Signal transducer and activator of transcription 4 (STAT4) is a critical mediator of interleukin-12 (IL-12)-stimulated inflammatory immune responses. Despite extensive analysis of the immune responses of STAT4-deficient mice, there is still very little understood about STAT4-dependent gene induction. IL-12 stimulated increases in IL-2 receptor α chain gene (CD25) mRNA levels and surface expression require STAT4. In this report, we utilize chromatin immunoprecipitation assays to analyze IL-12-stimulated and STAT4-dependent changes in chromatin remodeling of the CD25 gene. Gene activation requires binding of STAT4 to the PRRIII upstream regulatory element, the recruitment of the CREB-binding protein (CBP), and chromatin remodeling including increased acetylation and decreased methylation of histones within the CD25 promoter. Evidence suggests that STAT4 also facilitates binding of other factors to the CD25 promoter including c-Jun. Thus, these results provide a model for STAT4-dependent gene induction and a mechanism for cytokine-induced expression of the CD25 gene.

Interleukin-12 (IL-12)\(^1\) is a pleiotropic cytokine produced from macrophages and dendritic cells, which induces several responses in T cells including increased proliferation, increased cytotoxic activity, and Th1 differentiation (1). IL-12 activates several signaling pathways that may mediate these biological activities including p38 MAPK and the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (2–5). Upon binding of IL-12, both chains of the IL-12 receptor (IL-12R\(^{B1}\) and IL-12R\(^{B2}\)) heterodimerize and activate the associated JAKs, TYK2, and JAK2. The IL-12R\(^{B2}\) chain is subsequently tyrosine phosphorylated and recruits STAT4 to a specific docking site where it is itself phosphorylated (6). The phosphorylated STAT4 monomers then homodimerize and translocate into the nucleus. STAT4 is required for many of the functions of IL-12 including the induction of IFN-γ and the differentiation of Th1 cells (7, 8).

Once in the nucleus, STAT4 binds to a cognate binding sequence within IL-12 responsive genes to subsequently mediate activation of transcription (9–11). Several IL-12 responsive genes that require STAT4 for transcriptional activation have been identified including IFN-γ (7–9, 12), IL-18Ra (13, 14), ERM (15) and IRF-1 (10, 11). STAT4 may interact with other transcription factors at a promoter through several domains, including the C-terminal “transactivation domain.” However, both STAT4 isoforms, STAT4\(^{α}\) and STAT4\(^{β}\) (16), the latter of which lacks the C-terminal 40 amino acids encompassing the putative transcription activation domain, are able to activate transcription and mediate many IL-12 responses. This suggests that interactions of other transcription factors with the STAT4 transactivation domain are not required for all STAT4-dependent responses. Furthermore, the events that STAT4 initiates at a promoter, and the kinetics with which they occur have not been characterized.

CD25 is the α chain of the high affinity IL-2 receptor complex. CD25 is only expressed upon T cell activation, and expression is further modulated by IL-2 stimulation. Transcription of CD25 is regulated by multiple transcription factors that bind to elements termed positive regulatory regions (PRR) in the CD25 promoter and intronic regions. PRR is located around 260 nucleotides upstream of the major transcription initiation sites in the mouse gene (−276 to −244 in human) (17–19) and binds NF-κB and serum response factor (20). PRR-II is around −100 in the mouse gene (−137 to −64 in human) and binds Elf-1 and HMG proteins (21). PRRIII, also known as the IL-2 responsive element (IL-2RE), is located −1306 to −1387 in the mouse gene (−3780 to −3703 in human), and one of the tandem STAT5 binding sites overlaps a binding site for Elf-1 (22–26). In the mouse gene, an additional regulatory element at −576 to −687 that contains binding sites for NFAT and AP-1 proteins is also required for T cell receptor-mediated induction of IL-2Rα chain expression (27). Recently, two additional elements in the human gene have been identified, a CD28 responsive element (CD28RE) at −8.5 kb that binds CREB/ATF/AP-1 family members (28) and an intron 1 enhancer at +3389 to +3596 (homologous to +2539 to +2740 in the mouse gene) that binds STAT5 and AP-1 proteins (29, 30). Intermolecular interactions between the complexes bound to PRRI and PRRII, and presumably factors binding to other elements as well, result in a highly ordered stereospecific complex thought to be crucial to regulated CD25 transcription (21).

IL-2-mediated induction of CD25 expression requires PRRIII. Mouse PRRIII consists of two STAT sites (termed site I and II) that are weak binding sites for STAT5A and STAT5B, and an overlapping Ets binding site (site III) that binds Elf-1.
(22, 24): All three sites are required for IL-2 responsiveness. Upon IL-2 stimulation, STAT5 dimers bind to the STAT motifs in a cooperative manner (25, 26) forming a tetrameric STAT5 complex necessary to achieve maximal activation of CD25 expression (26). IL-2-induced CD25 expression is greatly reduced in mice deficient in STAT5a or STAT5b, demonstrating the importance of STAT5 in gene induction (31–33). It has not been determined whether other additional cytokines or STAT proteins can function through this element.

In this report we demonstrate that CD25 is an IL-12 responsive gene and is induced in a STAT4-dependent manner. The CD25 gene was chosen for further study because the promoter was well characterized and STAT-responsive elements have been clearly defined. Using chromatin immunoprecipitation (ChIP) assays we show that CD25 transcription activation by STAT4 involves the recruitment of additional transcription factors and chromatin remodeling thus providing a kinetic model of gene activation by STAT4.

EXPERIMENTAL PROCEDURES

Mice—Wild-type BALB/c mice were purchased from Harlan Bioproducts (Indianapolis, IN). The generation of the STAT4+/- CD2/STAT4a and STAT5a-null mice has previously been described (34, 35). FACS Analysis of CD25 Expression—Spleen cells were activated with 2 μg/ml anti-CD3 and cultured for 72 h. Activated T cells were purified over Histopaque, washed, and incubated overnight in the absence or presence of 5 μg/ml anti-IL-2 (S4B6). Cells were then incubated in the presence or absence of 2 ng/ml IL-12 (Genetics Institute, Cambridge, MA) or 20 units/ml IL-2 (Roche Applied Science) overnight or for the number of hours described in the text. Cells were then stained with fluorescein isothiocyanate-anti-CD25 and PE-anti-CD4 (BD Pharmingen, San Diego, CA). The intensity of CD25 fluorescence on CD4 cells was determined using a FACScan.

Northern Blot Analysis of CD25 mRNA—Cells were activated as described above and treated in the presence or absence of IL-12 for the indicated amount of time. Total RNA was isolated using Trizol (Invitrogen). Total RNA (10 μg) was fractionated by electrophoresis through a 1% denaturing agarose gel, transferred to a nylon membrane (Schleicher and Schuell, Keene, NH), and UV cross-linked. The membranes were pre-hybridized for 3 h at 42 °C, and hybridization was performed with a 32P-labeled CD25 probe for 20 h at 42 °C. The membranes were sequentially washed in 2× SSC containing 0.1% SDS at 60 °C for 20 min and in 0.1× SSC containing 0.1% SDS at 60 °C for 20 min, and then exposed to x-ray film at −80 °C. The membranes were stripped and re-hybridized with either a T cell receptor or a glyceraldehyde-3-phosphate dehydrogenase probe to confirm equal RNA loading.

Affinity Purification of DNA-Binding Proteins—A 5′ biotinylated oligonucleotide sequence (TGGTGAGCAACTGAGGATCCAGCCTTGTGATAGAGA) was annealed to a complementary non-biotinylated oligonucleotide to create a double-stranded biotinylated probe containing both of PRRIII STAT binding sites. Non-biotinylated competitors encompassing the same region (Competitor A) or only a single STAT element (Competitor B) were also annealed to form double stranded competitor probes. Nuclear extracts (400 μg) prepared from cytokine-stimulated activated T cell cultures were incubated in lysis buffer (50 mM Tris, pH 8, 0.5% Igepal, 15 mM NaCl, 0.1 mM EDTA, 10% glycerol, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μM leupeptin) in the presence or absence of 2 μg of the annealed non-biotinylated competitors at 4 °C for 1 h. Biotinylated probe (2 μg) was subsequently added and the mixture incubated overnight at 4 °C. The probe and proteins were recovered by incubation with 30 μl of streptavidin-agarose beads. The beads were washed twice in lysis buffer and then boiled in gel loading buffer (2% SDS, 10% glycerol, 50 mM Tris, pH 6.8, 2% β-mercaptoethanol). The eluted proteins were separated by SDS-PAGE on 10% gels and detected by Western blotting using anti-STAT4 and anti-STAT5 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Chromatin Immunoprecipitation Assays (ChIP)—Following the indicated cytokine stimulation, cells (5 × 106/ml) were incubated in 1% formaldehyde for 10 min and the cross-linking subsequently quenched by the addition of glycine to a final concentration of 0.125 M. Cells were washed in phosphate buffered saline, harvested by centrifugation, and resuspended in cell lysis buffer (as described above but containing 150 mM NaCl) on ice for 10 min. The soluble chromatin was sheared by sonication to yield fragments less than 500 bp. Following centrifugation the soluble chromatin supernatant (from 8 × 106 cells) was subject to immunoprecipitation with 5 μg of the indicated antibody (antibodies to STAT4, STAT5, and c-Jun) were purchased from Santa Cruz; antibodies to modified histones and CBP were purchased from Upstate Biotechnology, Lake Placid, NY) overnight at 4 °C as described previously (34). Immune complexes were harvested by incubation with 50 μl of protein A-Sepharose beads (Upstate Biotechnology) at 4 °C for one hour and a series of washes as follows: twice with 1 ml of 20 mM Tris, pH 8, 2 mM EDTA, 0.2% SDS, 0.5% Triton X-100, and 150 mM NaCl, twice with 20 mM Tris, 0.2% SDS, 0.5% Triton X-100 and 500 mM NaCl, and twice with 1 ml of 20% TE. Immunoprecipitated material was eluted twice from the beads with 50 μl of 20% TE containing 2% SDS at 37 °C for 10 min each. A total of 0.5 μl of 20% TE was added to the pooled eluates, and proteins were digested by the addition of 100 μg proteinase K (Roche Applied Science). Following incubation at 37 °C for 30 min, formaldehyde cross-links were reversed by incubation at 65 °C for 4 h. 3 mM sodium acetate (40 μl) was added, and the mixture was extracted with phenol-chloroform. Glyoxen (5 μl of a 10 mg/ml solution) was added as a carrier, and DNA was recovered by ethanol precipitation and resuspended in 10 μl of TE for subsequent PCR analysis. Controls were performed with the PCR4-CD25 plasmid, which contains the regions between −1740 to +117 relative to the murine CD25 promoter in the PCR4-TOPO vector (Invitrogen).

Quantitative PCR—Quantitative PCR reactions were performed as previously described (35). 7% PAGE (23 cycles of 95 °C for 0.5 min, 60 °C for 0.5 min, and 1 min at 72 °C) were performed. Aliquots of PCR mixtures were subjected to electrophoresis on 8% polyacrylamide gels in 1× TBE. Signals were then visualized by autoradiography. The primers used were as follows: PRRIII forward (5′-AAAGGAGGACATCTGGTGCAGATA-3′) and PRRIII reverse (5′-GTTCACTGTATAAAAGCCTGAGCTC-3′) amplifying a fragment from 1417 to 6340 °C for 20 min and in 0.1 M HEPES, 10 mM MgCl2, 0.2 mM MgCl2, 0.2% SDS, 0.5% Triton X-100, and 150 mM NaCl, twice

Quantitation of ChIP Assays Using Real-time PCR—Immunoprecipitated DNA samples from the ChIP assays (1 μl) were analyzed using real-time PCR (ABI Prism 7700 Sequence detection system). FAM-labeled LUX fluorogenic primers (Invitrogen) were created to amplify both the PRRIII and TSS regions (PRRIII forward, 5′-GGTGGACCATCTCTCTATGTTA-3′; PRRIII reverse, 5′-ACACCTGACTTCTCTCTCTGCCTCTCTCTC-3′) and 5′-UTR reverse (5′-GTTGAGCAACTTCTCTTGGTGATAGA-3′). PCR reactions were performed using Platinum Quantitative PCR SuperMix-UDG with Ross reference dye (Invitrogen) and the amount of product determined relative to a standard curve.

Chromatin Immunoprecipitation—Extracts were incubated with c-Jun antibody (Santa Cruz Biotechnology) and precipitated as previously described (35). Immunooblots were performed with antibodies to Stat4, phosphorylated Stat4, and c-Jun (BD Biosciences).

RESULTS

IL-12 Induces CD25 Expression in a STAT4-dependent Manner—It has been previously shown that IL-12 stimulation of activated lymphocytes leads to increased expression of CD25 (36–38). To assess the requirement for STAT4 proteins in gene induction we examined the IL-12-stimulated expression of CD25 on CD4+ T cells. Spleen cells from wild-type and STAT4-deficient mice were activated with anti-CD3 for 72 h. Cells were then washed and incubated overnight with anti-IL-2 and in the presence or absence of IL-12. The cells were then stained with antibodies to CD4 and CD25 and analyzed by FACS to examine expression of CD25 on CD4+ T cells. As expected from previous studies, IL-12-induced expression of CD25 (Fig. 1A). This increased expression is STAT4-dependent as no induction is observed in STAT4-deficient (c-Jun−/−) cells. By contrast, IL-2-induced similar expression of CD25 in wild-type and
STAT4 activates T cells (35). Northern analysis of IL-12-stimulated wild-type activated T cells demonstrates that CD25 mRNA levels increase in cells post-IL-12 stimulation with levels peaking after four hours (Fig. 1B). This high level gradually decreases and returns to background between 12–24 h post-IL-12 stimulation. Northern blot analysis of activated T cells stimulated for four hours in the absence or presence of IL-12 showed a marked increase in the level of CD25 mRNA in wild-type cells, contrasting only minimal changes in STAT4−/− cells (Fig. 1C). To determine whether both STAT4 isoforms are capable of inducing this response, cells from CD2:STAT4−/− and CD2:STAT4α and STAT4β transgenic mice generated as in A following a 4-h incubation with 5 μg/ml anti-IL-2 and in the presence or absence of 2 ng/ml IL-12. Following transfer to nylon membranes, RNA was hybridized with a radiolabeled CD25 cDNA probe. The blot was stripped and re-probed with T cell receptor α as a control for loading.

STAT4 binds PRRIII in vitro — As described in the Introduction, the promoter of the CD25 gene is well characterized. An IL-2 response element (PRRIII) has been shown to bind STAT5, which is required to mediate gene induction (23, 25, 39). Having established that CD25 expression is induced by IL-12 in a STAT4-dependent manner, we next wanted to identify whether STAT4 binds motifs contained within PRRIII. Nuclear extracts were prepared from wild-type activated splenocytes incubated with either anti-IL-2, in the presence or absence of IL-12, or treated with IL-2. To determine whether STAT4 could bind PRRIII sites in vitro, each of the nuclear extracts was incubated with biotinylated oligonucleotide encompassing both of the STAT binding sites contained within the murine PRRIII (Fig. 2A). Bound proteins were recovered and characterized by Western blotting. STAT4 protein was recovered from cell extracts treated with IL-12, but not from extracts treated with either anti-IL-2 or IL-2 (Fig. 2B, upper panel). Thus, STAT4 can bind to PRRIII in vitro. Because STAT5 is known to bind to these STAT binding sites in response to IL-2 stimulation, as a positive control we also immunoblotted with anti-STAT5. STAT5 proteins were recovered from cell extracts treated with IL-2 (Fig. 2B, lower panel). In both cases this binding was eliminated if extracts were first incubated with a non-biotinylated competitor oligonucleotide encompassing both of the STAT binding sites contained within the murine PRRIII (Fig. 2A). Bound proteins were recovered using Trizol. Following transfer to nylon membranes the RNA was probed with radiolabeled CD25 cDNA. The blot was stripped and re-probed with glyceraldehyde-3-phosphate dehydrogenase as a control for loading. C, total RNA was isolated from activated T cells derived from wild-type, STAT4-deficient or STAT4α and STAT4β transgenic mice generated as in A following a 4-h incubation with 5 μg/ml anti-IL-2 and in the presence or absence of 2 ng/ml IL-12. Following transfer to nylon membranes, RNA was hybridized with a radiolabeled CD25 cDNA probe. The blot was stripped and re-probed with T cell receptor α as a control for loading.
STAT4 and STAT5 could still be purified using the biotinylated oligonucleotide (Fig. 2B). This suggests that the presence of both sites is required for STAT binding, presumably due to the formation of tetramers to stabilize binding (25, 26). This correlates with previously published data (23, 25) that demonstrated neither STAT binding site individually could activate transcription and that both sites are required for IL-2 responsiveness.

**STAT4 Binds to PRRIII and the TSS in Vivo**—To confirm that STAT4 exerts its effect on CD25 transcription by binding to PRRIII, we studied the distribution of STAT4 in vivo using ChIP assays. Activated splenocytes were generated as in the figure and incubated with anti-IL-2, in the presence or absence of IL-12, or treated with IL-2. The cells were stimulated with the appropriate cytokines for a period of four hours as this is the time point at which maximal amounts of CD25 mRNA are observed by Northern blot analyses (Fig. 3). These cells were then treated with formaldehyde and a soluble chromatin fraction prepared. This chromatin was subsequently sheared by sonication into fragments with an average size of 600 bp and subjected to immunoprecipitation using various antibodies. DNA was recovered from the precipitated chromatin and subjected to quantitative PCR analysis using primer pairs specific to a region spanning the TSS and to PRRIII. Control reactions containing decreasing amounts of cloned target DNA (pCR4-CD25) are shown for each primer pair used (Fig. 3), enabling validation of the quantitative nature of the PCR reactions.

At the TSS and PRRIII regions strong positive signals were observed in anti-STAT4 ChIP assays from wild-type cells treated with IL-12 (Fig. 3). The specificity of these signals was confirmed by the lack of product obtained in anti-STAT4 ChIP assays using wild-type cells that were either unstimulated or treated with IL-2. No signal was observed from anti-STAT4 ChIP assays from IL-2-stimulated wild-type cells, but were absent from ChIP assays using unstimulated or IL-12-stimulated cells. STAT5 was also present at the TSS and PRRIII regions in IL-2-stimulated cells from STAT4-deficient mice.

ChIP assays were also performed using antisera against acetylated histone H4. Low levels of product were observed using primers specific to the PRRIII region in unstimulated wild-type and STAT4-deficient cells. Strong positive signals were seen at both the TSS and PRRIII regions in IL-12 and IL-2-stimulated wild-type cells (Fig. 3). No increase in histone H4 acetylation was observed in IL-12-stimulated, STAT4-deficient cells (Fig. 3), consistent with the lack of increased CD25 mRNA observed under the same conditions (Fig. 1C). However, an increase in histone H4 acetylation is still observed in IL-2-stimulated STAT4-deficient cells. Taken together these results demonstrate that both IL-12- and IL-2-mediated induction of CD25 is associated with STAT protein binding and an increase in histone acetylation within the CD25 promoter.

**Upon IL-12 Stimulation the Association of STAT4 to PRRIII and the TSS Correlates with Chromatin Remodeling**—To look in greater detail at the events involved in CD25 induction post-IL-12 stimulation, ChIP assays were performed over a 24-hour time course, using anti-CD3 activated wild-type cells. PCR reactions were performed using each set of primers and a cloned plasmid containing the target sequence, as well as 10% of the input DNA to evaluate the linear range of the signals observed (Fig. 4A, rows 1 and 2). Additionally, the specificity of the signals obtained was confirmed by the low level of signal produced using pre-immune sera (Fig. 4A, row 3).

In unstimulated cells, no STAT4 is found at either the TSS or PRRIII. At 2 h post-IL-12 stimulation STAT4 is observed associated with both of these regions. This binding, although reduced, is still maintained for a further 2 h but is dramatically reduced by 6 h post-stimulation (Fig. 4). Similarly, in unstimulated cells, CBP, a histone acetyltransferase known to associate with several STAT proteins (40–44), was not associated with the TSS or PRRIII regions. However, at 2 h post-stimulation, CBP was found at the TSS and PRRIII regions, coinciding with the appearance of STAT4. Over the 24-h time course the pattern of CBP binding was similar to that of STAT4. To more accurately quantify these changes in TSS and PRRIII occupancy, we used real-time PCR on STAT4 and CBP ChIP assays.
and observed the same pattern of binding (data not shown). Thus, STAT4 and CBP are transiently associated with the CD25 promoter.

As previously mentioned low levels of acetylated histone H4 are observed at PRRIII in unstimulated cells. However, levels at both the TSS and PRRIII increase dramatically at 2 h post-stimulation and these increased levels are maintained over a 24-hour period. A similar pattern is observed for acetylated histone H3 but at the PRRIII region the increase in signal is less pronounced due to high basal levels of acetylation observed in unstimulated cells. In unstimulated cells significant amounts of dimethylated histone H3K9 are observed in ChIP assays at PRRIII. The signal observed decreases 4 h post-stimulation and remains low up to 24 h after IL-12 treatment. Much lower levels of PCR product are obtained at the TSS, but these low levels are eliminated by 4 h post-stimulation and have still not been restored after 24 h. Taken together this data suggest a kinetic model of the events involved in IL-12-mediated induction of CD25 transcription.

c-Jun Interacts with STAT4 at PRRIII in Vivo—The expression of most genes is activated only when several transcription factors bind to distinct sites within the enhancer regions, and in turn bind coactivators, which facilitate contact with the transcription machinery. We wanted to investigate whether STAT4 was interacting with other transcription factors to mediate CD25 transcription. STAT4 is known to cooperatively interact with c-Jun to induce several genes such as IFN-γ and IRF-1 (16, 45).

ChIP assays were performed using anti-c-Jun antibodies and both wild-type and STAT4-deficient cells that had been stimulated with IL-12 for varying periods of time (Fig. 5 A). PCR products were detected using primer pairs specific to PRRIII, and as a negative control, to the 3′-UTR. No signal was detected using the primers for the 3′-UTR. Surprisingly, strong positive signals were detected at PRRIII in wild-type cells post-IL-12 stimulation, which were absent in STAT4-deficient cells. As there are no recognizable AP-1 binding sites within the PRRIII region this suggests that c-Jun may be present in the immunoprecipitate due to an interaction with STAT4. To demonstrate the interaction of Stat4 and c-Jun in vivo, activated splenocytes were treated with or without IL-12 and protein extracts were immunoprecipitated with anti-c-Jun. Immunoblotting of precipitates demonstrated that Stat4 was present in c-Jun immunoprecipitates, regardless of the activation state of Stat4 (Fig. 5, B and C). Thus, Stat4 interacts with c-Jun and can recruit c-Jun to an IL-12 responsive promoter.

DISCUSSION

Despite IL-12 being a critical regulator of inflammation and immune responses to infectious disease, very little is known...
about how STAT4 activates gene expression. We and others (35, 38) have identified CD25 as an IL-12 inducible and STAT4-dependent gene. We have now further explored the events coincident with STAT4 transactivation using CD25 as a model gene. CD25 was attractive as a model gene because its promoter elements have been extensively characterized and STAT5 is known to be critical for IL-2-induced expression. In this report, we have shown that STAT4 binds PRRIII in vitro and in vivo. Both isoforms of STAT4, STAT4α and STAT4β, are capable of mediating CD25 induction, suggesting that the C-terminal transactivation domain is not required for gene activation. Although there is histone acetylation in IL-12-stimulated wild-type cells, there is a lack of histone acetylation following IL-12 stimulation of STAT4-deficient T cells, suggesting that STAT4 mediates chromatin remodeling. Kinetic analysis of the IL-12-stimulated response demonstrates STAT4 promoter occupancy coincident with recruitment of CBP, histone acetylation, and remodeling of methylated histones. Furthermore, STAT4 is required for stable association of c-Jun to the CD25 promoter. Thus, STAT4 mediates gene induction by recruiting acetyltransferases allowing histone acetylation and also by interacting with other factors bound within a promoter complex.

CBP is well known as a coactivator that stimulates target gene transcription by promoting interactions between the basal transcription machinery and enhancer bound transcription factors, and for generating histone acetylation patterns that correlate with transcriptionally active chromatin (reviewed in Refs. 46 and 47). STAT4 has not been demonstrated to directly interact with the CBP. However, several STAT proteins associate with CBP and other histone acetyltransferases (40–44). Nmi (originally cloned as an N-myc interactor) interacts with STAT4, as it does with many STAT proteins and stabilizes the interaction of STAT1 and STAT5 with CBP (48), suggesting that it may function similarly with STAT4. The association of both STAT4 and CBP with PRRIII and the TSS region simultaneously would suggest that STAT4 is recruiting CBP to this gene to subsequently mediate histone acetylation.

Di-methylation of histone H3 (lysine 9) (dmH3K9) is associated with the formation of heterochromatin and long-term transcription repression. A subset of inflammatory genes have been reported to contain low constitutive levels of this modification at their promoter regions, which is erased upon transcriptional activation and restored as gene expression decreases (49). A similar pattern is observed within the TSS region. Low levels of dmH3K9 are eliminated by 4 h post-IL-12 stimulation at the TSS region. Much higher amounts of dmH3K9 are observed at the PRRIII initially and these levels are also decreased following IL-12 stimulation. Because there are no known histone demethylases, it remains unclear how the removal of dmH3K9 occurs at inducible loci. There may be an active process of methylated histone exchange for unmethylated histones over the course of gene induction. In that respect, it is interesting to note that several histone genes are induced by IL-12 stimulation (16).

STAT4 is known to interact cooperatively with c-Jun to enhance its binding at the IRF-1 and IFN-γ promoters (16, 45). We observed binding of c-Jun to PRRIII, which does not contain any AP-1 sites (Fig. 5A). These results suggest that c-Jun is present at PRRIII due to an interaction with STAT4, supported by coimmunoprecipitation of c-Jun and STAT4 in primary activated T cells (Fig. 5B). Because c-Jun was previously reported to interact with CBP/p300 (50, 51) it may also play a role in STAT4-mediated histone acetylation. The association of c-Jun at the PRRIII element, where an AP-1 element has not been described, as well as the association of STAT4 at the TSS, where a STAT binding site has not been identified, further suggests a higher order promoter structure wherein STAT4, c-Jun, CBP, and the transcriptional machinery are physically close to one another. A model of the proposed complex is presented in Fig. 6. The interaction of these factors may mediate looping of the promoter allowing apposition of distant elements, such as PRRIII to the transcriptional start site (21) (reviewed in Refs. 52 and 53).
We have demonstrated that the IL-12-induced expression of CD25 is dependent upon STAT4. The kinetics of STAT4 binding to the CD25 promoter is somewhat different from what has been seen for STAT1 and STAT2 promoter binding. ChIP assays demonstrated that STAT1 binding to the CD25 promoter is much stronger and reaches maximal levels within 30 min, falling thereafter (54, 55). We observed little STAT4 binding within the first hour, high levels bound by 2 h with levels decreasing thereafter (54, 55). We observed little STAT4 binding within the first hour, high levels bound by 2 h with levels decreasing thereafter (54, 55). We observed little STAT4 binding within the first hour, high levels bound by 2 h with levels decreasing thereafter (54, 55). We observed little STAT4 binding within the first hour, high levels bound by 2 h with levels decreasing thereafter (54, 55). 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STAT4 Is Required for Interleukin-12-induced Chromatin Remodeling of the CD25 Locus
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