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Changes in Chromatin Accessibility Across the GM-CSF Promoter upon T Cell Activation Are Dependent on Nuclear Factor κB Proteins

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Abstract

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a key cytokine in myelopoiesis and aberrant expression is associated with chronic inflammatory disease and myeloid leukemias. This aberrant expression is often associated with constitutive nuclear factor (NF)-κB activation. To investigate the relationship between NF-κB and GM-CSF transcription in a chromatin context, we analyzed the chromatin structure of the GM-CSF gene in T cells and the role of NF-κB proteins in chromatin remodeling. We show here that chromatin remodeling occurs across a region of the GM-CSF gene between −174 and +24 upon T cell activation, suggesting that remodeling is limited to a single nucleosome encompassing the proximal promoter. Nuclear NF-κB levels appear to play a critical role in this process. In addition, using an immobilized template assay we found that the ATPase component of the SWI/SNF chromatin remodeling complex, brg1, is recruited to the GM-CSF proximal promoter in an NF-κB–dependent manner in vitro. These results suggest that chromatin remodeling across the GM-CSF promoter in T cells is a result of recruitment of SWI/SNF type remodeling complexes by NF-κB proteins binding to the CD28 response region of the promoter.

Key words: transcription • chromatin remodeling • Brg1 • cytokine • RelA

Introduction

GM-CSF is a key cytokine involved in the production and function of myeloid cells (1–3). GM-CSF is induced in a variety of cell types, including T cells, macrophages, endothelial cells, and fibroblasts in response to immune and inflammatory signals (1–3). In T cells, GM-CSF expression is rapidly and transiently induced after T cell receptor stimulation and costimulation through CD28:B7 interactions (4). Dysregulated expression of GM-CSF is most likely a contributing factor in a range of pathological conditions including leukemias (5, 6) and chronic inflammatory diseases such as rheumatoid arthritis (7). These diseases are often associated with constitutive expression of transcription factors such as NF-κB (8, 9).

The production of GM-CSF is regulated primarily at the level of transcription, via proximal promoter and enhancer regions (10–12), although posttranscriptional control also contributes to the final level of mRNA and protein (13). The GM-CSF proximal promoter (+1 to −100) consists of a complex array of well characterized transcription factor binding sites (see Fig. 1 A; for a review, see reference 14). The NF-κB family of transcription factors plays an important role in the induction of GM-CSF expression via a region of the proximal promoter known as the CD28 response region (CD28RR; Fig. 1 A). This region consists of a variant NF-κB site, known as CK-1 or CD28RE, a classical NF-κB site and an adjacent Sp1 site (15) which all serve to integrate signals from the T cell receptor and the CD28 costimulatory signal (4, 16, 17). The state of chromatin condensation across specific genes in the nucleus forms an important level of control in the regulation of inducible gene transcription (for reviews, see references 18 and 19). The formation of DNase I hypersensitive (DH)* sites has been observed in the enhancer

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**Abbreviations used in this paper:* CHART-PCR, chromatin accessibility by real-time PCR; DH, DNase I hypersensitive.

and promoter regions of the GM-CSF gene upon T cell activation (11, 20), suggesting that changes in chromatin structure occur across these regions. Furthermore, the CD28RR has been implicated in these events at the promoter (21). A mutation in the NF-κB/Sp1 region of the CD28RR blocked induction of the DH site in the human GM-CSF promoter in a transgenic mouse model resulting in an inactive transgene (21). Interestingly, this mutation in the CD28RR reduced activation of the human GM-CSF gene only when it was integrated into chromosomal DNA (21), indicating that factors which bind to or are recruited to the CD28RR may play a key role in the reorganization of chromatin structure across the GM-CSF promoter.

Currently, there are two general mechanisms by which chromatin structure is known to be altered to allow gene transcription to occur (for reviews, see references 19 and 22). First, multi-subunit ATP-dependent remodeling complexes have been identified (for a review, see reference 23), which use the energy of ATP hydrolysis to disrupt histone–DNA interactions. Second, histone modifying complexes which alter histone NH_2-terminal tails are thought to regulate gene transcription by disrupting chromatin structure and/or providing a histone code that is targeted by other proteins (24–26). One model suggests that chromatin modifying/remodeling complexes are recruited to specific promoter regions through interactions with DNA bound transcription factors which are able to access their binding sites in a chromatin context (for reviews, see references 23 and 25).

In this study, we show that upon T cell activation changes in chromatin structure occur across a specific region of the GM-CSF proximal promoter, limited to a region of less than 200 bp. NF-κB proteins appear to play a critical role in these chromatin remodeling events, which precede GM-CSF gene transcription. Using an in vitro recruitment assay, we show that the NF-κB sites of the CD28RR may play a critical role in recruitment of brg1-containing complexes to the GM-CSF promoter.

Materials and Methods

Plasmids. A pGMSelect plasmid, containing a 4.5 kb BamHI-EcoRI fragment of the human GM-CSF gene was used to mutate the GM-CSF promoter (indicated in Fig. 6 A) using Altered site II (Promega). pCMV4sIκBαS32/36Aflag was provided by Dr. D. Ballard, Vanderbilt University School of Medicine, Nashville, TN (27). The mouse GM-CSF constructs, AOGM and pMGM2.4luc, were provided by Dr. P. Cockerill, University of Leeds, Leeds, UK (28).

Cell Culture and Preparation of Stable Cell Lines. Jurkat T cells and EL-4 T cells were grown as described previously (15). Cells were stimulated with 20 ng/ml PMA (Boehringer), 1 μM calcium ionophore (A23187; Sigma-Aldrich), 10 μg/ml murine CD28 receptor antibody (clone 37NF1; provided by Dr. A. Strasser) and pretreated with 1 μM MG-115 (Carbobenzoxy-L-leucyl-L-leucyl-norvalinal; Boehringer) for 1 h before stimulation. Cells were transfected by electroporation with 10 μg of the plasmid of interest and 1 μg of pcDNA3.1 for selection, as described previously (15). Transfected cells were selected in media containing 800 μg/ml of G418 then maintained in 400 μg/ml G418.

Nuclear Extracts. Nuclear extracts were prepared by the method of Schreiber et al. (29). Protein concentrations were determined by Bradford Assay (Bio-Rad Laboratories).

RNA Isolation and Real-Time PCR Analysis. Total RNA was isolated from 5×10^6 cells using Tri Reagent (Sigma-Aldrich). cDNA was prepared and SYBR Green PCR reactions performed as described previously (30) with 50 ng cDNA in a total volume of 25 μl. The GM-CSF primer set +II (Table I) was used. PCR reactions conducted in parallel using GAPDH primers (sense: 5′-CATGGAGAAGGCTGGGGCTC-3′ and antisense: AACGGATACATTGGGGGTAG-3′) were used to normalize for differences in cDNA synthesis and RNA input.

Chromatin Accessibility by Real-Time PCR. Accessibility of DNA to digestion with restriction enzymes and MNase was analyzed using chromatin accessibility by real-time PCR (CHART-PCR) as described previously (30). EL-4 T cell nuclei (5×10^6 nuclei per 100 μl) were treated with 80 units HaeIII or 100 units HinfI enzyme (Boehringer) at 37°C for 45 min or with 50 units MNase (Boehringer) for 5 min at 20°C. Genomic DNA (50 ng) was isolated and analyzed by SYBR Green real-time PCR (30). The primer sets used are shown in Table I. Accessibility was determined by correlating the Ct values from the amplification plots to a standard curve generated with genomic DNA, and was ex-

Table I. *Primer Sequences for SYBR Green Real-Time PCR Analysis*

Set	Sense primer (5′–3′)[a]	Antisense primer (5′–3′)[a]
+II	AAGGTCCTGAGGAGGATGTG	GAGGTTCAGGGCTTCTTTGA
+I	GAGTTCTGTGGTCACCATTAATCA	CACATCCTCCTCAGGACCTT
−I	GCCTGACAACCTGGGGGAAG	TGATTAATGGTGACCACAGAACTC
−II	AAAAGGAGAGGCTAGCCAGA	TAAGCCCTTCCAAGAACTGG
−III	CCAGGAAATCCAAATATGCC	CAGTCTGACCCAGCCTCTG
−IV	GAACAGCAGGTGCTATGGAA	GGCATATTTGGATTTCCTGG
−V	TGGAATGAGCCACCAGAGTA	GGCTCTTGCTTCCATAGCAC
−VI	CATTGGGCTGGACCTTATTT	TACTCTGGTGGCTCATTCCA
−VII	TCATTCTCACTGCTCCCAAG	ATAAGGTCCAGCCCAATGAC

[a]The primer sequences were designed using the murine GM-CSF gene sequence (GenBank/EMBL/DDBJ accession number X03020).

pressed as a percentage of undigested genomic DNA for each primer set.

PCR Amplification from Mononucleosomal DNA. Mononucleosomal DNA was prepared from formaldehyde-fixed EL-4 T cells as described previously (31). EL-4 T cells (5×10^5 cells) were fixed for 10 min with 1% formaldehyde and the reaction stopped by addition of 0.125 M glycine. Cells were washed and incubated in 0.5 ml buffer A (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA. 0.5% NP-40, containing protease inhibitors) containing 60 ng/ml spermine and 315 ng/ml spermidine for 5 min at 4°C. Cell nuclei were recovered by centrifugation at 800 *g* for 5 min at 4°C, resusupended in MNase buffer, incubated with 50 units MNase for 10 min at 37°C, and then incubated in 0.3 M NaCl at 65°C for 16 h to reverse the cross-links. Mononucleasome fragments (150–200 bp) were purified from an agarose gel and resuspended in 30 μl of MilliQ water. PCR amplification was performed with 4 μl of mononucleosomal DNA, 20 ng of genomic DNA, or 100 pg pMGM2.4luc plasmid DNA using 0.5 U Taq DNA Polymerase (Fisher Biotech).

Nucleosome Assembly. Chicken long chromatin was prepared as described previously (32). A fragment of the mouse GM-CSF promoter (−179 to +24) was amplified by PCR from the pAOGM plasmid using a biotinylated sense primer. After digestion at restriction enzyme sites in the primers, the PCR product was radiolabeled with ^{32}Pα-dATP using Klenow DNA polymerase. Nucleosomes were assembled onto the DNA by the salt gradient dialysis method (33). Mock assembled and nucleosome assembled DNA was incubated with 10–40 units of HinfI restriction enzyme, electrophoresed through 5% polyacrylamide/1× TBE and visualized using the Fuji PhosphorImager.

Immobilized Template Assay. Template assays were performed by a modification of the method of Ranish et al. (34). A fragment of the human GM-CSF promoter (−120 to −45) was amplified by PCR from the pGMselect wild-type and mutant plasmids using a biotinylated sense primer. The PCR product was purified by gel electrophoresis and eluted using the QIAGEN QIAquick gel extraction kit. The template (150 ng per reaction) was bound to 15 μl Dynabeads M280 Streptavidin (Dynal) as described previously (34). The prepared template was blocked for 15 min at room temperature in 50 μl binding buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.01% NP-40, 10% glycerol) containing 0.1 mg/ml BSA. Alternatively assembled or mock assembled nucleosome reactions were incubated with Dynabeads overnight at 4°C, washed three times in binding buffer, and blocked similarly.

After blocking, beads were washed three times in binding buffer and resuspended in 10 μl binding buffer. Nuclear extracts (250 μg per reaction) were diluted fourfold and supplemented so that the reaction conditions were equivalent to those in binding buffer. Reaction mix was supplemented with 4.5 μg poly(dI:dC), 4.5 μg sheared salmon sperm DNA and protease inhibitors, and incubated on ice for 10 min. The DNA template was added to the nuclear extracts and incubated for 2 h at 4°C with mixing. The beads were washed three times with binding buffer containing 1 mM DTT, 1 mg/ml BSA, and protease inhibitors. Proteins were eluted from the beads in SDS load buffer, resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to Western analysis using anti-RelA (Santa Cruz Biotechnology, Inc.), anti-Sp1 (Santa Cruz Biotechnology, Inc.), anti-CBP (Santa Cruz Biotechnology, Inc.), and anti-brg1 (35) antibodies. Proteins were detected using SuperSignal Chemiluminescent substrate (Pierce Chemical Co.), visualized using the Fuji luminescent image analyzer (Las-1000 plus), and quantified using the Fuji Image Gauge software.

Results

Chromatin Is Remodeled Across the Proximal Promoter Region of the GM-CSF Gene After T Cell Activation. To investigate changes in chromatin structure across the GM-CSF gene upon T cell activation, accessibility of the gene to micrococcal nuclease (MNase) or restriction enzyme digestion after T cell activation was measured using a real-time PCR assay (CHART-PCR; reference 30) and nine primer sets which amplify regions of ~100 base pairs from −633 to +164 (Fig. 1 A, primer sets −VII to +II). PCR amplification was monitored by SYBR green incorporation (36). The amount of PCR product generated from MNase digested samples was plotted as a percentage of that generated from undigested samples for each primer set.

In nonstimulated cells accessibility of different regions of the GM-CSF gene to MNase digestion was not uniform and ranged from ~25 to 60% accessibility across the region of the gene examined (see Fig. 1 B, open bars; Fig. 1 C, black line). There were two regions which were less accessible to digestion with MNase, one centered in the vicinity of the proximal promoter (~−100 bp) and another further upstream at about −350 to −400 bp. These regions of relative inaccessibility to MNase may indicate the preferred positions of nucleosomes on the uninduced GM-CSF promoter.

To further investigate nucleosome positioning across the GM-CSF promoter, mononucleosomal DNA of average size 150 bp, was prepared by MNase digestion of formaldehyde cross-linked EL-4 T cells (Fig. 1 D) and used in PCR reactions with the primer sets indicated in Fig. 1 A. While all the primer sets amplified appropriate size products from genomic DNA or from a plasmid containing the GM-CSF promoter (Fig. 1 D), only specific primer sets gave strong PCR products from the mononucleosomal DNA (Fig. 1 E). It should be noted that primer set +I cannot amplify from the plasmid because the insert does not contain this part of the GM-CSF gene. Primer set −I gave a strong PCR product from the mononucleosomal DNA whereas flanking primer sets −II and +I gave little or no product (Fig. 1 E). The lower band seen in primer sets −I and −II most likely represent primer dimers that are also seen in the control sample lanes. Similarly, primer set −V yielded a strong PCR product from mononucleosomal DNA whereas sets −IV and −VI did not (Fig. 1 E). These data support the possibility that nucleosomes are preferably positioned at regions centered at −100 and −400.

Upon T cell stimulation the region covered by primer set −I (−155 to −40 spanning the CD28RR, see Fig. 1 A) became significantly more accessible (~3.4-fold) to MNase digestion (Fig. 1 B, open bars). The CD28RR is important for induction of GM-CSF gene expression upon T cell activation (37–39). Set +I (−63 to +43) that spans the transcription start site became slightly more accessible (~1.4-fold) to MNase digestion upon T cell activation (Fig. 1 B).

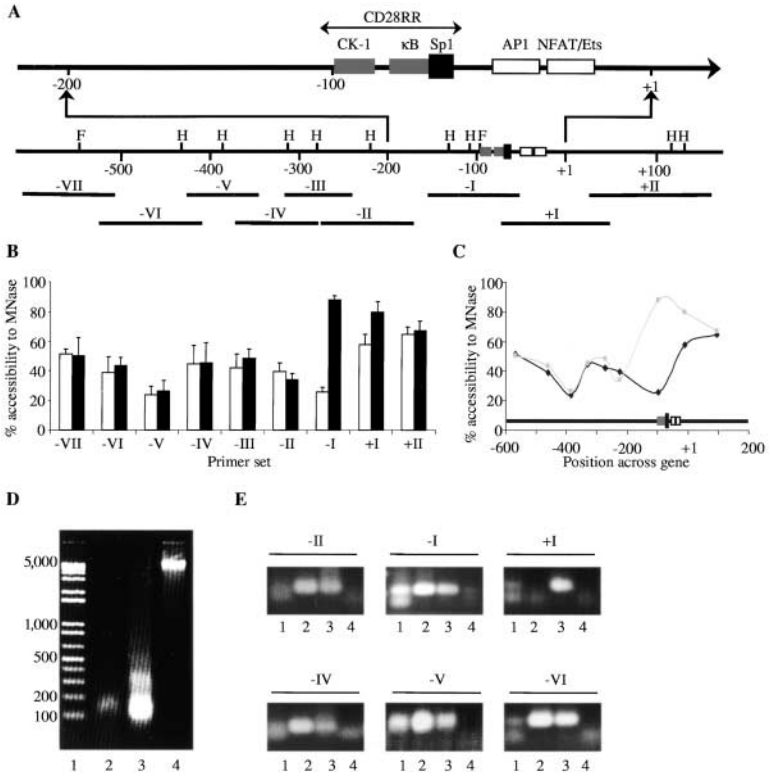

Figure 1. Accessibility of regions of the GM-CSF gene to MNase digestion. (A) Schematic representation of the GM-CSF gene showing transcription factor binding sites and the CD28RR of the promoter. DNA fragments amplified by primer sets −VII to +II and HinfI (F) and HaeIII (H) restriction enzymes sites are indicated. (B) Nuclei from nonstimulated (white bars) or 4 h P/I stimulated (black bars) EL-4 T cells were incubated with MNase. Genomic DNA was analyzed by real-time PCR using the indicated primer sets. The mean and standard error of three replicate assays are shown. (C) Percentage accessibility determined in B for nonstimulated (black line) and stimulated (gray line) nuclei was plotted against position on the GM-CSF gene corresponding to the midpoint of each PCR product. Transcription factor binding sites in the GM-CSF promoter are represented. (D) Agarose gel purified mononucleosome-size DNA fragments generated by MNase digestion of formaldehyde cross-linked EL-4 T cells (lane 2), genomic DNA from formaldehyde cross-linked cells digested with MNase to generate mononucleosome-size fragments (lane 3) and genomic DNA from untreated cells were run on a 2% agarose gel. Lane 1 contains a DNA marker ladder. (E) PCR amplification with primer sets from A as indicated using mononucleosomal DNA (lane 1), pMGM2.4luc plasmid DNA (lane 2), genomic DNA (lane 3), and no DNA as a control (lane 4). The PCR products are shown for each primer set after agarose gel electrophoresis. Faster migrating bands are likely to represent primer dimers.

This primer set covers AP-1 and NFAT/Ets binding sites, which are also important for promoter activity (37, 40, 41). The region downstream of the transcription start site, covered by primer set +II (+24 to +164) and the regions upstream of the proximal promoter covered by primer sets −II through to −VII did not show any significant changes in accessibility after T cell stimulation (Fig. 1 B). Therefore, changes in MNase accessibility upon T cell stimulation were limited to a 200 bp region assayed with primer sets −I and +I, which covers the proximal promoter elements and the transcription start site (Fig. 1 C).

To confirm the changes in accessibility of the GM-CSF gene upon T cell stimulation, the accessibility of HinfI (F) and HaeIII (H) restriction enzyme sites across the GM-CSF gene were also analyzed (see Fig. 1 A). The HinfI sites at −97 and −548 (as assayed using primer set −I and −VII, respectively) showed intrinsic accessibility (∼30% at both sites, see Fig. 2 A), in agreement with the intrinsic accessibility to MNase digestion. Upon T cell stimulation the HinfI site at −97 became more accessible again supporting the data generated using MNase (Fig. 2 A). Importantly, this HinfI site is adjacent to the CK-1 site within the CD28RR of the GM-CSF promoter. In contrast, the distal HinfI site at −548 did not become more accessible to restriction enzyme digestion upon T cell stimulation (Fig. 2 A).

The accessibility of 2 HaeIII restriction enzyme sites (at −105 and −127, both amplified by primer set −I) before and after T cell stimulation was also monitored using CHART-PCR. Using this primer set, accessibility to HaeIII digestion upon T cell activation increased (Fig. 2 B) implying that either one or both of these HaeIII sites became more accessible to digestion upon T cell stimulation. In contrast, the HaeIII site further upstream at −219, displayed no change in accessibility upon T cell stimulation (Fig. 2 B, primer set −II) as was also seen with HaeIII sites at −281, −383, and −483 (Fig. 2 C). Similarly, HaeIII sites downstream of the transcription start site (at +122 and +141) assayed with primer set +II did not display any changes in accessibility upon T cell stimulation (Fig. 2 B), in keeping with data generated with MNase digestion. These data for both MNase and restriction enzyme accessibility clearly demonstrate that significant changes in chromatin structure are limited to a region encompassing the proximal GM-CSF promoter.

Put together, these data point to the presence of a nucleosome spanning the GM-CSF promoter in resting T cells, which becomes remodeled upon T cell activation.

Characteristics of Chromatin Remodeling across the GM-CSF Promoter after T Cell Stimulation. The timing of chromatin remodeling events across the proximal promoter region of the GM-CSF gene after T cell stimulation, and the relationship of these events to transcription from the GM-CSF gene were investigated. As described earlier, in nonstimulated cells, the region covered by primer set −I displayed a low level of inherent accessibility to digestion (Fig. 3 A), which was unchanged at 30 min after stimulation. However, 1 h after stimulation, accessibility at this site had increased approximately threefold (Fig. 3 A) and this was maintained for up to 6 h when levels of accessibility started to decline (Fig. 3 A).

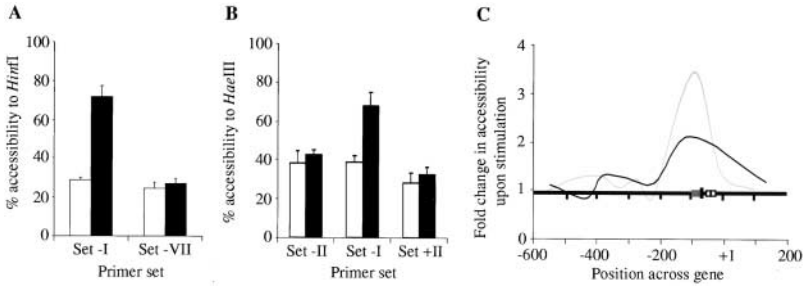

Figure 2. Accessibility of regions of the GM-CSF gene to restriction enzyme digestion. Nuclei from nonstimulated (white bars) or 4 h P/I stimulated (black bars) EL-4 T cells were incubated with HinfI (A) or HaeIII (B) restriction enzyme. Genomic DNA was analyzed by real-time PCR, using the indicated primer sets. The mean and standard error of three replicate assays are shown. (C) Changes in accessibility in 4 h P/I stimulated nuclei compared with nonstimulated EL-4 T cell nuclei as determined with MNase (gray line) and restriction enzymes (black line) were plotted against position across the GM-CSF gene. For restriction enzymes the position of each cutting site was plotted and for MNase the position corresponding to the midpoint of each PCR product was plotted. Transcription factor binding sites in the GM-CSF promoter are represented.

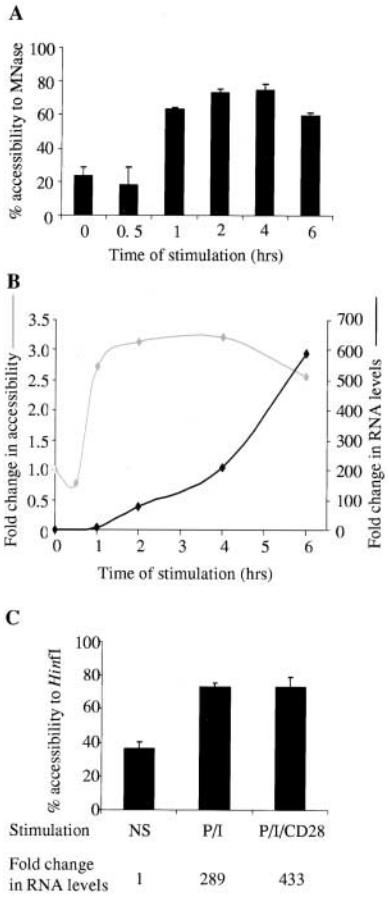

Figure 3. Characteristics of chromatin remodeling at the GM-CSF promoter in stimulated EL-4 T cells. (A) Nuclei from nonstimulated EL-4 T cells and cells stimulated with P/I for the indicated time periods, were incubated with MNase. Genomic DNA was analyzed by real-time PCR analysis, using primer set −I. (B) Data generated in A was graphed as fold change in accessibility at each time point compared with nonstimulated (gray line). GM-CSF mRNA levels were determined by real-time PCR analysis of cDNA prepared from EL-4 T cells treated as in A, using primer set +II. Data is graphed as fold change in RNA levels at each time point compared with nonstimulated (black line). (C) Nuclei from EL-4 T cells either nonstimulated or stimulated with P/I and P/I/antiCD28 antibody for 4 h, were incubated with HinfI. Genomic DNA was analyzed as in A. GM-CSF mRNA levels in cells treated in parallel, were determined as in B and are indicated below.

To correlate these remodeling events with transcription, GM-CSF mRNA levels were analyzed from EL-4 T cells stimulated in parallel by quantitative real-time PCR using primer set +II (+24 to +164), which covers a transcribed region of the GM-CSF gene (see Fig. 1 A). Little increase in GM-CSF gene transcription was detected 1 h after stimulation (Fig. 3 B). However, RNA levels dramatically increased after this time with a 70-fold increase seen after 2 h stimulation and ~600-fold after 6 h simulation. This is in keeping with previous studies, where maximum levels of transcription were detected after 8–10 h of stimulation using RNase protection (11). Chromatin remodeling events across the promoter, therefore, precede significant transcription from the gene and begin to decline before the time of maximum mRNA accumulation.

We next examined the stimulation conditions required for chromatin accessibility changes across the GM-CSF promoter. P/I stimulation lead to increased accessibility for HinfI digestion across the proximal promoter but treatment with a CD28 activating antibody together with P/I, did not increase the level of HinfI accessibility (Fig. 3 C). In contrast, levels of GM-CSF mRNA were increased by CD28 activation (Fig. 3 C), implying that the signals generated by P/I are sufficient to lead to changes in chromatin accessibility but that CD28 signals increase the levels of mRNA via a distinct mechanism.

Remodeling of the GM-CSF Promoter Is Dependent on NF-κB Proteins. As the CD28RR region of the human GM-CSF promoter has been previously implicated in the formation of an inducible DH site (21), the role of NF-κB proteins in generating MNase or RE accessibility across the GM-CSF promoter after stimulation was examined. EL-4 T cells were pretreated with the proteasome inhibitor MG-115 for 1 h before stimulation, preventing degradation of the IκB proteins and subsequent translocation of NF-κB proteins to the nucleus (unpublished data; reference 42). The MG-115 inhibitor had no effect on the inherent basal level of accessibility seen in nonstimulated cells (unpublished data). However, in stimulated cells treatment with MG-115 reduced the increase in accessibility by almost fourfold (Fig. 4 A). To further assess the effect of inhibiting NF-κB translocation to the nucleus, EL-4 T cells were stably transfected with a mutant IκBα construct which cannot be phosphorylated and thus prevents NF-kB translocation

to the nucleus (27, 43). Levels of RelA protein were reduced by ~50% in stimulated EL-4-IκBα nuclear extracts compared with extracts from cells transfected with the empty vector (Fig. 4 B, compare lanes 4 and 2). Levels of the transcription factor Sp1 or the ATPase component of the SWI/SNF chromatin remodeling complex, brg1, were unaffected in the nuclei of the EL-4-IκBα cells (Fig. 4 B).

Primer set −I was used to assay the accessibility of the HaeIII sites in the proximal promoter region of the GM-CSF gene in nonstimulated EL-4-IκBα cells and cells stimulated with P/I for 2 h. The change in accessibility normally seen upon stimulation was reduced by 40% in the EL-4-IκBα cell line (Fig. 4 C), in line with the 50% reduction observed in RelA levels. The level of GM-CSF mRNA was also reduced by ~40% in the IκBα-transfected cells (Fig. 4 D) compared with the control CMV-transfected cells (set at 100%) consistent with the reduction in promoter accessibility.

Therefore, reduction of NF-κB protein levels in the nucleus lead to a reduction in chromatin remodeling at the GM-CSF promoter as well as a reduction in transcription from the gene. This suggests that NF-κB proteins play a role in chromatin remodeling events at the GM-CSF promoter upon T cell activation.

Brg1 Can Be Recruited to the GM-CSF Promoter In Vitro. From the data above, it is possible that the NF-κB proteins and their cognate binding sites in the CD28RR of the GM-CSF promoter are involved in recruiting chromatin remodeling activities to the GM-CSF promoter as has been shown for certain transcription factors on other genes (44, 45). To assess this possibility, we used an in vitro recruitment assay to determine if chromatin modifying complexes can be recruited to the GM-CSF promoter in a NF-κB–dependent manner. An immobilized GM-CSF template, consisting of a 75 bp region of the proximal promoter from −120 to −45, encompassing the CD28RR (17; see Fig. 1 A) was incubated with nuclear extracts from nonstimulated and P/I-stimulated Jurkat T cells. After washing, bound proteins were eluted in SDS load buffer, subjected to SDS-PAGE, and analyzed by Western blotting, using antibodies to specific proteins.

Little RelA is present in nuclear extracts of nonstimulated Jurkat T cells, but is detected in extracts stimulated with P/I for 30 min as expected from previous results (Fig. 5 A, lanes 1 and 2, top panel). As expected, RelA bound to the GM-CSF template from stimulated but not nonstimulated extracts (Fig. 5 A, lanes 3 and 4). Sp1 protein was detected in both nonstimulated and stimulated nuclear extracts at approximately equal levels (Fig. 5 A, lanes 1 and 2, middle panel), but significant binding of Sp1 to the GM-CSF template was only detected from stimulated extracts (Fig. 5 A, lane 4). In the experiments shown in Figs. 5 and 6 there are variable levels of Sp1 binding in the unstimulated extracts. The reason for this variation is not clear, but may arise because the Sp1 protein binds only weakly to the Sp1 site in the absence of NF-κB proteins and is stabilized by binding of NF-κB proteins to the adjacent sites. Recruitment to the GM-CSF template of the ATPase component of the SWI/SNF chromatin remodeling complex, brg1, and the histone acetyl transferase CBP, representing the two classes of chromatin altering activities was then examined. Approximately equal amounts of brg1 protein was detected in both nonstimulated and stimulated nuclear extracts (Fig. 5 A, lanes 1 and 2, bottom panel); however, significant binding of brg1 to the GM-CSF template was only detected from stimulated extracts (Fig. 5 A, lane 4). In contrast, although CBP protein was present in nonstimulated and both 30 min and 6 h stimulated nuclear extracts at approximately equal levels (Fig. 5 B, lanes 1–3, bottom panel), it did not bind to the GM-CSF promoter (lanes 4–6). Therefore, brg1 but not CBP is recruited to the GM-CSF CD28RR template in vitro after T cell activation.

To determine the timeframe of recruitment of brg1 to the GM-CSF template, nuclear extracts that had been stimulated with P/I for various times between 0–8 h were examined using the template assay. Localization of RelA to the nucleus upon T cell stimulation is highest at 30 min after stimulation and continues at lower levels through to 8 h (Fig. 5 C, top panel, lanes 1–6). This is reflected in binding of RelA to the GM-CSF template (lanes 7–12). Levels of Sp1 protein in the extracts do not change post stimulation (Fig. 5 C, middle panel, lanes 1–6). However, Sp1 binding

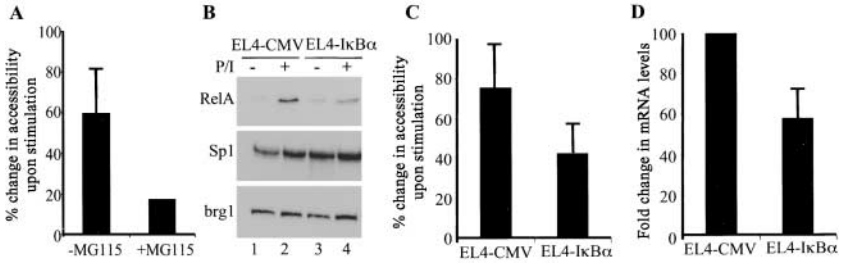

Figure 4. Chromatin remodeling at the GM-CSF promoter is dependent on NF-κB proteins. (A) EL-4 T cells were either untreated or pretreated for 1 h with the proteasome inhibitor MG115, then incubated with or without P/I for 4 h. Nuclei were incubated with HaeIII restriction enzyme and the genomic DNA analyzed by real-time PCR analysis using primer set −I. The percentage change in accessibility in 4 h P/I stimulated nuclei compared with nonstimulated nuclei was graphed. The mean and standard error of three replicate assays are shown. (B) Nuclear extracts (50 μg) from CMV-transfected (lanes 1 and 2) and mutant IκBα-transfected (lanes 3 and 4) EL-4 T cells, either unstimulated (lanes 1 and 3) or stimulated for 2 h with P/I (lanes 2 and 4) were subjected to SDS-PAGE and analyzed by Western blotting with the indicated antibodies. (C) Nuclei from CMV-transfected and mutant IκBα-transfected EL-4 T cells, either left unstimulated or stimulated for 2 h with P/I, were incubated with HaeIII restriction enzyme and the genomic DNA analyzed by real-time PCR as in A. (D) GM-CSF mRNA levels were determined by real-time PCR analysis of cDNA prepared from cells treated as in C, using primer set +II. The increase in GM-CSF mRNA levels upon stimulation in EL4-IκBα cells was plotted relative to control (EL4-CMV) cells set at 100%. The mean and standard error of three replicate assays are shown.

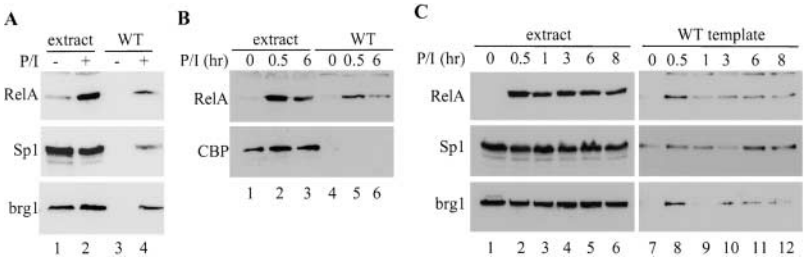

Figure 5. Recruitment of brg1 to the GM-CSF promoter in vitro. (A) Nuclear extracts prepared from Jurkat T cells, either nonstimulated (lane 1) or stimulated with P/I for 30 min (lane 2) were incubated with WT GM-CSF template (lanes 3 and 4). Bound proteins were resolved by SDS-PAGE and subjected to Western blotting using the indicated antibodies. Lanes 1 and 2 represent 1/5th of the input extract. (B) Nonstimulated (lane 1), 0.5 h (lane 2), and 6 h P/I stimulated (lane 3) Jurkat T cell nuclear extracts were incubated with WT GM-CSF template (lanes 4–6). Bound proteins were analyzed by Western blotting with the indicated antibodies. Lanes 1 to 3 represent 1/5th of input extract. (C) Nonstimulated Jurkat T cell nuclear extracts (lane 1) or extracts from cells stimulated with P/I for the indicated time periods (lanes 1–6) were incubated with WT GM-CSF template (lanes 7–12). Bound proteins were analyzed as in A.

to the GM-CSF template appeared to mimic that of RelA, with little binding from nonstimulated extracts and binding seen at all time points after stimulation although variable levels of binding are seen across the time course examined (lanes 7–12). Brg1 levels in the nucleus also remained constant after T cell stimulation (Fig. 5 C, bottom panel, lanes 1–6), but maximum brg1 binding to the template was detected at 30 min of stimulation (Fig. 5 C, lane 8). Lower levels of brg1 binding were detected at all other time points (Fig. 5 C, lanes 9–12) in parallel with RelA binding. Similar results were obtained when nuclear extracts from EL-4 T cells were used with a mouse GM-CSF template (unpublished data), which has a high degree of conservation with the human sequence across the CD28RR (Fig. 6 A).

Thus, brg1 can bind in vitro to the GM-CSF promoter template from stimulated but not unstimulated T cell extracts and binding appears to parallel that of RelA and Sp1.

Brg-1 Recruitment to the GM-CSF Template Is Dependent on NF-κB Proteins Binding to the CD28RR. To determine whether brg1 recruitment to the GM-CSF promoter was in fact dependent on NF-κB and/or Sp1 binding sites and proteins, binding studies were conducted using mutant DNA templates. Templates were generated with mutations in either the NF-κB (κBm) or CK-1 (CK-1m) sites, both NF-κB and CK-1 sites (CK-1/κBm) and the Sp1 site (Sp1m) of the CD28RR (Fig. 6 A). Binding to these templates from nonstimulated and 30 min P/I stimulated extracts was examined. Mutation of the Sp1 site, while reducing Sp1 binding, had no effect on RelA or brg1 binding (Fig. 7 C). Mutation of the individual NF-κB binding sites (κBm and CK-1m) reduced RelA binding and in parallel reduced brg1 binding but appeared to have no effect on Sp1 binding (Fig. 7 B). In contrast, in the absence of both NF-κB sites (CK-1/κBm) binding of RelA, Sp1, and brg1 were dramatically reduced (Fig. 7 D). Thus, brg1 binding to the GM-CSF template is dependent on intact NF-κB but not Sp1 binding sites, suggesting that NF-κB proteins may recruit brg1 to the GM-CSF promoter template. In addition, Sp1 binding is dependent on intact NF-κB binding sites but not vice versa.

To further test the possibility that NF-κB proteins were involved in recruiting brg1 to the GM-CSF template, extracts were prepared from Jurkat T cells stably expressing the mutant IκBα protein. Stable expression of this mutant considerably reduced the level of RelA in the nucleus (Fig. 7 E, compare lanes 2 and 4) and as expected binding of RelA to the GM-CSF template was dramatically reduced in these extracts compared with extracts from cells trans-

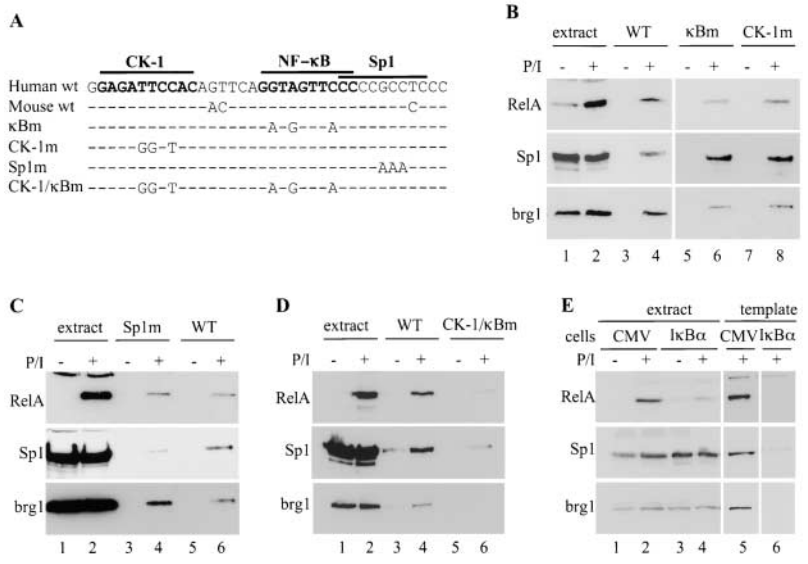

Figure 6. Recruitment of brg1 to the GM-CSF promoter is dependent on NF-κB proteins. (A) Nucleotide sequence of the human and mouse GM-CSF CD28RR is shown with CK-1, NF-κB, and Sp1 binding sites indicated. Bases that are different in the mouse sequence or that were mutated to generate the mutant templates are indicated. (B) Nuclear extracts from nonstimulated (lane 1) and 30 min P/I stimulated (lane 2) Jurkat T cells were incubated with GM-CSF templates as indicated (lanes 3–8). Bound proteins were resolved by SDS-PAGE and subjected to Western blotting using the indicated antibodies. Lanes 1 and 2 represent 1/5th of the input extract. (C and D) Nuclear extracts from nonstimulated (lane 1) and 30 min P/I stimulated (lane 2) Jurkat T cells were incubated with the indicated GM-CSF templates (lanes 3–6) and analyzed as in A. (E) Nuclear extracts prepared from CMV-transfected (CMV, lanes 1 and 2) and mutant IκBα-transfected (IκBα, lanes 3 and 4) Jurkat T cells were left unstimulated (lanes 1 and 3) or stimulated for 30 min with P/I (lane 2 and 4). Stimulated nuclei were incubated with WT GM-CSF template (lanes 5 and 6) and analyzed as in A.

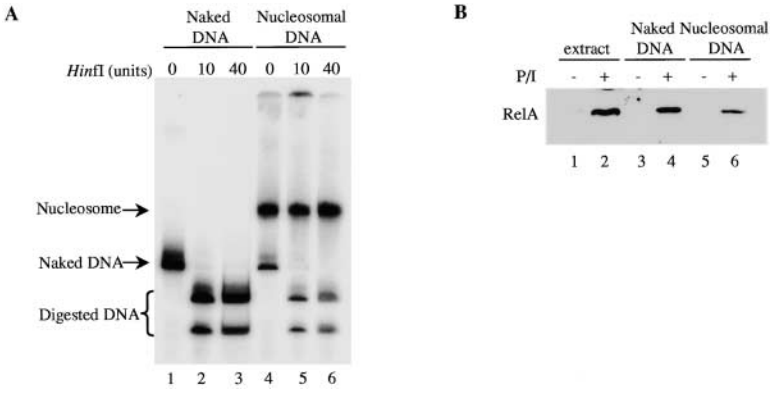

Figure 7. NF-κB can bind to the nucleosome assembled GM-CSF promoter, in vitro. (A) A 197 bp GM-CSF promoter template, either naked DNA (lanes 1–3) or nucleosome assembled DNA (lanes 4–6) was incubated with varying amounts of HinfI restriction enzyme, as indicated. The DNA was electrophoresed through 5% polyacrylamide gel. The positions of the nucleosomal DNA, naked DNA, and digested DNA fragments are indicated. (B) Nuclear extracts prepared from Jurkat T cells, either nonstimulated (lane 1) or stimulated with P/I for 30 min (lane 2) were incubated with a naked GM-CSF template (lanes 3 and 4) or nucleosomal assembled GM-CSF template (lanes 5 and 6). Bound proteins were resolved by SDS-PAGE and subjected to Western blotting using the indicated antibodies. Lanes 1 and 2 represent 1/5th of the input extract.

fected with the empty CMV vector (Fig. 7 E, compare lanes 5 and 6). This reduction in RelA binding was paralleled by a similar reduction in Sp1 and brg1 binding (Fig. 7 E, lane 6).

Taken together, these experiments show that in vitro binding of brg1 to the GM-CSF promoter is dependent on prior binding of NF-κB proteins to two sites in the CD28RR.

NF-κB Bind to a Nucleosome Assembled GM-CSF Promoter, In Vitro. To test the possibility that NF-κB proteins are able to bind to the GM-CSF promoter in a chromatin context before nucleosome remodeling, a 197 bp fragment of the GM-CSF promoter (−173 to +24) was assembled into a nucleosome and binding of RelA examined. This fragment contains the HinfI restriction site at −97 and corresponds to the region which displayed an increase in accessibility following T cell activation (see Fig. 1 C). The GM-CSF template was radiolabeled and assembled into a single nucleosome as assessed by a shift in the migration of the DNA through a polyacrylamide gel (Fig. 7 A, lane 4), compared with mock assembled DNA (lane 1). The naked DNA was digested by the HinfI enzyme into two fragments of 121 and 76 bp, as expected (Fig. 7 A, lanes 2 and 3). In contrast, the HinfI restriction site in the nucleosome assembled DNA was protected from digestion (Fig. 7 A, lanes 5 and 6) but the small amount of naked DNA remaining in the assembly reaction digested as expected. Therefore, the GM-CSF promoter template could be assembled into a single nucleosome which protected the HinfI restriction site from digestion.

The HinfI restriction site is adjacent to the CK-1 element in the GM-CSF promoter so binding assays were conducted to determine whether the nucleosome could also prevent NF-κB binding (Fig. 7 B). Unstimulated and 30 min P/I stimulated Jurkat T cell extracts where incubated with the DNA templates. RelA bound to the naked DNA template from stimulated extracts as expected (Fig. 7 B, lane 4). RelA also bound to the nucleosome assembled template from stimulated extracts, although with reduced affinity (Fig. 7 B, lane 6). Therefore, the RelA protein is able to access its binding sites in the GM-CSF promoter despite the presence of a nucleosome.

Discussion

A key control point in inducible gene expression involves reorganization of chromatin structure across regulatory regions of genes to allow access for the transcriptional machinery. Here we have mapped chromatin accessibility across the promoter region of the GM-CSF gene in unstimulated and stimulated EL-4 T cells and several important points have emerged. First, the entire region that was mapped from −633 to +164 showed intrinsic accessibility to both MNase and restriction enzymes. This is in stark contrast to a recent study on the IL-2 promoter in the same cell type where we showed that there is no accessibility across a large region of the IL-2 gene implying a more "closed" chromatin configuration for this gene (30). Second, two regions of the GM-CSF upstream sequence were relatively inaccessible to digestion in these assays and such regions may represent preferred nucleosome positions. The possibility that nucleosomes are preferentially located in these positions (centered on −100 and −400) was confirmed by the ability to amplify these regions and not flanking regions from cross-linked mononucleosomal DNA. While one of these proposed nucleosomes would span the GM-CSF promoter in resting T cells, the fact that this region still displays some inherent accessibility may indicate a fairly flexible nucleosome arrangement that allows some access of transcription factors to their binding sites. GM-CSF, in contrast to IL-2, has a pattern of expression that may require flexible access to DNA binding sites in chromatin, being expressed in a wide variety of cell types in response to many different signals (for a review, see reference 1). In addition, some myeloid cell types display constitutive transcription from the GM-CSF gene with mRNA levels being controlled at the stability level (46). Recently, the chromatin structure across the promoters of several immune function genes has been investigated. For example, the IL-12 gene assembles a nucleosome across important control regions including NF-κB and C/EBP binding sites (47). In contrast, the HIV and IFNβ promoters contain important transcription factor binding sites in nucleosome-free regions (48, 49). Positioning of nucleosomes across the promoter regions of inducible genes appears, therefore, to be gene-specific and such differences may reflect the time-

frame or signal requirements for the response of these genes to cell activation.

The third important finding was that the proximal promoter region of the GM-CSF gene undergoes an increase in accessibility to MNase and restriction enzymes after activation of the cells with P/I. The region that becomes remodeled stretches between −174 to + 24 across the transcription start site and encompasses the CD28RR as well as more proximal AP-1 and NFAT/Ets binding sites. This suggests that a single nucleosome positioned across this region may be remodeled after activation, which is similar to the situation described for the IL-12 p40 promoter upon activation of macrophages (47). Furthermore, the remodeling events at both the GM-CSF and IL-12 promoters occur 1 h after stimulation (47). In addition, chromatin changes in both genes are dependent upon a primary stimulus (P/I for GM-CSF and LPS for IL-12) but not enhanced by a secondary stimulus (CD28 for GM-CSF and IFN-γ for IL-12) (47). Importantly, an increase in GM-CSF mRNA levels is detected at 2 h and nuclear run-on assays have shown that transcription is induced in 2 h (50), only after the chromatin remodeling events have occurred. Therefore, chromatin remodeling events at the GM-CSF promoter may be a critical initiating step in activation of GM-CSF gene transcription.

Several pieces of evidence have previously shown the importance of the CD28RR in GM-CSF promoter function particularly in a chromatin context. First, a mutation in the CD28RR, that altered the specific NF-κB family members that bound to the GM-CSF κB site, eliminated promoter activity in transgenic mice and in stable cell lines but not in transient transfections (21). This mutation also specifically prevented the formation of a DNase I hypersensitive site in the promoter that was normally detected in the wild-type transgene (21) and in the native human gene (20). Second, a triplex-forming oligonucleotide directed against the NF-κB/Sp1 region efficiently inhibited constitutive GM-CSF expression in Juvenile Myelomonocytic Leukemic cells (9) as well as inducible expression in Jurkat T cells (51), suggesting an important role for this region in transcription from the endogenous gene.

These results presented here suggest that specific NF-κB family proteins bound to the GM-CSF promoter are important for recruiting chromatin remodeling activities which bring about changes in chromatin structure. The SWI/SNF chromatin remodeling complex is a likely candidate for such an activity, given that we have now shown that brg1 can be recruited to the GM-CSF promoter in vitro in an NF-κB–dependent manner. From these in vitro binding experiments, a model can be proposed whereby NF-κB proteins first interact with the CD28RR. This in turn leads to both Sp1 binding and brg1 recruitment to the promoter although brg1 recruitment does not appear to depend on Sp1 binding. While Sp1 was not required for recruitment of brg1 in vitro it may play a role in stabilizing the complex in a chromatin context or in the recruitment of coactivators or the transcription machinery after chromatin remodeling and further studies are required to determine this role. Similarly, while the histone acetyl transferase CBP was not recruited to the CD28RR region of the GM-CSF promoter, in vitro, this does not rule out the involvement of CBP or other histone acetylases in GM-CSF promoter activation.

It has previously been shown that a brg1-containing complex binds more tightly to chromatin after Jurkat T cell activation (52) in keeping with the possibility that brg1 is recruited to the GM-CSF promoter in vivo. SWI/SNF complexes have been shown to be recruited to other promoters through interaction with specific transcription factors (44, 45) but this is the first example of brg1 complexes being dependent on NF-κB for binding at least in vitro. This model would suggest that NF-κB proteins may be able to access their binding sites in the CD28RR in a chromatin context. In support of this is the finding that a nucleosome can be assembled across the GM-CSF promoter region in vitro and that RelA can still bind, although with reduced affinity, to the nucleosome-assembled GM-CSF promoter. This is in contrast to a recent study on the IL-2 gene, where binding of NF-κB proteins was completely inhibited by the assembly of a nucleosome over the promoter (53).

Aberrant expression of NF-κB is often a feature of pathological conditions such as leukemia, other cancers, and chronic inflammatory diseases. For example, the constitutive activation of NF-κB proteins has been reported as a distinguishing characteristic of leukemic stem cells (54). It is possible then that in such conditions a key checkpoint in the control of GM-CSF expression may be circumvented so that the chromatin structure of the GM-CSF promoter is in a constitutively 'open' state. This may contribute to the inappropriate expression of GM-CSF often observed in these conditions. Thus, defining the mechanism by which chromatin remodeling complexes are recruited to genes such as GM-CSF may provide novel therapeutic targets for these diseases.

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References

1. Gasson, J.C. 1991. Molecular physiology of granulocyte-macrophage colony-stimulating factor. *Blood*. 77:1131–1145.
2. Wada, H., Y. Noguchi, M.W. Marino, A.R. Dunn, and L.J. Old. 1997. T cell functions in granulocyte/macrophage colony-stimulating factor deficient mice. *Proc. Natl. Acad. Sci. USA*. 94:12557–12561.
3. Lang, R.A., D. Metcalf, R.A. Cuthbertson, I. Lyons, E. Stanley, A. Kelso, G. Kannourakis, D.J. Williamson, G.K.

Klintworth, T.J. Gonda, and A.R. Dunn. 1987. Transgenic mice expressing a hemopoietic growth factor gene (GM-CSF) develop accumulations of macrophages, blindness, and a fatal syndrome of tissue damage. *Cell.* 51:675–686.
4. Fraser, J.D., and A. Weiss. 1992. Regulation of T-cell lymphokine gene transcription by the accessory molecule CD28. *Mol. Cell. Biol.* 12:4357–4363.
5. Young, D.C., and J.D. Griffin. 1986. Autocrine secretion of GM-CSF in acute myeloblastic leukemia. *Blood.* 68:1178–1181.
6. Young, D.C., K. Wagner, and J.D. Griffin. 1987. Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. *J. Clin. Invest.* 79:100–106.
7. Williamson, D.J., C.G. Begley, M.A. Vadas, and D. Metcalf. 1988. The detection and initial characterization of colony-stimulating factors in synovial fluid. *Clin. Exp. Immunol.* 72: 67–73.
8. Karin, M., Y. Cao, F.R. Greten, and Z. Li. 2002. NF-κB in cancer: From innocent bystander to major culprit. *Nat. Rev. Cancer.* 2:301–310.
9. Kotchetkova, M., P.O. Iversen, A.F. Lopez, and M.F. Shannon. 1997. Deoxyribonucleic acid triplex formation inhibits granulocyte macrophage colony-stimulating factor gene expression and suppresses growth in Juvenile Myelomonocytic Leukemic cells. *J. Clin. Invest.* 99:3000–3008.
10. Chan, J.Y., D.J. Slamon, S.D. Nimer, D.W. Golde, and J.C. Gasson. 1986. Regulation of expression of human granulocyte/macrophage colony-stimulating factor. *Proc. Natl. Acad. Sci. USA.* 83:8669–8673.
11. Cockerill, P.N., M.F. Shannon, A.G. Bert, G.R. Ryan, and M.A. Vadas. 1993. The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. *Proc. Natl. Acad. Sci. USA.* 90:2466–2470.
12. Nimer, S.D., E.A. Morita, M.J. Martis, W. Wachsman, and J.C. Gasson. 1988. Characterization of the human granulocyte-macrophage colony-stimulating factor promoter region by genetic analysis: correlation with DNase I footprinting. *Mol. Cell. Biol.* 8:1979–1984.
13. Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell.* 46:659–667.
14. Shannon, M.F., L.S. Coles, M.A. Vadas, and P.N. Cockerill. 1997. Signals for activation of the GM-CSF promoter and enhancer in T cells. *Crit. Rev. Immunol.* 17:301–323.
15. Himes, S.R., L.S. Coles, R. Katsikeros, R.K. Lang, and M.F. Shannon. 1993. HTLV-1 tax activation of the GM-CSF and G-CSF promoters requires the interaction of NF-κB with other transcription factor families. *Oncogene.* 8:3189–3197.
16. Himes, S.R., R. Katsikeros, and M.F. Shannon. 1996. Co-stimulation of cytokine gene expression in T cells by the human T leukemia/lymphotropic virus type 1 trans activator Tax. *J. Virol.* 70:4001–4008.
17. Himes, S.R., L.S. Coles, R. Reeves, and M.F. Shannon. 1996. High mobility group protein I(Y) is required for function and for c-Rel binding to CD28 response elements within the GM-CSF and IL-2 promoters. *Immunity.* 5:479–489.
18. Struhl, K. 1999. Fundamentally different logic of gene regulation in eukaryotes and prokaryotes. *Cell.* 98:1–4.
19. Workman, J.L., and R.E. Kingston. 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu. Rev. Biochem.* 67:545–579.
20. Cockerill, P.N., A.G. Bert, D. Roberts, and M.A. Vadas. 1999. The human granulocyte-macrophage colony-stimulating factor gene is autonomously regulated in vivo by an inducible tissue-specific enhancer. *Proc. Natl. Acad. Sci. USA.* 96:15097–15102.
21. Cakouros, D., P.N. Cockerill, A.G. Bert, R. Mital, D.C. Roberts, and M.F. Shannon. 2001. A NF-κB/Sp1 region is essential for chromatin remodeling and correct transcription of a human granulocyte-macrophage colony-stimulating factor transgene. *J. Immunol.* 167:302–310.
22. Wu, J., and M. Grunstein. 2000. 25 years after the nucleosome model: chromatin modifications. *Trends Biochem. Sci.* 25:619–623.
23. Vignali, M., A.H. Hassan, K.E. Neely, and J.L. Workman. 2000. ATP-dependent chromatin-remodeling complexes. *Mol. Cell. Biol.* 20:1899–1910.
24. Hansen, J.C., C. Tse, and A.P. Wolffe. 1998. Structure and function of the core histone N-termini: more than meets the eye. *Biochemistry.* 37:17637–17641.
25. Strahl, B.D., and C.D. Allis. 2000. The language of covalent histone modifications. *Nature.* 403:41–45.
26. Struhl, K. 1998. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.* 12:599–606.
27. Brockman, J.A., D.C. Scherer, T.A. McKinsey, S.M. Hall, X. Qi, W.Y. Lee, and D.W. Ballard. 1995. Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. *Mol. Cell. Biol.* 15:2809–2818.
28. Osborne, C.S., M.A. Vadas, and P.N. Cockerill. 1995. Transcriptional regulation of mouse granulocyte-macrophage colony-stimulating factor/IL-3 locus. *J. Immunol.* 155:226–235.
29. Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.
30. Rao, S., E. Procko, and M.F. Shannon. 2001. Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the IL-2 gene. *J. Immunol.* 167:4494–4503.
31. Lomvardas, S., and D. Thanos. 2001. Nucleosome sliding via TBP DNA binding *in vivo*. *Cell.* 106:685–696.
32. Drew, H.R., and C.R. Callidine. 1987. Sequence-specific positioning of core histones on an 860 base-pair DNA. *J. Mol. Biol.* 195:143–173.
33. Varga-Weisz, P.D., E.J. Bronte, and P.B. Becker. 1999. Analysis of modulators of chromatin structure in Drosophila. *Methods Enzymol.* 304:742–757.
34. Ranish, J.A., N. Yudkovsky, and S. Hahn. 1999. Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB. *Genes Dev.* 13: 49–63.
35. Wang, W., T. Chi, Y. Xue, S. Zhou, A. Kuo, and G.R. Crabtree. 1998. Architectural DNA binding by a high-mobility-group/kinesin-like subunit in mammalian SWI/SNF-related complexes. *Proc. Natl. Acad. Sci. USA.* 95:492–498.
36. Overbergh, L., D. Valckx, M. Waer, and C. Mathieu. 1999. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine.* 11:305–312.
37. Jenkins, F., P.N. Cockerill, D. Bohmann, and M.F. Shannon. 1995. Multiple signals are required for function of the human granulocyte-macrophage colony-stimulating factor

gene promoter in T cells. *J. Immunol.* 155:1240–1251.
38. Kaushansky, K., C. O'Rork, S.G. Shoemaker, and J. McCarty. 1996. The regulation of GM-CSF is dependent on a complex interplay of multiple nuclear proteins. *Mol. Immunol.* 33:461–470.
39. Shannon, M.F., S.R. Himes, and L.S. Coles. 1995. GM-CSF and IL-2 share common control mechanisms in response to costimulatory signals in T cells. *J. Leukoc. Biol.* 57:767–773.
40. Thomas, R.S., M.J. Tymms, L.H. McKinlay, M.F. Shannon, A. Seth, and I. Kola. 1997. ETS1, NF-κB and AP1 synergistically transactivate the human GM-CSF promoter. *Oncogene.* 14:2845–2855.
41. Thomas, R.S., M.J. Tymms, A. Seth, M.F. Shannon, and I. Kola. 1995. ETS1 transactivates the human GM-CSF promoter in Jurkat T cells stimulated with PMA and ionomycin. *Oncogene.* 11:2135–2143.
42. Palombella, V.J., O.J. Rando, A.L. Goldberg, and T. Maniatis. 1994. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell.* 78:773–785.
43. Baldwin, A.S., Jr. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14:649–683.
44. Kadam, S., G.S. McAlpine, M.L. Phelan, R.E. Kingston, K.A. Jones, and B.M. Emerson. 2000. Functional selectivity of recombinant mammalian SWI/SNF subunits. *Genes Dev.* 14:2441–2451.
45. Kowenz-Leutz, E., and A. Leutz. 1999. A C/EBP beta isoform recruits the SWI/SNF complex to activate myeloid genes. *Mol. Cell.* 4:735–743.
46. Koeffler, H.P., J. Gasson, and A. Tobler. 1988. Transcriptional and posttranscriptional modulation of myeloid colony stimulating factor expression by tumor necrosis factor and other agents. *Mol. Cell. Biol.* 8:3432–3438.
47. Weinmann, A.S., S.E. Plevy, and S.T. Smale. 1999. Rapid and selective remodeling of a positioned nucleosome during the induction of IL-12 p40 transcription. *Immunity.* 11:665–675.
48. Agalioti, T., S. Lomvardas, B. Parekh, J. Yie, T. Maniatis, and D. Thanos. 2000. Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. *Cell.* 103:667–678.
49. Verdin, E., P. Paras, Jr., and C. Van Lint. 1993. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *EMBO J.* 12: 3249–3259.
50. Ryan, G.R., S.E. Milton, A.F. Lopez, P.G. Bardy, M.A. Vadas, and M.F. Shannon. 1991. Human interleukin-3 mRNA accumulation is controlled at both the transcriptional and posttranscriptional level. *Blood.* 77:1195–1202.
51. Kotchetkova, M., and M.F. Shannon. 1996. DNA triplex formation selectively inhibits granulocyte-macrophage colony-stimulating factor gene expression in human T cells. *J. Biol. Chem.* 271:14438–14444.
52. Zhao, K., W. Wang, O.J. Rando, Y. Xue, K. Swiderek, A. Kuo, and G.R. Crabtree. 1998. Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. *Cell.* 95: 625–636.
53. Attema, J.L., R. Reeves, V. Murray, I. Levichkin, M.D. Temple, D.J. Tremethick, and M.F. Shannon. 2002. The human IL-2 gene promoter can assemble a positioned nucleosome that becomes remodeled upon T cell activation. *J. Immunol.* 169:2466–2476.
54. Guzman, M.L., S.J. Neering, D. Upchurch, B. Grimes, D.S. Howard, D.A. Rizzieri, S.M. Luger, and C.T. Jordan. 2001. Nuclear factor-kB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood.* 98:2301–2307.