REVIEW

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Regulation of cytokine transcription in the context of chromatin

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Abstract

Understanding the transcriptional regulation of an important class of innate and adaptive immune system effector molecules, the cytokines, is increasingly important given the promise cytokine regulation holds for treating various autoimmune and inflammatory diseases. Studies defining the mechanisms regulating cytokine transcription initially focused on identifying the *cis*-acting elements and *trans*-acting factors that activate cytokine promoters and enhancers. In the past, these studies were largely completed in the absence of constraints instituted by cellular chromatin. Over the past decade it has become obvious that changes in chromatin accessibility critically control, rather than simply correlate with, the transcriptional activation of most genes, including cytokines. Hence candidate transcriptional activators are being re-evaluated for potency in the context of cellular chromatin. Several distinct mechanisms for manipulating the generally repressive context of chromatin have been identified for cytokine genes. Most recently, single nucleotide polymorphisms in cytokine transcriptional regulatory elements have been shown to play measurable roles in regulating cytokine levels in the context of naturally selected haplotypes. Overall, subtle differences in DNA sequence and nucleoprotein complex composition, including protein post-translational modification, come together in cell type-specific combinations to explain the normal variation in cytokine transcription throughout the human populace.

Key words: cytokine transcription, chromatin remodeling, haplotypes.

Abbreviations: IL – interleukin, TNF – tumor necrosis factor, IFN – interferon, TBP – TATA-binding protein, LPS – lipopolysaccharide, IRF – IFN regulatory factor, SNPs – single nucleotide polymorphisms, GM-CSF – granulocyte macrophage colony-stimulating factor, HSSs – DNAase I hypersensitive sites.

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THE GENERAL PARADIGM FOR INDUCIBLE CYTOKINE TRANSCRIPTION

The general rules for inducible cytokine transcription determined outside the context of chromatin by transient transfection/reporter and DNA binding assays are similar to paradigms established for many other classes of developmentally regulated and inducible genes. Simplistically, a combination of tissue-restricted and ubiquitous sequence-specific DNA binding factors activate cytokine promoters and enhancers in combination with the general transcription machinery. Although the details defined in these many analyses are important in identifying potential candidates both to understand and to pharmacologically regulate the transcription of endogenous genes, cytokines were apparently not regulated by mechanisms fundamentally different from other classes of genes identified in these early studies. The interesting aspects of cytokine mRNA production instead lie in the phenomenon of rapid transcriptional activation due to defined changes in chromatin packaging and the tissue-specific mechanisms used to activate the same gene in different cell types.

Experiments to examine cytokine transcription in a natural chromatinized context have resulted in a general paradigm that explains how an initially repressive chromatin structure prevents promoter activation in resting cells and is overcome upon cellular stimulation. In general, activation of signaling pathways by external stimuli, generally pathogens or their products, results in "remodeling" of a nucleosome positioned over the transcription start site. Nucleosome remodeling can be measured as a longitudinal shift of the histone octamer, as shown for the interleukin (IL)-12 p40 and interferon (IFN)-β promoters in macrophages and epithelial cells, respectively [1, 77]. Although the idea of DNA sliding

over the surface of the histone octamer to result in a positional shift is both intuitive and experimentally supported (reviewed in ref. [51]), the energy required to break each of the dozens of non-covalent bonds formed between the histone octamer and the DNA suggests a mechanism that is substantially more complicated than sliding between, for example, two smooth surfaces. Alternatively, remodeling can be defined as a complete loss of histone octamer association upon cellular stimulation, as demonstrated for the IL-2 and granulocyte macrophage colony-stimulating factor (GM-CSF) promoters in T cells. Interestingly, the loss of histone packaging on the IL-2 promoter is reversible and independent of histone tail acetylation [17]. Furthermore, inducible chromatin accessibility, as measured by nucleosome remodeling, histone tail acetylation, or endonuclease cleavage, may represent only one of multiple steps required for inducible cytokine production, as demonstrated for tumor necrosis factor (TNF)-α and GM-CSF [13, 40].

Undoubtedly the most complete mechanistic analysis of cytokine transcriptional initiation has been completed on the IFN-β promoter. This work illustrated the important role promoter nucleosome remodeling plays in transcriptional activation. A series of chromatin immunoprecipitation assays coupled with elegant nucleosome positional mapping showed that a complex orchestration of multiple steps leads to promoter activation. The first step stimulated by viral infection (of HeLa cells) is association of the enhanceosome, a collection of sequence-specific DNA binding proteins, on a nucleosome-free region upstream of the IFN-β transcription start site. The next steps include recruitment of a histone acetyl transferase and RNA polymerase II, SWI/SNF chromatin remodeler association, and, finally, TATA-binding protein (TBP) binding [1, 46]. TBP binding results in promoter DNA bending [34], then nucleosome sliding culminates in IFN-β transcription [1, 46]. Interestingly, the precise order of these detailed steps can vary from gene to gene both within and beyond the cytokine family. Regardless, the critical step for most cytokine promoters involves remodeling a repressive nucleosome that blocks the transcription start site. Although this general model of transcription-factor-targeted chromatin remodeling followed by recruitment of additional co-activators has not been as thoroughly detailed for the IL-4, IFN-γ, IL-12 p35, IL-13/IL-5, IL-10, IL-6, IL-21, TGF-β, GM-CSF, CSF, and MCP-1 promoters, transcription start sites of these cytokines appear to be packaged into a relatively inaccessible chromatin structure in resting cells, and become more accessible by one or more measures in stimulated cells [1, 4–6, 9, 11, 12, 21, 25, 30, 31, 33, 36, 41, 45, 47, 57, 63, 69, 74, 77].

Although a repressive chromatin structure can prevent premature cytokine transcription, a second mechanism of regulation through epigenetic changes has also been characterized at the murine IFN-γ gene [78, 79]. Specifically, the IFN-γ promoter DNA is demethylated as naïve T cells are stimulated to differentiate into T_H1 cells, a major source of IFN-γ *in vivo*. A similar demonstration of the role changes in DNA methylation play in cytokine transcription has been completed at the IL-4 promoter. T_H2 cells with low levels of DNA CpG methylation over the IL-4 locus quantitatively produce more IL-4 than cells with more methylated loci. Interestingly, locus demethylation is not required for inducible chromatin remodeling [2, 26]. This finding suggested the two processes are not tightly coupled. Overall, as has been found for other genes, cytokine promoter demethylation introduces an additional layer of transcriptional regulation. Although DNA and histone methylation, the latter of which can activate or repress transcription depending on the histone tail target, are mechanistically linked [23, 24, 70], the importance of this link for cytokine gene transcription has not been reported.

CYTOKINE TRANSCRIPTION FROM A POISED PROMOTER STRUCTURE

Recent evidence indicates that inducible remodeling of a nucleosome followed by newly established accessibility of the transcription start site to RNA polymerase II does not regulate the transcription of all cytokines. Studies on the IL-8 promoter mapped a nucleosome that encompassed DNA at positions +39 to +186. Hence, a repressive chromatin structure does not block the transcription start site at +1. Viral stimulation leads to transcription factor binding to the upstream non-nucleosomal DNA [46], suggesting the histone-free structure surrounding the transcription start may be important for IL-8 transcriptional activation. Other examples suggested some cytokine promoters may be constitutively packaged into accessible chromatin. In one series of assays, Smale and colleagues measured changes in the chromatin structure of multiple promoters in resting versus stimulated macrophages [62]. The genes fell into three categories: 1) immediate early (transcript level increases at least 5-fold within 30 min of stimulation), 2) immediate late (cycloheximide insensitive and transcribed <2-fold over background 30 min post-stimulation), and 3) secondary (cycloheximide sensitive) response genes. The immediate early cytokine genes tested, TNF-α and IL-1β, were inducibly transcribed in the absence of the major chromatin remodelers Brg-1 and Brm. Despite normal transcription in the absence of these ATPases, chromatin immunoprecipitation assays demonstrated that Brg/Brm associate with immediate early genes in resting cells. One possible explanation for these seemingly contradictory findings is that the measured Brg/Brm association is a vestige of a requirement for Brg/Brm-mediated chromatin remodeling during macrophage development [62]. This possibility remains to be tested. Regardless, the production of transcript in the absence of these remodelers suggested the promoters are packaged into a relatively permissive/accessible chromatin structure. Direct measurements

of TNF-α chromatin accessibility in resting versus induced cells by these authors and others show either modest [62] or significant [40] changes in endonuclease-mediated promoter cleavage upon lipopolysaccharide (LPS) stimulation. However, association of a TNF-α promoter binding protein, C/EBPβ, in both resting and stimulated cells [62] strongly suggests this immediate early promoter is not entirely inaccessible in resting macrophages. Therefore, along with the IL-8 promoter, the TNF-α promoter may fail to follow the general model for inducible cytokine transcription, perhaps by being packaged in a "ready" or "poised" structure in resting cells. In contrast, immediate late (IFN-β) and secondarily activated cytokines (IL-12 p40 and IL-6) require Brg/Brm for transcriptional activation [62], hence are likely to follow the general paradigm of cytokine transcription from a repressive chromatin context.

Perhaps the most thorough study of immediate early cytokine gene transcription has been completed on IL-1β. IL-1β is packaged into a poised chromatin structure in monocytes and macrophages as indicated by restriction endonuclease accessibility and lack of histone octamer packaging at the transcription start site. The locus is also sensitive to digestion with the endonuclease micrococcal nuclease (MNase), which preferentially cleaves nucleosome-free DNA [43, 62]. These findings are consistent with the ability of this cytokine to be inducibly transcribed in the absence of the Brg/Brm chromatin remodelers [62]. Interestingly, multiple sequence-specific DNA binding factors, including the *ets* transcription factor PU.1, constitutively associate with both the promoter and the enhancer in resting monoctyes, forming a "poised promoter architecture" [15, 43]. However, both RNA polymerase II and IFN regulatory factor (IRF)-4 are recruited to IL-1β regulatory elements upon monocyte stimulation. Because IRF-4 recruitment requires PU.1 phosphorylation [60], and cellular stimulation results in activation of a PU.1 kinase, CK2, we have proposed that phosphorylation of constitutively DNA-bound PU.1 is important for inducible IL-1β transcription. Extension of this mechanistic explanation for immediate early gene activation awaits further analysis of other rapidly inducible targets packaged into accessible chromatin structures. This model of gene activation, including a poised promoter architecture and a critical role for post-translational modification of constitutively associated DNA binding proteins, has been previously identified only for genes outside the cytokine family [29, 42, 61, 68, 75].

Although we and others have speculated that the poised chromatin structure facilitates rapid gene activation under physiological stimulatory conditions, evidence for the abnormal formation of a poised structure at cytokine promoters under disease conditions is beginning to emerge. For example, the NF-κB subunit p65 is constitutively associated with the TNF-α and IL-6 promoters in cells exposed to chronic hyperglycemia in a model of human diabetic monocytes [52]. The histone acetyltransferase CBP and the p65 partner p50 also associate with the TNF-α promoter in this model in the absence of additional stimuli, and histones packaging the promoter become hyperacetylated within 16 h of glucose stimulation. Hence hyperglycemia may be an important mediator of cytokine activation. Additional work shows that the hyper-responsive IFN-γ promoter in NOD mice, a model for type 1 diabetes, correlates with packaging of the locus into hyperacetylated histones in NOD-derived CD8 T cells [80]. This finding suggests that chronic hyperglycemia can facilitate cytokine activation by increasing chromatin accessibility. Finally, although IL-1β is required for joint inflammation in mouse models of rheumatoid arthritis [32] and in humans with juvenile idiopathic arthritis [19], and has been widely implicated in autoimmune phases of type 1 diabetes [20, 50, 72], the precise role of the poised structure in normal versus pathological IL-1β production remains to be determined.

TISSUE-SPECIFIC CYTOKINE GENE TRANSCRIPTION

Multiple cytokines are produced by more than one cell type, and recent analyses support the idea that different cell types may activate the same cytokine regulatory elements using fundamentally different mechanisms. For example, the highly conserved non-coding sequence of the RAD50/IL13/IL4 locus is activated by different mechanisms in two hematopoietic cell types, T cells and mast cells. Specifically, DNAase I mapping has shown that some hypersensitive sites (HSSs, indicative of a more accessible chromatin structure and hyperacetylated histones [18, 56, 66]) are shared between the two cell types, while other HSSs are cell type specific [54]. The functional significance of HSS formation is indicated by the demonstration that a T cell-specific HSS is required for cytokine expression in T cells but is dispensable for cytokine transcription in mast cells, which lack that particular HSS [54]. Similarly, two DNAase hypersensitivity sites are required for activation of the IL-3 promoter in T cells; however, only one of these sites is required for IL-3 transcription in mast cells [28]. IFN-γ is a third cytokine regulated by two different mechanisms in two hematopoietic cell types. In T cells, relatively long-term stimulation and cell cycling is required for IFN-γ transcription, which correlates with gain of histone tail acetylation in promoter nucleosomes [10, 21]. In contrast, IFN-γ DNA is constitutively demethylated (an indication of an active gene structure) in natural killer cells and rapidly activated in the absence of notable changes in HSS formation [71]. IL-12 p40 remodeling is also achieved by different stimuli in two hematopoietic cell types, macrophages and dendritic cells, suggesting fundamentally different mechanisms of nucleosome shifting at a single cytokine locus [3]. Interestingly, this locus, once remodeled, can be activated by multiple combinations of the NF-κB family proteins, and usage of different family members correlates with susceptibility to different autoimmune

diseases (lupus versus diabetes) [44], consistent with the proposed relationship between differential cytokine activation and disease. Because IL-12 p40 promoter remodeling occurs in the absence of at least some NF-κB proteins [76], tissue-specific regulation of IL-12 p40 by NF-κB proteins is likely independent of chromatin regulatory mechanisms. In another example, IL-10 is activated by mechanisms consistent with the general paradigm for cytokine transcriptional activation outlined above [47]. However, the finding that the locus contains DNAase I HSSs in macrophages but not T cells suggests that, like IL-4, IFN-γ, and IL-12 p40, different immune cells utilize unique mechanisms to activate the same cadre of chromatin remodelers and transcriptional regulatory elements at the IL-10 locus [65]. Likewise, different DNAase I HSS patterns over the TNF-α promoter differ in monocyte versus T cell lines, despite transcription of the cytokine by each, strongly suggesting that multiple chromatin structures can be transcriptionally active [9]. Finally, the cytokine IL-1β is activated from a poised promoter architecture in monoctyes; in contrast, B cells, which can be stimulated to transcribe IL-1β in at least some experimental systems (Sardi and Nikolajczyk, unpublished observation), package the promoter into an MNase-inaccessible structure [43].

These data could be generalized to support the thesis that mechanisms controlling cytokine production are fundamentally different in cells of the innate versus the adaptive immune system; however, additional studies refute this possibility. Studies of chromatin structure in cytokine gene loci during naïve T cell activation to form two T helper cell subsets, T_H1 and T_H2, demonstrate that these two closely related cell types use differences in chromatin structure to achieve cell-type-specific cytokine transcription. For example, the T_H2 gene cluster takes on a DNAase-accessible chromatin structure in T_H2 (but not T_H1) T cells [21, 22, 48]. Further analyses demonstrate that transcriptionally permissive marks are established over the T_H2 cluster in naïve T cells, but these are compounded or tempered by additional chromatin accessibility marks and DNA demethylation as cells are grown in T_H1 versus T_H2 skewing conditions [2, 6, 7, 13, 21, 26, 37–39, 46, 49, 58, 64, 81]. Similar findings have been noted in the IL-10 promoter, which is inaccessible in naïve T cells, but attains hypersensitivity in differentiated T_H2 cells. Alternatively, the promoter acquires repressive chromatin marks in T_H1 T lymphocytes [31]. Furthermore, although $CD4^+$ T cells remodel and transcribe the IL-2 gene in response to stimuli, a second type of T cell, the regulatory T cell, maintains the locus in an inaccessible chromatin structure, mechanistically explaining why the latter cells do not produce IL-2 [67].

HAPLOTYPE-DETERMINED REGULATION OF CYTOKINE GENES

Many studies have genetically linked polymorphisms in human cytokine genes, including TNF-α, IL-1β, IL-8, and IL-6 (among others) to disease susceptibility [8, 14, 27, 35, 53, 55]. However, mechanistic analyses to understand how to correct the defects introduced by naturally occurring polymorphisms in cytokine genes are in their infancy. Quantitative differences in transcriptional activation (and corresponding differences in protein levels) due to polymorphisms in the transcriptional regulatory elements of human cytokine genes have only been reported for the IL-8 and IL-1β genes. For each of these promoters, single nucleotide polymorphisms (SNPs) in isolation can result in substantial differences in transcription factor/promoter association in EMSA assays [16, 27]. However, careful examination of the IL-1β promoter demonstrated that differences in protein association are functionally discernible only in the context of a subset of naturally occurring haplotypes, or SNP combinations. Interestingly, of the 16 theoretically possible combinations of 4 promoter SNPs identified within 4 kb upstream of the IL-1β transcription start site, >98% of a human population representing diverse genetic backgrounds use only 4 of these haplotypes; furthermore, of these 4 haplotypes, the predominant haplotype(s) varies based on genetic background [16]. These data are consistent with the speculation that differential IL-1β transcription correlates with human genetic fitness over the course of evolution, with the net result being the retention of only 25% of the theoretically possible IL-1β promoter haplotypes in a large majority of the population.

Studies in mouse models of autoimmune disease strongly link differences in IL-1β haplotypes to disease susceptibility and strengthen the case for further analyzing the biological effects of cytokine non-coding SNPs in humans. Through a series of mouse crosses combined with selection for inducible hyper-production of IL-1β, the Mathis/Benoist lab mapped susceptibility to a rheumatoid arthritis-like disease to the IL-1β locus [59]. Reminiscent of the limited usage of possible IL-1β haplotypes in the human population, this work demonstrated that, based on a sampling of 16 SNPs in more than 10 mouse strains, only two IL-1β haplotypes are represented in mice. One haplotype, characterized most extensively in BALB/c mice, transcribed IL-1β at 10–20--fold higher levels upon *in vivo* LPS stimulation as compared to the second haplotype. SNPs in the IL-1β locus identified in this work clustered in the non-coding regions of the gene, including some in the upstream promoter, and did not result in changes in the amino-acid sequence of IL-1β protein. The finding that transcript levels correlated with higher serum IL-1β levels by ELISA further emphasized the biological importance of the differences in non-coding SNPs at the whole-organism level. Most importantly, mouse strains harboring the hyperactive haplotype were (with 1 exception) more susceptible to arthritic inflammation in the serum transfer model [32] as measured by time of onset and severity of the disease. Lower susceptibility to arthritic symptoms was noted in mice harboring the hypoactive haplotype, as exemplified by the strain C57BL/6. Interestingly, a second mouse strain commonly used for rheumatoid

arthritis studies, DBA, carries the hyperactive IL-1β haplotype. Although this study highlighted for the first time the critical role IL-1β transcription likely plays in autoimmune disease, the presence of a hyporesponsive IL-1β haplotype in a classical model of another autoimmune disease, the NOD mouse, underscores the multifactorial nature of such diseases [59].

CONCLUDING REMARKS

Cytokine gene transcription is regulated by precise combinations of multiple mechanisms that likely enable exquisite control based on cell source, stimulus, and individual genetic variation. The presence of unique regulatory mechanisms provides numerous opportunities for exquisite regulating cytokine production in autoimmune or inflammatory disease while avoiding the negative immunological consequences of eliminating any one cytokine in its entirety. Promising, though often partial, effects of moderating some cytokines (for example, TNF-α and IL-1β [73, 74]) in patients with autoimmune or inflammatory disease at the level of protein function suggest that further mechanistic analyses will be fruitful towards more effectively regulating these important immune system mediators during both acute and chronic phases of multiple human maladies.

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