

# The Basis for IL-2-Induced IL-2 Receptor $\alpha$ Chain Gene Regulation: Importance of Two Widely Separated IL-2 Response Elements

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## Summary

The interleukin-2 receptor  $\alpha$  (IL-2R $\alpha$ ) chain is an essential component of high-affinity IL-2 receptors. Accordingly, IL-2R $\alpha$  expression helps to regulate T cell growth and other lymphoid functions. Lineage-restricted and activation-dependent IL-2R $\alpha$  transcription is controlled by three upstream positive regulatory regions (PRRs). We now describe an additional IL-2 response element, PRRIV, within intron 1, in humans and mice. PRRIV activity requires GAS motifs that bind Stat5 proteins and additional upstream HMG-I(Y) binding sites. Moreover, IL-2 induces the binding of HMG-I(Y), Stat5a, and Stat5b in vivo to PRRIV and PRRIII, which also functions as an IL-2 response element. Thus, the IL-2 inducibility of the IL-2R $\alpha$  gene is unexpectedly mediated by two widely separated regulatory Stat5-dependent elements, located both upstream and downstream of the transcription initiation sites.

## Introduction

Interleukin-2 (IL-2) is the principal growth factor for T lymphocytes and regulates the magnitude and duration of the T cell immune response following antigen encounter (Lin and Leonard, 1997). Three classes of IL-2 receptors exist, binding IL-2 with low ( $K_d = 10^{-8}$  M), intermediate ( $K_d = 10^{-9}$  M), and high ( $K_d = 10^{-11}$  M) affinity. The low-affinity receptors contain only the IL-2 receptor  $\alpha$  chain (IL-2R $\alpha$ ), intermediate-affinity receptors contain IL-2R $\beta$  and the common cytokine receptor  $\gamma$  chain  $\gamma_c$ , and high-affinity receptors contain all three chains (Lin and Leonard, 1997). The intermediate- and high-affinity receptors are the functional receptor forms, and heterodimerization of the IL-2R $\beta$  and  $\gamma_c$  cytoplasmic domains is necessary and sufficient for IL-2 signaling (Nakamura et al., 1994; Nelson et al., 1994). In contrast to the larger IL-2R $\beta$  and  $\gamma_c$  cytoplasmic domains, the highly inducible  $\alpha$  chain has a very short cytoplasmic domain and presumably mainly functions to increase the affinity for IL-2, allowing cellular responsiveness to the low levels of IL-2 that are physiologically produced in vivo. IL-2R $\alpha$  is essential, as evidenced by the autoimmunity in patients (Sharfe et al., 1997) and mice (Willerford et al., 1995) lacking this protein.

IL-2R $\alpha$  expression is undetectable on resting T cells. Its expression is triggered by antigen, mitogen lectins, or antibodies to the T cell receptor (TCR). These signals also result in secretion of IL-2, which in turn can increase and prolong IL-2R $\alpha$  expression, thus acting as a positive

feedback regulator of its own high-affinity receptor (Waldmann, 1989).

IL-2R $\alpha$  is mainly regulated at the level of transcription. The IL-2R $\alpha$  promoter contains three positive regulatory regions (PRRs): PRRI (between nucleotides  $-276$  and  $-244$  relative to the major transcription initiation sites) (Bohnlein et al., 1988; Cross et al., 1987, 1989; Leung and Nabel, 1988; Lin et al., 1990; Ruben et al., 1988; Toledano et al., 1990), PRRII ( $-137$  to  $-64$ ) (John et al., 1995), and PRRIII ( $-3780$  to  $-3703$ ) (John et al., 1996; Lecine et al., 1996; Sperisen et al., 1995). Both PRRI and PRRII are required for mitogenic stimulation of the IL-2R $\alpha$  gene, while PRRIII is an IL-2 response element. PRRI binds to NF- $\kappa$ B1, c-Rel, and serum responsive factor (SRF) (Ballard et al., 1988; Toledano et al., 1990), 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). Intermolecular interactions between proteins that bind to PRRI and PRRII appear to result in a highly ordered stereospecific complex that regulates IL-2R $\alpha$  promoter activity upon mitogenic stimulation (John et al., 1995). PRRIII can bind to Stat5 proteins, Elf-1, HMG-I(Y), and a GATA-1-like protein (John et al., 1996).

We now describe the identification of a fourth positive regulatory region (PRRIV) in the human IL-2R $\alpha$  gene, which was found by examining intron 1 sequences for DNase I hypersensitive sites (HSs). One strong site, HS4, is induced in peripheral blood lymphocytes (PBL) following treatment with IL-2. The region spanning the site contains binding sites for Stat5a, Stat5b, and HMG-I(Y) proteins that are important for PRRIV function in response to IL-2. Thus, in addition to the previously described 5' upstream IL-2-response element (PRRIII), we now unexpectedly find that there is a second IL-2 response element (PRRIV) in the first intron, indicating more of the complexity of the molecular regulation of the IL-2R $\alpha$  gene in response to IL-2. The implications of this finding are discussed.

## Results

### Identification of Four DNase I Hypersensitive Sites in the Human IL-2R $\alpha$ Gene

To further evaluate in vivo the significance of the positive regulatory regions previously identified in the human IL-2R $\alpha$  promoter region (PRRI, PRRII, and PRRIII) and to potentially identify novel regulatory elements, sequences flanking exon 1 were examined for DNase I HSs. We used a 14.1 kb segment, extending from an EcoRI site located 8.9 kb 5' and a HindIII site in the first intron located 5.2 kb 3' of the major transcription start sites (Figure 1A). PBL were preactivated, rested, and then either not stimulated or stimulated with IL-2 for 1 hr. Nuclei were then isolated and subjected to DNase I hypersensitivity assays. A total of four HSs were located (Figure 1A). Hybridization of Southern blots of EcoRI-digested genomic DNA with the probe allowed detection of HS1 (Figure 1B), whereas blots of HindIII-digested

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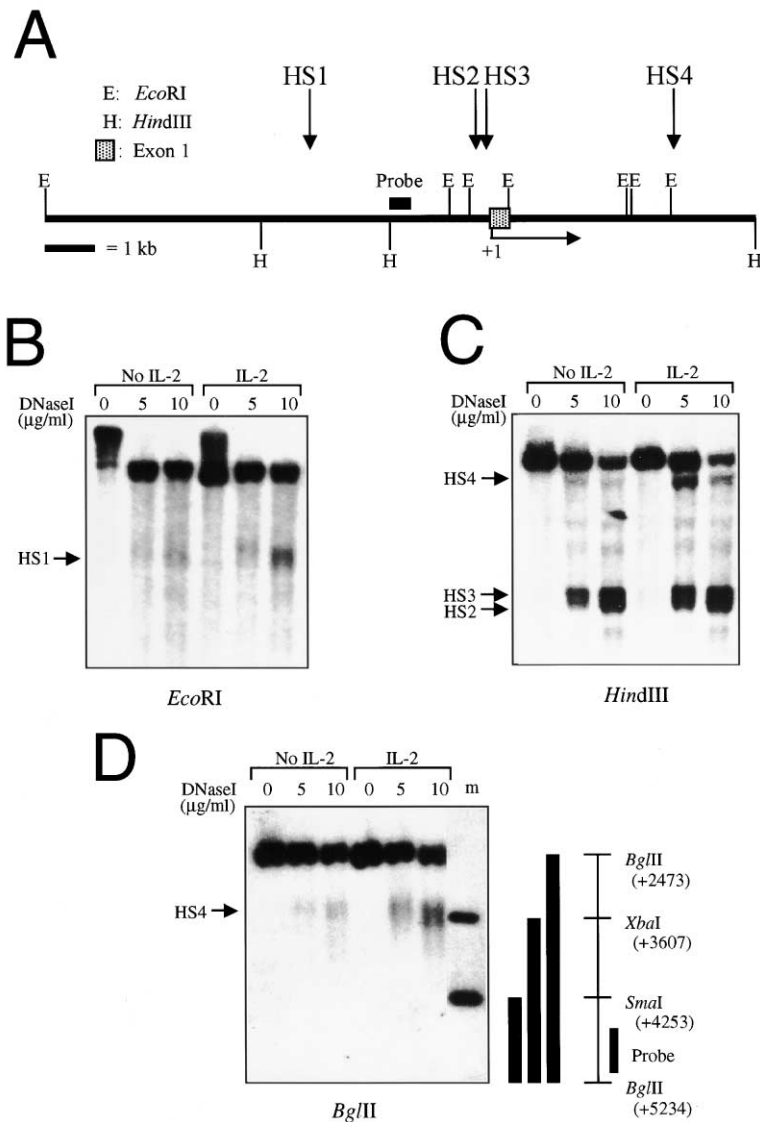


Figure 1. Characterization of DNase I HSs of the Human IL-2R $\alpha$  Gene

(A) A schematic showing locations of DNase I HSs in the human IL-2R $\alpha$  gene. The thick bar represents the probe. The transcriptional start site (+1) is indicated by the horizontal arrow.

(B and C) Nuclei were isolated from preactivated PBL, cultured for 1 hr in the presence and absence of IL-2, and digested with DNase I as indicated. DNA was extracted, digested with the indicated restriction enzymes, and analyzed on 0.6% agarose gels. HS1 was mapped by hybridizing EcoRI-digested DNA with a 301 bp (–2106 to –1806) probe (B); HS2, HS3, and HS4 were identified by hybridizing HindIII-digested DNA with the same probe (C).

(D) Nuclei were isolated from YT cells, cultured for 1 hr in the presence or absence of IL-2, and digested with DNase I as indicated. DNA was extracted and then digested with BglIII. HS4 was identified by probing with a 242 bp fragment (+4800 to +5040). Size markers are shown on the right.

DNA with the same probe revealed HS2, HS3, and HS4 (Figure 1C). HS2 and HS3 were detected with similar efficiency in preactivated PBL whether or not the cells were stimulated with IL-2, but HS1 and HS4 were stronger in cells treated with IL-2. HS1 was located ~3.5 kb upstream of the transcription initiation sites and thus probably corresponds to PRRIII. The higher DNase I sensitivity of HS1 in IL-2-treated PBL (Figure 1B) indicates that IL-2 induced the opening of this region of chromatin and supports previous data that PRRIII is an IL-2 response element (John et al., 1996; Lecine et al., 1996; Sperisen et al., 1995). HS2 and HS3 were located near to the transcription initiation sites and thus might correspond to the promoter region and PRRI and PRRII. This region of chromatin containing the core promoter/PRRI/PRRII region is constitutively open, probably resulting from preactivation with phytohemagglutinin (PHA) (Figure 1C). Thus, HS1, HS2, and HS3 likely reflect *cis*-acting elements in PRRIII, PRRI, and PRRII, respectively, indicating the importance of chromatin structure for positive regulation of the IL-2R $\alpha$  gene. HS4 was located in the

first intron ~3.5 kb 3' to the transcription start sites (Figure 1C), and we investigated this site as a putative novel IL-2 response element.

#### The Four DNase I Hypersensitive Sites Correlate with IL-2R $\alpha$ Gene Expression

To further assess the relationship between the four hypersensitive sites and human IL-2R $\alpha$  gene transcription, assays were performed using HeLa cells, which do not express this gene, and MT-2 cells, which constitutively express human IL-2R $\alpha$ . HS1, HS2, HS3, and HS4 were barely detectable in HeLa cells but were readily detected in MT-2 cells (data not shown), correlating the DNase hypersensitivity of these sites with human IL-2R $\alpha$  expression. Given the strong inducibility of HS4, we further characterized this site. A 241 bp fragment encompassing the +4800 to +5040 region was used to probe BglIII-digested DNA, and BglIII/SmaI and BglIII/XbaI fragments were used as size markers. HS4 was mapped close to the XbaI site, which in turn is 3607 bp down-

stream of the major transcription initiation site (+1), thus refining the mapping of the HS4 location (Figure 1D).

To further characterize HS4, BAC clones spanning the human and mouse IL-2R $\alpha$  gene were identified. The 7 kb BamHI fragment spanning human HS4 and the 7 kb NcoI fragment spanning the first intron of the murine IL-2R $\alpha$  gene were subcloned and sequenced on both strands (GenBank Accession Number AF243502 and Number AF243503). A comparison of the human and murine intron 1 sequences revealed that the HS4 region was the most conserved (Figure 2A). Analysis of this region (Figure 2B) revealed several potential consensus sequences for factor binding, including two consensus  $\gamma$ -interferon activated sequence (GAS) motifs and one nonconsensus GAS motif.

#### IL-2-Responsive Activity of the HS4 Region

The function of the IL-2-responsive HS4 sequences was evaluated by transient transfection analyses using a luciferase reporter construct. The 651 bp (+3214 to +3864) region spanning human HS4 was generated by PCR and cloned in both orientations 5' as well as 3' of the human IL-2R $\alpha$  promoter. The resulting constructs were transfected into YT cells and analyzed for transcriptional activity in the absence or presence of IL-2. All constructs conferred IL-2 inducibility to the human IL-2R $\alpha$  promoter, suggesting that HS4 sequences contained an IL-2 response element (Figure 3A). Similar results were observed when the +3214 to +3864 fragment was cloned upstream and downstream of the SV40 promoter instead of the IL-2R $\alpha$  promoter (Figure 3B). The corresponding murine sequences (+2350 to +2955) also conferred IL-2 inducibility to the murine IL-2R $\alpha$  and SV40 promoters in a position- and orientation-independent fashion (Figures 3C and 3D).

#### The GAS Motifs in HS4 Are Important for IL-2-Induced PRRIV Activity

To characterize the IL-2 response element in HS4, a number of 5' and 3' deletion fragments were generated and inserted 5' to the SV40 promoter. The resulting constructs were transfected into YT cells (Figure 4A). The +3204/+3596 construct, which contains both of the consensus GAS motifs as well as the nonconsensus GAS motif, mediated  $\sim$ 5.3-fold IL-2 inducibility. However, when the 3' end was deleted to +3546 so that GAS<sub>CII</sub> is selectively eliminated (Figure 2B), IL-2 inducibility decreased to 2.5-fold (Figure 4A). Further deletion to +3516, which eliminated all GAS motifs, abrogated IL-2 responsiveness, indicating their importance for IL-2 inducibility.

Deletion of 5' sequences between +3204 and +3389 had only a modest decrease in IL-2 inducibility ( $\sim$ 4-fold inducible), but deletion to +3450 and +3509 decreased the inducibility down to 2.4-fold and 1.4-fold, respectively. Interestingly, the +3509/+3596 fragment conferred very little inducibility by itself, even though it contains all three GAS motifs, indicating that the GAS motifs were not sufficient by themselves and that the upstream region was also important. Thus, we defined the +3389 to +3596 region as a novel fourth IL-2R $\alpha$  positive regulatory region, PRRIV.

To clarify the functional significance and the relative

contribution of each GAS motif, we performed site-directed mutagenesis (Figure 4B) and assayed the IL-2 responsiveness of each mutant construct in YT cells (Figure 4C). While the wild-type construct showed 4.6-fold IL-2 inducibility in YT cells, selective mutation of the GAS<sub>C</sub> (M1), GAS<sub>N</sub> (M2), and GAS<sub>CII</sub> (M3) motifs modestly lowered IL-2 inducibility. Double mutation of GAS<sub>C</sub> and GAS<sub>N</sub> (M4) or of GAS<sub>N</sub> and GAS<sub>CII</sub> (M5) more potently decreased IL-2 inducibility, and simultaneous mutation of GAS<sub>C</sub> and GAS<sub>CII</sub> (M6) or of all the GAS motifs (M7) abrogated IL-2 inducibility. These results suggest that all of the GAS motifs are required for maximal IL-2 inducibility. As shown in Figure 2B, PRRIV also contains AP-1 sites; however, mutagenesis of these sites did not diminish IL-2-inducibility (data not shown).

#### Stat5a and Stat5b Bind to PRRIV

As the deletion and mutagenesis analyses indicated the importance of the GAS motifs in PRRIV, we investigated factor binding to the GAS motifs using electrophoretic mobility shift assays (EMSAs). For these experiments, nuclear extracts were prepared from unstimulated or IL-2 stimulated YT cells and assayed for factor binding using probes containing the GAS<sub>C</sub> or GAS<sub>CII</sub> motifs (Figure 5A). When the GAS<sub>C</sub> probe was assayed, one slowly migrating complex was generated with extracts from IL-2 stimulated cells but not from unstimulated cells (Figure 5B, lanes 1 and 2). When the GAS<sub>CII</sub> probe was assayed, two complexes were generated with extracts from IL-2-stimulated cells but not from unstimulated cells (Figure 5B, lanes 7 and 8). All complexes were competed by a 125-fold excess of either unlabeled wild-type GAS<sub>C</sub> or GAS<sub>CII</sub> competitor (Figure 5B, lanes 3, 5, 9, and 11). However, mutant GAS oligonucleotides (TTCN<sub>3</sub>GAA mutated to TTGN<sub>3</sub>NAAA; see M1 and M3 in Figure 4B) could not compete complex formation, indicating that these complexes were specific (Figure 5B, lanes 4, 6, 10, and 12). We confirmed the binding of Stat5a and Stat5b proteins by supershift assays (Figure 5C). Although a monoclonal antibody against Stat3 did not supershift any of the complexes, it partially diminished complex C3 (Figure 5C, lane 8 versus 7), suggesting that Stat3 might also bind to PRRIV.

To define the nucleotides involved in Stat5 binding, we used uracil interference assays, which identify T nucleotides involved in protein-DNA interactions. Using a DNA fragment spanning from nucleotide +3474 to +3622 of the PRRIV, uracil interference assays identified three GAS motifs, two of which were canonical TTCN<sub>3</sub>GAA motifs (Figures 5D and 5E). In these GAS motifs, thymines at positions 1 and 9 in both strands (+3519 and +3527 of GAS<sub>C</sub> and +3576 and +3584 of GAS<sub>CII</sub>) showed a strong affinity for Stat5a, whereas the thymines at positions 2 and 8 (+3520 and +3526 of GAS<sub>C</sub>m and +3577 and +3583 of GAS<sub>CII</sub>) showed weaker interaction with Stat5a (Figures 5D and 5E). Thymines at positions at 1 and 8 of nonconsensus GAS motif also showed weaker interaction with Stat5a. Thus, Stat5a could bind to nonconsensus GAS motif as well as two consensus GAS motifs in PRRIV, although GAS<sub>N</sub> has lower affinity for Stat5a than GAS<sub>C</sub> and GAS<sub>CII</sub>.

To determine whether Stat5 proteins could transactivate PRRIV in vivo, dominant-negative Stat5aY694F or



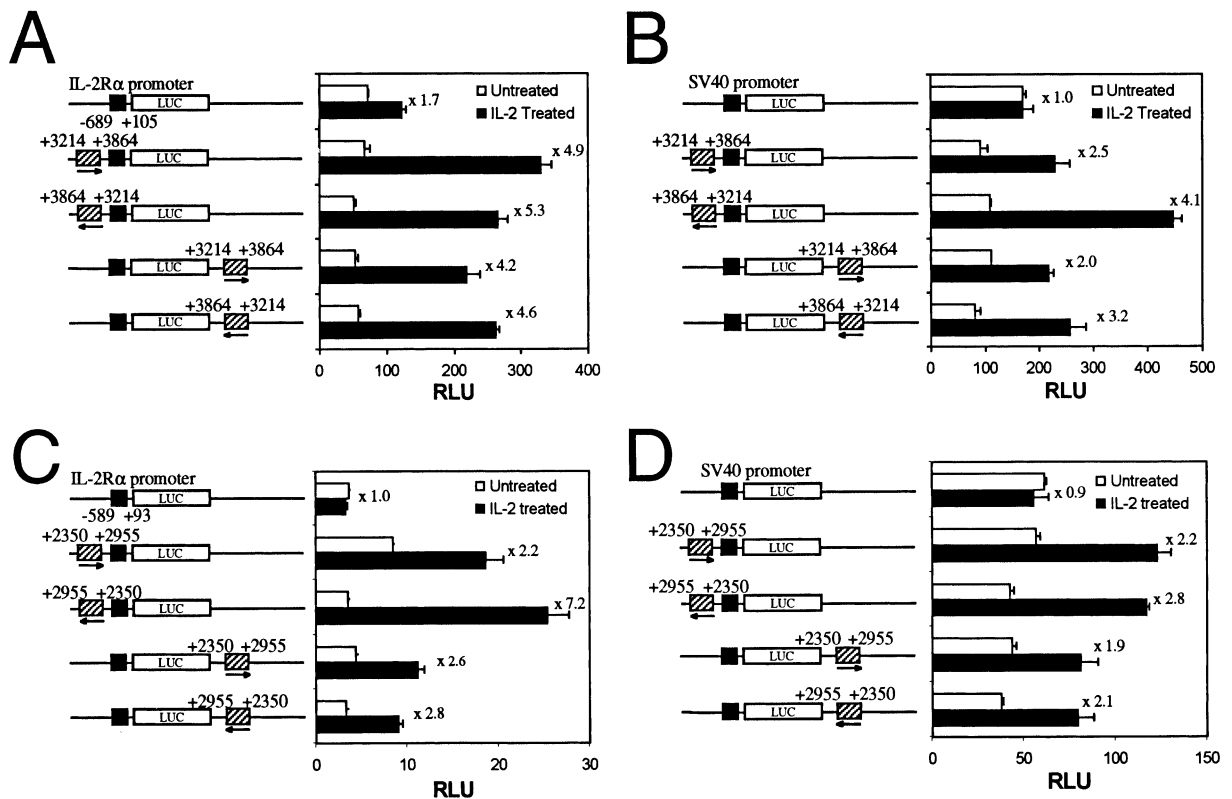


Figure 3. IL-2 Responsiveness of the HS4 Region

(A) Fragments spanning HS4 (+3214 to +3864) were generated by PCR and cloned in both orientations 5' and 3' to the human IL-2R $\alpha$  promoter (-689 to +105) in pGL3-Basic. These constructs were transfected into YT cells and evaluated for their IL-2 responsiveness.

(B) As in (A), except that the fragments spanning HS4 were cloned in both orientations 5' and 3' to the SV40 promoter in pGL3-Promoter.

(C) Fragments spanning +2350 to +2955 of murine IL-2R $\alpha$  gene were generated by PCR and cloned in both orientations 5' and 3' to the murine IL-2R $\alpha$  promoter (-589 to +93) in pGL3-Basic. The resulting constructs were transfected into PC60 cells and evaluated for IL-2 responsiveness.

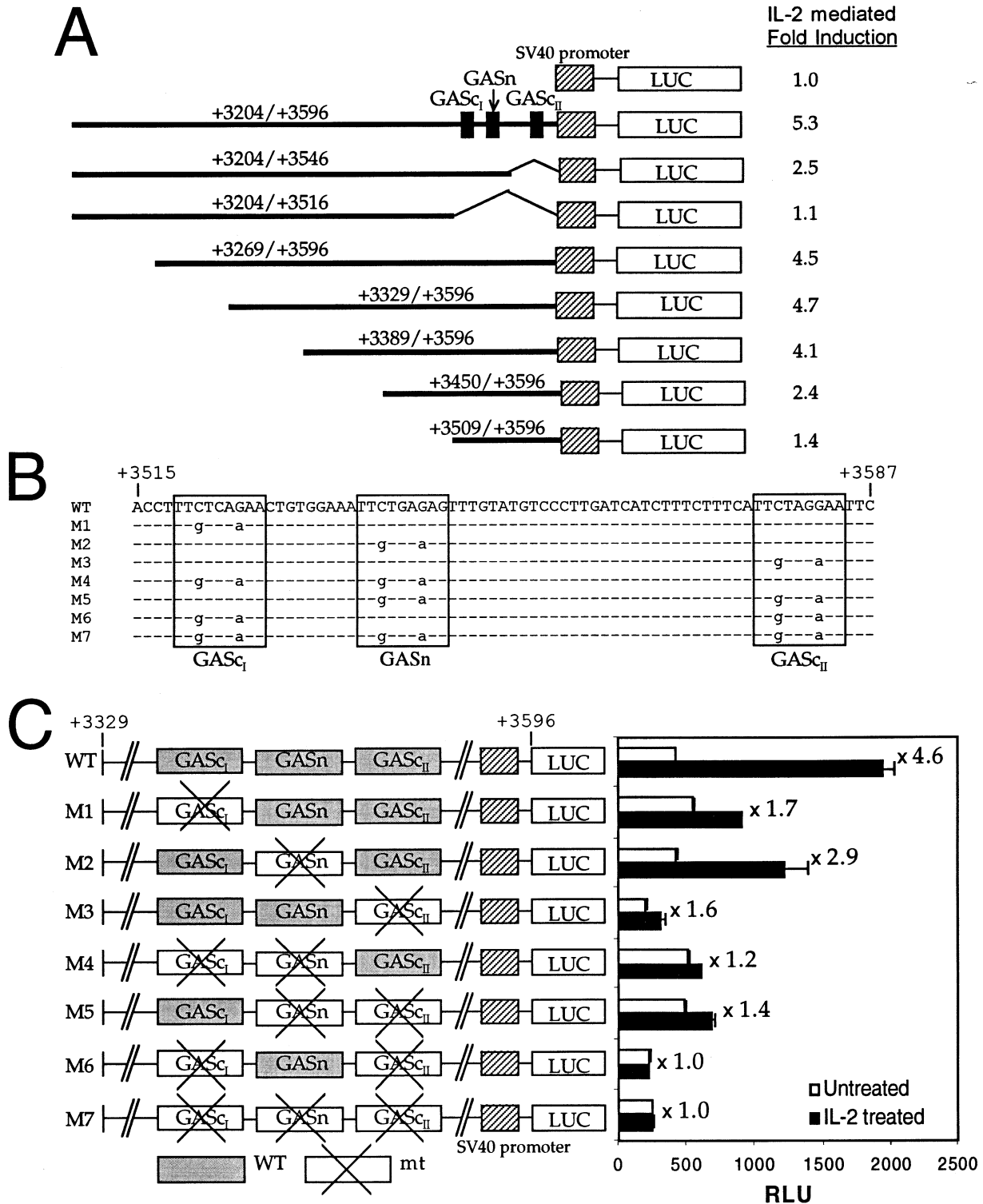
(D) As in (C), except that the +2350 to +2955 fragment was cloned in both orientations 5' and 3' to the SV40 promoter in pGL3-Promoter.

heterologous endogenous Stat5 protein relative to the level of the transfected dominant-negative Stat5 constructs. These findings are consistent with observations in *Stat5a*<sup>-/-</sup> (Nakajima et al., 1997) and *Stat5b*<sup>-/-</sup> (Imada et al., 1998) mice, as well as in *Stat5a*<sup>-/-</sup>*Stat5b*<sup>-/-</sup> double knockout mice (Moriggl et al., 1999 and Figure 6), where defects in IL-2-induced IL-2R $\alpha$  expression were noted. Together, these studies explain the basis for the vital role of Stat5 proteins in IL-2-induced IL-2R $\alpha$  expression.

#### HMG-I(Y) Binds to Multiple Elements within the PRRIV Upstream Region and Contributes to IL-2-Induced PRRIV Activity

In addition to the importance of the GAS motifs, the region upstream of the GAS motifs was also important for maximal IL-2-induced activity of PRRIV. Careful examination of the sequence of the +3329 to +3509 region revealed that it was >60% A:T rich and contained many potential binding sites for the high mobility group HMG-I(Y) proteins. HMG-I and HMG-Y are small nonhistone proteins that bind in the narrow minor groove of A:T-rich B form DNA. HMG-I (107 amino acids) and HMG-Y (96 amino acids) derive from alternatively spliced transcripts of the same gene with indistinguishable biological

properties. These "architectural" factors can induce structural changes in DNA substrates (Lehn et al., 1988; Nissen and Reeves, 1995) that, in turn, can lead to alterations in the assembly of transcription factors into higher order functional complexes (Bustin and Reeves, 1996). In addition, HMG-I(Y) appears to play a critical role in chromatin architecture (Reeves and Nissen, 1995), perhaps by interacting directly with isolated nucleosome core particles (Reeves and Nissen, 1993), altering the rotational setting of DNA on the surface of nucleosomes (Reeves and Wolffe, 1996) and antagonizing H1-mediated transcriptional repression (Kas et al., 1993; Zhao et al., 1993). It has been proposed that HMG-I(Y) involvement may be a general feature of the architecture of inducible gene promoters or enhancers (Thanos and Maniatis, 1995). In the IL-2R $\alpha$  promoter region, HMG-I(Y) proteins are important for the function of the PRRIV region (John et al., 1995). To determine whether HMG-I(Y) binds to the PRRIV region, DNase I footprinting was performed using purified HMG-I protein and +3269/+3596 DNA fragments labeled on either the top (Figure 7A) or the bottom (Figure 7B) strand. Footprints for HMG-I were observed at a number of locations in PRRIV (vertical bars a to k in Figure 7A and 7B, summarized in Figure 7C).



**Figure 4. Two Consensus and One Nonconsensus GAS Motifs Are Required for Maximal IL-2 Responsiveness of PRRIV**  
 (A) HS4 sequences were subjected to systematic 5' and 3' deletions, and the fragments were inserted 5' to the SV40 promoter in pGL3-Promoter. The resulting constructs were transfected into YT cells and their IL-2 inducibility was evaluated.  
 (B) Sequences of wild-type (WT) and mutant (M1, M2, M3, M4, M5, M6, and M7) constructs. The positions of GAS<sub>cI</sub>, GAS<sub>n</sub>, and GAS<sub>cII</sub> are indicated. For the mutant constructs, only the mutations are shown; hyphens indicate identity to the wild-type sequence.  
 (C) Mutations in the GAS motifs in PRRIV were made in the context of a +3329/+3596 human IL-2R $\alpha$ -luciferase reporter construct. Constructs were transfected into YT cells, followed by no stimulation or stimulation with IL-2.

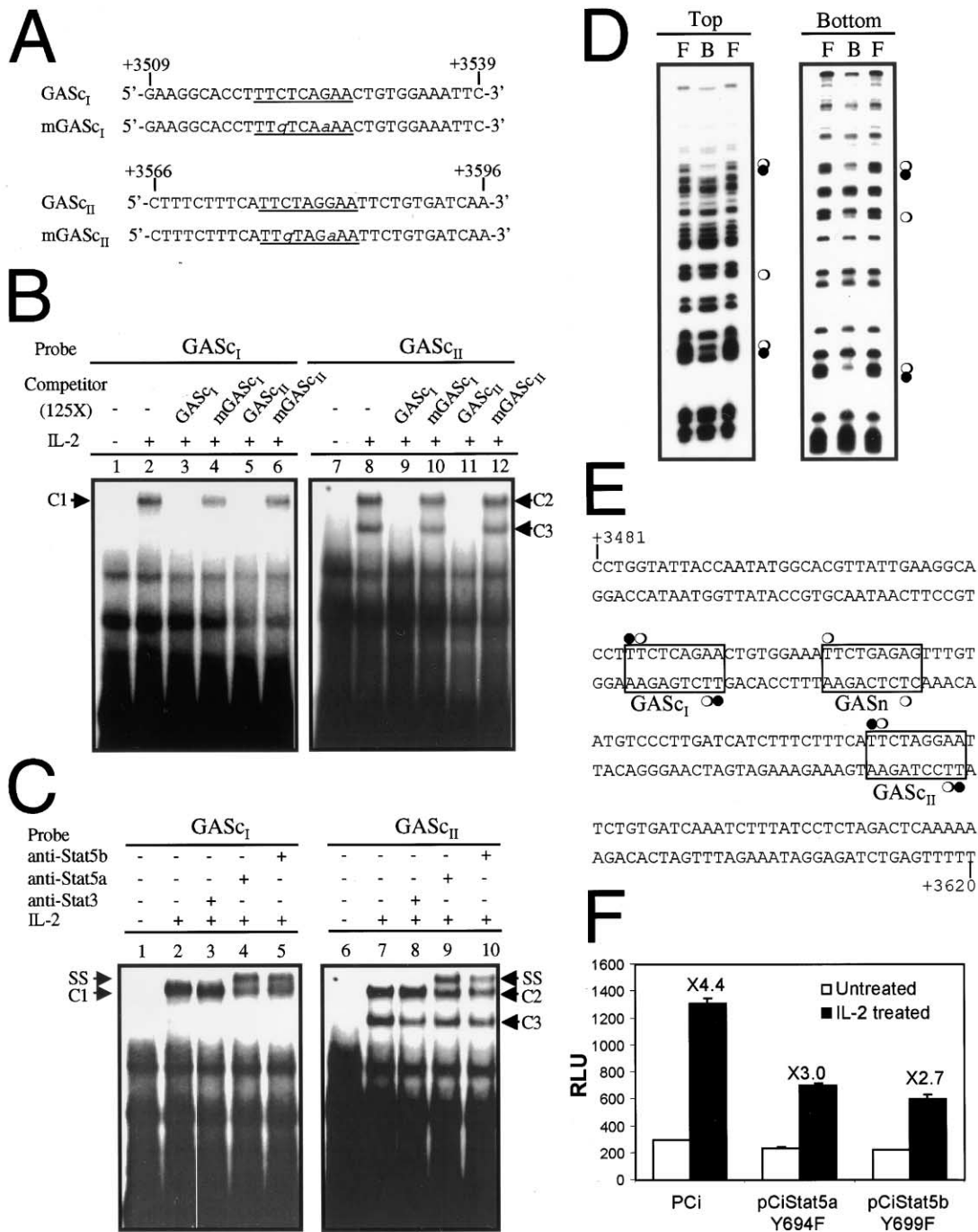


Figure 5. Stat5 Binds to and Regulates PRRIV Activity

(A) Sequences of the +3509/+3539 (GASc<sub>I</sub>) and +3566/+3596 (GASc<sub>II</sub>) oligonucleotides used in (B) and (C). For mGASc<sub>I</sub> and mGASc<sub>II</sub>, the GAS motifs are underlined, and the mutated nucleotides are in lowercase italics.

(B) EMSAs using wild type or mutant probes (GASc<sub>I</sub>, mGASc<sub>I</sub>, GASc<sub>II</sub>, and mGASc<sub>II</sub>) from PRRIV and extracts from untreated YT cells (lanes 1 and 7) or extracts from IL-2-treated cells (lanes 2 to 6 and 8 to 12). In lanes 3 to 6 and 9 to 12, a 125-fold molar excess of the indicated oligonucleotides was added.

(C) EMSAs using the GASc<sub>I</sub> and GASc<sub>II</sub> probes and nuclear extracts from YT cells not stimulated (lanes 1 and 6) or stimulated with IL-2 (lanes 2 to 5 and 7 to 10). In lanes 3 to 5 and 8 to 10, EMSAs were performed in the presence of antibodies to Stat3, Stat5a, or Stat5b, as indicated.

(D) Uracyl interference analysis of Stat5a binding to PRRIV. +3474 to +3622 probes labeled on the top or bottom strand were generated by PCR (see Experimental Procedures), and recombinant Stat5a protein was bound. Free (F) and bound (B) probes were excised from the gel, treated with piperidine, resolved on 6% denaturing gels, and autoradiographed.

(E) Summary of data from panel (D). Strong (solid circles) and weak (open circles) interferences are indicated.

(F) Dominant-negative Stat5 constructs inhibit PRRIV activity. YT cells were transfected with 10  $\mu$ g of +3329/+3596 human IL-2R $\alpha$ -luciferase reporter construct together with 10  $\mu$ g pCi, pCiStat5A Y694F, or pCiStat5b Y699F. Transfected cells were either not stimulated or stimulated with IL-2.

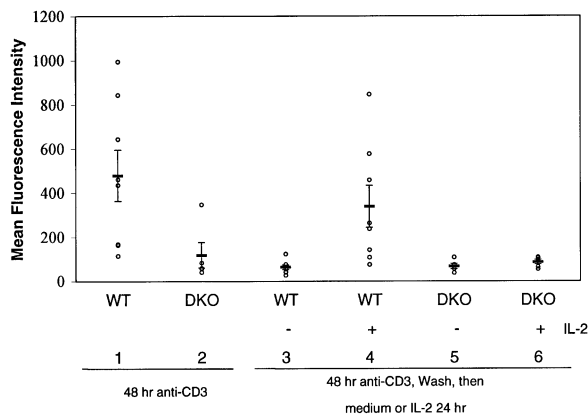


Figure 6. Essential Role for Stat5 Proteins for IL-2-Induced IL-2R $\alpha$  Gene Expression

Wild-type (WT) or Stat5a/Stat5b double knockout mouse (DKO) splenocytes were stimulated with anti-CD3 for 48 hr. As is evident, higher IL-2R $\alpha$  expression was seen in the WT mice (lane 2 versus 1). The cells were then washed three times and then were cultured for 24 hr additional time in the absence (lanes 3 and 5) or presence of IL-2 (lanes 4 and 6). Shown is IL-2-induced IL-2R $\alpha$  expression on CD8-gated cells. IL-2R $\alpha$  was induced by IL-2 in the WT but not in the Stat5a/Stat5b DKO mice.

To confirm that these sites were contacted by HMG-I, we performed EMSAs with wild-type and mutant +3317/+3478 DNA fragments (Figure 7D). Three complexes of different affinities could form with the wild-type probe (Figure 7E, lane 1). Complex C1 represented a protein-DNA interaction of high affinity since it was apparent with the addition of lower amounts of HMG-I, while slower mobility complexes C2 and C3 were observed only at higher concentrations of HMG-I, indicating lower affinity interactions (data not shown). The formation of C3 was diminished by mutation of HMG-I(Y) binding sites g/h (mutant MH1), d/e/f (mutant MH2), or b/c (mutant MH4), while mutation of four or five HMG-I(Y) binding sites (MH3, MH5, and MH6) decreased complex C2, and mutation of all sites (b to h) simultaneously (MH7) resulted in the complete abrogation of complexes C1, C2, and C3.

To investigate the functional significance of the HMG-I(Y) binding sites, we made mutations in the HMG-I(Y) binding sites in PRRIV in the context of a +3329/+3596 human IL-2R $\alpha$ -luciferase reporter construct and assayed the IL-2 responsiveness of each mutant construct in YT cells. In the experiment shown in Figure 7F, while the wild-type construct showed 3.7-fold IL-2 inducibility in YT cells, mutation of two HMG binding sites (MH1, MH2, and MH4) showed  $\sim$ 2.6-fold, 3.1-fold, and 3.0-fold IL-2 inducibility, respectively. Mutation of four or five HMG binding sites (MH3, MH5, and MH6) showed  $\sim$ 2.0-fold, 2.3-fold, and 2.6-fold IL-2 inducibility, respectively. Simultaneous mutation of all sites, b to h, (MH7) diminished IL-2 inducibility to  $\sim$ 1.9-fold. The MH7 construct showed approximately comparable IL-2 inducibility to the +3450/3596 human IL-2R $\alpha$ -luciferase reporter construct (2.4-fold; Figure 4A). There was a slightly greater decrease (1.7-fold inducibility) when the i and j regions were additionally mutated (data not shown). Thus, the HMG-I(Y) binding sites appear to substantially explain why the construct containing upstream sequences has more activity than those containing only

GAS motifs as shown in Figure 4A. Thus, for maximal IL-2 inducibility, multiple HMG-I(Y) binding sites as well as GAS motifs in PRRIV are required.

### Stat5 and HMG-I(Y) Proteins Bind to PRRIII and PRRIV In Vivo

To further investigate factor binding to PRRIV in vivo, we performed chromatin immunoprecipitation (ChIP) experiments. Chromatin was prepared from YT cells that were crosslinked with formaldehyde. DNA was sheared to an average size of  $\sim$ 400 bp, and immunoprecipitations were performed with specific antisera. The complexes were harvested using magnetic beads, crosslinking was reversed, and the DNA was then recovered and PCR amplified with primer pairs that separately amplified PRRIII, PRRI/PRR2, and PRRIV. Consistent with in vitro binding of Stat5 proteins and HMG-I(Y) to both PRRIII (John et al., 1996) and PRRIV (this study), treatment with IL-2 induced an increase in occupancy of both PRRIII and PRRIV by Stat5a, Stat5b, and HMG-I(Y) (Figure 8A). Consistent with the lack of known GAS motifs in the PRRI/II region, ChIP analyses did not reveal any significant increase in Stat5a and Stat5b occupancy of PRR1 and PRR2, indicating the specificity of the reaction. Although HMG-I(Y) protein was bound by PRR1 and PRR2, the binding was not increased by treatment with IL-2. Thus, IL-2 specifically increases both HMG-I(Y) and Stat5 binding to PRRIII and PRRIV.

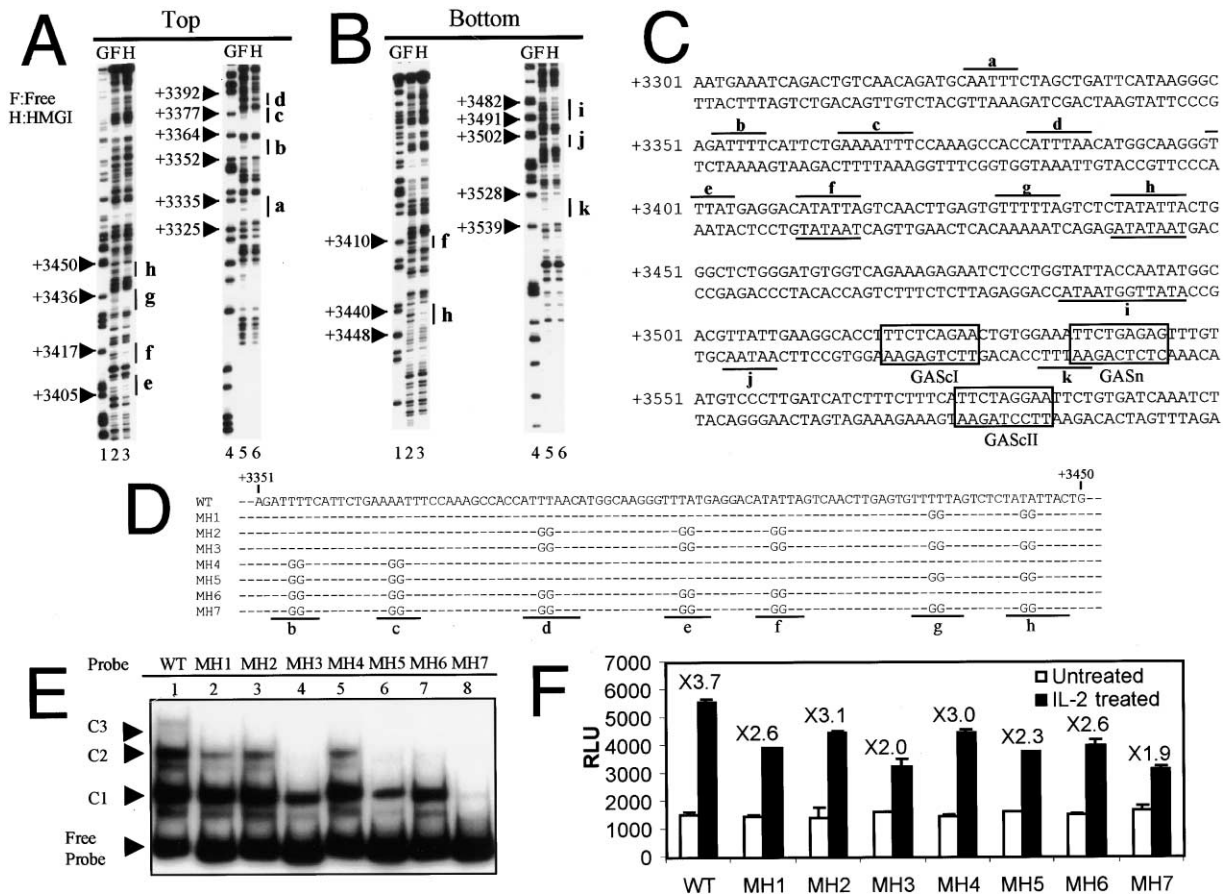
### Both PRRIII and PRRIV Are Required for Maximal IL-2-Induced IL-2R $\alpha$ Promoter Activity

Given that both PRRIII and PRRIV bind HMG-I(Y) and Stat5, we analyzed their relative contributions to IL-2-induced IL-2R $\alpha$  promoter activity. The PvuII/PstI human IL-2R $\alpha$  fragment (-4059 to +109) was inserted upstream of the luciferase reporter gene, and the adjacent PstI fragment (+109 to +5039) was subcloned downstream of the luciferase gene. The consensus and nonconsensus GAS motifs in PRRIII and both GAS<sub>C1</sub> and GAS<sub>C2</sub> in PRRIV were separately mutated. In YT cells, the wild-type construct showed  $\sim$ 3.2-fold IL-2 inducibility (Figure 8B). Mutation of either PRRIII or PRRIV slightly diminished IL-2 inducibility, and simultaneous mutation of PRRIII and PRRIV abrogated IL-2 inducibility (Figure 8B). Cooperativity of PRRIII and PRRIV was more evident in experiments in PC60 cells using the murine IL-2R $\alpha$  regulatory elements. The SphI/PstI fragment (-2543 to +93) was inserted upstream of the luciferase reporter gene and the adjacent PstI fragment (+93 to +4533) was subcloned downstream of luciferase gene. In PC60 cells, the wild-type construct showed  $\sim$ 7.9-fold IL-2 inducibility (Figure 8C). Mutation of either PRRIII or PRRIV diminished IL-2 inducibility down to 3.1-fold and 2.0-fold, respectively, but simultaneous mutation of PRRIII and PRRIV abrogated IL-2 inducibility. These data indicate that both PRRIII and PRRIV are required for maximal IL-2-induced IL-2R $\alpha$  promoter activity in both the human and murine IL-2R $\alpha$  genes.

### Discussion

IL-2R $\alpha$  is required for the formation of high-affinity IL-2 receptors, which allows cells to respond to the low levels of IL-2 that are physiologically produced in vivo. Mice





**Figure 7. Functionally Important HMG-I(Y) Sites in PRRIV**  
 (A and B) DNase I footprinting of HMG-I on PRRIV. The DNA fragment was labeled at either +3269 (A) or +3596 (B). Recombinant HMG-I proteins were added as indicated (lanes 3 and 6 in each panel). A Maxam-Gilbert “G” chemical cleavage of the naked DNA served as a sequence reference marker (lanes 1 and 4). Lanes 1 to 3 and 4 to 6 are two different loadings of the same samples that were electrophoresed for different lengths of time. The nucleotide numbers are relative to the major transcription start site. The vertical solid lines (labeled a, b, c, d, e, f, g, and h) indicate areas protected by HMG-I.  
 (C) Position of HMG-I footprints relative to GAS motifs in PRRIV.  
 (D) Schematic showing the HMG-I(Y) mutants (MH1 to MH7) evaluated in (E) and (F). Only the mutations are shown; hyphens indicate WT sequence.  
 (E) EMSAs were performed using 4 ng of recombinant HMG-I protein and <sup>32</sup>P-labeled +3317 to +3478 WT or mutant (MH1 to MH7) probes.  
 (F) Effect of mutating HMG-I(Y) binding sites in PRRIV in the context of a +3329/+3596 human IL-2R $\alpha$ -luciferase reporter construct. Constructs were transfected into YT cells, followed by no stimulation or stimulation with IL-2.

lacking IL-2R $\alpha$  exhibit profound autoimmunity and premature death, underscoring the importance of this protein. IL-2R $\alpha$  is not expressed by resting lymphocytes but is potently induced after antigen encounter. Although it is less well appreciated, the expression of IL-2R $\alpha$  is also potently induced after stimulation with IL-2. This induction serves as a mechanism by which IL-2 can upregulate expression of its own high-affinity receptor (Lin and Leonard, 1997; Waldmann, 1989). The physiological significance of this regulation is suggested by analysis of Stat5a knockout mice that exhibit a defect in IL-2-induced IL-2R $\alpha$  expression and proliferation in vitro and defective expansion of V $\beta$ 8<sup>+</sup> T cells in vivo in response to the superantigen, Staphylococcus enterotoxin B (Nakajima et al., 1997). Because of its immunological importance and its strong inducibility, the molecular regulation of the IL-2R $\alpha$  gene has been extensively investigated. Previous studies have characterized three important elements upstream of the major transcription

initiation sites. We now have identified an additional positive regulatory region, PRRIV, which is located within the first intron. PRRIV was initially identified as an IL-2-inducible DNase I hypersensitive site (HS4), and it fulfills the criteria of an IL-2 responsive enhancer, as it can increase transcriptional activity following IL-2 treatment in a promoter- and orientation-independent fashion. Interestingly, all of the hypersensitive sites, including HS4, were constitutively detected in MT-2 cells, which constitutively express the IL-2R $\alpha$  gene and have constitutively activated nuclear Stat5 even without IL-2 stimulation (Migone et al., 1995).

Analysis of PRRIV revealed a total of three GAS motifs, including one nonconsensus and two consensus sites. Each of these sites contributes to PRRIV activity, as the IL-2 inducibility decreased significantly when any of them was mutated. In addition to the importance of the GAS motifs, a deletion analysis revealed that additional sequences are also required for the full function of

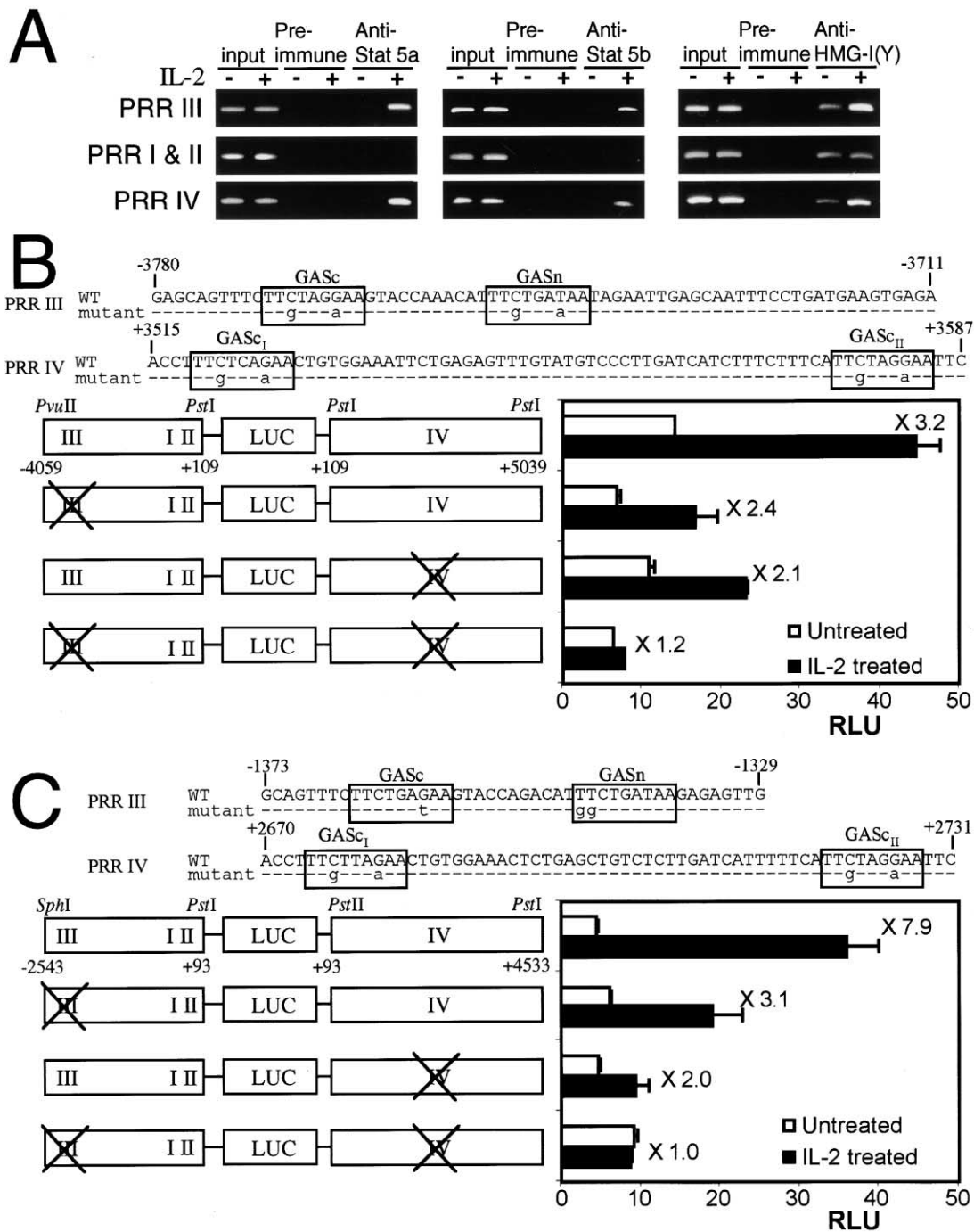


Figure 8. Binding of Stat5a, Stat5b, and HMG-I(Y) to Both PRRIII and PRRIV In Vivo Correlates with the Importance of Both PRRIII and PRRIV for Maximal IL-2-Induced Reporter Activity

(A) ChIP assays were performed using YT cells. DNA was purified and used as a template for PCR reactions with pairs of primers that amplify PRRIII, PRR I and PRRII, or PRRIV, as indicated.

(B and C) Both PRRIII and PRRIV are required for maximal IL-2-induced IL-2R $\alpha$  promoter activity. Regions from human (B) and murine (C) PRRIII and PRRIV are shown to indicate the WT and mutant sequences. PRRIII and PRRIV fragments were incorporated into -4061 to +109 and +109 to +5039 human IL-2R $\alpha$ -luciferase reporter constructs and transfected into YT cells (B). Analogous murine PRRIII and PRRIV fragments were incorporated into -2543 to +93 and +93 to +4533 murine IL-2R $\alpha$ -luciferase reporter constructs and transfected into PC60 cells (C). Cells were then either not stimulated or stimulated with IL-2.

PRRIV. Using DNase I footprinting analysis and EMSAs, we identified multiple HMG-I(Y) binding sites in the upstream region of PRRIV and found that simultaneous

mutation of these sites substantially decreases IL-2 inducibility even when the GAS motifs are intact.

Analysis of the activity of luciferase reporter con-

structs in which PRRIII, PRRI, and PRRIV were upstream of the reporter and PRRIV was downstream revealed the importance of both PRRIII and PRRIV for maximal IL-2-induced activity. Previously, human and murine PRRIII were found to confer IL-2 inducibility to the IL-2R $\alpha$  gene (Bucher et al., 1997; John et al., 1996; Soldaini et al., 1995). Given the existence of PRRIII, our discovery of PRRIV as a second IL-2 response element was unexpected. The existence of PRRIV indicates that two widely spatially separated regulatory regions can cooperate and/or are at least partially redundant in their ability to mediate IL-2 inducibility of the IL-2R $\alpha$  gene. Using chromatin immunoprecipitation assays, we demonstrate that both PRRIII and PRRIV bind Stat5a, Stat5b, and HMG-I(Y) in an IL-2-inducible fashion. This not only clarifies the mechanism for PRRIV action but also studies some of the factors binding to PRRI, PRRII, PRRIII, and PRRIV *in vivo*. Importantly, the chromatin immunoprecipitation assays revealed that IL-2 induced the recruitment of HMG-I(Y), Stat5a, and Stat5b to both PRRIII and PRRIV but not to PRRI or PRRII. This complexity is consistent with the importance of the inducibility of this gene in response to IL-2. For both human and murine IL-2R $\alpha$  constructs, maximal activity required the presence of both PRRIII and PRRIV. These findings explain the basis for the markedly defective IL-2-induced IL-2R $\alpha$  expression in mice lacking Stat5a and Stat5b. Although HMG-I(Y) binding was augmented after stimulation with IL-2, a basal level of HMG-I(Y) binding was detected even before IL-2 stimulation and recruitment of Stat5. Thus, these observations plus the binding assays in Figure 7E with purified HMG-I indicate that HMG-I(Y) binding was not strictly dependent on the presence of Stat5. Presumably, HMG-I(Y) exerts its positive effect at least in part by inducing DNA bending.

Important regulatory elements have long been appreciated to exist in introns. For example, the immunoglobulin  $\kappa$  chain gene enhancer is located in an intron (Picard and Schaffner, 1984). For STAT proteins, most important elements have been found in promoter regions, although the IFN- $\gamma$  gene is an example where GAS motifs have been found in an intron (Xu et al., 1996). The IL-2R $\alpha$  gene provides an example of a gene in which IL-2 responsiveness (and perhaps responsiveness to any cytokine) has been demonstrated to depend on multiple elements that collectively are located both in the 5' regulatory region and the first intron. Each of the two IL-2 response elements has more than one GAS motif, with PRRIII binding Stat5 as a tetramer and PRRIV involving a total of three GAS motifs (two of which are consensus motifs). Thus, IL-2 inducibility of this gene involves greater complexity and likely greater functional redundancy than was previously appreciated. Although it is uncertain why the IL-2 responsiveness of the IL-2R $\alpha$  gene is controlled by two widely separated elements, one benefit of such an organization is that it might conceivably help to protect an important function during evolution. For example, intronic elements might minimize potentially deleterious consequences if there were rearrangements or translocations that selectively affect the far upstream PRRIII regulatory sequences. These types of considerations may be important for many genes with important regulatory elements contained within the first intron. In the case of the IL-2R $\alpha$

gene, it is important to realize that PRRIII and PRRIV may not simply be redundant elements. In addition to HMG-I(Y) and Stat5 binding sites, PRRIII is critically dependent on an Ets binding site that can bind the lineage-restricted factor, Elf-1, the expression and phosphorylation of which are increased by antigenic stimulation. As such, PRRIII may be activated in a lineage- and activation-dependent manner. PRRIV instead may be an important "amplifier" that perhaps is not as dependent on lineage. Another possible difference between these sites is that PRRIII is unlikely to be affected by Pol II during active transcription, whereas one can envision that factors binding to PRRIV might be displaced by Pol II as transcription proceeds through the first intron, which would necessarily suggest that the role of PRRIV might be more transient while that of PRRIII could be more sustained. In summary, we have further clarified the complexity of IL-2-induced IL-2R $\alpha$  expression by demonstrating that at least two widely separated elements, each containing GAS motifs and HMG-I(Y) binding sites, help to regulate this vital function. These studies clarify the basis for the critical role of Stat5 proteins in regulating IL-2-induced IL-2R $\alpha$  gene expression.

#### Experimental Procedures

##### Cell Culture

The cell lines YT and PC60 were maintained at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G, and 100  $\mu$ g/ml streptomycin.

##### Antibodies and Recombinant Proteins

Recombinant HMG-I protein and anti-HMG-I(Y) antibodies were gifts from R. Reeves (Washington State University, Pullman, WA). Recombinant Stat5a protein was purified as described (John et al., 1999). Antibodies to Stat5a, Stat5b, and Stat3 were from Zymed Laboratories, Inc. (South San Francisco, CA).

##### DNase I Hypersensitivity Assay

DNase I hypersensitivity assays were performed using isolated nuclei as previously described (Enver et al., 1985) with some modification. Peripheral blood lymphocytes were preactivated with phytohemagglutinin (PHA) (Lin et al., 1995) and then not stimulated or stimulated with IL-2 for 1 hr. Cells in logarithmic-phase growth ( $2-5 \times 10^5$ /ml, 160 ml) were harvested by centrifugation and washed once in RPMI 1640 medium. The cells were resuspended in 2 ml ice-cold lysis mix (50% glycerol, 50 mM Tris [pH 7.9], 100 mM KCl, 5 mM MgCl<sub>2</sub>, 0.05% saponin, 200 mM 2-mercaptoethanol, and 1 mM EGTA) and left on ice for 10 min. The lysed mixture was spun at 5500 rpm for 15 min at 4°C. The supernatant was removed, and the nuclear pellet was resuspended in 0.8 ml ice-cold buffer A (50 mM Tris [pH 8.0], 100 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.1 mM AEBF, 1 mM CaCl<sub>2</sub>, and 1 mM EGTA) and repelleted at 3000 rpm for 5 min at 4°C. The nuclear pellet was resuspended in 600  $\mu$ l ice-cold Buffer A. To 200  $\mu$ l aliquots of this suspension was added 2  $\mu$ l of differing dilutions of DNase I in buffer A. These reactions were incubated for 5 min at 37°C before termination by addition of 2  $\mu$ l 0.5 M EDTA. The genomic DNA was purified with the Wizard genomic DNA purification kit (Promega, Madison, WI). The DNA was digested with appropriate restriction enzymes and analyzed on Southern blots (Nytran Plus, Schleicher & Schuell, Keene, NH) using random primer-labeled probes and Quickhyb solution (Stratagene, La Jolla, CA).

##### Isolation of HS4

DNA sequences containing HS4 were isolated by screening a human genomic BAC library (Genome Systems, Inc., St. Louis, MO) with a probe corresponding to -16 to +221 of the human IL-2R $\alpha$  gene. A 7 kb BamHI fragment from the BAC DNA was subcloned to pBluescript SK(+) and sequenced on both strands. DNA sequences spanning

the murine IL-2R $\alpha$  gene were isolated by screening a mouse genomic BAC library (Genome Systems, Inc.) with a probe corresponding to -257 to +95 of the murine IL-2R $\alpha$  gene. A 7 kb NcoI fragment from the BAC DNA was subcloned to pLITMUS28 and was sequenced on both strands.

#### Sequence Analysis

The GCG sequence analysis package (Wisconsin Package, Oxford Molecular Group, Inc.) was used for dot plot analysis of nucleotide sequences. Sequence alignments were made with Clustal W (accessible via <http://www2.ebi.ac.uk/clustalw/>) (Thompson et al., 1994).

#### Enhancer Constructs

To test enhancer activity of human and murine PRRIV, we used either the human (-689 to +109) or murine (-589 to +93) IL-2R $\alpha$  promoter fragments, respectively, each of which was cloned in the HindIII site of the polylinker in the pGL3-Basic luciferase reporter vector (Promega, Madison, WI). PCR fragments containing human or murine PRRIV were inserted either upstream, between the KpnI and SacI sites in the polylinker, or downstream, in the Sall site. Human and murine PRRIV were also similarly cloned 5' or 3' of the SV40 promoter, which was inserted into the BglIII site of pGL3-Basic.

Site-directed mutagenesis of these PRRIV-pGL3 plasmids was performed with a kit (QuikChange, Stratagene, La Jolla, CA). Three mutagenic primers (labeled AM for  $\alpha$ -mutagenic primers), AM1 (+3509 to +3539; 5'-GAAGGCACCTTTGTCAAACCTGTGGAAATTC-3'), AM2 (+3525 to +3557; 5'-GAAGTGTGAAATTGTGAAAGTTGTATGTGCC-3'), and AM3 (+3566 to +3596; 5'-CTTCTTTCATTGTAGAAATCTGTGATCAA-3'), were used, respectively, to introduce the indicated (underlined) 2 bp changes into the GAS $\alpha$ , GASn, and GAS $\beta$  motif of human PRRIV. Analogously, primers AM4 (-3782 to -3748; 5'-CTGAGCAGTTTCTGTAGAAAGTACCAACATTTC-3') and AM5 (-3758 to -3714; 5'-CCAAACATTTGTGAAATAGAATTGAGCAATTTCTGTGAAGTG-3'), were used to introduce the indicated 2 bp changes into the GAS $\alpha$  and GASn motifs of human PRRIII. Primers AM6 (+3417 to +3459; 5'-GTCAACTTGAGTGTGGTAGTCTCTAGGTTACTGGGCTC TGGG-3'), AM7 (+3372 to +3429; 5'-CCAAAGCCACCATGGAACATGGCAAGGGTTGGTGAGGACATGGTAGTCAACTTGAGTG-3'), and AM8 (+3338 to +3382; 5'-GATTGATAAGGGCAGATGGTCACTTCTGAAAGTTTCCAAAGCCACC-3') were used to introduce the indicated changes into the HMG-(Y) binding sites of human PRRIV. Primers AM9 (+2662 to +2696; 5'-CTGAAGCCACCTTTGTAAACTGTGGAAACTC-3') and AM10 (+2708 to +2744; 5'-GATCATTTTTTATTGTAGAAATCTGTAAATCAAATCC-3'), were used to introduce the indicated 2 bp changes into murine GAS $\alpha$  and GAS $\beta$  motifs in murine PRRIV. Primer AM11 (-1373 to -1329; 5'-GCAGTTTCTGTGATAAGTACCAGCATGGCTGATAA GAGAGTTG-3') was used to introduce the indicated 3 bp changes into the GAS $\alpha$  and GASn motifs of murine PRRIII. All mutants were verified by sequencing.

#### Transient Transfections and Luciferase Assays

Transient transfections of YT cells and PC60 cells were performed by the DEAE-dextran technique (Sompayrac and Danna, 1981). In each case,  $5 \times 10^6$  cells in logarithmic growth phase were transfected with 10  $\mu$ g supercoiled test plasmid and 40 ng pRL-SV40 as a transfection efficiency control; cells were then allowed to recover for 24 hr at 37°C. Transfected cells were stimulated with either medium alone or 2 nM human recombinant IL-2 for 18 hr, and the cells were harvested and analyzed for luciferase activity using Analytical Luminescence Laboratory equipment and the Dual luciferase assay system kit (Promega, Madison, WI).

#### Electrophoresis Mobility Shift Assays

Nuclear extracts were prepared as described (Schreiber et al., 1989) from untreated YT cells or cells that had been treated with recombinant IL-2 (2 nM) for 30 min at 37°C. EMSAs were performed as described previously (John et al., 1996) by using glycerol-containing 5% polyacrylamide gels (29:1) containing 0.5 $\times$  Tris-borate-EDTA buffer. For supershifting assays, nuclear extracts were preincubated for 10 min with antibodies to Stat3, Stat5a, or Stat5b (Zymed, South San Francisco, CA). Oligonucleotide sequences from PRRIV used as probes are shown in figures.

#### Uracil Interference Analyses

The uracil interference assay was performed essentially as described (Ausubel et al., 1987). Thymine was randomly replaced by deoxyuracil in DNA fragments containing GAS motifs by PCR amplification using the following oligonucleotides as primers: UI1 (+3474 to +3502; 5'-AGAATCTCCTGGTATTACCAATATGGCAC-3') and UI2 (+3622 to +3599; 5'-AGAATTATTTTGTAGCTAGAGGATAAAG-3'). The oligonucleotides were labeled with [ $\gamma$ -<sup>32</sup>P]ATP. Two PCR reactions were performed (15 cycles with the following profile: 94°C, 60 s; 50°C, 10 s; 72°C, 60 s); each reaction contained a different labeled oligonucleotide in order to label each DNA strand separately. The amplified PCR products were separated in a 5% native polyacrylamide gel, recovered by electroelution, and used as probes ( $3 \times 10^5$  cpm) for EMSA. The probes were incubated separately with recombinant Stat5a protein, and binding reactions were loaded in a 5% native polyacrylamide gel. The free probes and the protein-bound DNA fragments were excised from the gel and recovered by electroelution. The DNA fragments were treated for 1 hr with one unit of uracil-N-glycosylase (Roche Molecular Biochemicals, Indianapolis, IN) at 37°C in 50  $\mu$ l of PCR buffer, ethanol precipitated, and cleaved with 1 M piperidine for 30 min at 90°C. Then, the same amount (in cpm) of the protein-bound and -free DNA fragments was loaded on a 6% polyacrylamide sequencing gel.

#### In Vitro DNase I Footprinting

The +3269 to +3596 fragment, radiolabeled at either the top strand or the bottom strand, was incubated with recombinant HMG-I and digested with DNase I, as described previously (Reeves and Nissen, 1993). The optimal enzyme concentration and digestion time were determined empirically. Single-stranded DNA cleavage products were fractionated by electrophoresis on a 6% sequencing gel with Maxam-Gilbert "G-lane" chemical cleavage products of naive DNA fragments serving as reference standards.

#### Analysis of Stat5a/Stat5b Double Knockout Mice

Heterozygous Stat5a/Stat5b double knockout mice were obtained from Dr. James Ihle and mated to generate homozygous knockout mice and wild-type littermate controls, as determined by PCR. Mice were evaluated at 8–12 weeks of age. All experiments were performed under protocols approved by the National Institutes of Health Animal Use and Care Committee and followed the National Institutes of Health guidelines "Using Animals in Intramural Research." Single-cell suspensions from spleens were prepared, and splenocytes ( $1 \times 10^6$ /ml) were stimulated in Falcon 3003 plates coated with 7.5 mg/ml 2C11 anti-CD3 $\epsilon$ mAb (PharMingen, San Diego, CA) in RPMI 1640 containing 10% FBS, 2 mM glutamine and antibiotics for 48 hr. The cells were then cultured in the presence or absence of IL-2 for an additional 48 hr. Splenocytes were stained after the initial 48 hr culture as well as after the additional 48 hr culture with anti-CD4-PE, anti-CD8-APC, and anti-IL-2R $\alpha$ (CD25)-FITC (all from PharMingen) and analyzed using a FACSort with CELLQuest software (Becton Dickinson, San Jose, CA).

#### Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described (Moreno et al., 1999). YT cells were incubated for 10 min at room temperature in tissue culture medium containing 1% formaldehyde. Cells were then pelleted, resuspended in phosphate-buffered saline, repelleted, and lysed in lysis buffer (5 mM PIPES [pH 8.0], 85 mM KCl, 0.5% NP40, and 1 $\times$  protease inhibitor cocktail [Roche Molecular Biochemicals, Indianapolis, IN]) for 10 min on ice. Nuclei were pelleted and lysed in nuclei lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS, and 1 $\times$  protease inhibitor cocktail). Lysed nuclei were sonicated using a microtip until the average DNA fragment was  $\sim$ 400 bp. Chromatin samples were diluted in 1 $\times$  RIPA buffer (1% Triton X-100, 0.1% deoxycholic acid, 0.1% SDS, 140 mM NaCl, and 1 $\times$  protease inhibitors) followed by immunoclearing with 20  $\mu$ g sheared salmon sperm DNA, 50  $\mu$ g BSA, 20  $\mu$ l preimmune serum, and secondary antibodies linked to magnetic beads (DynaL, Lake Success, NY). Immunoprecipitations were performed with 5  $\mu$ g primary antibody overnight at 4°C. Immune complexes were harvested with secondary antibodies linked to magnetic beads, and washed twice for 5 min in each of the

following buffers: 1 $\times$  RIPA buffer, IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.0], and 167 mM NaCl), dialysis buffer (2 mM EDTA, 50 mM Tris-HCl [pH 8.0], and 0.2% Sarkosyl), TSE-500 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], and 500 mM NaCl), and IP wash buffer (100 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1% NP40, and 1% deoxycholic acid), followed by a final wash with TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). Immune complexes were disrupted with elution buffer (50 mM NaHCO<sub>3</sub> and 1% SDS), and covalent links were reversed by adding NaCl to a final concentration of 300 mM and heating to 65°C for 6 hr. DNA was ethanol precipitated and further purified by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. DNA pellets were dissolved in 50  $\mu$ l water, and 2  $\mu$ l were used as template for PCR reactions. The primers used in this study were as follows: CH1-1, 5'-AGAAGTGCTTGCTCACCCTAC-3' (-300 to -280) and CH1-2, 5'-TGCCTAGCACTCTCTCTCTC-3' (-58 to -78) were used to amplify PRR1 and PRR2, CH2-1, 5'-ACGCTAGAAAGAAAGTGGTC-3' (-3850 to -3830) and CH2-2, 5'-ACTAGGTTCCAGGACAGC-3' (-3667 to -3687) were used to amplify PRR3, and CH3-1, 5'-TTTCTAGCTGATTCATAAGG-3' (+3329 to +3348), and CH3-2, 5'-TTGATCACA GAATTCCTAG-3' (+3596 to +3578) were used to amplify PRR4.

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#### Accession Numbers

The GenBank accession numbers for the sequences reported in this paper are AF243502 and AF243503.