enhanceosome structures.

The apparent presence of GATA-1 in the IL-2-inducible complex (C4) was surprising since GATA-1 has only been

shown to be expressed in cells of the erythroid lineage. We have not detected GATA-1 transcripts or protein in preactivated PBL extracts (our unpublished observations) and therefore hypothesize that complex C4 may contain a novel protein that shares an epitope with GATA-1. Indeed, in EMSAs performed with extracts prepared from COS-7 cell transfectants expressing murine GATA-1, human Stat5A, Stat5B and Elf-1 proteins (each alone or all in combination), we found that although GATA-1 could bind to PRRIII we could not reconstitute complex C4 (unpublished observations) reinforcing the idea that the C4 may contain a GATA-1-related rather than authentic GATA-1 protein. Thus, the exact identity of the protein in this complex remains unclear; however, our data indicate that its binding activity is induced by IL-2 stimulation.

Interestingly, the human IL-2 response element has evolved to incorporate an additional level of control as compared to its murine counterpart in that the consensus GAS motif contains an overlapping EBS. Mutation of this site (mutant M1.1) affected the formation of all four complexes C1–C4; interestingly however, only complex C1 was abrogated by this mutation although all four complexes had revealed protection of this site in methylation interefence analyses. Since mutation of this EBS alone results in a dramatic increase in the basal levels of transcriptional activity of the IL-2Rα promoter, it is possible that the proteins that comprise complex C1 can function to negatively regulate IL-2Rα promoter activity. Surprisingly however, a related mutant, M1.2, which contains a simultaneous mutation of this EBS and the consensus GAS motif did not show elevated basal levels of promoter activity. While the reason for this difference in functional activities between M1.1 and M1.2 is unclear, it is possible that although mutants M1.1 and M1.2 only show an apparent difference in the ability to form complex C4, the *in vivo* occupancy of these two mutant IL-2Rα promoter constructs may be different from that detected by *in vitro* gel mobility shift assays. Thus the precise molecular basis for the differences in the activities of these mutants awaits future studies aimed at the identification and characterization of the proteins contained within complex C1 and a more detailed study of the role played by this EBS in the negative regulation of the IL-2Rα promoter.

Thus, the IL-2Rα promoter contains multiple regulatory regions which together orchestrate a coordinated response to mitogenic or growth factor stimulation. In this study, we have characterized in detail the factors binding to PRRIII and shown that they can both positively and negatively regulate IL-2-induced IL-2Rα transcriptional activity. We demonstrated previously protein–protein interactions between proteins binding to PRRI and PRRII and suggested that IL-2Rα transcription might depend on the formation of activation-induced nucleoprotein complexes (John *et al.*, 1995). In the current study, we characterize PRRIII as an additional important regulatory element. The fact that PRRIII is located at a large distance from the transcription start site further suggests that DNA bending or looping may be required for the transcriptional activation of the IL-2Rα promoter. It will be important to elucidate how the signal from PRRIII is integrated with signals from other regions of the promoter and whether PRRIII is important in regulating IL-2Rα promoter activity in response to other signals in addition to IL-2.

Materials and methods

Cell culture

YT natural killer-like cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Preactivated PBLs were prepared as previously described (Lin *et al.*, 1995).

Nuclear extracts, antibodies and purified protein

Nuclear extracts were prepared essentially as described (Dignam, 1983). Protein concentrations were determined using the Bio-Rad protein assay kit. The anti-human Elf-1, GATA-1, GATA-3, Stat5A and Stat5B antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

EMSAs

Binding reactions (20 μl) contained 2 μl of *in vitro* translated proteins or 5–10 μg of nuclear extracts from PBL, 10 000 c.p.m. of probe (0.1–0.2 ng), 2 μg of poly(dI-dC), in 10 mM Tris–HCl (pH 7.5), 10 mM HEPES, 50 mM KCl, 1.25 mM DTT, 1.1 mM EDTA and 15% glycerol. Following incubation on ice for 30 min, DNA–protein complexes were analyzed on 5% polyacrylamide gels (40:1 acrylamide:bisacrylamide) run in Tris–borate buffer at 150 V for 2.5 h at room temperature. In antibody supershift assays, 1 μl of specific antiserum was preincubated with proteins for 30 min on ice. Probe was then added and incubations continued for 15 min before analysis on gels.

Plasmids and oligonucleotides

The IL-2Rα promoter sequence was determined by double-stranded DNA sequence analysis using Sequenase and by fluorescent double-stranded DNA sequence analysis using an Applied Biosystems Model 377 fluorescent sequencer with a modified ABI protocol (Robbins *et al.*, 1996). All oligonucleotides were synthesized with *Hin*dIII (5′) and *Bam*HI (3′) ends. The double-stranded PRRIII oligonucleotide (–3780 to –3703, see Figure 1A and B) was subcloned into pBLCAT2 (Luckow and Schutz, 1987) and the DNA sequence verified. The *Bgl*II–*Pst*I IL-2Rα –4103 to +109 fragment was cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in pUC13. The control plasmid, pBLCAT3 was a promoterless plasmid that contained the CAT gene (Luckow and Schutz, 1987). The IL-2Rα –4103 to +109 promoter mutants were constructed using the MORPH site specific DNA mutagenesis kit (5 Prime-3 Prime, Inc.) The DNA sequence of each mutant was confirmed. The Elf-1 expression vector, pcDNA3-Elf-1, was constructed by cloning the *Eco*RV–*Xho*I Elf-1 cDNA fragment from pcDElf-1 (Thompson *et al.*, 1992) into pcDNA3 (Invitrogen).

Transfection and CAT assays

YT cells were transfected using DEAE–dextran (Sompayrac and Danna, 1981), 12 μg of plasmid DNA from IL-2Rα promoter constructs and 5 μg of the PRRIII–TKCAT reporter construct. After transfection, cells were maintained in RPMI medium with 10% FBS for 24–30 h, stimulated overnight with 2 nM IL-2, and cell extracts prepared by three cycles of freeze–thawing in 0.25 M Tris–HCl, pH 7.5. Extracts were clarified at 65°C for 10 min followed by centrifugation. Protein concentrations of the supernatants were determined, and equivalent amounts of protein were used for CAT assays. Acetylated forms of chloramphenicol were separated by thin layer chromatography and quantitated using a Molecular Dynamics PhosphorImager.

Methylation interference assays

PRRIII–TKCAT was linearized at either end with *Hin*dIII or *Bam*HI and end-labeled with [α-^{32}P]dATP and [α-^{32}P]dGTP (both >3000 Ci/mmol; Amersham) by a fill-in reaction with Klenow enzyme (New England Biolabs). The probe was released by a second digestion with *Bam*HI or *Hin*dIII, as appropriate, and purified on non-denaturing polyacrylamide gels. Typically, 0.5×10^6 c.p.m. of end-labeled probe and 25 μg of nuclear extracts were used in each binding reaction. Methylation interference assays were performed as described (Baldwin, 1987).

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