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*J Immunol* 2011; 187:3292-3299; Prepublished online 10 August 2011;
doi: 10.4049/jimmunol.1003173

http://www.jimmunol.org/content/187/6/3292

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/08/10/jimmunol.1003173.DC1

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Two-Step Binding of Transcription Factors Causes Sequential Chromatin Structural Changes at the Activated IL-2 Promoter

Satoru Ishihara*† and Ronald H. Schwartz*

Most gene promoters have multiple binding sequences for many transcription factors, but the contribution of each of these factors to chromatin remodeling is still unclear. Although we previously found a dynamic change in the arrangement of nucleosome arrays at the IL2 promoter during T cell activation, its timing preceded that of a decrease in nucleosome occupancy at the promoter. In this article, we show that the initial nucleosome rearrangement was temporally correlated with the binding of NFAT1 and AP-1 (Fos/Jun), whereas the second step occurred in parallel with the recruitment of other transcription factors and RNA polymerase II. Pharmacologic inhibitors for activation of NFAT1 or induction of Fos blocked the initial phase in the sequential changes. This step was not affected, however, by inhibition of c-Jun phosphorylation, which instead blocked the binding of the late transcription factors, the recruitment of CREB-binding protein, and the acetylation of histone H3 at lysine 27. Thus, the sequential recruitment of transcription factors appears to facilitate two separate steps in chromatin remodeling at the IL2 locus. The Journal of Immunology, 2011, 187: 3292–3299.

T he initiation of gene transcription requires the binding of transcription factors to promoters and/or enhancers in the target gene. These regulatory cis elements are usually packaged into chromatin, which presents an obstacle to their accessibility for transcription factor binding. The open/close switch for access cannot be simple, because DNA strands are folded into chromatin at several levels (1, 2). This process has been studied using inducible transcription systems. For example, cultured cells carrying a high-copy number locus with a hormone-responsive element revealed enlargement of stained foci cytologically, in parallel with binding of nuclear receptors after hormone exposure (3, 4). This chromatin decondensation appears to be at the level of the 30-nm fiber or higher order structures. In contrast, the binding of a complex including RelA to the promoter of the IFN-β gene caused repositioning of a nucleosome to expose the TATA box to basal transcription factors after viral infection (5, 6). RAR/RXR nuclear receptors are also known to be capable of sliding nucleosomes along a DNA strand after binding their agonists (7).

The IL-2 gene (IL2) is induced in T cells activated through the TCR by Ag displayed on the surface of an APC. It functions as a differentiation and growth factor for the T cell itself, as well as for other cells (8). T cell activation is known to trigger chromatin structural changes around the IL2 promoter region, such as alterations in nucleosome occupancy or an increase in accessibility to endonucleases (9–14). We recently described a new fractionation assay, based on the size of sheared, formaldehyde–cross-linked chromatin (designated the “SEVENS” [sedimentation velocity method followed by normalization in the size of the DNA] assay), that can be used to examine chromatin structure at promoter regions (15). This technique revealed a dynamic change in chromatin configuration at the IL2 locus after anti-CD3 and anti-CD28 stimulation of previously activated CD4+ mouse T cells (15). The change occurred predominantly around the minimal promoter/enhancer region, located near the transcription start site (TSS), where many well-known transcription factors bind for IL2 induction (16). Although cooperative occupancy by these factors has been observed with in vivo footprinting (17, 18), the relationship between these chromatin structural changes and the binding of the transcription factors is still unclear.

In this study, the kinetics of recruitment of NFAT1 and AP-1 is shown to correspond to the timing of the nucleosome rearrangement process seen in the SEVENS assay. After this initial step, occupancy of nucleosomes and linker histones was diminished at the IL2 promoter region. This second event was accompanied by the binding of other critical transcription factors and RNA polymerase II (Pol II), as well as the binding of the histone acetyltransferase (HAT) CREB-binding protein (CBP). Thus, there appears to be a two-step recruitment process in which “early-” and “late-binding” transcription factors are responsible for the sequential changes in chromatin structure observed around the IL2 promoter during T cell activation.

Materials and Methods

Mouse T cells and chemicals

All T cells were derived from B10.A, TCR-SC.C7 transgenic, Rag-2–deficient mice (female, 5–8 wk old) (19). Naive T cells were collected from...
lymph nodes. Preactivated resting T (PR-T) cells were prepared as described previously (15). For (re)activation of these cells, culture dishes precoated with 10 μg/ml anti-CD3ε Ab (clone 145-2C11; BD Biosciences) and a 1:2500 dilution of an ascites preparation of an anti-CD28 mAb (clone 37.51) were used (15). Cycloheximide (CHX), FK506, and SP600125 were used at 10 μg/ml, 10 nM, and 30 μM, respectively. CHX was added at time 0, whereas FK506 and SP600125 were added 30 min before activation. All of these chemicals were purchased from Sigma-Aldrich.

**IL-2 secretion assay**

An IL-2 secretion assay was performed using a Mouse IL-2 Secretion Assay Detection Kit (Miltenyi Biotec). Naïve T cells and PR-T cells were also stained with anti-CD4 Ab (clone GK1.5; BD Biosciences) and 7-amino actinomycin D (BD Biosciences). After gating out dead cells stained by the 7-amino actinomycin D, IL-2– and CD4-expressing cells were detected using a FACS Calibur (BD Biosciences).

**Western blotting**

A cell lysate in SDS lysis buffer (1% SDS, 50 mM Tris-HCl [pH 8.0], and 10 mM EDTA. Complete Protease Inhibitor Cocktail [#4693124; Roche]) was run on SDS-PAGE and then blotted onto a nitrocellulose membrane. After exposure to a primary Ab, a biotinylated secondary Ab (GE Healthcare), and a streptavidin-conjugated alkaline phosphatase (GE Healthcare), sequentially, the membrane was developed using Western Blue (Promega). The primary Abs used are shown in Supplemental Table I.

**Quantitative RT-PCR**

Extraction of mRNA and preparation of cDNA were performed as described previously (15). For quantitative PCR (qPCR), 25 (IL-2, β-actin, and 18S rRNA) or 250 pg (CD3δ, brain-derived neurotrophic factor [BDNF], and adenosine deaminase domain-containing 1 [Adad1]) cDNA was applied to one reaction using a QuantiFast SYBR Green PCR Kit (Qiagen) in a real-time PCR machine (ABI7900HT; Applied Biosystems). Because different primers have different PCR amplification efficiencies, we could not directly compare the values we measured by qPCR among different genes. Instead, we used mouse genomic DNA diluted serially (0.76–12,500 ng) as a standard for comparison. In addition, we performed qPCR for three different regions of each mRNA using primers specific for three different exons. Therefore, all primers for PCR were designed to amplify a coding region within a single exon. The relative amount of cDNA of interest was estimated from its cycle threshold (Ct) value plotted on a standard curve acquired from the Ct values of a diluted series of genomic DNA. These quantified amounts were normalized to the amount of ribosomal 18S RNA. The primers used are shown in Supplemental Table II.

**Chromatin immunoprecipitation assay**

For assays using anti-histone H3 Ab, PR-T cells were treated with 1% formaldehyde in PBS at room temperature for 10 min. In the other assays, PR-T cells were cross-linked with formaldehyde in PBS at 37 °C, brain-derived neurotrophic factor [BDNF], and adenosine deaminase domain-containing 1 [Adad1]) cDNA was applied to a 1:2500 dilution of an ascites preparation of an anti-CD28 mAb (clone 37.51) were used (15). Cycloheximide (CHX), FK506, and SP600125 were used at 10 μg/ml, 10 nM, and 30 μM, respectively. CHX was added at time 0, whereas FK506 and SP600125 were added 30 min before activation. All of these chemicals were purchased from Sigma-Aldrich.

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**The SEVENS assay**

The details of the SEVENS assay are described in our previous report (15). Primers for the specific DNA sequences used in these assays are shown in Supplemental Table II.

**Statistical analysis**

Statistical analysis was performed with Microsoft Excel. All data with error bars are presented as mean ± SD for at least three independent experiments.

**Results**

**Large induction of IL-2 mRNA from most preactivated T cells stimulated with anti-CD3ε and anti-CD28**

We used CD4+ cells isolated from TCR transgenic mice to study chromatin structural changes in inducible IL2 transcription. We activated the cells with plate-bound anti-CD3ε and anti-CD28 Abs, which only react with the T cells in the population and mimic stimulation through the TCR and the costimulatory receptor, CD28, respectively. To evaluate the homogeneity of activation of the T cell population for IL-2 production, we measured IL-2 secretion by flow cytometry using anti-IL-2 Ab. As shown in Fig. 1A (upper right panel), only ~7% of naïve T cells made IL-2 after 4 h of activation. To circumvent this low-response problem, we prepared a population of PR-T cells. When these cells were restimulated for 4 h, 84% of them secreted IL-2 (Fig. 1A, lower right panel). No secretion was observed before activation. Thus, PR-T cells appeared suitable for biochemical analysis of chromatin structural changes at the IL2 locus after synchronized T cell activation.

The kinetics of IL-2 mRNA induction was measured using a quantitative RT-PCR method, in which the qPCR values are normalized to the genomic DNA standard for each primer pair (Fig. 1B; see Materials and Methods). As shown in the red lines of Fig. 1, IL-2 mRNA was not detected in the PR-T cell population before activation. In 1 h of stimulation with anti-CD3ε and anti-CD28, the mRNA was easily detected. At 2 h, the induced level reached 53–60% of maximum in all three assays using different primer sets. The levels then peaked at 3 h. Although the signals from the primers for the fourth exon were 2- to 4-fold less than those from the first or third exon primers, all of the PCR values showed the same induction kinetics. To evaluate the relative amount of IL2 transcription, we also performed qPCR using primers annealing to β-actin (blue lines) and CD3δ (green lines). β-Actin and CD3δ mRNAs were expressed constitutively, although the mRNA level of β-actin was estimated to be ~40-fold that of CD3δ. The latter appeared to decrease ~4-fold after 4 h of T cell activation. We also carried out qPCR using primer sets for BDNF (21) or Adad1 (22). Expression of these repressed genes was not observed at all during the 4-h-stimulation period. Importantly, the induced level of IL-2 mRNA was comparable with the constitutive level of β-actin mRNA when the qPCR was performed with primers to either the first or third exons.

**Recruitment kinetics of Pol II and TATA-binding protein**

To evaluate transcriptional activity at the IL2 locus after T cell activation, we performed a ChIP assay using anti-Pol II Ab, which recognizes all Pol II proteins regardless of the phosphorylation state of the carboxy-terminal domain. As shown in Fig. 1C, the IL2 promoter in the precipitate from unactivated PR-T cells was at a background level, equivalent to the promoter of the nonexpressing Adad1 gene. This indicates that no Pol II proteins were bound to the IL2 TSS region before T cell activation, as reported previously (23). Subsequent activation, however, increased the Pol II level, reaching a maximum binding at 3 h. To confirm this timing, we also performed a ChIP assay using an anti-TATA-binding protein (anti-TBP), one component of the preinitiation complex (Fig. 1D). Its binding kinetics was identical to that of Pol II. These ChIP assays showed a good correlation with the expression level of IL-2 mRNA.

**Timing of alterations in nucleosome and linker histone occupancy at the IL2 promoter during T cell activation**

In our previous report, we used the new “SEVENS” assay to show that stimuli through the TCR and CD28 receptors on PR-T cells made nucleosomes around the IL2 promoter alter their arrangement. This structural change begins 1 h after stimulation and is almost complete by 2 h (15). Although the occupancy of nucleosomes at the IL2 promoter is known to diminish after T cell activation (13, 14), the relationship between this event and changes observed in the SEVENS assay was not clear. To explore this, we performed ChIP assays with an anti–pan-histone H3 Ab, using our PR-T cells and the anti-CD3ε/CD28 stimulus (Fig. 2A). Pre-
The precipitation of the \(\text{Il2}\) promoter region remained constant up to 1 h. Beyond this point, however, the ChIP value began to decrease, leveling off by 3–4 h at \(\sim 50\%\) of the starting level. We also performed ChIP assays using an anti-histone H1 Ab. As shown in Fig. 2B, the amount of the \(\text{Il2}\) promoter in the precipitate did not significantly change up to 2 h. Incubation for a 3rd hour, however, showed a 50–60% decrease in the level of the precipitated promoter, and this lower level was maintained at 4 h. This kinetics is similar to what was seen with the anti-histone H3 Ab. These observations suggest that both the nucleosomes and the linker histones alter their occupancy at the \(\text{Il2}\) promoter after T cell activation. Importantly, it took the core and linker histones 3 h to be altered, whereas the structural change seen in the SEVENS assay is almost complete within the first 2 h of activation (15). Therefore, we conclude that structural activation at the \(\text{Il2}\) promoter is composed of at least two separable changes: an initial remodeling event and a subsequent alteration in nucleosome occupancy.

Kinetics of the recruitment of transcription factors to the \(\text{Il2}\) promoter

The first 300-bp region of the \(\text{Il2}\) promoter upstream from the TSS has been well characterized, and many binding sites for transcription factors have been mapped there, as shown in Fig. 3A (16). To explore the relationship between the binding of these transcription factors and the sequential chromatin structural changes, we investigated the recruitment kinetics of the transcription factors using ChIP assays.

For the NF-\(\kappa\)B/Rel family, the recruitment of RelA and c-Rel to the \(\text{Il2}\) promoter was examined (Fig. 3B,3C). Before activation, both Abs hardly precipitated the \(\text{Il2}\) promoter region. Stimulation with anti-CD3\(\varepsilon\)/CD28 increased the binding of both proteins to the \(\text{Il2}\) promoter, reaching a maximum level by 3 h. No enhancement was seen at the \(\text{Adad1}\) promoter over the same period. Similar results were observed for Oct2 and NFAT2 (Fig. 3D,3E). Fig. 3I shows the percentage binding occupancy of these proteins over time (see legend for Fig. 3 for calculation). In all cases for this group of proteins, \(\sim 25\%\) of the maximal level was bound to the \(\text{Il2}\) promoter after 1 h of activation. Although binding levels increased over the next hour, occupancy was still only 46–71% of the maximum, which was only reached at 3 h. These kinetics are similar to that for the formation of the Pol II complex (summarized in Fig. 3I). Importantly, because changes in occupancy of nucleosomes at the \(\text{Il2}\) promoter also required activation for 3 h (Fig. 2A), the recruitment of these transcription factors appeared...
to occur in parallel with or just before this nucleosomal alteration rather than with the initial rearrangement of nucleosomes seen in the SEVENS assay.

We next examined the recruitment kinetics of NFAT1 (Fig. 3F). The binding of NFAT1 to the Il2 promoter was greatly increased within 1 h, and the level for 2-h-activated cells was 83% of the maximum seen after activation for 3 h. Surprisingly, this pattern was not comparable with that of NFAT2 (Fig. 3F versus 3E). Thus, the recruitment of NFAT1 apparently preceded that of NFAT2. Finally, to examine the recruitment of AP-1 (Fos/Jun heterodimer), we performed ChIP assays using anti–pan-Jun and antifra-2 Abs (Fig. 3G, 3H). For both proteins, activation for only 1 h significantly increased the precipitated Il2 promoter, and the recruitment at 2 h almost reached the maximum level (95% for Jun and 87% for Fra-2). The recruitment of FosB showed a similar kinetics (data not shown). The parallel recruitment of Fos and Jun proteins suggests that we are observing the binding of AP-1 to the Il2 promoter. In addition, its kinetics are similar to that of NFAT1, implying that the well-known NFAT1/AP-1 complex assembles early on the activated Il2 promoter (24). These kinetics are also similar to the early chromatin remodeling seen with the SEVENS assay, and thus raised the possibility that these two events were linked. As summarized in Fig. 3I, these careful ChIP analyses clearly subdivide the DNA-binding transcription factors required for Il2 transcription into two discernible groups: early- and late-binding proteins.

**Inhibition of protein synthesis prevents the nucleosome rearrangement at the Il2 promoter**

Because the Jun and Fos proteins are known to be induced by various extracellular stimuli such as hormones and stress (25), we evaluated the level of expression of these proteins, as well as the other transcription factors, in the PR-T cells (Fig. 4A). Only Fos proteins, observed with an anti–pan-Fos Ab, were completely absent in unactivated PR-T cells. After activation, Fos proteins were detected first at 0.5 h, and both c-Fos and FosB/Fra-2 (indistinguishable bands) reached a maximum between 2 and 3 h. All the transcription factors other than Fos proteins were constitutively expressed, although the levels of Jun, c-Rel, and Oct2 increased somewhat during the activation period. To verify that AP-1 is required for the initial nucleosome rearrangement, we used CHX, a general translation inhibitor, to create activated PR-T cells lacking all Fos proteins. Under these conditions, Il2-transcripts were not detected (Supplemental Fig. 1A). Fig. 4B shows that 1-h-activated PR-T cells treated with CHX did not show induction of any Fos proteins, although Jun proteins were still expressed at a basal level. In the SEVENS assay (Fig. 4C), the Il2 promoter was distributed evenly throughout all the fractions of the gradient (Fig. 4C, closed bars). This pattern is different from that observed in 1-h-activated PR-T cells without CHX, showing enrichment of the Il2 promoter in the upper fractions (Fig. 4C, open bars), similar to the open chromatin pattern seen for the Actb promoter (Supplemental Fig. 2A). These observations indicate that CHX...
blocked the structural changes seen in the SEVENS assay around the Il2 promoter after T cell activation.

Contribution of dephosphorylation of NFAT1 to the initial chromatin rearrangement around the Il2 promoter after T cell activation

After TCR signaling, NFAT1 is dephosphorylated by calcineurin and then translocated to the nucleus (26, 27). This event is well-known to be blocked by the pharmacologic inhibitor, FK506 (26, 28). Western blotting experiments were performed to confirm its effect on PR-T cells (Fig. 5A). The transcript from the Il2 gene was barely detectable in the drug-treated cells (Supplemental Fig. 1B). In the SEVENS assay, FK506 treatment resulted in an even fractional distribution of the Il2 promoter in 3-h-activated cells (Fig. 5C, yellow bars). This is different from FK506-un treated but activated cells (Fig. 5C, blue bars) and corresponds to what is seen in unactivated PR-T cells (Fig. 5C, red bars). In contrast, the distribution pattern of the Actb promoter was not affected by the drug (Supplemental Fig. 2B, open bars). Thus, FK506 specifically blocked the nucleosome rearrangement around the Il2 promoter. Taken together with the rapid recruitment of NFAT1 to the promoter, the data argue that activation of this transcription factor is required for the initial change in the chromatin structure at the Il2 promoter. In addition, changes in occupancy of nucleosomes and linker histones at the Il2 promoter were not observed, consistent with their being downstream events (Fig. 5D, arrows over the yellow bars).

An inhibitor of Jun phosphorylation prevents the changes in nucleosome occupancy, CBP and late transcription factor binding, and histone acetylation, but not the initial chromatin rearrangement

The activity of c-Jun is believed to require phosphorylations at serine residues 63 and 73 by JNK to respond to various stimuli (29, 30). To explore the contribution of these phosphorylation events to the chromatin structural changes, we used SP600125, a chemical that inhibits JNK activity (31). The effect of this drug was confirmed in Western blotting (Fig. 5B). When the drug-pretreated, activated cells were applied to the SEVENS assay, we still observed enrichment of the Il2 promoter into the upper fractions of the gradient (Fig. 5C, green bars). This indicates that SP600125 did not greatly affect this chromatin structural change, despite its 90% inhibition of IL-2 transcription (Supplemental Fig. 1B). Thus, although induction of Fos, the partner of Jun in an AP-1 complex, is required for the initial chromatin changes (Fig. 4C), the phosphorylation of c-Jun is not essential for this step. Instead, SP600125 blocked the changes in nucleosome and linker histone occupancy at the Il2 promoter after T cell activation (Fig. 5D, arrowheads over the green bars). In addition, the drug blocked the enhanced binding of all the “late-binding” transcription factors as measured in ChIP assays 3 h after T cell activation (Fig. 6B–E, green bars). Pol II and TBP also did not bind to the promoter in the drug-treated cells (data not shown). The selective effect of SP600125 supports a two-step model for the chromatin remodeling in which the phosphorylation of serines 63/73 in c-Jun is required for the transition between the first and second steps.

Phosphorylated c-Jun is known to bind to CBP, which possesses HAT activity (30). To evaluate the contribution of this protein, we performed ChIP assays using an anti-CBP Ab. Recruitment of CBP to the Il2 promoter was indeed observed in 3-h-activated PR-T cells (Fig. 6F, blue bar). Furthermore, treatment with SP600125 prevented CBP from being recruited (Fig. 6F, green bar). In addition, ChIP assays with an Ab specific for histone H3 acetylation at lysine 27 showed an enhancement in this histone modification 3 h after T cell stimulation (Fig. 6G). This too was blocked by SP600125. In contrast, Brahma-related gene 1 (Brg1), a SWI/SNF chromatin remodeling factor, and histone H2A.Z, a variant for histone H2A, which are both associated with chromatin structural changes at activated loci, were not enriched at the Il2 promoter on T cell activation (Fig. 6H, 6I). These observations suggest that CBP and its possible product, acetylation of lysine residue at position 27 of histone H3 (H3K27ac), are involved in facilitating the binding of the late transcription factors and diminishing the nucleosomal occupancy at the activated Il2 promoter.

Discussion

We have recently described a new assay, designated “SEVENS”, which is able to estimate the local compactness of chromatin via the frequency of chemical cross-linking (15). Using this assay, we found that the density of nucleosomes in the vicinity of the Il2 promoter begins to decrease within 1 h when PR-T cells are stimulated with anti-CD3ε and anti-CD28 Abs. This change is almost complete in the cell population by 2 h (15). There is also a decrease in the nucleosome occupancy at the Il2 promoter as observed in a ChIP assay with anti-histone H3 Ab; however, this process takes 3 h to show a significant change (Fig. 2). This 1-h time lag indicates that the alteration in nucleosome occupancy follows the structural change seen in the SEVENS assay, suggesting that two distinct changes in the chromatin structure are occurring sequentially. Because the DNA helix in the cell is folded into high-order structures such as 30-nm fibers and chromatin...
The factors required for IL-2 transcription have been reported to work cooperatively (17, 18). Our ChIP assays, however, suggest a two-step process in transcription complex assembly, with early- (NFAT1, Jun, and Fos) and late-binding factors (Oct2, RelA, c-Rel, and NFAT2). The Jun and Fos families, known as immediate early genes, harbor a paused preinitiation complex even before the cells receive a stimulus (32, 33). In our PR-T cells, the Jun proteins are already expressed before activation. Thus, the rate-limiting step is the induction of the Fos proteins, which begin expressing by 0.5 h after stimulation (Fig. 4A). In contrast, expression of IL-2 mRNA appears to take a longer time. RNA polymerase is not dwelling on this promoter at time 0 (Fig. 1C), indicating that this gene is transcribed as a secondary response gene in T cell activation. In addition, the factor binding required for its transcription occurs in two sequential steps. The early-binding factors reach >80% of maximum levels within 2 h of IL-2 induction (Fig. 3I). This recruitment kinetics corresponds to that of the initial nucleosome rearrangement seen in the SEVENS assay. Disruption of Fos production (by CHX) or prevention of nuclear mobilization of NFAT (by FK506) blocked this initial nucleosome rearrangement (Figs. 4C, Fig. 5C, yellow bars), suggesting that these two factors are required for the event. Perhaps this is a general function of poised genes. After this step, the late-binding factors seem to be capable of recognizing their target sequences in the preopened chromatin. This, in turn, presumably results in the alterations in nucleosome occupancy at the II2 locus. This second step is blocked by the JNK inhibitor SP600125, which prevents Jun phosphorylation and CBP binding. However, why there is a second rate-limiting step, which delays the late-factor binding for an average of an hour, is still not clear. A similar two-component process for the binding of transcription factors to the II2 promoter (observed by EMSA) was reported after forskolin addition to EL4 cells (17). The elevation of cAMP disrupted NF-κB and TGGGC binding activities after T cell activation, but not that of NFAT and AP-1. Thus, there appears to be a checkpoint after NFAT1/AP-1 binding where activation of the II2 locus can be prevented by inhibiting the second step in various ways. Finally, transcriptional activation at the IL-1β promoter also occurs in a multistep process involving first the recruitment of PU.1 and C/EBPβ, next the recruitment of a third factor IFN regulatory factor 4, and then the binding of RNA polymerase (34). Thus, sequential binding of transcription factors might be the normal series of events for activation of inducible cytokine genes.

How do the early-binding transcription factors work for the initial chromatin rearrangement around the II2 proximal promoter? The NFAT1, c-Jun, and c-Fos proteins have been reported to interact with CBP, a coactivator that includes an HAT activity (30, 35, 36). Although CBP does, in fact, get recruited to the II2 promoter after T cell activation (Fig. 6F), this binding was inhibited by SP600125, a drug that could not block the early changes in the SEVENS assay (Fig. 5C). Therefore, CBP and its H3K27 acetylation are dispensable for the first step. We also did not detect any increase in acetylation of histone H3 at lysines 9/14 loops (1, 2), sequential changes in structure to unveil the II2 locus after induction would seem plausible. For example, after relieving compression of chromatin structure through decompaction, the nucleosomes could then be free to reposition their location on the DNA or alter their exchange rates.

The prevention of dephosphorylation of NFAT1 in FK506-treated PR-T cells was confirmed by Western blotting. The arrow or the arrowhead denotes the dephosphorylated or the phosphorylated form of NFAT1, respectively. B. The prevention of phosphorylation of c-Jun in SP600125-treated cells was confirmed by Western blotting using anti–phospho-c-Jun (serine 63). C. The SEVENS assays for 3-h–activated PR-T cells revealed that the pretreatment with FK506 (yellow bars), but not SP600125 (green bars), blocked the enrichment of the II2 promoter (PCR position: −77 to +39) in the upper fractions. For comparison, the distribution of the II2 promoter in unactivated (red bars) or 3-h–activated drug-nontreated cells (blue bars) is shown. The distribution of the Actb promoter was not affected by either of the drugs (Supplemental Fig. 2B). D. ChIP assays using anti-histone H3 (left panel) or H1 (right panel) showing that FK506 and SP600125 prevented core/linker histones from being removed from the II2 promoter after 3-h activation (arrows and arrowheads, respectively). As controls, the constant level of these histones binding to the Actb or Adad1 promoters was monitored. Because of different lots of the Abs used, the actual values in this figure are not the same as those shown in Fig. 2.
FIGURE 6. ChIP assays to examine the recruitment of the “late-binding” transcription factors and chromatin-related factors to the Il2 promoter (PCR position: −220 to −111). A. The culture conditions are represented in this key by four different colors. Red is cells without activation and drugs; blue is cells activated without drugs for 3 h; yellow is cells activated for 3 h in the presence of 10 nM FK506; and green is cells activated for 3 h in the presence of 30 μM SP600125.

B–E, The recruitment of the late-binding factors RelA (B), c-Rel (C), Oct2 (D), and NFAT2 (E) was blocked by SP600125, as well as FK506. F, CBP, a coactivator with HAT activity, bound to the Il2 promoter on T cell activation. Treatment with either FK506 or SP600125 blocked this recruitment. G, Histone H3 was acetylated at lysine 27 in the activated PR-T cells. Similar to the pattern for CBP, the level of acetylation was not increased when either of the drugs was added to the culture. These values are normalized by total amount of histone H3. H and I, TCR activation did not enhance Brg1 binding (H) or enrich for the presence of H2A.Z (I) at the Il2 locus.

or histone H4 at lysines 5/8/12/16 (15). Thus, acetylation of histones is unlikely to be responsible for the chromatin rearrangement, although there is still the possibility that other histone modifications we have not examined contributed to this change. rAP-1 has been reported to make reconstituted nucleosomes accessible to a nuclease, before the binding of another transcription factor (37). Taken together with the whole-genome analysis showing colocalization of AP-1 with a pioneer factor, FoxA1 (38), AP-1 may collaborate with such a factor to loosen chromatin structure at the activated Il2 promoter. Interestingly, the initial nucleosome rearrangement appeared to require NFAT1, but not NFAT2, as reflected in their different recruitment kinetics (Fig. 3f). Such separable binding of NFAT family members has also been observed in human Jurkat cells stimulated with PMA and ionomycin (39). Only NFAT1 has a unique C-terminal domain, which is reported to function as a transactivation domain, as well as an interaction domain with Jun (39, 40). This domain may contribute to the initial change at the Il2 promoter. In addition, NFAT1 is known to interact with Brg1 and to recruit it to the promoter region of the IFN-γ locus after activation of T cells (41). However, our ChIP assays failed to detect the recruitment of Brg1 to the Il2 promoter after activation (Fig. 6H). Nonetheless, other family members, such as Brahma, could bind to the Il2 promoter of activated T cells and contribute to the change seen in the SEVENS assay. Finally, NFAT1 has been reported to facilitate the upregulation of c-Rel expression by binding directly to its promoter (42). Thus, NFAT1 could be responsible for the late enhancement of c-Rel expression we observed (Fig. 4A), and this, in turn, could be the second rate-limiting step for IL-2 transcription (14).

The nucleosomes were diminished around the Il2 proximal promoter as the second change detected after T cell activation. This structural change occurred with a similar kinetics to that of Pol II binding to the Il2 promoter. This reciprocal relationship is consistent with whole-genome analyses in human T cells showing that expressed loci generally lack nucleosomes just upstream of the TSS, which instead is occupied by a Pol II complex (43). This change was blocked by both FK506 and SP600125 (Fig. 5D). The FK506 effect presumably results from disruption of the initial chromatin remodeling step and thus also prevents any further downstream events. SP600125, however, hardly affects the initial step (Fig. 5C, green bars); instead, the Jun phosphorylations seem to be required for the second step. The major target of JNK phosphorylation is c-Jun (44); however, its DNA binding to target genes does not depend on its phosphorylation (45). Therefore, it is possible that c-Jun proteins have a dual function. First, nonphosphorylated c-Jun would bind and loosen the chromatin structure, and then, after JNK phosphorylation, operate to recruit other remodeling proteins. JNK-mediated phosphorylation of c-Jun has been reported to result in recruitment of the CBP coactivator (30), which is capable of catalyzing acetylation at lysine 27 of histone H3 (46). In this article, we show that T cell activation resulted in the recruitment of CBP to the Il2 promoter and an increase in H3K27-acetylation (Fig. 6F, 6G). Importantly, the pharmacologic block of the c-Jun phosphorylation inhibited both of these events, suggesting that the H3K27ac might be required for late transcription factor binding and changes in nucleosome occupancy at the Il2 promoter. This idea is supported by previous reports showing that some lysine residues of histone H3 are acetylated at MNase hypersensitive sites in the Il2 promoter of fully activated T cells (12, 14). Alternatively, either of those two events could be the rate-limiting step because they were also blocked by SP600125. Finally, IL-2 transcription is also negatively regulated by the transcription factors CREM/ICER, Nil-2a, and Ikaros (47–51), which we have not examined in this article. Although whole-genome analyses have not shown any apparent enrichment of methylation of histone H3 at lysine 27 (a repressive epigenetic mark that opposes transcriptional activation) at the Il2 promoter in resting murine CD4+ T cells (52), the negatively regulating transcription factors might facilitate the deposition of other repressive marks such as methylation of lysine 9 on histone H3. TCR stimulation might then be needed to erase such marks by a process that could also be rate limiting for the second step in IL-2 gene activation.
Acknowledgments
We are grateful to Dr. Ananya Rao for anti-NFAT1 Ab. We also thank Drs. Ellen Rothenberg, Gary Felsenfeld, Carl Wu, Wendy Bickmore, Keiko Ozato, Rajat Varma, and Nevil Singh for constructive criticisms of the experiments presented in this article.

Disclosures
The authors have no financial conflicts of interest.

References
**Supplementary Table S1.** Primary antibodies for Western blotting and ChIP analyses.

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### Supplementary Table S2. Primers for the SEVENS, ChIP, and RT-PCR assays.

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Supplementary Figure S1. Inhibition of IL-2 expression in PR-T cells treated with three chemicals. A, No IL-2 transcripts were observed by RT-PCR in 1 hr-activated PR-T cells treated with CHX. The expression level of non-treated cells following 1 hr activation is designated as 100%. B, Both FK506 and SP600125 strongly blocked IL-2 transcription in 3 hr-activated PR-T cells, although small amounts of IL-2 message were detected (1.7% for FK506 and 10.9% for SP600125). The expression level of non-treated cells following 3 hr activation is designated as 100%.
Supplementary Figure S2. Fractional distributions of the Actb promoter in the SEVENS assay. A, The Actb promoter region including its TSS in 1 hr-activated PR-T cells treated with CHX was enriched in the slowly sedimenting fractions (#2 and #4), and excluded from the quickly sedimenting fractions (#6 to #18). B, Three hr-activated PR-T cells treated with either FK506 (open columns) or SP600125 (closed columns) showed a similar pattern for the Actb promoter to that in A.