Rapid and Selective Remodeling of a Positioned Nucleosome during the Induction of IL-12 p40 Transcription

Amy S. Weinmann,* Scott E. Plevy,¹
and Stephen T. Smale*³

Howard Hughes Medical Institute
Department of Microbiology, Immunology, and Molecular Genetics
University of California, Los Angeles
Los Angeles, California 90095-1662

Immunobiology Center
The Mount Sinai School of Medicine
New York, New York 10029-6574

Summary

Nucleosomes are important for gene regulation, but comprehensive studies of nucleosome positioning, remodeling, and transcription factor binding at inducible mammalian promoters have not been reported. We have analyzed the IL-12 p40 promoter, which is induced in macrophages by bacterial products. High-resolution micrococcal nuclease analyses revealed that a positioned nucleosome, nucleosome 1, spans the promoter, with three positioned nucleosomes further upstream. Upon activation, nucleosome 1 was rapidly and selectively remodeled in a protein synthesis-dependent manner. In primary macrophages, IFNγ synergistically enhanced p40 expression, but little effect on remodeling or promoter occupancy was observed. These results suggest that remodeling complexes are selectively targeted to a single, promoter-encompassing nucleosome and that IFNγ influences an event that is independent or downstream of remodeling.

Introduction

Genomic DNA in eukaryotes is incorporated into chromatin, the most basic unit of which is the nucleosome. Nucleosomes can serve as a barrier for transcription factor access to DNA. Thus, the chromatin environment within which a gene resides can play a crucial role in transcriptional regulation.

For genes that are rapidly induced in response to extracellular signals, nucleosomes appear to contribute regulatory functions that are particularly significant. These contributions have been studied in the greatest detail at the Saccharomyces cerevisiae Pho5, human immunodeficiency virus (HIV), and murine mammary tumor virus (MMTV) promoters. In brief, the Pho5 promoter is contained within positioned nucleosomes flanking a short DNase I hypersensitive region that contains a binding site for the Pho4 activator (Almer and Horz, 1986). Upon induction with low phosphate, Pho4 is dephosphorylated, ultimately allowing DNA binding and the selective remodeling of four nucleosomes in the vicinity of the promoter (Almer et al., 1986; McAndrew et al., 1998). The HIV-1 promoter is nucleosome free, but nucleosomes are positioned upstream of the promoter and downstream of the start site (Verdin et al., 1993). Upon T cell activation, transcription factors bind the promoter, leading to the remodeling of the downstream nucleosome. Finally, the MMTV promoter, which is induced by the glucocorticoid receptor, is contained within a nucleosome array, with one nucleosome spanning the receptor binding sites (Richard-Foy and Hager, 1987; Fragoso et al., 1995). Upon ligand addition, this nucleosome is selectively remodeled, facilitating the binding of additional factors required for transcription. Recent findings suggest that an interaction between the glucocorticoid receptor and a remodeling protein, BRG1, is essential for MMTV remodeling, with different receptor domains needed for the subsequent transcriptional activation (Fryer and Archer, 1998).

BRG1 is among a growing list of proteins that have been implicated in nucleosome remodeling (Workman and Kingston, 1998). Histone acetyltransferases and kinases have also been shown to play critical roles in remodeling by modifying core histone tails. Remodeling complexes and histone acetyltransferases have been implicated in the induction of a large number of genes on the basis of their ability to interact with inducible transcription factors (Workman and Kingston, 1998).

Two recent studies further support the hypothesis that nucleosome remodeling is important for inducible transcription in mammalian cells. First, upon T cell activation, the BRG1 complex becomes stably associated with chromatin (Zhu et al., 1998), suggesting that it plays a global role in gene induction. Second, histones within approximately 500 bp of the interferon γ promoter become rapidly acetylated upon induction, providing a direct link between acetylation and inducible transcription (Parekh and Maniatis, 1999).

Interleukin-12 (IL-12) is a heterodimeric cytokine that is produced by activated macrophages and dendritic cells and is required for the development of T helper 1 (Th1) cells (Trinchieri, 1995). Th1 cytokines enhance macrophage activation and cytolytic T cell maturation, thus contributing to the generation of an effective cell-mediated immune response (Abbas et al., 1996). The importance of IL-12 expression for cell-mediated immunity was established by experiments in which IL-12 activity was eliminated by disruption of the genes encoding the IL-12 receptor or IL-12 subunits, or by addition of IL-12 antibodies (Gately et al., 1998). In each case, cell-mediated immunity was diminished.

The p40 subunit of IL-12 is strongly induced in macrophages and dendritic cells by bacterial products, including lipopolysaccharide (LPS) and heat-killed Listeria monocytogenes (HKLM) (D’Andrea et al., 1992; Hsieh et al., 1993). In transfection assays, the p40 promoter was sufficient for transcriptional induction (Murphy et al., 1995; Ma et al., 1996; Plevy et al., 1997). Analysis of mutations in the murine promoter revealed contributions by multiple DNA elements, the two most important of which bind C/EBP and Rel family members (Murphy et al., 1995; Plevy et al., 1997).
To further understand the regulation of IL-12 p40 transcription, the endogenous gene must be studied in its native nucleosomal environment to examine important regulatory events that are not manifested in the transfection assays. An independent reason for studying the p40 chromatin environment is the paucity of published analyses of the nucleosomal contribution to mammalian gene induction. Although studies of nucleosome positioning and/or remodeling at inducible mammalian genes have been performed (see the Discussion), none approach the level of detail and resolution established in the yeast and viral studies cited above. The need for mammalian model systems, particularly in the immune system, has become increasingly apparent, as exemplified most recently by evidence that chromatin structure and epigenetic events contribute to the regulation of cytokine genes during T helper cell differentiation (Agarwall and Rao, 1998; Bix and Locksley, 1998; Hollander et al., 1998).

In this study, the contribution of nucleosomes to the induction of the endogenous murine p40 promoter was analyzed. The results reveal a positioned nucleosome upstream of the start site, encompassing the promoter elements required for induction. Upon macrophage activation, this nucleosome is rapidly and selectively remodeled in a protein synthesis-dependent manner, suggesting that selective remodeling is essential for p40 induction. Interestingly, the synergistic enhancement of p40 expression by IFN-γ appears to be independent of remodeling and of the occupancy of at least one critical promoter element, suggesting that IFN-γ targets an independent or downstream event.

Results

Nucleosome Positioning at the Murine IL-12 p40 Locus

To understand the influence of chromatin on p40 transcription, the nucleosome organization of the endogenous promoter in a transcriptionally inactive state must first be determined. Micrococcal nuclease (MNase) preferentially digests protein-free DNA including linker regions between nucleosomes. Thus, digestion of a nucleosomal array with limiting MNase will produce a ladder of DNA fragments corresponding to multiples of the nucleosome particle (approximately 180 bp). Isolated nuclei from RAW264.7 macrophages were subjected to limiting digestion with MNase. Genomic DNA was then purified and analyzed by Southern blot, using a probe containing nucleotides 2333 to 2697 (Figure 1B). The gel image reveals diffuse bands that appear to represent a nucleosome ladder (Figure 1A, lane 7), suggesting that the p40 promoter may be assembled into a nucleosomal array.

To confirm the presence of nucleosomes and to determine whether the nucleosomes are consistently positioned, MNase-treated genomic DNA from RAW264.7 nuclei was further digested with restriction enzymes that cleave in the promoter region. In vitro digestion creates DNA fragments possessing a restriction enzyme–cleaved constant end and an MNase-cleaved variable end. Positioned nucleosomes are visualized as a ladder of specific hybridization products that differ in length by a constant amount from the ladder observed in the absence of restriction digestion (unless the restriction enzyme cleaves in a nucleosomal linker).

Southern blot results obtained with three restriction enzymes are shown in Figure 1A. With each enzyme, nucleosome ladders were apparent that were offset from the ladder observed without restriction enzyme digestion (compare lanes 1–6 to lane 7). For example, Bsu36I digestion yielded bands that migrate at intermediate positions relative to the nucleosome ladder (compare lane 1, Bsu36I bands 1' and 2' to lane 7, bands 2 and 3). Although this low-resolution technique does not allow nucleosome positions to be determined with precision, the results suggest that three nucleosomes are
positioned between −300 and −900 (Figure 1B, diagram). Interestingly, the DNA in the vicinity of −900 was strongly hypersensitive to MNase cleavage (e.g., lanes 1 and 2, band 3'). This hypersensitivity, which was also observed following DNase I digestion, but not by in vitro restriction enzyme digestion in the absence of in vivo MNase or DNase I cleavage (data not shown), is likely to represent a nucleosome-free region.

High-Resolution MNase Analysis of the p40 Promoter Region

The above results provide evidence that positioned nucleosomes exist upstream of the p40 promoter. However, the resolution of the Southern blot method is inherently low, making it difficult to determine the precise locations of the nucleosomes. More importantly, the Southern blot method did not provide direct evidence of the nucleosome organization within the region surrounding the functionally important promoter elements because a radiolabeled probe hybridizing to sequences between −333 and +56 failed to reveal a nucleosome ladder following restriction enzyme digestion (data not shown); the dashed ovals in Figure 1B merely represent a hypothetical extrapolation of the data described above. To determine if the important promoter elements are assembled into positioned nucleosomes and to obtain higher-resolution information, MNase-digested genomic DNA was analyzed by ligation-mediated PCR (LM-PCR; Garrity and Wold, 1992; McPherson et al., 1993). In brief, when isolated nuclei are treated with MNase, cleavage within nucleosomal linker regions creates double-stranded breaks with unphosphorylated 5' ends. Following the purification of genomic DNA, the 5' ends are phosphorylated by T4 polynucleotide kinase and ligated to a unidirectional linker, which provides a common 5' sequence for annealing of a PCR primer. PCR amplification is then performed using a 3' gene-specific primer and a primer complementary to the linker. A subsequent PCR step with a radiolabeled 3' gene-specific primer is used to detect the amplified MNase cleavage products.

To analyze the region of the promoter containing the control elements known to be involved in inducible transcription, MNase/LM-PCR experiments were performed with uninduced RAW264.7 and J774 macrophage cell lines using a radiolabeled primer that hybridizes to p40 sequences spanning nucleotides −4 to −33 (Figure 2A). In comparison to purified genomic DNA digested with MNase in vitro (lane 1), MNase treatment of isolated nuclei from either the RAW264.7 (lane 2) or J774 (lanes 3-5) cells yielded strong hypersensitive sites at nucleotides −175 and −185. These nucleotides were cleaved with MNase much more frequently than nucleotides further downstream, even in the presence of high concentrations of MNase. This cleavage pattern suggests that nucleotides −175 and −185 are located in a nucleosome linker, with the sequences downstream of −175 containing a positioned nucleosome. This putative nucleosome, which encompasses all of the promoter elements that have been identified (Figure 3), will be referred to as nucleosome 1.

IL-12 p40 Expression in Macrophage Cell Lines

To study nucleosome alterations upon p40 induction, a cell line is needed in which the p40 gene is efficiently induced. Analysis of the J774 cell line by ELISA revealed...
that substantial quantities of p40 protein were secreted following induction with HKLM or LPS (Figure 4A). In contrast, the amount of p40 protein secreted by the RAW264.7 line was highly variable in our hands and at least 3-fold less than the amount produced by J774 cells, even when the RAW264.7 cells were primed with IFN-γ (data not shown); in fact, in some experiments, p40 protein was undetectable in supernatants from activated RAW264.7 cells. For this reason, J774 cells were used for the remodeling experiments described below. IFN-γ stimulation was not used for these initial experiments because its effects were generally small and highly variable. Instead, primary cells were employed to monitor the effect of IFN-γ (see below).

Intracellular flow cytometry revealed that less than one-third of the J774 cells within a clonal population produced p40 protein upon activation with optimal concentrations of HKLM (Figure 4B) or LPS (data not shown). Interestingly, subcloning of the J774 line by limiting dilution failed to separate the expressing and nonexpressing cells, demonstrating that the inefficient activation is an inherent property of the J774 line (M. Studley and S. T. S., unpublished data). By primer extension analysis of cytoplasmic RNA isolated from J774 cells, p40 transcripts were readily detected following activation (Figure 4C, lanes 1–4). Furthermore, consistent with previous studies (Aste-Amezaga et al., 1998), p40 transcription was inhibited by incubation with the protein synthesis inhibitor, cycloheximide (CHX; Figure 4C, lanes 5–8).

Selective Remodeling of Nucleosome 1

To determine if nucleosomes within the p40 promoter are altered upon cell activation, the MNase digestion pattern within nucleosome 1 was examined in J774 nuclei isolated before and after activation. MNase cleavage...
Nucleosome Remodeling at the IL-12 p40 Promoter

of the first enzyme’s recognition site. The genomic DNA cleavage products were then analyzed by LM-PCR. The in vitro cleavage is critical for normalization of the reactions, as the results are typically presented as the ratio of nuclear cleavage to in vitro cleavage. An increase in this ratio upon cell activation would suggest that a nucleosome becomes more accessible to nuclear cleavage and, thus, remodeled.

J774 activation led to a strong and reproducible increase in nuclear cleavage by SpeI, MseI, and DdeI, all of which cleave within nucleosome 1 (Figure 5B, compare the ratio of the nuclear cleavage products at –82, –133, and –166 to the control cleavage products in lanes 2–7 versus lanes 8–13). In most experiments, the ratio of nuclear cleavage versus the control cleavage increased by at least 10-fold following activation with either HKLM (Figure 5B) or LPS (see below). These results provide strong support for the hypothesis that nucleosome 1 is remodeled upon p40 induction.

Of note, nuclear cleavage at the SpeI (-102), MseI promoter (-212), and DdeI (-2102) sites remained inefficient following induction (Figure 5B; the latter two cleavage sites remain difficult to detect in the figure but are apparent after a longer exposure of the gel). The inefficient cleavage at –102, the only one of these sites within nucleosome 1, may be due to the rotational phasing of the nucleosome, which may limit restriction enzyme access both before and after remodeling. However, a more likely explanation for the inefficient cleavage at all three sites is that, in activated cells, enhanced nuclear cleavage at the downstream sites by the same restriction enzymes reduces the probability that nuclear cleavage will occur only at the upstream sites. In other words, a genomic DNA molecule that is cleaved at both sites will yield a band only at the downstream site following LM-PCR analysis.

To further characterize the remodeling event, its kinetics and requirement for de novo protein synthesis were monitored. The results reveal substantial remodeling 1 hr after addition of the inducer (Figure 5C, lanes 5 and 11), with the remodeling completely inhibited by cycloheximide (lanes 4 and 11), or anisomycin (data not shown). Assays performed 2 hr post induction revealed was slightly enhanced within nucleosome 1 in nuclei from activated cells (data not shown). However, because the effects were small, the results provided only weak evidence of remodeling. One contributing factor to the small effects may be the inefficient induction of p40 expression.

Because the MNase assay did not provide compelling evidence of remodeling, a restriction enzyme accessibility assay was employed as a more sensitive alternative for monitoring remodeling events. J774 cell nuclei were treated with a limiting concentration of an appropriate restriction enzyme. The cleaved genomic DNA was then purified and digested in vitro to completion with a different restriction enzyme that recognizes a site upstream

Figure 3. Summary of Nucleosome Organization of the IL-12 p40 Promoter

The approximate positions of nucleosomes are indicated by open ovals. Also shown are the locations of the macrophage-specific hypersensitive region, the transcription start site (+1), and the LM-PCR primer binding sites. At the bottom, the position of nucleosome 1 is shown relative to the locations of the three important promoter elements defined by transient transfection (Murphy et al., 1995; Plevy et al., 1997). Three additional elements that appear to be of lesser importance are shown (Plevy et al., 1997).

Figure 4. Characterization of IL-12 p40 Expression in the J774 Macrophage Cell Line (A) J774 cells were unstimulated or stimulated with HKLM or LPS for 24 hr. Culture supernatants were analyzed by ELISA. The results shown were from three separate experiments performed simultaneously. (B) Intracellular flow cytometry was performed with J774 cells that were unactivated or activated with HKLM for 24 hr. Cells were stained with a PE-conjugated IgG control antibody (light gray) or a PE-conjugated antibody specific for the p40 protein (black). The x-axis is a logarithmic representation of the PE fluorescence emission with the y-axis representing the number of cells with the given fluorescence level. (C) Total cytoplasmic RNA was isolated from J774 cells that were unstimulated (lane 1) or stimulated with LPS for 4 (lane 2), 8 (lane 3), or 10 (lane 4) hr. In addition, total RNA was isolated from J774 cells pretreated with CHX (lanes 6 and 8) or left untreated (lanes 5 and 7). These cells were then stimulated with LPS (lanes 7 and 8) or left unstimulated (lanes 5 and 6). Thirty micrograms of RNA was analyzed by primer extension. The arrows indicate the expected 59 bp product.
no further increase in remodeling (lanes 6 and 13) and exhibited the same sensitivity to protein synthesis inhibitors (lanes 7 and 14). Thus, nucleosome 1 remodeling appears to be complete within 1 hr of cell activation and is dependent on de novo protein synthesis.

It may be noteworthy that Figure 5C provides an example of an experiment performed with LPS-activated cells, whereas HKLM activation was used for the experiment shown in Figure 5B. Consistent with the ELISA results in Figure 4A, these results demonstrate that the two agents are equally effective inducers of both p40 remodeling and expression.

To examine whether remodeling occurs throughout the promoter region or is confined to nucleosome 1, restriction enzymes that cleave at sites on either side of nucleosome 1 and within nucleosomes 2 and 3 were tested (Figure 6). Accessibility to restriction enzyme cleavage within nucleosome 2 (HaeIII) and nucleosome 3 (AluI) remained unchanged upon p40 induction. Accessibility also remained unchanged at the distal end of the extended linker between nucleosomes 1 and 2 (PstI, lanes 5 and 6). Each of these negative results was obtained in multiple independent experiments, with each experiment performed with a positive control demonstrating efficient remodeling of nucleosome 1 (data not shown). Interestingly, however, accessibility increased substantially at nucleotide −197, at the proximal end of the extended linker separating nucleosomes 1 and 2 (AatII, lanes 2 and 3). This enhanced accessibility is intriguing because of the general absence of knowledge regarding the organization of DNA sequences immediately flanking a nucleosome undergoing remodeling. Finally, analysis of the region downstream of nucleosome 1 revealed a slight increase in accessibility at −32 and −18 upon cell activation and a more substantial increase at −20 (BstNI, lanes 14 and 15), which maps to the proximal edge of nucleosome 1. Collectively, these data suggest that nucleosome remodeling, as measured by an activation-dependent increase in restriction enzyme accessibility, is confined to nucleosome 1.

**Nucleosome Remodeling and Promoter Occupancy in Primary Macrophages**

The above results establish the status of the chromatin environment at the IL-12 p40 promoter in murine macrophage cell lines. However, we were unable to use the cell lines to study one important aspect of p40 regulation: namely, its synergistic activation by bacterial products and IFN-γ (e.g., D’Andrea et al., 1992; Murphy et al., 1995;
Ma et al., 1996). As mentioned above, a substantial IFN-γ effect was sometimes observed in the RAW264.7 cells in our hands, but the maximal p40 expression level remained well below that observed with LPS or HKLM alone in J774 cells. Furthermore, in our hands, IFN-γ had only a small effect on p40 expression in J774 cells, regardless of the time of addition or amount added (data not shown).

To study the effect of IFN-γ, we therefore examined thioglycollate-elicited peritoneal macrophages from C57BL/6 mice. In addition to providing information about the mechanism of IFN-γ enhancement, the use of primary cells provides a test of the validity of the results obtained in transformed cell lines. MNase/LM-PCR analysis of the p40 locus confirmed that nucleosome 1 was at the same position as in RAW264.7, J774, and VL3-3M2 cells (Figure 7C). In addition, ELISA results revealed strong induction of p40 expression by LPS, with synergistic induction when IFN-γ was added according to the procedures of Murphy et al. (1995) and Magram et al. (1996) (Figure 7A). Intracellular flow cytometry showed that, upon activation with LPS alone, a peak representing the vast majority of cells shifts significantly to the right, suggesting that most cells express p40 at low levels (Figure 7B, LPS). Upon activation with LPS plus IFN-γ, this peak shifts further to the right, revealing a 3.9-fold increase in the mean fluorescence of the entire population (Figure 7B, LPS + IFN-γ).

An analysis of nucleosome remodeling using the restriction enzyme accessibility assay revealed a strong increase in nuclear cleavage upon p40 induction with LPS (Figure 7D, compare lane 1 to lanes 3 and 5). Interestingly, despite the strong effect of IFN-γ on p40 expression levels, no effect of IFN-γ on nuclear cleavage was observed at the 1 hr time point (compare lanes 3 and 4), with only a small enhancement observed at the 4 hr time point (lanes 5 and 6).

An examination of promoter occupancy by DNase I genomic footprinting revealed strong protection of the C/EBP site upon stimulation with LPS alone (Figure 7E, lanes 2 and 3). The DNase I cleavage pattern was also altered at other promoter locations, in particular on occupancy of the C/EBP site (Figure 7E, lanes 2-5), similar to the small effect of IFN-γ on remodeling.

The highly efficient remodeling and transcription factor binding observed following activation with LPS alone strongly suggests that the endogenous promoters are remodeled and occupied in the vast majority of cells in the absence of IFN-γ. These results are consistent with the intracellular staining results, which suggest that most or all cells express at least low levels of p40 protein following activation with LPS alone. Taken together, these results suggest that IFN-γ does not influence nucleosome 1 remodeling or the occupancy of the C/EBP site. Rather, it must target an event that is downstream or independent of these steps.

Discussion

This study provides the foundation for a detailed analysis of the mechanism by which chromatin contributes
Figure 7. Analysis of Peritoneal Macrophages Activated with LPS and IFN-γ

(A) ELISA results from the primary macrophage population used in (D) and (E) are shown. Cells were untreated, treated with IFN-γ, LPS, or LPS and IFN-γ. Culture supernatants were harvested after 6 hr.

(B) Intracellular staining was performed with cells that were left untreated or were treated with IFN-γ, LPS, or LPS and IFN-γ. Profiles relative to the IgG control are shown.

(C) Nuclei from primary macrophages were treated with increasing concentrations of MNase (lanes 2 and 3). Radiolabeled primer A was used for LM-PCR. pBR322 digested with MspI (lane 1) was used as a size standard. The MNase hypersensitive sites at -175 and -185 are indicated.

(D) Primary macrophages were left untreated (lane 1), treated with IFN-γ (lanes 2, 4, and 6), LPS for 1 (lanes 3 and 4) or 4 (lanes 5 and 6) hr. Nuclei were digested with SpeI at 37°C for 10 min. Purified DNA was digested with AatII as a control. Arrows to the right and left indicate nuclear and control cleavage products, respectively.

(E) Primary macrophages were untreated (lane 2) or were treated with IFN-γ (lane 5), LPS (lane 3), or LPS and IFN-γ (lane 4) for 4 hr. Nuclei were digested with DNase I (0.15 μg) followed by LM-PCR with primer A. Radiolabeled pBR322 digested with MspI (lane 1) was the size standard.

The data reveal that positioned nucleosomes occupy the IL-12 p40 promoter region, with nucleosome 1 spanning DNA elements that are important for transcriptional induction. An extended linker region appears to be present upstream of nucleosome 1, followed by three additional positioned nucleosomes and a region devoid of nucleosomes in macrophage cell lines. Nucleosome 1 was rapidly and selectively remodeled upon macrophage activation in a protein synthesis-dependent manner. Analysis of peritoneal macrophages confirmed the above results and suggested that IFN-γ targets an event that is independent or downstream of remodeling.

IFN-γ could enhance p40 gene transcription by targeting any of a number of events. For example, it could stimulate the activity of a distant enhancer, allowing it to further stimulate transcription from the remodeled and occupied promoter. Alternatively, IFN-γ could stimulate the activity of a coactivator complex that acts after remodeling and promoter occupancy. Another alternative, as suggested by Murphy et al. (1995) and Ma et al. (1997), is that IFN-γ could stimulate the binding of proteins to the Rel site or to an upstream Ets site, which appears to be important for activity of the human p40 promoter. Although our DNase I footprinting experiments showed no effect of IFN-γ on the DNase I cleavage pattern at the Rel site, it is not clear if the footprint at the Rel site following LPS activation corresponds to Rel protein binding or altered histone contacts with the DNA. Therefore, IFN-γ-inducible interactions with the Rel site may exist that were not detected by the DNase I genomic footprinting technique. It is noteworthy that if the Rel or Ets proteins bind inefficiently in the absence of IFN-γ, then these proteins are unlikely to be necessary for remodeling of nucleosome 1 or for occupancy of the C/EBP site.

Our results can be compared and contrasted with the yeast and viral model systems described in the Introduction. Nucleosome positioning at the p40 promoter exhibits the greatest similarity to the MMTV promoter; in both promoters, a single nucleosome spans the DNA elements known to be important for induction. Nucleosome positioning at the Pho5 and HIV-1 promoters appears to be fundamentally different because, in both cases, at least one binding site for an inducible activator is in a nucleosome-free region (Almer and Horz, 1986; Verdin et al., 1993). Although the p40 promoter appears to be similar to the MMTV promoter, it is important to note...
that the DNA-binding proteins that stimulate remodeling at the p40 promoter have not yet been determined; the functional elements that have been identified are within nucleosome 1, but an element may exist elsewhere that stimulates remodeling.

The similarity between the p40 and MMTV promoters extends beyond nucleosome positioning to the basic characteristics of remodeling. At both promoters, only one nucleosome encompassing the promoter appears to be remodeled. This suggests that remodeling and/or histone modification complexes are targeted with precision. In contrast, nucleosome alterations are apparent at four nucleosomes in the Pho5 promoter region (Almer et al., 1986). HIV-1 remodeling is restricted to one nucleosome, but the nucleosome is downstream of the start site (Verdin et al., 1993).

In addition to the three well-characterized promoters described above, several other inducible promoters have been analyzed with respect to nucleosome positioning and/or remodeling, although in less detail. For example, using low-resolution MNase/Southern blot techniques, positioned nucleosomes have been reported in the vicinity of endogenous promoters for the c-fos and interferon β genes (Sagar et al., 1985; Herrera et al., 1997). Remodeling events have been observed near a number of inducible promoters on the basis of enhanced DNase I hypersensitivity. However, in most instances, the locations of the nucleosomes and the selectivity of remodeling were not analyzed. Restriction enzyme accessibility and the loss of MNase resistance have been used to study remodeling at only a few mammalian promoters, including the c-fos and IL-2 promoters (Herrera et al., 1997; Ward et al., 1998).

The simultaneous analysis of nucleosome remodeling and transcription factor binding at the p40 promoter provides an initial step toward the principle goal of defining the order of events that lead to inducible transcription. Time-course experiments revealed that both remodeling and promoter occupancy were nearly complete 1 hr after cell activation, and both required protein synthesis (Figures 5 and 7; data not shown). This close correlation suggests that one event immediately precedes the other; remodeling may be required before the binding of C/EBP and Rel proteins, or the binding of these factors may recruit a remodeling complex to the promoter. Although Rel and C/EBP proteins have been reported to interact with histone acetylases (Mink et al., 1997; Perkins et al., 1997), our preliminary studies using mice lacking the Rel proteins that are required for p40 induction suggest that remodeling occurs before the binding of either protein (A. S. W. et al., unpublished data).

A determination of the order of events leading to inducible p40 transcription is one of several key issues that remain to be addressed. Other issues include the identification of the complex that induces remodeling and elucidation of the relative functions of ATP-dependent remodeling complexes and histone modification complexes at the p40 promoter (e.g., which type of complex is responsible for the enhanced restriction enzyme accessibility). Furthermore, if remodeling occurs before C/EBP and Rel binding, it will be important to identify the protein that recruits the remodeling complex and to determine the mechanism by which remodeling is targeted with a high degree of specificity to a single nucleosome. To address these issues, the endogenous p40 locus will need to be studied in greater depth and complemented by in vitro studies using chromatin assembly/transcription assays. For both types of studies, the foundation provided by the current analysis will be invaluable.

Experimental Procedures

Cell Culture

RAW264.7 and J 774 murine macrophage cell lines (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), low endotoxin, Omega Scientific) and 1× penicillin/streptomycin (P/S, Omega Scientific). The VL3-3M2 murine T cell line was a gift of C. Guidos (University of Toronto) and was grown in RPMI supplemented with 10% FBS and 1× P/S. RAW264.7 and J 774 cells were grown to confluence for most experiments. Macrophage activation was accomplished by adding either LPS (10 μg/mL, Sigma) or HKLM (Pievy et al., 1997) to the cell culture medium. CHX (10 μg/mL) and anisomycin (10 μg/mL) were purchased from Sigma.

Primary Macrophages

C57BL/6 mice were injected intraperitoneally with thioglycollate and, after 3 days, the peritoneal exudate cells were isolated. Cells were allowed to adhere to tissue culture plates and were washed on consecutive days 3 times with 1× PBS. The cells were then activated as indicated with recombinant murine IFNγ (10-50 units/mL, PharMingen) and LPS (10 μg/mL).

Flow Cytometry

Phycocerythrin (PE) anti-mouse IL-12 p40/p70 and PE rat IgG1 isotype control immunoglobulin (PharMingen) were used for flow cytometry. Six hours prior to harvesting cells, brefeldin A (5 μg/mL, Sigma) was added to the culture media. The staining protocol provided with the antibodies (PharMingen) was followed with the exception that antibody incubations were carried out at room temperature instead of 4°C.

ELISA

IL-12 p40 production was measured by sandwich ELISA. Purified rat anti-mouse IL-12 p40/p70 (0.5 μg/mL) and biotin anti-mouse IL-12 p40/p70 (200 ng/mL) were from PharMingen, and poly-HRP80-streptavidin (1:5000) was from Research Diagnostics. The ELISAs were developed using the ABTS microwell peroxidase substrate system (Kirkgaard and Perry Laboratories).

Nuclei Preparation

Cells grown to confluence were scraped and pelleted at 1500 rpm in a clinical centrifuge. Cells were washed once in ice-cold 1× PBS. The cell pellet was resuspended in ice-cold NP-40 lysis buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM MgCl2, 0.1% NP-40, 0.15 mM spermine, and 0.5 mM spermidine) and incubated on ice for 5 min. Nuclei were pelleted at 1000 rpm followed by washing in the respective digestion buffer (without CaCl2).

Nuclease Digestions

For DNase I genomic footprinting, cell nuclei were resuspended in buffer A (100 mM NaCl, 50 mM Tris [pH 8.0], 3 mM MgCl2, 0.15 mM spermine, and 0.5 mM spermidine) supplemented with CaCl2 (1 mM). DNase I (0.15 μg/mL) was added and the reactions (in a total volume of 100 μL) incubated at 37°C for 2 min. Reactions were stopped by adding 2 μL 0.5 M EDTA, 100 μL buffer A, 3 μL proteinase K (25 mg/mL), and 10 μL 20% SDS, and were incubated overnight at 37°C. DNA purification proceeded with phenol/chloroform and chloroform extractions, taking care not to shear the genomic DNA. RNase A (20 μg/mL) was added, and samples were incubated at 37°C for at least 2 hr followed by phenol/chloroform and chloroform extraction. DNA precipitated with ethanol was resuspended gently in water.

MNase digestion proceeded as described by Richard-Foy and
Hager (1987) with minor modifications. In brief, cell nuclei were resuspended in MNase digestion buffer (10 mM Tris [pH 7.4], 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine) containing CaCl \(_2\) (1 mM). MNase (stock solution = 25 units/\mu L) was added, and the reactions (in a total volume of 100 \mu L) were incubated at room temperature for 5 min. Reactions were terminated by addition of 80 \mu L MNase digestion buffer, 20 \mu L MNase stop buffer (100 mM EDTA and 10 mM EGTA), 3 \mu L proteinase K (25 mg/mL), and 10 \mu L 20% SDS. Samples were incubated overnight at 37°C. DNA purification proceeded as described for DNase I.

For restriction enzyme digestion, cell nuclei were washed in RE buffer (10 mM Tris [pH 7.4], 50 mM NaCl, 10 mM MgCl \(_2\), 0.2 mM EDTA, 0.2 mM EGTA, 1 mM \(\beta\)-mercaptoethanol, 0.15 mM spermine, and 0.5 mM spermidine) (Reik et al., 1991). Nuclei were resuspended in the recommended New England Biolabs (NEB) buffer for restriction enzyme digestion (50 \mu L). Digestion reactions were initiated with 2 \mu L of the restriction enzyme with incubation at 37°C for 10 min (unless otherwise noted). Reactions were stopped by addition of an equal volume of \(2\times\) proteinase K buffer (100 mM Tris [pH 7.5], 200 mM NaCl, 2 mM EDTA, and 1% SDS) and incubated at 55°C for 1 hr. Samples were then treated with 50 \mu L of \(2\times\) proteinase K buffer, 50 \mu L RE buffer, and 3 \mu L of proteinase K (25 mg/mL) and incubated overnight at 37°C. DNA purification proceeded as described for DNase I.

Southern Blot
Purified, MNase-digested DNA (20 \mu g) was incubated overnight at 37°C with an excess of restriction enzyme. Samples were analyzed by electrophoresis on a 1% agarose gel followed by transfer and hybridization with a radiolabeled probe specific for the p40 promoter (−333 to −697).

LM-PCR
LM-PCR was performed as previously described (Garrity and Wold, 1992; McPherson et al., 1993) with the following modifications. All PCR reactions were carried out with the DNA polymerase pfu. First strand synthesis reactions were performed for DNase I- and restriction enzyme-treated DNA as described, but MNase-treated DNA did not undergo this step. Instead, MNase-treated DNA (\(1 \mu g\)) was kinased, followed directly by ligation with the unidirectional linker. The amplification PCR consisted of 18 (restriction enzyme), 22 (DNase I), or 23 (MNase) PCR cycles with an extension time starting at 5 min plus 15 s for each additional cycle. The labeling PCR consisted of three cycles for all of the in vivo DNA treatments. Primers complementary to the coding strand of the IL-12 gene were used as the 3' primer. The approximate locations of the labeling primers are shown in Figure 3. Radiolabeled size markers were derived from pBRR322 digested with MspI (New England Biolabs).

Primer Extension
The expression of IL-12 p40 mRNA was analyzed by primer extension as previously described (Plevy et al., 1997). In brief, total RNA was isolated by the NP-40 lysis method. Thirty micrograms of total RNA was hybridized with a \(3\beta\)-labeled IL-12 p40-specific primer (5'-TTACCTGTGGTTGTCGACGTCG 3') at 60°C for 1.5 hr. Following reverse transcription, products were analyzed on an 8% denaturing polyacrylamide gel.

Acknowledgments
We thank Paul Garrity and Ken Zaret for helpful suggestions and Deborah Mitchell for performing intracellular staining of the primary macrophages. We are also grateful to Michael Grunstein, Michelle Studley, and Shomi Sanjabi for critical reading of the manuscript. This work was supported by United States Public Health Service National Research Service Award GM07185 (to A. S. W.). S. T. S. is an investigator with the Howard Hughes Medical Institute.

Received May 19, 1999; revised November 10, 1999.

References


