Nucleosome remodeling at the IL-12 p40 promoter is a TLR-dependent, Rel-independent event

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Lipopolysaccharide (LPS) induction of the gene encoding interleukin 12 p40 requires remodeling of a promoter-encompassing nucleosome and the Toll-like receptor (TLR)–mediated activation of a c-Rel–containing complex. Analysis of TLR4-mutant mice revealed that remodeling requires TLR signaling. However, Rel proteins and other proteins required for transcription of an integrated p40 promoter were insufficient for remodeling. c-Rel was also unnecessary for remodeling, as remodeling was observed in c-Rel$^{-/-}$ macrophages, which lack p40 transcripts. These results suggest that remodeling requires TLR signaling pathways that diverge from the c-Rel activation pathways. The factors that stimulate remodeling may represent, therefore, newly identified targets of TLR signaling and of agents that regulate inflammatory responses and T_H1 development.

An inflammatory response can be initiated by the interaction between a microbial pathogen and host antigen presenting cells, including macrophages[1]. This interaction leads to the transcriptional induction of several proinflammatory cytokine genes, which then generate the inflammatory response. Toll-like receptors (TLRs) comprise a family of mammalian cell-surface proteins that stimulate proinflammatory cytokine gene transcription in response to lipopolysaccharide (LPS) and microbial lipoproteins[2–6]. TLRs stimulate transcription through various signaling pathways. The most prominent of these are the Rel (also called nuclear factor κB or NF-κB) and JNK pathways[7–10].

Consistent with the induction of the Rel and JNK pathways by TLR signaling, the promoters for most proinflammatory cytokine genes contain binding sites for Rel proteins and adaptor protein 1 (AP-1) as well as binding sites for other common transcription factors, such as CCAAT enhancer–binding protein β (C/EBP-β). However, several observations suggest that our knowledge of the regulatory mechanisms for these genes remains at a preliminary stage. For example, the common transcription factors that regulate proinflammatory cytokine genes regulate many other genes, which suggests that the specificity of induction is dictated by DNA-binding proteins that have not been identified. Also, several signaling pathways in addition to the Rel and JNK pathways have been implicated in gene induction in response to LPS stimulation—for example, p38 and extracellular signal–regulated kinase (ERK) pathways—yet the transcription factor targets of these pathways have not been fully defined[10,11]. Finally, several agents that enhance and suppress proinflammatory cytokine gene transcription, including interferon γ (IFN-γ) and interleukin 10 (IL-10), have little or no impact on the known activators of proinflammatory cytokine genes[12], which indicates that new approaches may be required to dissect the mechanisms that underlie these immunoregulatory events.

IL-12 is an important example of a proinflammatory cytokine produced by macrophages whose proper regulation is critical for an effective immune response. This heterodimeric cytokine, which is composed of inducible p40 and p35 subunits, serves as a bridge between innate and adaptive immune responses by stimulating the development of type 1 T helper cells[13,14]. The gene encoding IL-12 p40 is of particular interest because it is regulated by many of the same agents that regulate other proinflammatory cytokine genes as well as by other agents that specifically regulate T helper cell differerentiation[12].

The murine IL-12 p40 promoter is induced by LPS and microbial lipoproteins through TLR signaling pathways and contains multiple DNA elements that contribute to induction in a transient transfection assay, including Rel and C/EBP elements[5,15,16]. The Rel site appears to interact with a c-Rel–containing complex, as demonstrated by the selective absence of p40 expression in c-Rel$^{-/-}$ macrophages[17]. Before induction, the endogenous IL-12 p40 promoter region is assembled into positioned nucleosomes, with nucleosome 1 encompassing the critical Rel and C/EBP elements[18]. This nucleosome is selectively remodeled upon activation of macrophages with LPS or heat-killed *Listeria monocytogenes* (HKLM)[18], which suggests that a remodeling complex is selectively targeted to this nucleosome during gene induction.

We show here that remodeling at the p40 promoter requires TLR signaling events. We also questioned whether remodeling is stimulated by the Rel or C/EBP proteins that are required for p40 transcription or whether it is an independent inducible event. This question is biologically important because, if remodeling is an independent event, it may represent a new route toward identifying targets of TLR signaling pathways and of positive and negative regulators of inflammation. From a mechanistic perspective, this question is equally relevant because the relationship between nucleosome remodeling and transcription factor binding

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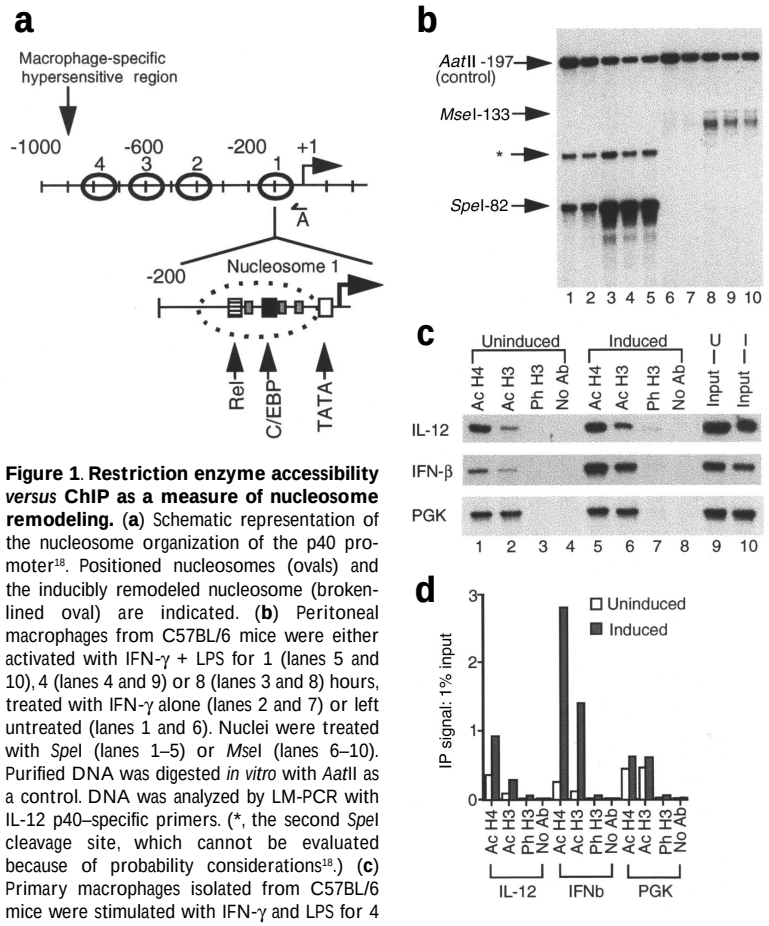

Figure 1. Restriction enzyme accessibility *versus* ChIP as a measure of nucleosome remodeling. (**a**) Schematic representation of the nucleosome organization of the p40 promoter[18]. Positioned nucleosomes (ovals) and the inducibly remodeled nucleosome (broken-lined oval) are indicated. (**b**) Peritoneal macrophages from C57BL/6 mice were either activated with IFN-γ + LPS for 1 (lanes 5 and 10), 4 (lanes 4 and 9) or 8 (lanes 3 and 8) hours, treated with IFN-γ alone (lanes 2 and 7) or left untreated (lanes 1 and 6). Nuclei were treated with *Spe*I (lanes 1–5) or *Mse*I (lanes 6–10). Purified DNA was digested *in vitro* with *Aat*II as a control. DNA was analyzed by LM-PCR with IL-12 p40–specific primers. (*, the second *Spe*I cleavage site, which cannot be evaluated because of probability considerations[18].) (**c**) Primary macrophages isolated from C57BL/6 mice were stimulated with IFN-γ and LPS for 4 h (lanes 5–8 and 10) or left untreated (lanes 1–4 and 9). Histone modifications were then monitored by ChIP using antibodies to AcH4 (lanes 1 and 5), AcH3 (lanes 2 and 6), PhH3 (lanes 3 and 7) or no antibody (lanes 4 and 8). A sample (1%) of the input was used as a control (lanes 9 and 10). PCR amplification was done, using the same DNA samples, with primers specific to the IL-12 p40, IFN-β and PGK promoters. (**d**) Phosphorimager quantification of the data from **c** is shown as the ratio of the signal following immunoprecipitation (IP) *versus* 1% of the input.

has not been thoroughly addressed at mammalian promoters. Rel and C/EBP family members can interact with histone modification and/or ATP-dependent remodeling complexes[19–21] but the importance of these interactions for remodeling and gene activation has been examined only in the context of overexpressed proteins. Our results suggest that TLR-dependent remodeling at the p40 promoter occurs in the absence of c-Rel and may also be independent of C/EBP proteins and other proteins required for activity of a stably integrated promoter. The biological and mechanistic implications of these results are discussed.

Results

Restriction enzyme accessibility *versus* ChIP

Micrococcal nuclease and restriction enzyme accessibility assays demonstrated that a single nucleosome in the vicinity of the murine IL-12 p40 promoter, nucleosome 1, is remodeled in LPS- or HKLM-activated macrophages (**Fig. 1a**)[18]. For analysis using restriction enzymes, peritoneal macrophages were obtained from C57BL/6 mice. Nuclei from unactivated and LPS and IFN-γ–activated cells were isolated and treated with *Spe*I or *Mse*I, which cleave within nucleosome 1. The cleaved genomic DNA was purified and digested to completion *in vitro* with the restriction enzyme *Aat*II, which recognizes an upstream site and serves as a normalization control. Using p40-specific primers, the approximate ratios of the nuclear and *in vitro* cleavage products were then revealed by ligation-mediated polymerase chain reaction (LM-PCR). Nuclear cleavage by *Spe*I at nucleotide -82 and by *Mse*I at nucleotide -133 was more efficient in activated macrophages than in unactivated macrophages (**Fig. 1b**). Cleavage by *Spe*I at nucleotide -102 was enhanced to a lesser extent, most likely due to probability considerations related to the presence of two recognition sites for this enzyme in the region analyzed[18]. The hypothesis that nucleosome 1 is remodeled during IL-12 p40 induction is supported by the enhanced cleavages observed in this experiment. For all other restriction enzyme experiments described, only the *Spe*I cleavage results are shown. However, all results were qualitatively and quantitatively similar when *Mse*I cleavage was monitored (data not shown).

The restriction enzyme assay has the potential to reveal chromatin alterations induced by both ATP-dependent remodeling complexes and histone-modification complexes. To determine whether inducible histone modifications may contribute to p40 remodeling, chromatin immunoprecipitation (ChIP) experiments were done. Formaldehyde cross-linked chromatin samples were prepared from unactivated and activated peritoneal macrophages. Antibodies to acetylated histone H4 (AcH4), AcH3 and phosphorylated histone H3 (PhH3) were used for immunoprecipitation. After reversal of the cross-links and DNA purification, PCR was done to monitor the degree of enrichment of specific DNA fragments in the immunoprecipitated samples.

Maximally, a two to threefold increase in the abundance of an IL-12 p40 promoter fragment was observed in the activated samples relative to the unactivated samples after precipitation with either the AcH4 or AcH3 antibodies (**Fig. 1c**,**d**). Smaller increases or no increase were observed in some experiments (data not shown). The same samples were subjected to PCR amplification with primers to the gene encoding phosphoglycerate kinase (PGK), which serves as a negative control for induction because it is constitutively active in these cells. The abundance of this PCR product was not altered in the activated samples when the AcH4 and AcH3 antibodies were tested. With the PhH3 antibodies, a similar increase in both the IL-12 p40 and PGK PCR products was observed in the activated samples, which suggested that this increase was unrelated to p40 induction (**Fig. 1c,d**).

As a positive control for inducible acetylation, PCR primers to the IFN-β promoter were used to analyze the same DNA samples. The promoter of this gene, which is induced by LPS in macrophages (data not shown)[22], was previously found to acquire a substantial increase in H4 and H3 acetylation upon induction in HeLa cells by Sendai virus[23]. Consistent with the previous results, the abundance of the IFN-β PCR product increased eight to tenfold after precipitation with the AcH4 and AcH3 antibodies (**Fig. 1c,d**). The abundance of this PCR product also increased in the PhH3 immunoprecipitates but remained close to the limit of detection.

Although the increase in histone acetylation at the p40 promoter may be necessary for remodeling, the small magnitude of the increase suggests that p40 remodeling may be independent of inducible acetylation or less dependent than remodeling at the IFN-β promoter. The

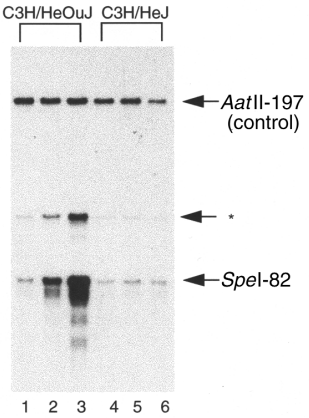

Figure 2. Nucleosome remodeling at the p40 promoter is a TLR-dependent event. Peritoneal macrophages from C3H/HeOuJ (lanes 1–3) or C3H/HeJ (lanes 4–6) mice were either left untreated (lanes 1 and 4) or treated with IFN-γ + LPS (10 ng/ml) for 1 h (lanes 2 and 5) or 4 h (lanes 3 and 6). Cell nuclei were digested with *Spe*I then the DNA was purified and digested *in vitro* with *Aat*II. IL-12 p40 specific primers were used for LM-PCR analysis.

inducible restriction enzyme accessibility we observed could be, therefore, more dependent on an ATP-dependent remodeling complex or on histone modifications that were not detected by the three antibodies used for these experiments (**Fig. 1b**). It was perhaps not unexpected that the magnitude of inducible histone acetylation varies from gene to gene because genetic analyses in yeast have revealed the presence of both SAGA-dependent and SAGA-independent genes[24]. Because the restriction enzyme assay yielded results that were far more compelling than the ChIP results, and may take into account the activities of both ATP-dependent remodeling complexes and histone-modification complexes, we concentrated on the use of this assay as a measure of nucleosome remodeling at the p40 promoter.

TLR signaling required for p40 remodeling

Activation of NF-κB and JNK by LPS requires signal transduction pathways that originate at the TLR4 protein on the macrophage cell surface[4]. However, it is not known whether all LPS-inducible events require TLR4 signaling. To determine whether TLR4 signaling is essential for nucleosome remodeling at the p40 promoter in response to LPS, peritoneal macrophages from C3H/HeJ mice were analyzed with the restriction enzyme accessibility assay. These mice contain a single amino-acid substitution in TLR4, which leads to LPS hyporesponsiveness[4]. IL-12 p40 protein was undetectable by enzyme-linked immunosorbent assay (ELISA) analysis of supernatants from C3H/HeJ macrophages (detection limit, 15 pg/ml); after stimulation with the same concentrations of LPS and IFN-γ, wild-type C3H/HeOuJ macrophages secreted 152,000 pg/ml of p40 protein.

Restriction enzyme accessibility did not increase in the C3H/HeJ macrophages after induction with LPS and IFN-γ, which indicated that remodeling did not occur (**Fig. 2**). In a parallel experiment, potent remodeling was observed in macrophages from the wild-type C3H/HeOuJ mice. Greatly reduced remodeling in response to LPS and IFN-γ was also observed in macrophages from C57BL/10ScCr mice, which lack the locus that encodes TLR4 (data not shown)[4]. These results demonstrate that inducible remodeling at the p40 promoter is a target of TLR4 signaling pathways.

Rel and C/EBP insufficient for p40 remodeling

We determined next whether the Rel and C/EBP transcription factors that are required for p40 promoter activity in transfection assays are sufficient for nucleosome remodeling at the p40 promoter. Insight was provided by a comparison between the endogenous locus that encodes p40 and p40 promoter–reporter plasmids integrated into the genomes of two macrophage cell lines, J774 and RAW264.7. Both of these lines express p40 mRNA after activation with LPS or HKLM[16,18].

As a first step, clonal RAW264.7 lines were generated to contain stably integrated p40 promoter fragments (nucleotides -350 to +55) fused to a chloramphenicol acetyltransferase (CAT) reporter gene. A genomic footprinting analysis revealed that the C/EBP site within the integrated promoters was efficiently occupied after cell activation, which was reminiscent of the results obtained with primary peritoneal macrophages[18] (**Fig. 3a**). Occupancy of the Rel site was not convincingly detected in the stable integrants or the peritoneal macrophages[18], possibly because the Rel complex is subject to dynamic exchange as has been shown for the glucocorticoid receptor[25].

Consistent with the inducible occupancy of the integrated promoters, inducible transcription was detected by primer extension using a radiolabeled primer that was complementary to the CAT mRNA (**Fig. 3b**). To determine the efficiency with which the stably integrated promoters could be induced, J774 and RAW264.7 lines were prepared containing p40 promoters fused to a green fluorescent protein (GFP) reporter gene. In multiple J774 and RAW264.7 clones, GFP expression was strongly induced in virtually all of the cells within the population (**Fig. 3c**). The activity of stably integrated p40 promoter-reporter plasmids is completely dependent on intact Rel and C/EBP sites[16]. Therefore, because GFP expression was detected in the entire population, the Rel and C/EBP activators of p40 transcription must have been induced in the entire population. This hypothesis is consistent with the efficient occupancy of the C/EBP site within the stably integrated promoters.

Although the factors required for activity of the stably integrated promoters were induced in all cells within the RAW264.7 or J774 populations, promoter occupancy and expression of the gene encoding endogenous p40 was inefficient. In activated RAW264.7 cells, C/EBP-site occupancy at the endogenous promoter was not detectable by genomic footprinting (**Fig. 3a**). In activated J774 cells, one band at the proximal edge of the C/EBP site was weakly and reproducibly protected but no sites were efficiently occupied. Consistent with these results, endogenous p40 protein was expressed in only 10–30% of an activated J774 population, as determined by intracellular flow cytometry (**Fig. 3d**)[18]. Subcloning of the J774 cells revealed that the inefficient induction is an intrinsic property of the line and is not due to heterogeneity within the population, as the gene encoding p40 was inefficiently induced in all subclones analyzed (M. N. Bradley, unpublished data). In an activated RAW264.7 population, a much smaller percentage of the cells expressed p40 protein (**Fig. 3d**). (The reason J774 cells express the gene encoding p40 more efficiently than RAW264.7 cells is not known. However, this observation is consistent with other properties of these lines, which includes the reduced expression of IL-10 in J774 cells relative to RAW264.7 cells, data not shown).

One possible explanation for the inefficient induction of the endogenous gene encoding p40 relative to the integrated p40 promoter-reporter plasmids is that the locus may be maintained in a heterochromatic state in most cells within a clonal population. This possibility cannot be excluded but it appears unlikely because a strong, macrophage-specific nuclease-hypersensitive region 840-bp upstream of the p40 transcription start site has been identified in both RAW264.7 and J774 cells[18]. Hypersensitive cleavage within this region presumably corresponds to the macrophage-specific decondensation of the locus. Because this strong hypersensitivity was observed in unactivated RAW264.7 cells, in which a very small percentage of the cells are competent for p40 expression after cell activation, assembly into heterochromatin does not appear to be responsible for inefficient induction of the endogenous p40 promoter. In other words, if assembly of the locus into heterochromatin in the vast majority of cells within a RAW264.7 population were responsible for the inefficient induction,

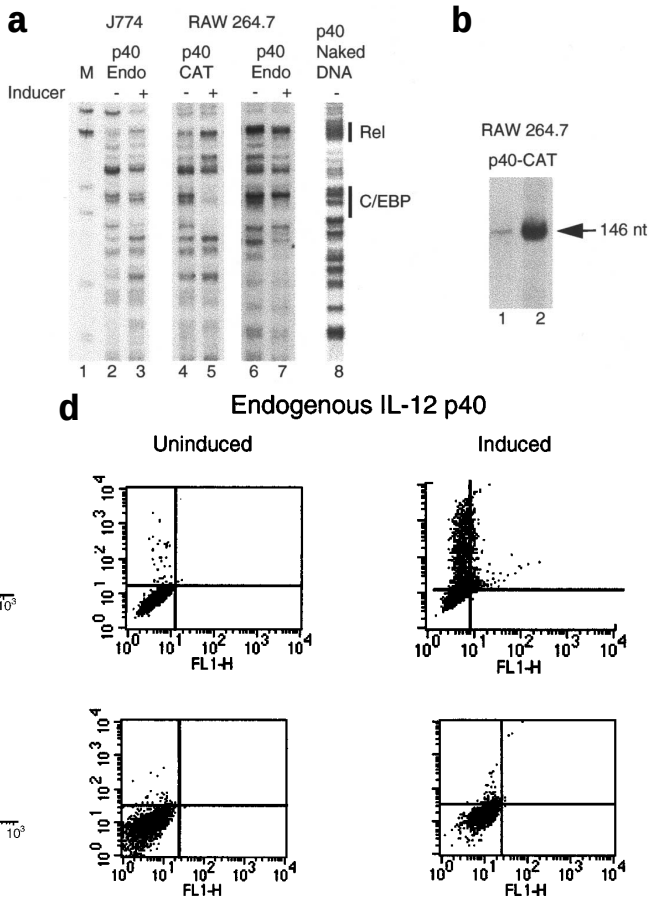

Figure 3. Comparison of endogenous and stably integrated IL-12 p40 promoters. (**a**) J774 cells (lanes 2 and 3), a RAW264.7 p40-CAT stable transfectant (lanes 4 and 5) and RAW264.7 cells (lanes 6 and 7) were untreated (lanes 2, 4 and 6) or treated with HKLM for 8 h (lanes 3, 5 and 7). Isolated nuclei were treated with DNase I. As a control, p40 plasmid DNA (lane 8) was digested with DNase I. Digested samples were analyzed by LM-PCR using primers specific for the endogenous p40 promoter (lanes 2, 3, 6–8) or the p40 promoter-CAT reporter (lanes 4 and 5). The same radiolabeled primer was used for all samples. Markers were pBR322×*Msp*I (lane 1). Locations of the p40 promoter elements, Rel and C/EBP, are indicated. (**b**) Primer extension analysis of total RNA isolated from RAW264.7 cells containing an integrated p40 promoter-CAT reporter. Cells were unstimulated (lane 1) or stimulated with LPS for 4 h (lane 2). Total RNA (30 μg) was analyzed using a radiolabeled primer specific to the CAT gene. Representative flow cytometry data examining (**c**) p40-GFP expression or (**d**) endogenous p40 expression are shown. J774 (upper panels) and RAW 264.7 (lower panels) lines were analyzed before (uninduced) or after (induced) activation with LPS. Similar results were obtained for endogenous p40 expression and GFP expression in multiple clonal lines.

nuclease hypersensitivity at position -840 would most likely be weak or absent.

An alternative explanation for the inefficient induction of the endogenous locus is that nucleosome 1 is inefficiently remodeled, with the stably integrated promoter remodeled much more efficiently. To examine this possibility, the endogenous and stably integrated p40 promoters were analyzed *via* restriction enzyme accessibility. The accessibility of the endogenous promoter in J774 cells was strongly enhanced after activation (**Fig. 4**)[18]. This enhancement is consistent with the induction of the gene encoding p40 in a substantial fraction (10–30%) of the cells, although the actual percentage of cells that have undergone remodeling cannot be determined from these data. However, the endogenous promoter in RAW264.7 cells was resistant to cleavage both before and after activation (**Fig. 4**). These results suggested that remodeling occurred in a very small fraction of the RAW264.7 cells, which provided a close correlation between inefficient remodeling and inefficient p40 expression.

In contrast, an analysis of the stably integrated p40 promoters fused to CAT or GFP reporter genes revealed that they were highly sensitive to cleavage in RAW264.7 cells, even in the absence of activation (**Fig. 4**). These results suggest that the stably integrated promoter exists in a constitutively accessible state. Similar results were obtained when stably integrated promoters in J774 cells were analyzed (data not shown). Although multiple copies of the plasmid are likely to be integrated in each line, it should be noted that normalization for promoter copy number is achieved by the *in vitro* cleavage step of the restriction enzyme assay. This normalization step allows the results obtained with the endogenous and integrated plasmids to be compared. The reason for the constitutive accessibility is unclear but it may result from integration into a region of the genome that is highly permissive for expression or from the proximity of the SV40 enhancer, which drives expression of the dominant selectable marker gene. Alternatively, the reporter construct used for the stable transfections may lack a region of the endogenous locus that is responsible for limiting the induction efficiency.

The results obtained in this series of experiments provide insight into the question of whether Rel and C/EBP proteins are sufficient for remodeling. The restriction enzyme data obtained with the endogenous locus in RAW264.7 cells suggest that p40 remodeling is inefficient in this cell line, leading to expression of the endogenous gene in only a small fraction of the cells. Because the proteins required for transcription of the stably integrated promoter are induced in the entire population, these results suggest that additional proteins are required for remodeling. The proteins required for activity of the stably integrated promoter, including Rel and C/EBP proteins, are therefore insufficient for remodeling. In addition, because occupancy of the C/EBP site can be monitored by genomic footprinting but was not observed at the endogenous locus, the results suggest that the relevant C/EBP protein cannot gain access to its site until remodeling has occurred.

Nucleosome remodeling in absence of c-Rel or p50

The stable transfection data suggest that Rel proteins are insufficient for remodeling. However, it remains possible that Rel proteins are necessary for inducible remodeling in concert with other inducible proteins that remain undefined. To explore this possibility, remodeling was examined in cells that lack the Rel proteins required for p40 transcription. If a critical Rel activator is unnecessary for remodeling, it should be possible to detect efficient remodeling in its absence. In contrast, if this protein is essential for remodeling, remodeling will be abolished when it is absent.

To do this analysis, the specific Rel proteins required for p40 promoter activity must first be identified. Biochemical approaches and an analysis of p40 transcription in mice that are deficient for specific Rel family members have addressed this issue[17]. p40 mRNA and protein, as monitored by RNase protection and ELISA, respectively, were essentially absent in activated macrophages from c-Rel$^{-/-}$ mice but were reduced by only a modest extent in macrophages from p65$^{-/-}$ mice[17].

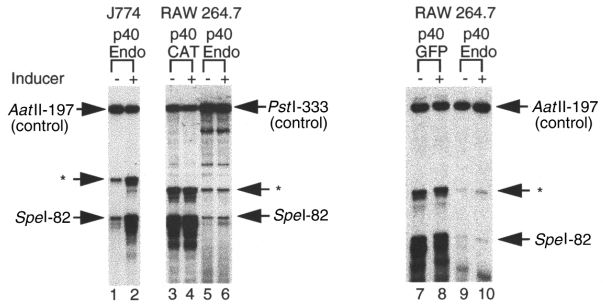

Figure 4. Comparison of restriction enzyme accessibility at the endogenous and stably integrated p40 promoters. J774 cells (lanes 1 and 2) or RAW264.7 cells containing the integrated p40 promoter-CAT reporter (lanes 3–6) or GFP reporter (lanes 7–10) were untreated (lanes 1, 3, 5, 7 and 9) or treated with LPS for 4 h (lanes 4, 6, 8 and 10) or HKLM for 8 h (lane 2). Nuclei were treated with *Spe*I for 10 min at 37 °C. Purified DNA was digested with *Pst*I (lanes 3–6) or *Aat*II (lanes 1, 2, 7–10) as a control. Primers specific for the integrated promoter-CAT reporter (lanes 3 and 4), GFP reporter (lanes 7 and 8) or the endogenous promoter (lanes 1, 2, 5, 6, 9 and 10) were used for LM-PCR. The same radiolabeled primer was used for all samples.

Analysis of p40 mRNA using a primer extension assay highlights the magnitude of the reduction in the absence of c-Rel (**Fig. 5a**). Quantification of the data revealed that p40 mRNA was reduced by more than 98% in c-Rel$^{-/-}$ macrophages, which was consistent with a 99.6% average reduction in p40 protein by ELISA[17]. These data suggest that a c-Rel–containing complex is a direct activator of p40 transcription. This is supported by the observation that several other proinflammatory cytokines were induced to wild-type amounts in c-Rel$^{-/-}$ macrophages[17], which demonstrates that the mutant cells do not possess a general activation defect. Biochemical studies suggest that the activator of p40 transcription is likely to be a p50–c-Rel heterodimer[17].

The restriction enzyme accessibility assay was used to monitor remodeling at the p40 promoter in the c-Rel$^{-/-}$ macrophages. Despite the dramatic reduction in p40 transcription, a strong increase in restriction enzyme accessibility upon activation was observed, which indicated that efficient remodeling had occurred (**Fig. 5b**). Although quantification of the results is of limited value in an LM-PCR–based assay, the increase in restriction enzyme cleavage upon cell activation was generally reduced by only 50–75% in c-Rel$^{-/-}$ macrophages relative to wild-type macrophages. This reduction suggests that a c-Rel complex may indeed contribute to remodeling efficiency or may help to stabilize the remodeled state after the initial remodeling event. Nevertheless, the results clearly separate remodeling from transcription and suggest that c-Rel is much more important for a subsequent step in the transcriptional activation process than for remodeling. These results demonstrate also that other TLR-inducible factors are capable of stimulating remodeling in the complete absence of the critical c-Rel complex.

To examine the consequences of eliminating c-Rel on promoter occupancy by C/EBP proteins, a DNase I genomic footprinting experiment was done. The results reveal clear occupancy of the C/EBP binding site at the endogenous p40 promoter in c-Rel$^{-/-}$ macrophages that is comparable to that observed in wild-type peritoneal macrophages[18] and stably integrated reporter plasmids (**Figs. 3** and **5c**). The inducible DNase I hypersensitive sites both upstream and downstream of the C/EBP site were observed in wild-type macrophages[18] and are consistent with the hypothesis that nucleosome remodeling accompanies activation (because DNase I cleaves remodeled nucleosomes much more efficiently than unremodeled nucleosomes). These data suggest that occupancy of the C/EBP site at the endogenous p40 promoter does not require the c-Rel complex that is critical for activation.

The c-Rel complex that stimulates p40 transcription is likely to be a p50–c-Rel heterodimer[17]. In p50$^{-/-}$ macrophages, p40 mRNA and protein expression were reduced by 90–94% (**Fig. 5a**)[17]. Most likely, the residual IL-12 p40 transcription observed in these cells was stimulated by a p52–c-Rel complex because the p50 and p52 members of the Rel family are partially redundant (A. Hoffmann, unpublished data)[26]. Consistent with the presence of substantial concentrations of p40 mRNA in the p50$^{-/-}$ macrophages, a strong increase in restriction enzyme cleavage was observed 1 h after cell activation (**Fig. 5d**). This increase was comparable to that observed in c-Rel$^{-/-}$ macrophages (data not shown). However, the efficiency of nuclear cleavage by two different restriction enzymes was reduced by 4 h after activation. This reduction was consistent and was never observed in wild-type or c-Rel$^{-/-}$ macrophages, even 8 h after activation (**Figs. 1b** and **5b** and data not shown)[18]. A similar reduction at the 4 h time point was observed in homozygous p50$^{-/-}$c-Rel$^{-/-}$ macrophages (data not shown), in which p40

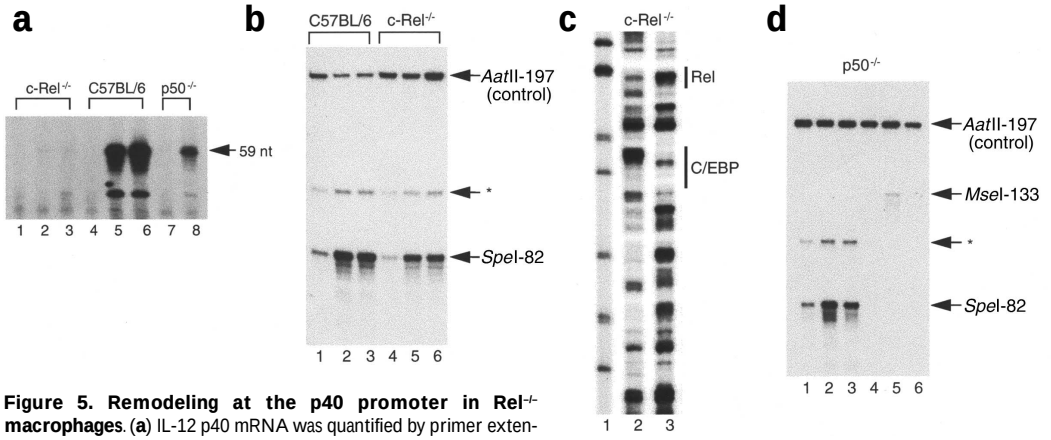

Figure 5. Remodeling at the p40 promoter in Rel$^{-/-}$ macrophages. (**a**) IL-12 p40 mRNA was quantified by primer extension analysis. RNA samples were derived from peritoneal macrophages isolated from c-Rel$^{-/-}$ (lanes 1–3), C57BL/6 (lanes 4–6) or p50$^{-/-}$ (lanes 7–8) mice. Cells were left untreated (lanes 1, 4 and 7) or were treated with IFN-γ + LPS for 4 h (lanes 2, 3, 5, 6 and 8). (**b**) The endogenous p40 promoter in peritoneal macrophages from C57BL/6 (lanes 1–3) or c-Rel$^{-/-}$ (lanes 4–6) mice was analyzed using the restriction enzyme accessibility assay. Cells were either unactivated (lanes 1 and 4) or activated with IFN-γ and LPS for 1 h (lanes 3 and 6) or 4 h (lanes 2 and 5). Nuclei were treated with *Spe*I. Purified DNA was cleaved *in vitro*, with *Aat*II as a control, followed by LM-PCR analysis. (**c**) The endogenous p40 promoter in peritoneal macrophages from c-Rel$^{-/-}$ mice was analyzed by DNase I genomic footprinting. Cells were either unactivated (lane 2) or activated with LPS for 4 h (lane 3). The markers were radiolabeled pBR322 digested with *Msp*I (lane 1). (**d**) Peritoneal macrophages isolated from p50$^{-/-}$ mice were unactivated (lanes 1 and 4) or activated with IFN-γ and LPS for 1 h (lanes 2 and 5) or 4 h (lanes 3 and 6). Nuclei were treated with *Spe*I (lanes 1–3) or *Mse*I (lanes 4–6). Purified DNA was digested *in vitro* with *Aat*II then LM-PCR analysis was done.

mRNA is undetectable[17]. The reduced accessibility at late time points in p50$^{-/-}$ and p50$^{-/-}$c-Rel$^{-/-}$ macrophages suggests that the remodeled nucleosome may be in a dynamic unstable state in activated cells.

Discussion

Our results lead to the following model for induction of the gene encoding IL-12 p40. Before activation of differentiated macrophages, the locus that encodes p40 appears to be in a relatively "open" chromatin environment[18]. The promoter region is assembled into positioned nucleosomes, with nucleosome 1 spanning the control elements identified by transient transfection. Upon cell activation, a remodeling or histone-modification complex is rapidly targeted to nucleosome 1 by DNA-binding proteins distinct from those that are required for promoter activity in transfection assays. Remodeling may depend on the activity of a histone acetyltransferase but the modest increase in acetylation suggests that other types of remodeling complexes are more important. The p50–c-Rel complex may be able to bind the Rel site before remodeling but a more likely scenario is that remodeling is essential for both the p50–c-Rel and C/EBP complexes to gain access to their sites. Once bound, these complexes may help to stabilize the remodeled state and make major contributions to transcriptional activation by recruiting coactivators or general transcription factors.

One critical unresolved issue is the identity of the DNA-binding protein that recruits the remodeling complex to the promoter. Both the stable transfection and c-Rel$^{-/-}$ data suggest that a c-Rel complex is not required for this step, despite its importance for p40 transcription. It remains a formal possibility, however, that a p50-p65 complex binds the Rel site and induces remodeling but is incapable of stimulating transcription. In addition, although the stable transfection data suggest that C/EBP proteins are insufficient for remodeling, an analysis of C/EBP-deficient macrophages will be necessary to determine whether remodeling requires C/EBP proteins. It was found previously that a domain within a large C/EBP-β isoform interacts with a mammalian (SWI-SNF) complex and enhances the stimulation of endogenous target genes when the isoform is overexpressed[19]. It is unlikely that this interaction is involved in p40 remodeling, however, because the large C/EBP-β isoform is not expressed in detectable amounts in murine macrophages (M. N. Bradley, unpublished results). The only C/EBP complexes in macrophages that can bind the p40 promoter contain the smaller C/EBP-β isoforms, which were incapable of associating with SWI-SNF[13].

In retrospect, it perhaps is not unexpected that the DNA-binding proteins required for promoter activity in transient transfection assays are insufficient for recruitment of a remodeling complex. Between nucleotides -342 and +9, the DNA sequences of the murine and human p40 promoters are 78% conserved, which suggests that numerous proteins may contribute to p40 transcription by binding this region. A comprehensive mutant analysis of the promoter revealed that the C/EBP and Rel sites, along with the TATA box, are the most important elements for promoter activity in a transient transfection assay, with three other elements making modest contributions[16]. However, because transiently transfected plasmids do not appear to assemble into typical nucleosomes[27], the elements and DNA-binding proteins identified using this assay may primarily be those that are important for the direct stimulation of transcription by RNA polymerase II. At some mammalian promoters, such as the IFN-β promoter, these same proteins appear to recruit histone acetyltransferase complexes[28]. However, it has not been determined whether the complexes recruited to any of these promoters contribute directly to the remodeling of promoter-encompassing nucleosome. We may, therefore, find that other elements in the conserved region between nucleotides -342 and +9 bind the proteins that recruit remodeling complexes to nucleosome 1. The nucleosome-free region upstream of nucleosome 1, which extends from approximately -175 to -350[18], is a particularly attractive location for a protein that recruits the remodeling complex because it would not be necessary for this protein to bind nucleosomal DNA.

To identify the DNA elements and transcription factors that recruit the remodeling complex, a systematic mutant analysis of the promoter region will first be required. The goal of this analysis will be to identify promoter mutations that eliminate remodeling. The results presented here suggest that a simple stable transfection assay with a functional promoter fragment may be insufficient for the analysis of remodeling because the stably integrated promoters were constitutively accessible to restriction enzyme cleavage. It is possible that an episomal stable transfection assay will recapitulate the results obtained with the endogenous locus.

A second issue that remains unresolved is the identity of the complex that remodels nucleosome 1. It may be possible to use a ChIP assay to identify a known ATP-dependent remodeling complex or histone-modification complex that associates with the promoter. A major limitation of this approach, however, is that it is restricted to an analysis of known complexes. A more unbiased approach would be to first identify the DNA-binding protein that recruits the remodeling complex and then to identify the complex that functionally interacts with this protein.

Finally, the evidence that remodeling at the p40 promoter is a TLR-dependent event suggests that remodeling is a target of one or more signal transduction pathways that originate at the cell-surface TLR proteins. It has become clear that LPS induces a large number of signal transduction pathways in macrophages that are essential for the activation of important target genes including the genes encoding IL-12 and other proinflammatory cytokines[10]. It appears that only a subset of these pathways influences the activities of common regulators of genes such as NF-κB, AP-1 and C/EBP. One or more of these pathways must be responsible for inducing the events that lead to nucleosome remodeling. The inefficient remodeling observed in J774 and RAW264.7 cells relative to the efficient induction of Rel and C/EBP proteins suggests that remodeling may serve as a particularly important mechanism for modulating IL-12 expression. Although the gene encoding p40 is expressed in 100% of the cells within a population of LPS-stimulated peritoneal macrophages[18], it is expressed in only a fraction of the cells within a population of bone marrow–derived macrophages (D. M. Mitchell, unpublished data). Because inefficient expression is observed in primary cells, we can speculate that regulation of the p40 expression efficiency through nucleosome remodeling may help modulate an immune response. Further analysis of the factors that stimulate remodeling should, therefore, enhance our understanding of TLR signaling pathways and of the mechanisms by which inflammatory responses and T_H1 development are regulated.

Methods

Cell culture and isolation of peritoneal macrophages. RAW 264.7 and J774 cell lines (ATCC, Rockville, MD) were maintained as described[18]. Cells were activated with 10 μg/ml of LPS (Sigma, St. Louis, MO) or HKLM[16]. Primary macrophages were isolated from the peritoneal cavity of thioglycollate-treated mice. C57BL/6, C3H/HeJ, C3H/HeOuJ (Jackson Laboratories, Bar Harbor, ME), c-Rel$^{-/-}$, p50$^{-/-}$ and p50$^{-/-}$c-Rel$^{-/-}$ mice were used as indicated[26,29]. Activation of primary macrophages involved recombinant IFN-γ (10–50 U/ml, PharMingen, San Diego, CA) and LPS (10 μg/ml), except where indicated otherwise.

Stable transfections and flow cytometry. For stable transfections with the p40-CAT reporter plasmid (p40 sequences from nucleotides -350 to +55)[16], RAW264.7 or J774 cells were cotransfected with the reporter plasmid and pSV2his as described[16]. Histidinol-resistant cells were selected as described[16]. For stable transfection experiments with GFP reporter plasmids, the p40 promoter (-350 to +55) was removed from p400GL2B[16] and

inserted into pd2EGFP-Basic (Clontech, Palo Alto, CA) *via* a 5′ *Asp*718 site and a 3′ *Bgl*II site. To generate RAW 264.7 cell lines, 5×10^6 cells were cotransfected with 10 μg of reporter plasmid and 1 μg of pBabe-puro[30] using Superfect (Qiagen, Valencia, CA), according to the manufacturer's protocol. After 48 h, cells were selected with puromycin (3.5 μg/ml) in 96-well plates. Single colonies were expanded and screened for inducible enhanced green fluorescent protein (EGFP) expression after LPS activation. To generate J774 cell lines that stably express the GFP reporter, 7.5×10^6 cells were cotransfected with 20 μg of the reporter plasmid and 2 μg of pBabe-puro *via* electroporation as described[16], except that a pulse of 270 V was used. After 72 h, cells were selected in puromycin (1.5 μg/ml), expanded and screened, as above.

EGFP fluorescence was analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with a 488 nm excitation laser and a 530/30 band pass detection filter. IL-12 p40 protein was monitored by intracellular flow cytometry as described[18].

Primer extension, restriction enzyme accessibility and DNase I genomic footprinting. IL-12 p40 mRNA was detected by primer extension analysis as described[18]. Total RNA (30 μg) was hybridized with either a radiolabeled IL-12 p40 specific primer or a CAT specific primer (5′– CAACGGTGGTATATCCAGTG–3′) as indicated. The restriction enzyme accessibility and DNase I genomic footprinting assays were done using primer sets specific for the endogenous genes encoding IL-12 p40, CAT or EGFP[18].

ChIP assay. The ChIP procedure was done following the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). Briefly, thioglycollate-elicited peritoneal macrophages from C57BL/6 mice were treated with 1% formaldehyde for 10 min at 37 °C. Nuclei were prepared and subjected to sonication to obtain DNA fragments ranging from 200–1000 bp. Chromatin fractions were precleared with protein A agarose beads followed by immunoprecipitation overnight at 4 °C with either AcH4, AcH3 or PhH3 antibodies. Cross-linking was reversed for 4 h at 65 °C and was followed by proteinase K digestion. DNA was purified and subjected to 18 PCR cycles with gene-specific primers followed by 3 cycles with a radiolabeled 3′ primer. Samples were precipitated and analyzed by electrophoresis on an 8% denaturing polyacrylamide gel.

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