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Chromatin Remodeling, Measured by a Novel Real-Time Polymerase Chain Reaction Assay, Across the Proximal Promoter Region of the IL-2 Gene

Sudha Rao, Erik Procko, and M. Frances Shannon

The structure of chromatin and its remodeling following activation are important aspects of the control of inducible gene transcription. The IL-2 gene is induced in a cell specific manner in T cells following an antigenic stimulus. We show, using a novel real-time PCR assay, that significant chromatin remodeling of the IL-2 proximal promoter region occurred upon stimulation of both the murine EL-4 T cell line and primary CD4+ T cells. Chromatin remodeling appears to be limited to the first 300 bp of the proximal promoter region as measured by micrococcal nuclease and restriction enzyme accessibility. Time course studies indicated that chromatin remodeling was observed at 1.5 h postinduction and was maintained for up to 16 h. The remodeling is reversible upon removal of the stimulus. The region immediately upstream from the transcription start site, however, remains accessible for up to 16 h. Upon restimulation, remodeling occurs much more rapidly, consistent with a more rapid rise in IL-2 mRNA levels. Using a number of pharmacological inhibitors we show that remodeling is dependent on the presence of specific transcription factors, but not on the modification of histones. The development of this novel chromatin accessibility assay based on real-time PCR has allowed rapid, sensitive, and quantitative measurements on the IL-2 gene following cellular activation in both T cell lines and primary cells. The Journal of Immunology, 2001, 167: 4494–4503.

Interleukin-2 is an important cytokine that drives the proliferation of T cells, B cells, and NK cells. The expression of this cytokine is controlled almost entirely at the transcriptional level (1, 2). The 300-bp region immediately upstream of the IL-2 transcriptional start site contains a minimal promoter/enhancer region that can drive expression in a stimulation-dependent, T cell-restricted fashion in T cell lines or clones in culture (3, 4). Binding sites for many inducible transcription factors have been characterized within the proximal promoter/enhancer region, including members of the NFAT, NF-κB, and AP1 families (5, 6) and constitutive factors such as OCT1 (5, 6).

Despite such extensive knowledge about the structure and function of the IL-2 gene and signals leading to its activation in T cells, relatively little information is available about regulation of the IL-2 gene at the chromatin level. Previous investigators have shown that the human IL-2 minimal enhancer undergoes specific increases in DNase I accessibility consistent with changes in chromatin structure upon activation (7). In contrast, a region between −313 and −570 is constitutively hypersensitive to DNase I in T cells and has been proposed to play a role in cell specificity (8). In addition, Ward et al. (8) have used Drai restriction enzyme accessibility studies to reveal that the murine IL-2 proximal promoter remains in a closed conformation until stimulation (8). There is no information available on the chromatin structure of the IL-2 gene in primary T cells nor how that structure may be remodeled in response to T cell activation in vivo.

The genomic DNA in the nucleus is organized into a highly complex structure known as chromatin, where the basic building block is the nucleosome (9–11). The higher order structure of chromatin represents an obstacle to the binding of transcription factors, the formation of preinitiation complexes at the TATA box and transcription start site and finally the elongation of RNA polymerase II. Recent studies have identified several ATP-dependent multiprotein complexes whose primary function is to alter chromatin structure so that its DNA sequence becomes transparent to the transcriptional apparatus (9–11). The modification of histone proteins by acetylation or phosphorylation is also thought to alter the packaging of nucleosomes (12–14).

It is now well documented that chromatin structure is perturbed in the neighborhood of expressed genes, and this is particularly significant in controlling their expression. Chromatin remodeling in response to cell activation has been well studied for the Saccharomyces cerevisiae Pho5 gene, HIV, and the murine mammary tumor virus (MMTV)2 promoters. Induction of the Pho5 promoter with low phosphate results in the selective remodeling of four nucleosomes in the vicinity of the promoter (15, 16). In a similar manner, induction of the MMTV promoter by the glucocorticoid receptor results in the selective remodeling of the nucleosome spanning the glucocorticoid receptor binding sites (17–19). By comparison, the HIV promoter is nucleosome free, but nucleosomes positioned downstream of the transcription start site undergo remodeling upon T cell activation (20, 21). These studies clearly establish that alterations in chromatin structure play an important role in the regulation of inducible transcription in eukaryotes.

Several recent studies have indicated that chromatin remodeling is important for transcription of cytokine genes in mammalian cells, including the genes that encode IL-4, IFN-γ (22–24), and IL-12 (25). Upon macrophage induction, a single nucleosome,
positioned upstream of the start site of the IL-12p40 promoter, is rapidly and selectively remodeled in a protein synthesis-dependent manner (25). Agrawal and Rao (23) have shown that chromatin remodeling of cytokine gene loci is functionally associated with acquisition of the Th cell phenotype. The differential expression of IL-4 and IFN-γ in Th cell clones has been shown to correlate completely with differential chromatin accessibility in specific regions of the IL-4 and IFN-γ genetic loci (23).

In this report, we describe a novel assay, based on real-time PCR analysis, to determine the chromatin remodeling events of the murine IL-2 gene in both EL-4 T cells and primary T cells. This assay has been designated as chromatin accessibility by real-time PCR (CHART-PCR) and provides a comparatively rapid, sensitive, and quantitative means of characterizing chromatin remodeling events for inducible genes in both cell lines and primary cells.

We show, using the CHART-PCR assay, that the murine IL-2 gene undergoes substantial changes in chromatin accessibility upon T cell activation in both T cell lines and, more importantly, primary T cells. This remodeling is limited to a region of ~300 bp across the proximal promoter region. The remodeling can be inhibited by several pharmacological agents that are known to inhibit specific transcription factors involved in IL-2 gene transcription. Furthermore, we found that the kinetics of remodeling appear to slightly precede IL-2 mRNA accumulation on both primary stimulation and restimulation following withdrawal of the stimulus.

Materials and Methods

Cell Culture

EL-4.E1.4 (EL-4) thymoma cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics. Cells in suspension were stimulated for an optimal period of 4 h at 1 × 106 cells/ml with PMA; Ca2+ ionophore, A23187 (I); an activating CD28 Ab (BD PharMingen, San Diego, CA); and plate-bound CD3e (BD PharMingen) at final concentrations of 10 ng/ml, 1 μM, 5 μg/ml, and 10 μg/ml, respectively. When inductions were performed in the presence of the inhibitors cyclosporin A (CSA; 0.5 μg/ml), CAMP (1 mM), and H89 (10 μM), cells were pretreated with the respective inhibitor for 30 min at 37°C before the addition of the stimulus. When using the deacteylase inhibitor, tricostatin A (TSA), cells were pretreated with TSA for 5 h before the addition of stimulus. A time course was initially conducted to determine this optimal period of 4 h for IL-2 mRNA production by semiquantitative PCR analysis.

Primary T Cell Preparation

All mice were maintained in a pathogen-free environment in barrier facilities. Spleens were isolated from C57BL/6 mice (5–6 wk old). The CD4+ cells were purified using MACS CD4+ (LT34) beads according to the manufacturer’s guidelines (Miltenyi Biotec, Auburn, CA). The cells were subsequently stained and analyzed by flow cytometry with T cell populations shown to be 90–95% pure using Abs against CD4+ T cells, CD8+ T cells, B cells, and macrophages.

 Primer design for real-time PCR analysis (sequence 7700 detector)

Primers were designed for SYBR Green PCR analysis with the sequence detector 7700 to perform 1) IL-2 mRNA quantification and 2) CHART-PCR assays using the computer program Primer Express (PerkinElmer/PE Biosystems, Foster City, CA). The sequences of all primer sets used are detailed in Table I. For amplification of IL-2 cDNA, primers were designed on an intron-exon junction to prevent coamplification of genomic DNA. The melting temperature was 57–60°C. The amplicon length was kept between 100 and 250 bp. For each primer set, primer concentrations (300–900 nM) were tested to optimize the PCR amplification. However for all primer sets identical thermocycler conditions were used: stage 1, 50°C for 2 min for one cycle; stage 2, 95°C for 10 min for one cycle; and stage 3, 95°C for 15 s and 60°C for 1 min for 40 cycles. Since PCR amplification was being monitored by SYBR Green, it was essential to confirm the absence of nonspecific amplification by analyzing the PCR amplification products on a 4% Nuseive (3:1) agarose gel (Perkin Elmer/PE Biosystems).

IL-2 mRNA quantification

RNA extraction and cDNA synthesis. Total RNA was extracted from stimulated and unstimulated T cells using the modified RNAzol B method described previously (26). The sample sizes were kept constant, with 1 × 107 cells in all experiments. RNA was quantified on a Gene Quant fluorimeter (Amersham Pharmacia Biotech, Piscataway, NJ), and the RNA (1–5 μg) was subsequently treated with DNase I (1 U/μg RNA). The DNase I-treated total RNA was reverse transcribed using 100 U Superscript II reverse transcriptase (Life Technologies, Grand Island, NY) as detailed in the manufacturer’s guidelines.

SYBR Green PCR amplification. PCR were performed on the Applied Biosystems PRISM 7700 sequence detector (PerkinElmer/PE Biosystems). SYBR Green PCR were performed in a total volume of 50 μl containing 10 μg of cDNA as detailed in the manufacturer’s guidelines (Perkin Elmer/PE Biosystems, protocol 04304965). Each PCR was performed in duplicate using the thermocycler conditions detailed above. Controls were included for all PCRs to exclude PCR amplification of contaminating genomic DNA and to ensure that amplification was not due to contamination of other components within the PCR mix. An aliquot of each sample was analyzed by quantitative PCR for β-actin to normalize for inefficiencies in cDNA synthesis and RNA input amounts. To correlate the threshold (Ct) values from the IL-2 cDNA amplification plots to copy number, a standard curve was generated using a plasmid incorporating murine IL-2 cDNA.

CHART-PCR assay

Accessibility assays. Stimulated and unstimulated T cells were pelleted by centrifugation at 1500 rpm at 4°C. Cells (5 × 106 cells/sample) were washed once in ice-cold PBS. The cell pellet was resuspended in 1 ml ice-cold Nonidet P-40 lysis buffer (10 mM Tris (pH 7.4), 1.0 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40, 0.15 mM spermine, and 0.5 mM spermidine) and incubated on ice for 5 min. The suspension was centrifuged at 3000 rpm for 5 min to pellet the nuclei. The nuclei were subsequently washed in the respective digestion buffer (without CaCl2). DNase I, micrococcal nuclease (MNase), and restriction enzyme accessibility assays were performed essentially as previously described (25). A control without the accessibility agent was included from both the stimulated and unstimulated cells to monitor endonuclease activity. The genomic DNA was isolated using a QIAamp blood kit (Qiagen, Valencia, CA).

CHART-PCR assay. The DNA samples from the accessibility assays described above were quantified using the Gene Quant Pro RNA/DNA calculator (Amersham Pharmacia Biotec, Arlington Heights, IL), and the RNA (1–5 μg) was subsequently treated with DNase I (1 U/μg RNA). The DNase I-treated total RNA was reverse transcribed using 100 U Superscript II reverse transcriptase (Life Technologies, Grand Island, NY) as detailed in the manufacturer’s guidelines.

Results

Novel assay to measure chromatin accessibility in cells

Studies were conducted to establish the time point for optimal IL-2 transcription in the EL-4 thymoma cell line, which has previously been shown to express IL-2 (5, 28), using traditional semiquantitative PCR and SYBR Green real-time PCR (Fig. 1). As shown in Fig. 1, low levels of IL-2 mRNA were first detected after 1 h of stimulation of EL-4 T cells with PMA/I. Real-time PCR analysis (Fig. 1B) indicated that an ~5-fold increase in IL-2 mRNA was observed compared with unstimulated cells. At 1.5 h poststimulation increased quantities of IL-2 mRNA were detected (~70-fold; Fig. 1B), reaching maximal levels (~1050-fold; Fig. 1B) at 4 h (Fig. 1) and declining to ~40-fold at 16 h poststimulation. The highest level of IL-2 mRNA was observed at 4 h for all the stimulants used with real-time PCR (data not shown and see Fig. 3E).

These data are in agreement with data obtained by previous investigators using Northern blotting and semiquantitative PCR analysis (28, 29).
A time course of IL-2 mRNA expression in EL-4 T cells. A. Semiquantitative PCR analysis was performed on cDNA from unstimulated and PMA/I-stimulated EL-4 T cells for the time points indicated. The PCR products were generated following 25 cycles of PCR amplification. The products were visualized on a ethidium bromide-stained agarose gel. The results were normalized using β-actin. B, SYBR Green real-time PCR analysis was performed on cDNA from unstimulated and PMA/I-stimulated EL-4 T cells for the time points. The Ct values generated for each time point were converted to copy numbers using a standard curve generated with an IL-2 cDNA construct. The results were normalized using β-actin. Data shown are the mean and SE for four separate experiments.

To examine the chromatin remodeling events across the control regions of the IL-2 gene with a novel in vivo assay was established using real-time PCR and the accessibility agents DNAse I, MNase, and restriction enzymes. Nuclei were prepared from unstimulated and stimulated cells and incubated with the appropriate accessibility agent, and genomic DNA was prepared according to standard procedures (25). One hundred nanograms of genomic DNA from the unstimulated and stimulated samples, including appropriate controls, was subjected to real-time PCR using primers encompassing the region of interest. The amount of product generated is inversely proportional to the amount of digestion occurring across the region amplified by the primers. Thus, if stimulation of the cells leads to an increase in accessibility and hence increased digestion with DNase I or other reagents, less PCR product will be generated (Fig. 2A). The procedure is standardized by the use of appropriate calibration controls described in Materials and Methods. This new assay procedure has been designated CHART-PCR. The primer sets A–F (Fig. 2B and Table I) were used to perform the CHART-PCR assay across the IL-2 gene. Using 100 ng genomic DNA from unstimulated cells, the Ct values for the six primer sets all fall within one Ct value of each other, ranging from 23 to 24 (Table I). This indicates that these regions of the IL-2 gene are amplified with similar efficiencies.

Studies performed by Ward et al. (8) have shown that a *Dra*I site (−169 to −164; Fig. 2B) located within the promoter of the murine IL-2 gene becomes accessible upon stimulation of T cells (8). Thus, to measure the effectiveness of the CHART-PCR assay, accessibility studies were initially performed with the *Dra*I enzyme. In vitro experiments were initially conducted with purified genomic DNA to determine the parameters of digestion and to generate a standard curve (Fig. 3A), which was linear from 600 ng down to 1.5 ng input DNA. This represents a range in cell numbers from $2.4 \times 10^3$ to $\sim 600$ cells. All experiments were conducted well within this linear range using 50–100 ng genomic DNA, which equates to $\sim 20,000–40,000$ cells. Genomic DNA isolated from EL-4 cells was incubated with *Dra*I for 0–60 min in vitro and the amount of *Dra*I digestion monitored by real-time PCR using primer set C, and the results are plotted as a percentage of the uncut sample (Fig. 3B). A substantial decrease in the amount of PCR product was observed at 15, 30, and 60 min of *Dra*I digestion, indicating that $\sim 30\%$ of the DNA was digested at 15 min, and almost complete digestion occurred at 60 min (Fig. 3B).

*Dra*I accessibility studies were then conducted in cells by incubating nuclei, prepared from stimulated and unstimulated EL-4 cells, with *Dra*I enzyme for 0, 30, and 60 min, respectively (Fig. 3C). We examined whether input DNA levels affect the percent accessibility and found that across a range of input DNA from 25 to 200 ng the percent change observed from unstimulated to stimulated samples remained the same. Since real-time PCR analysis was being monitored by SYBR Green, the absence of nonspecific amplification was confirmed by agarose gel electrophoresis where only one specific band was seen for unstimulated, digested samples following 40 cycles of PCR (Fig. 3D). One hundred nanograms of
Table I. Primer sequences for SYBR Green real-time PCR analysis

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Sequence (5' → 3')a</th>
<th>Ct0</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 cDNA</td>
<td>F, CCTGACAGGTTGGAATTACA</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>R, TCCAGAACTGCGCCAGAG</td>
<td></td>
</tr>
<tr>
<td>β-Actin cDNA</td>
<td>F, AGGGAGATGTGCTGAGAC</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>R, CAAATGTGACGCCTGCCCOT</td>
<td></td>
</tr>
<tr>
<td>Set A</td>
<td>F, CCTGCAGCATGACGATCAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R, CCGTGTGCAAGGAGAGCA</td>
<td></td>
</tr>
<tr>
<td>Set B</td>
<td>F, CACGGAGAATCTTGTGAAATATGTGTTAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R, CATGCGAGGAATTTATATCCTGTAATG</td>
<td></td>
</tr>
<tr>
<td>Set C</td>
<td>F, CCTAAATCCATTAGCAGTCAGTGATGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R, TGTGTTGCGAGAAGCATTACCT</td>
<td></td>
</tr>
<tr>
<td>Set D</td>
<td>F, CTGGGCGATCTCCACCCAAA</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>R, CACACTTATGAGGCGATTTTAATCAT</td>
<td></td>
</tr>
<tr>
<td>Set E</td>
<td>F, TGTTCTTAGCTTTATTTCTCTGCT</td>
<td>23.9</td>
</tr>
<tr>
<td></td>
<td>R, GGTCTTACCTTCTTTATCTCACACAATGAGC</td>
<td></td>
</tr>
<tr>
<td>Set F</td>
<td>F, CATGCAGATTTTTTGTGTTTTCTTAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R, GCTTAAAGGTCTCCTGCAAGAGAGA</td>
<td>23.3</td>
</tr>
</tbody>
</table>

a All of the primer sequences were designed using the murine IL-2 gene sequence (Genbank accession no. M39728) or the IL-2 cDNA sequence (Genbank accession no. X01772). F, Forward primer; R, reverse primer.

b The Ct values were generated from the real-time PCR plots with a threshold value of 0.282. The thermocycler conditions and the concentrations of primers used were identical for all primer sets.

genomic DNA from the stimulated and unstimulated EL-4 T cells was subjected to real-time PCR analysis. The results were plotted as a percentage of the undigested PCR product for each treatment. In unstimulated cells, no Dral digestion was observed, indicating that this region of the promoter is inaccessible (Fig. 3C). In EL-4 cells stimulated for 4 h with PMA/I there was an increase in Dral digestion, with ~28 and 69% cutting at 30 and 60 min of digestion, respectively (Fig. 3C). As an additional control, a region of IL-2 lacking a Dral site, encompassed by primer set F (Fig. 2B), was also amplified in unstimulated and stimulated cells, and as predicted there was no change in accessibility in all samples (data not shown).

As another measure of the effectiveness of the CHART-PCR assay, accessibility measurements were conducted with the HindII enzyme, a site for which is located close to the Dral site (~152–148; Fig. 2B). Fig. 3E shows that the HindII site becomes accessible to cleavage in activated EL-4 cells, and the extent of accessibility is dependent upon the nature of the stimulus. The order of HindII accessibility was I (25% cutting) < PMA (50% cutting) < PMA/I (53% cutting) < PMA/ICD28 (60% cutting). These changes in accessibility reflect the levels of IL-2 mRNA production that were measured in these cells (Fig. 3E). These results show that the CHART-PCR assay can be used to measure changes in restriction enzyme accessibility in chromatin, and the data agree with previous observations on Dral accessibility using primer extension assays (8).

Chromatin remodeling is restricted to a 300-bp region across the IL-2 promoter region

Changes in restriction enzyme accessibility have been interpreted as reflecting chromatin remodeling across a particular region of the DNA (8, 17, 25). Thus, the data above indicate that chromatin remodeling may be occurring across the proximal promoter region of the IL-2 gene upon stimulation of EL-4 cells. To generate a more comprehensive picture of changes in chromatin structure across the control regions of the IL-2 gene, primer sets were designed across the promoter (~14 to ~330, sets B–D, Fig. 2B), downstream of the start of transcription (~+40 to +110, set A, Fig. 2B), at a region shown to have T cell-specific constitutive DNase I footprints (~460 to ~550; set E, Fig. 2B), and in a far upstream region (~1890 to ~1980, set F, Fig. 2B).

MNase has a preference for digestion in regions of DNA that are nucleosome-free and can thus be used to measure nucleosome remodeling (25). The primer sets described above were used to measure MNase accessibility across the IL-2 control regions using the CHART-PCR assay. EL-4 cells were stimulated for 4 h with PMA/I. The nuclei were isolated and incubated with MNase for 5 min at room temperature. In unstimulated cells all the regions measured were inaccessible to MNase as shown by the fact that the real-time PCR curves parallel the curves for the samples without MNase digestion (data not shown). Since real-time PCR analysis was being monitored by SYBR Green the absence of nonspecific amplification was confirmed by agarose gel electrophoresis of samples from unstimulated, MNase-digested cells amplified for 40 cycles (Fig. 4B). When cells were stimulated for 4 h with PMA/I, significant changes in MNase digestion were observed with the primer sets that spanned the promoter (Fig. 4A, sets B–D), with increases of between 60 and 90% relative to unstimulated samples. Analysis of the regions between ~460 and ~550 and between ~1890 and ~1980 showed only a small change in digestion compared with the proximal promoter region (Fig. 4A, sets E and F). Interestingly, the region immediately downstream from the transcription start site also showed relatively little change in MNase sensitivity following PMA/I activation (Fig. 4A, set A). Thus, the major changes in MNase accessibility upon stimulation of cells are limited to a region of ~300 bp upstream from the transcription start site.

To further characterize the changes in chromatin structure across the control regions of the IL-2 gene, DNase I accessibility studies were conducted with primer sets A–F (Fig. 2B) DNase I accessibility across the IL-2 gene has been previously mapped by conventional Southern blot analysis and LM-PCR (5). To compare DNase I accessibility patterns across the IL-2 gene with the new CHART-PCR assay, EL-4 cells were stimulated with PMA/I for 4 h, and nuclei were isolated and incubated with 30 U DNase I for 5 min at 37°C. Digestion was measured using the CHART-PCR assays with each of the primer sets shown in Fig. 2B. Since real-time PCR analysis was being monitored by SYBR Green the absence of nonspecific amplification was confirmed by agarose gel electrophoresis of the amplified products (40 cycles) from unstimulated cells. (Fig. 4D). Unlike MNase, DNase I digested all regions of the gene in unstimulated cells, with levels of digestion ranging from ~30 to 60% compared with undigested samples (data not shown). This presumably reflects the fact that nucleosome-assembled DNA is still accessible to DNase I, but not MNase digestion. When cells were stimulated with PMA/I for 4 h,
the region spanning the proximal promoter became more sensitive to DNase I digestion (Fig. 4C, primer sets B–D) with increases in digestion, compared with unstimulated samples, of between 45% and 75%. In comparison, much smaller changes were seen across the digestion, compared with unstimulated samples, of between 45 and 75%. In comparison, much smaller changes were seen across the digestion, compared with unstimulated samples, of between 45 and 75%.

Kinetics of chromatin remodeling of the IL-2 gene

To further characterize the remodeling events, the kinetics was monitored by CHART-PCR assay using MNase (Fig. 5A) and restriction enzyme (Fig. 5B) accessibility. EL-4 cells were stimulated with PMA/I for various times ranging from 15 min to 16 h (Fig. 5, A and B). Nuclei were prepared from each time point and incubated with the appropriate accessibility agent, and the CHART-PCR assay was performed with primer sets B–D (Fig. 2B). As shown in Fig. 5A, significant MNase accessibility across the proximal promoter was first detected 1.5 h after addition of PMA/I. This remodeled state appears to be maintained for at least 6 h across the entire proximal promoter of the IL-2 gene (Fig. 5A, sets B–D). Increases of 80–100% MNase accessibility were observed during this time period across the promoter (+1 to −300; Fig. 5A, primer sets B–D). By 16 h poststimulation a decline in accessibility was observed. The region of the promoter detected by primer set C remained highly accessible (75% accessibility) even at 16 h poststimulation (Fig. 5A). In comparison, accessibility was reduced to 40–50% in the regions of the promoter detected by primer sets B and D (Fig. 5A). As a further measure of chromatin remodeling kinetics, accessibility studies were conducted at the Dra I site (−169 to −164, Fig. 2B) and the Hinfl sites (−152 to −148 and −102 to −98, Fig. 2B) respectively. As shown in Fig. 5B, digestion was first observed 1 h (Hinfl) or 2 h (Dra I), with maximum digestion at 3 h poststimulation and declining at 16 h. Thus, the remodeling kinetics observed with MNase and restriction enzymes closely parallel each other.

If the chromatin remodeling kinetics are plotted against the accumulation of IL-2 mRNA levels, it can be seen that chromatin remodeling slightly precedes the large increase in IL-2 mRNA that occurs at 2, 3, and 4 h poststimulation (Fig. 5C).

Chromatin remodeling occurs with altered kinetics in restimulated cells

It has been previously shown that upon stimulus removal and re-stimulation, IL-2 gene transcription occurs with faster kinetics (30, 31). It is possible that the faster kinetics of transcription following...
restimulation is related to maintenance of chromatin remodeling. We asked whether the chromatin structure of the IL-2 proximal promoter returned to its inactive state following stimulus withdrawal and also followed the kinetics of chromatin remodeling upon restimulation of the cells. EL-4 cells were stimulated with PMA/I for 4 h, when maximum levels of IL-2 mRNA were observed (Fig. 6). Next the stimulus was removed, and mRNA levels were measured by real-time PCR. Three hours following stimulus removal, IL-2 mRNA levels had fallen by ~5-fold, and by 16 h IL-2 mRNA levels were lower than those in resting cells (Fig. 6).

When these cells were restimulated with PMA/I, IL-2 mRNA was detected as early as 15 min, and at 1 h mRNA levels reached 3–4 times those seen at 4 h following the primary stimulation (Fig. 6).

When we examined the MNase accessibility of the proximal promoter following stimulus withdrawal, at all three promoter regions (primer sets B, C, and D) accessibility was reduced at 2 h (Fig. 6, W1). By 16 h following stimulus withdrawal, the set C and D regions (~110 to ~330) were completely inaccessible (Fig. 6, W2). Interestingly, the set B region closest to the transcription start site (~14 to ~115) remained partially accessible (~33%; Fig. 6, W2).

Within 15 min of restimulation each of the three regions began to increase in accessibility to MNase, with levels similar to the 4-h primary stimulation reached after 1 h of restimulation (Fig. 6). The gradual nature of the appearance of accessibility following restimulation is also in contrast with the primary stimulation where...
accessibility to almost maximum levels occurred between 1 and 1.5 h (compare Figs. 5 and 6). Similar results were observed with DraI accessibility and primer set C (data not shown).

These data show that the region of the IL-2 promoter closest to the transcription start site remains partially accessible following stimulus withdrawal and that chromatin remodeling occurs with distinct and faster kinetics following restimulation.

Cyclosporin A and cAMP inhibit chromatin remodeling across the IL-2 proximal promoter

Treatment of EL-4 cells with either CsA or cAMP has been shown to inhibit PMA/I activation of IL-2 gene transcription (5, 32). This has been shown to occur through the inhibition of specific transcription factors (5), which, in turn, may function to recruit chromatin remodeling complexes. To determine whether inhibition of specific transcription factors affected chromatin remodeling across the IL-2 gene, EL-4 cells were stimulated with PMA/I for 4 h with or without 30-min pretreatment with CsA or cAMP. The nuclei were prepared from these samples and incubated with DraI or HindIII restriction enzymes or MNase. As shown above, PMA/I leads to an increase in DraI or HindIII cutting of the genomic DNA in nuclei (60–70% compared with unstimulated cells; Fig. 7A).

When cells were treated with cAMP together with PMA/I a significant decrease in enzyme digestion was observed (down to 12–14% of unstimulated levels; Fig. 7A). Pretreatment with CsA before P/I activation for 4 h completely inhibited MNase accessibility for all three primer sets spanning the promoter. The data for primer set B is shown (Fig. 7B), and sets C and D gave identical results (data not shown). The inhibition of chromatin remodeling by CsA is paralleled by an inhibition of IL-2 gene transcription, as expected from previous studies (Fig. 7B). These results show that signals blocked by cAMP and CsA are required for initiation of chromatin remodeling. To determine whether signals blocked by CsA were also needed for maintenance of the remodeled state, cells were treated with CsA 2 h following P/I activation, at a time when chromatin remodeling has already occurred (see Fig. 5). In this case CsA reduced the amount of remodeling measured at 6 h postactivation to ~20% of the control value (Fig. 7B).

A similar pattern of inhibition was seen across the entire promoter (data not shown). Since treatment of preactivated cells with CsA has been shown to remove the NFAT proteins from the nucleus within 30 min of treatment (33), we asked whether chromatin remodeling was inhibited with the same kinetics. When cells were treated with CsA 2 h after P/I activation and chromatin remodeling was assayed 1, 3, and 6 h later, it was seen that chromatin reverted gradually over this time frame to a more inaccessible state (Fig. 7C).

These results imply that NFAT factors are likely to be involved in both the initiation and maintenance of chromatin remodeling across the IL-2 promoter, but that the promoter does not immediately revert to its inactive state when NFAT is removed from the nucleus. In addition, transcription factors inhibited by cAMP may be required for chromatin remodeling.

For some genes, chromatin remodeling has been shown to be dependent on the modification of histone proteins by acetylation or phosphorylation (34). It has not been shown whether these modifications are involved in cytokine gene transcription in T cells. We next examined the effect of TSA, a histone deacetylase inhibitor, on chromatin remodeling and IL-2 gene transcription. When EL-4 cells were treated for 1 h with P/I a very small increase (5-fold) in IL-2 mRNA levels was observed compared with that seen at 4 h of activation (~1000-fold) as described above (Fig. 7D). However, if cells are pretreated for 5 h with TSA (500 ng/ml) a much larger increase (127-fold) in mRNA levels was detected at 1 h post P/I stimulation (Fig. 7D). This implies that hyperacetylation of histone proteins, induced by inhibition of deacetylases, leads to increased IL-2 transcription. Chromatin remodeling is not normally observed at 1 h postactivation, so to determine whether the hyperacetylation induced by TSA pretreatment led to earlier chromatin remodeling, we measured MNase accessibility at 1 h in the presence or the

**FIGURE 7.** The chromatin remodeling of the proximal promoter region of the IL-2 gene is inhibited by cAMP and CsA, but not by TSA or H89. A, cAMP inhibits restriction enzyme accessibility of the IL-2 gene. Intact nuclei isolated from EL-4 cells left unstimulated (C), PMA/I stimulated (P/I), or PMA/I/cAMP stimulated (P/I/cAMP) were incubated with or without 100 U DraI or HindIII for 60 min at 37° C, respectively. One hundred nanograms of genomic DNA for each treatment was subjected to SYBR Green real-time PCR analysis. The Ct values generated were converted to DNA concentration using the standard curve (Fig. 3A). The DraI/HindIII accessibility were expressed as a percentage of uncut DNA and plotted for each treatment. B, MNase accessibility and IL-2 expression analysis of EL-4 T cells treated with CsA. Nuclei for MNase or cDNA for IL-2 expression analysis were prepared from EL-4 T cells stimulated with PMA/I alone (P/I), pretreated with CsA for 30 min and left unstimulated (C), or stimulated with PMA/I (CsA) for 6 h or for 2 h before the addition of CsA (CsA-2). The intact nuclei were incubated with or without 50 U MNase for 5 min at room temperature. One hundred nanograms of genomic DNA for each treatment was subjected to SYBR Green real-time PCR analysis using primer sets B–D. The Ct values generated were converted to DNA concentration using the standard curve (Fig. 3A). MNase accessibility was expressed as a percentage of the untreated, digested genomic DNA sample. cDNA samples for real-time PCR analysis were prepared as detailed in Materials and Methods. The fold change in IL-2 levels for each of the treatments relative to the control are shown. C, Kinetics of IL-2 promoter remodeling upon addition of CsA. Nuclei for MNase analysis were prepared from EL-4 T cells stimulated with PMA/I alone (P/I), pretreated with CsA and left unstimulated (C), or stimulated with PMA/I (CsA) for 6 h or for 2 h before the addition of CsA. Samples were taken for analysis after 1, 3, and 6 h, respectively. Samples were analyzed and data plotted as described in B above. D, MNase and IL-2 expression analysis of EL-4 T cells treated with TSA. Nuclei for MNase or cDNA for IL-2 expression analysis were prepared from EL-4 T cells stimulated with PMA/I alone (P/I), pretreated with TSA for 5 h and left unstimulated (C), or stimulated with PMA/I for 1 h (P/I h+TSA). Samples were analyzed and data plotted as described in C above. E, MNase and IL-2 expression analysis of EL-4 T cells treated with H89. Nuclei for MNase or cDNA for IL-2 expression analysis were prepared from EL-4 T cells stimulated with PMA/I alone (P/I), or stimulated with H89 for 30 min and left unstimulated (C), or stimulated with PMA/I for 4 h (P/I/1 h+H89). Samples were analyzed, and data plotted as described in C above. Data shown are the mean and SE for four separate experiments.
absence of TSA. Surprisingly, chromatin remodeling measured by either MNase or restriction enzyme accessibility (Fig. 7D and data not shown) was not increased at 1 h postactivation in the presence of TSA.

It has recently been shown the human MSK1 is a histone H3 kinase, and phosphorylation of histone H3 by MSK1 leads to immediate-early gene induction in response to a wide variety of stimuli (34). By searching mouse EST databases, we found ESTs that matched the human MSK1 sequence and designed primers to amplify the mouse MSK1 cDNA. We then showed that EL-4 T cells and CD4+ T cells expressed mRNA for MSK1 and that the level of mRNA was not increased by P/I activation (data not shown). Pretreatment of EL-4 T cells with H89, a specific inhibitor of MSK1 at the concentrations used (34), inhibited IL-2 mRNA levels by ~7-fold (Fig. 7E), but did not have any effect on chromatin remodeling across the promoter (Fig. 7E).

These data show that while histone acetylation and phosphorylation may play a role in IL-2 gene transcription, they do not affect the chromatin accessibility of the IL-2 promoter as measured by the CHART-PCR assay.

Chromatin remodeling is restricted to the IL-2 minimal promoter in primary T cells

The CHART-PCR assay described above is of significant value in assessing the chromatin structure of the IL-2 gene in primary T cells where the requirement for large numbers of cells has previously hampered detailed chromatin analysis. To test the efficacy of the method in primary T cells, DraI accessibility studies were performed using nuclei prepared from stimulated and unstimulated mouse primary splenic T cells. Treatment of splenic T cells with PMA/I, P/I and CD3 or CD3 and CD28 leads to increases in IL-2 mRNA levels (Fig. 8A). DraI accessibility studies of cells from two different populations of mice showed an increase in DraI digestion for each of the stimuli used (Fig. 8A). The combination of CD3 and CD28 activation was the most effective at remodeling chromatin at least across the set C region (Fig. 8), although it lead to the lowest level of mRNA accumulation. The level of accessibility in the CD4+ cells was generally lower than that seen in the EL-4 cell population, which may indicate that a smaller percentage of the primary cell population is activated to express IL-2. The results, however, indicate that it is possible to accurately measure chromatin changes in primary cells with the CHART-PCR assay.

MNase accessibility was also assessed across the IL-2 gene in primary T cells using primer sets A–F. Fig. 8B shows that the proximal promoter of the IL-2 gene (−14 to −330, primer sets B–D) is selectively remodeled upon stimulation in primary splenic T cells. As seen in EL-4 cells, the regions encompassed by primer sets A and E showed smaller changes in accessibility, and the region of primer set F was not altered by stimulation (Fig. 8B). Thus, for the first time we show that chromatin accessibility across the IL-2 proximal promoter changes following activation of primary T cells and that this change in accessibility is restricted to the 300-bp proximal promoter region.

Discussion

In this study, we show using a novel in vivo chromatin remodeling assay based on real-time PCR, CHART-PCR, that significant nucleosome remodeling of the IL-2 gene accompanies T cell activation and that this is limited to a region ~300 bp upstream of the transcription start site in both EL-4 T cells and, more importantly, primary T cells. Reproducing the DraI accessibility studies by Ward et al. (8) of the murine IL-2 gene with the mouse thymoma EL-4 cell line validated the CHART-PCR method. The CHART-PCR assay provides a rapid and sensitive means of identifying chromatin remodeling events for inducible genes, requiring only small numbers of cells. Thus, this assay should enable us in the future to perform chromatin remodeling studies on specific populations of primary T cell where only small numbers of cells are available.

A region of the promoter extending from −14 to −330 is specifically remodeled following T cell activation in either EL-4s or in CD4+ primary T cells. It is interesting that no remodeling was observed downstream of the start of transcription, implying that polymerase movement along the DNA does not bring about the
same type of disruption as does the assembly of transcription factor complexes. It has been shown that bacterial polymerases in vitro can transcribe through a nucleosome and move it backward along the DNA without complete disruption (35, 36). In eukaryotic systems it has been shown that chromatin disruption complexes accompany the RNA polymerase as it moves along the DNA (37), suggesting a possible transient disruption that may not be detected in this assay. The region of the IL-2 gene that becomes accessible to MNase corresponds closely with the region of the gene that has been shown to have altered sensitivity to DNase I and dimethyl sulfoxide in vivo (8). This 300-bp region has functional transcription factor binding sites across its entire length and corresponds to the minimal functional region in reporter assays (38).

Our results using MNase accessibility imply that nucleosomes may be located across the entire IL-2 gene in unstimulated cells and that those nucleosomes that are located across the proximal promoter region are selectively remodeled or disrupted following activation. We have recently shown that a highly positioned nucleosome can be assembled in vitro across the region from −60 to −200 of the IL-2 promoter (R. Reeves, J. Attema, and M. F. Shannon, unpublished data). Such a positioned nucleosome(s) may exist in cells and be remodeled following T cell activation as observed in the MNase and restriction enzyme accessibility assays described here. Recently it has been shown that nucleosomes positioned across the control regions of several genes, including the IL-12p40 gene (25), the IL-2Rα promoter (39), the IFN-β gene (40–42), the proto-oncogenes c-fos and c-myc (43), and the steroid-inducible MMTV promoter (17, 18), are all remodeled following cell activation. Thus, a common feature of many of the inducible genes studied to date, including now the IL-2 proximal promoter, is the selective remodeling of chromatin structure across the control regions upon cellular activation. This suggests that chromatin remodeling complexes are most likely recruited to these promoters with precision.

Time course experiments were conducted to determine the kinetics of the accessibility changes within the IL-2 proximal promoter. Upon exposure of T cells to PMA/I, significant remodeling was first observed 1.5 h postinduction. The chromatin remodeling observed following 1.5-h stimulation with PMA/I remained at a similar level for at least 6 h and started to decline after 16 h of exposure to stimulus. The kinetics of chromatin remodeling occur slightly in advance of in vivo transcription factor binding and IL-2 mRNA transcription kinetics (5). In vivo footprinting analysis indicates that maximal levels of regulatory region occupancy are obtained after 2 h of stimulation and persist for at least an additional 9 h (5). These results imply that chromatin remodeling is required before the assembly of active transcription factor complexes that, in turn, give rise to the large increase in IL-2 mRNA seen between 1.5 and 3 h. An accumulation of recent data suggests that gene-specific transcription factors are required for recruitment of chromatin remodeling complexes to promoter/enhancer regions, followed by the binding of other transcription factors to generate active transcription complexes (9, 10). In the few genes studied in detail, however, the order of events appears to be gene specific (41, 44).

Either of two models could explain the 1.5-h lag phase observed before the detection of IL-2 chromatin remodeling. In the first model chromatin remodeling is dependent on the presence of specific transcription factors that recruit remodeling complexes to the promoter region (45, 46). The binding of factors to the IL-2 promoter is strictly coordinated and probably occurs by a cooperative mechanism (8, 47–49). Thus, this implies that no factors bind stably to the IL-2 promoter unless all factors are present and competent to bind. Our finding that both cAMP and CsA block chromatin remodeling of the IL-2 minimal promoter provides support for the model above. cAMP has been shown to inhibit transcription by preventing the binding of NFAT and NF-kB factors to the IL-2 promoter (32). CsA has been shown to inhibit NFAT translocation to the nucleus by blocking calcineurin phosphatase activity (33, 50). Factors such as NF-kB and NFAT, translocate to the nucleus upon activation with very fast kinetics (6, 40). Thus, the availability of these proteins is unlikely to be the limiting event. In addition, brahma-related gene product-containing SWI/SNF complexes have been shown to rapidly translocate to chromatin compartments within 10 min of T cell activation (46). It is unlikely that this recruitment occurs specifically at the proximal promoter region within this time frame, since we do not observe remodeling until 1.5 h poststimulation. The limiting factor in this model may be the requirement of de novo synthesis of certain transcription factors. Factors such as Fos family proteins have been shown to be synthesized de novo upon T cell activation (6) and thus may constitute the limiting event in this model. CsA inhibits not only the initiation, but also the maintenance of chromatin remodeling. Thus, if transcription complexes are destabilized by the removal of a single transcription factor, in this case NFAT, then the chromatin appears to revert slowly to its resting state. These results agree with the results reported by Rothenberg and colleagues (5, 8, 47) showing that the formation of transcription factor complexes on the IL-2 promoter is highly cooperative, and in the absence of one factor the complexes become unstable.

In the second model recruitment of remodeling complexes is preceded by other events on the chromatin such as post-translational modifications, for example by acetylation or phosphorylation, of the histone tails of nucleosomes on the IL-2 promoter (41, 42). This would then facilitate the recruitment and subsequent binding of transcription factors on to the IL-2 proximal promoter, which, in turn, would lead to remodeling complex recruitment. Our results using inhibitors of histone deacetylation and phosphorylation do not support a role for these modifications in chromatin remodeling across the IL-2 proximal promoter, although there appears to be a role for these events in controlling the transcription rate from the IL-2 gene. We do not know, however, if this is a direct or an indirect effect, as the acetylation status of nucleosomes across the IL-2 gene has yet to be determined.

The remodeling of chromatin across the IL-2 promoter needs the continuous presence of signal. Once the stimulus was withdrawn, the chromatin reverted slowly to its resting state, with complete reversal seen by 16 h at least for the region of the gene from −100 to −300. The region from +1 to −100 did not revert to a resting state at least in the time frame assayed here. This agrees with the CsA results, which show that the continuous presence of transcription factors inhibited by CsA is required for maintenance of the remodeled state. A similar situation has been described for glucocorticoid induction of the tryosine aminotransferase gene (18). In this case removal of the stimulus led to a rapid reversal of DNase I-hypersensitive sites. The slow reversal seen on the IL-2 promoter implies that the factors involved in maintenance of remodeling have a longer half-life or that reversion may be related to cell division events.

It has been previously shown that transcription from the IL-2 gene occurs at an earlier time and at a higher level in restimulated T cells (31). Our data here support the idea that chromatin remodeling also occurs at an earlier time following restimulation. It is also interesting that remodeling occurs gradually from 15 min following restimulation. This implies that the limiting factors for remodeling gradually accumulate in the cell population following restimulation and allow remodeling to reach maximum levels by 1 h. When the stimulus was withdrawn after 4 h of activation, the
region of the gene immediately upstream from the start of transcription remained partially accessible to MNase digestion. Transcription factors or components of the basal transcription machinery may remain bound to this region of the promoter, which contains the TATA box and the ARRE1 region (binds OCT, NFAT, and API factors) (6). The partial accessibility remaining in this region may be important in the faster kinetics of chromatin remodeling and transcription from the IL-2 gene following restimulation.

In summary, in this study we show that significant chromatin remodeling occurs selectively across the IL-2 promoter upon T cell activation in both murine primary CD4+ T cells and EL-4 T cells. Overall, there appears to be an interdependence between chromatin remodeling and the formation and maintenance of stable transcription factor complexes on the promoter.

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