Preface

Cytokines, produced by immune cells, are soluble molecules by which the immune system communicates with other cell types within or outside the immune system. Cytokines form a central coordinating network of soluble effector molecules that plays a crucial role at every step of the development of autoimmune disease: in the generation of pathogenic or protective effector cells, in the trafficking of pathogenic cells to the target organ, and in mediating tissue damage or tissue tolerance in the target organ.

Upon activation, naive autoreactive T cells differentiate into cells that produce proinflammatory or anti-inflammatory cytokines. Cells producing proinflammatory cytokines are pathogenic and induce organ-specific autoimmune diseases, whereas autoreactive cells producing anti-inflammatory cytokines may inhibit autoimmune diseases, although they do not always do so. Thus, interventions targeted to inhibit the secretion of proinflammatory cytokines or the differentiation of cells to a proinflammatory phenotype, or alternatively to enhance anti-inflammatory cytokines or the differentiation of cells to an anti-inflammatory phenotype, may inhibit and/or reverse an autoimmune disease. This highlights the enormous therapeutic potential of cytokine modulation in the treatment of autoimmune diseases.

Over 18 different cytokines with both overlapping and distinct functions in the activation, expansion, and differentiation of other cell types in the immune system have been discovered. In addition there are over 20 different chemokines, molecules that are chemotactic and affect the accumulation of different types of cells at the sites of inflammation and tissue injury. In an immune response there is a complex interplay between these various cytokines and chemokines that determines the outcome of inflammation and the probability of developing autoimmune disease.

The current literature contains a large amount of primary data on the role of various cytokines in the induction and regulation of autoimmunity; however, there is no clear consensus. *Cytokines and Autoimmune*

Diseases aims to synthesize the available information on this single topic, the role of cytokines in autoimmunity, to help develop a clear understanding of how cytokines and chemokines are involved in the pathogenesis of autoimmune diseases.

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Chromatin Remodeling and Transcriptional Regulation of Cytokine Gene Expression in T Cells

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1. INTRODUCTION

The activation of T cells by antigen leads to the expression of a large number of cytokines that are important for the correct orchestration of an immune response. The specific array of cytokines produced depends on the antigen that is encountered and the environment of the T cell at the time of that encounter. These cytokines range from those required for T cell proliferation (e.g., interleukin-2 [IL-2]), activators of other cells of the immune system (e.g., IL-4 or granulocyte-macrophage colony-stimulating factor [GM-CSF]), to negative modulators of cell function (e.g., IL-10 or TGB-β). The aberrant expression of these cytokines can lead to immune related disease such as autoimmune disease or chronic inflammation and modulating the profile of cytokine expression or function has been long touted as a possible treatment for these diseases.

Cytokine expression is largely controlled at the level of gene transcription although there is significant evidence that mRNA stability as well as translational control also operate to govern the final level of protein produced. This chapter will focus entirely on the transcriptional control of cytokine gene expression. Cytokine genes are mostly maintained in a silent state until cells receive an appropriate activation signal. Then follows a transient burst of high level transcription followed by a return to baseline. This pattern of expression of gene transcription means that several control mechanisms have to play a role. First, the cytokine genes, while silent in resting T cells, are likely to be in a distinct chromatin configuration in the nucleus to those genes that are either permanently switched on or off in the cells. In other words, they may be "marked" in some way as genes that are silent but responsive to cell activation. The chromatin context of these inducible genes is currently an area of considerable interest. Second, they must be switched

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on in response to the correct set of environment signals, and this appears to be achieved by the assembly of a precise complex of transcription factors on promoter/enhancer regions. The requirement for a complex of transcription factors to switch on gene transcription means that the gene will not respond to signals that activate only one or two of the required transcription factors but will only respond when the correct array of factors is present. The third requirement, that the response be transient, may be a passive depletion of signaling or transcription factors or may require an active mechanism of repression but this is not yet well understood.

T cell activation is a complex process that depends on both activation of the T cell receptor (TCR) and so called costimulatory signals (reviewed in refs. *1,2*). While the TCR interacts with peptide MHC complexes on the surface of antigen-presenting cells (APCs), other cell surface molecules on APCs pair up with their counterparts on T cells to enhance the interaction of the APC with the T cells. The best-described costimulatory signal is the interaction of B7.1/B7.2 (CD80/CD86) on APCs with the CD28 receptor on T cells (reviewed in refs. *3,4*). CD28 signals serve to augment TCR signals and are thought to lower the threshold of TCR signal strength that is required for T cell activation. CD28 activation has been clearly shown to augment cytokine gene transcription in T cells to higher levels than that seen with TCR activation alone *(1–4)*. Blocking the interaction of B7 with CD28 alleviates the pathogenic response in many mouse models of autoimmunity and other immune-related diseases (reviewed in ref. *5*). The importance of this costimulatory pathway in regulating T cell activation has led to great interest in determining the mechanism of signal transduction by CD28 and the resultant mechanism of cytokine gene transcription.

While T cells produce a vast array of cytokines in response to activation, these cytokines can be divided into several groups depending on their time of production or their ultimate function (reviewed in refs. *6,7*). Activation of naive T cells leads first to the production of IL-2, GM-CSF, and IL-3 among others. These cytokines appear to have common patterns of activation although IL-2 and IL-3 are T cell specific, whereas GM-CSF can be produced by a variety of cells. The control of IL-2 production is a critical point in determining the outcome of the T cell response. If T cells are activated below a specific threshold and the IL-2 gene is not switched on, then the cells become anergic or unresponsive to further activation (reviewed in ref. *8*). On the other hand, too much IL-2 can lead to activation induced cell death *(8)*. Thus there is a critical range of IL-2 that must be produced to generate a productive immune response. Understanding the control of IL-2 gene transcription is crucial in understanding the mechanism of naive T cell

activation. Other cytokines produced in this initial phase of T cell activation such as GM-CSF and IL-3, while not directly governing the T cell response can control the activation or production of other immune-related cells such as APCs.

Depending on the immune stimulus and the environment, the dividing helper T cells will mature in to either Th1 or Th2 effector cells (reviewed in refs. *6,7*). Th1 cells are associated with inflammatory reactions and delayed-type hypersensitivity and are characterized by the production of interferon-γ (IFNγ). These cells are thought to mediate many autoimmune diseases. On the other hand, cell mediated immunity and allergy are associated with Th2 type T cells, which produce an array of cytokines (IL-4, IL-5, IL-13, etc.), the prototype of which is IL-4. The differentiation of Th cells and their ability to produce different population of cytokines has been well-reviewed and will not be discussed in detail here. It should be noted, however, that the Th1/Th2 paradigm is best understood at a population level and that individual cells appear have a great plasticity to produce both Th1 and Th2 type cytokines *(9)*. Cytokines produced by the Th subtypes appear to have overlapping but distinct requirements governing their transcription and differ also from the cytokines activated immediately following naive T cell activation.

This chapter will focus on the more recent advances in understanding cytokine gene expression as well as those aspects such as CD28 activation that are possible therapeutic targets in autoimmune disease.

2. MECHANISMS OF TRANSCRIPTIONAL RESPONSE TO T CELL ACTIVATION

As described earlier, one step in the induction of cytokine gene transcription is the assembly of a transcription-factor complex on the promoter/enhancer regions of the gene that allows the recruitment of the basal-transcriptional machinery and hence activation of the RNA polymerase activity. T cell activation leads to either the production or nuclear localization of several families of transcription factors, the most important of which appear to be the NFAT, NF-κB, and AP-1 families (reviewed in refs. *10,11*). A detailed understanding of the role of individual members of these families is being obtained from gene-deletion studies in mice and this information has been recently reviewed *(11)*. These activation-dependent transcription factors bind to promoter/enhancer regions and together with constitutive factors and architectural proteins form the transcriptional-activation complex. There have been many detailed studies documenting the transcription factors and their binding sites that can play roles in activating cytokine genes such as IL-2, GM-CSF, IFN-γ, and IL-4 and these data have been exten-

sively reviewed *(12–15)*. Several general principles have emerged from this work that are useful in terms of understanding the mechanisms of inducible cytokine gene expression.

For many of the T cell-expressed cytokine genes the proximal promoter region (i.e., the first 100–300 bp upstream from the start of transcription) is crowded with potential transcription factor-binding sites. Cooperation between the factors binding to many of these sites seems to be critical for promoter/enhancer activity. Many of the binding sites on these promoters are low-affinity sites for their cognate transcription factors and so do not have significant activity when tested in isolation. Furthermore, altering these low-affinity sites to high-affinity consensus sites not only leads to their ability to act alone but can disrupt the tissue-specific activity of the promoter. This has been well-demonstrated in the case of the IL-2 promoter and suggests that this cytokine promoter has been fine-tuned for both tissue specificity and induction in response to appropriate signals *(16,17)*. The need for cooperation between a number of transcription factors has been demonstrated in a number of ways. First, mutation of individual sites leads to a dramatic reduction in the activity of the promoter. This is illustrated by studies of the GM-CSF promoter where mutation of any of the known transcription factor-binding sites in the proximal promoter has a major effect on activity (reviewed in ref. *14*). Second, loss of a single factor can, in some cases, greatly reduce activity and this will be discussed in more detail later. Third, there is evidence from in vivo footprinting experiments that for the IL-2 promoter there is an all or nothing occupation of the promoter, implying cooperative promoter occupancy *(18)*. Finally, overexpression of transcription factors can lead to highly synergistic activation of promoter activity. This is illustrated by studies with the GM-CSF promoter where overexpression of Ets1, AP-1 (c-fos and c-jun), and NF-κB proteins (RelA and p50) showed highly synergistic activation in transient transfections in Jurkat T cells *(19)*.

Cooperation between transcription factors can be manifested in several ways. First, cooperative protein binding has been observed for some of the T cell transcription factors. A good example of this is the cooperative binding of NFAT and AP-1 proteins to many sites in the IL-2 and IL-4 promoters and the GM-CSF enhancer (reviewed in refs. *20,21*). For many of these sites, the individual factors bind very weakly but show strong cooperative binding. The precise positioning of the sites is essential for this cooperative binding and this leads to high levels of synergy at the level of function *(22)*. There are many other sites in cytokine genes that bind NFAT or AP-1 individually without any observation of cooperative binding or function, although it is not clear what the functional implications are.

Cooperation may also be manifested at the level of function and not DNA binding. A very good example of this is the activity of the CD28 response region (CD28RR) of IL-2. Here adjacent NF-κB and AP-1 sites are required for activity but show no cooperative binding of their cognate transcription factors *(23)*. Cooperative activation in such cases may be related to the ability of the combination of factors, but not the individual factors, to recruit coactivators or components of the basal machinery. The function of the CD28RR will be discussed in detail in Subheading 3.

Not just the presence of the correct set of proteins but the specific architecture of the complex appears to be important for optimal promoter activity. Altering the relative position of transcription factor-binding sites can have dramatic effects on transcription. This presumably disrupts the cooperative activities of the transcription factors either for DNA binding or recruitment of coactivators. The DNA structure can also alter transcription-factor interactions and the assembly of a functional complex. Architectural proteins that can alter DNA structure and also modulate transcription-factor binding have been implicated in cytokine expression. One such example is the HMGI(Y) family of architectural proteins that seem to play a major role in the activity of the IL-2 gene promoter *(24)*. These proteins are thought to be involved in the assembly of an active complex. Such an activation complex that forms on the IFN-β promoter in response to virus induction has been dubbed an enhanceosome (reviewed in ref. *25*). The formation of this enhanceosome allows the recruitment of coactivators such as CBP and also components of the basal machinery such as TFIIB to the promoter *(26,27)*. This recruitment appears to be the basis of enhanceosome activity. Such a complex structure may play an important role in allowing the promoter to respond only to the set of signals that result in the activation of the entire set of transcription factors required for enhanceosome assembly; for example, virus infection probably leads to the activation of an array of signal-transduction pathways that is interpreted at the IFN-β promoter by the assembly of the enhanceosome. The promoter is, thus, designed not to respond to the activation of only one of these signaling pathways. Similarly, T cell activation requires the precise interpretation of several cooperating signals to lead to the correct level and type of cytokine gene transcription. Given the examples described earlier of cooperative activation of cytokine promoters by different families of transcription factors, it is likely that enhanceosome-like complexes also form on many of the T cell cytokine promoters and dictate the response of the genes to T cell activation (*see* Fig. 1).

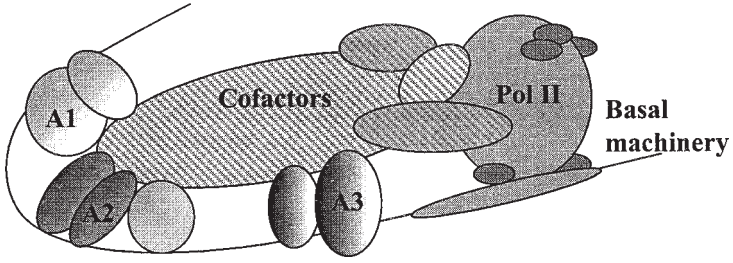

Fig. 1. A hypothetical model of the formation of an enhanceosome on a cytokine promoter, following T cell activation. Activated transcription factors (A1, A2, A3) bind to specific sequences on the DNA. This combination of factors serves as a recruitment "surface" for coactivators that may link the transcription factors to the basal transcriptional machinery represented by Pol II (RNA polymerase II) and associated factors. The cofactors may also possess chromatin-modifying activity as described in the text.

3. TRANSCRIPTIONAL RESPONSES TO COSTIMULATION IN T CELLS

The CD28 signal is clearly an important one in T cell activation as described earlier. It has been shown that inhibiting the interaction of CD28 with the B7 molecules has a significant impact on the pathology of experimental models of autoimmune disease. Therefore, a detailed understanding of the signal-transduction pathways that are activated by CD28 should provide many new targets for drugs to modify the immune response.

It has clearly been demonstrated that CD28 costimulation of T cells leads to an increase in transcription from the *IL-2* gene as well as many of the other cytokine genes expressed in T cells *(3,4)*. It is probably this increase in IL-2 expression and the subsequent T cell proliferation that is responsible for the ability of CD28 to overcome the anergic response that can be the result of TCR engagement in the absence of costimulation.

The mechanism of CD28 signal transduction is still a matter of debate but the end result appears to be an increase in the level or activity of some of the transcription factors that are known to activate cytokine gene transcription. The two best-described examples of transcription factor response to CD28 are the increased level of NF-κB family members and the increased activity of the AP-1 family (reviewed in refs. *3,4*). On the other hand, the NFAT family, which is also important in cytokine gene expression, does not appear to be affected. The ability of CD28 to increase the activity of AP-1 is most likely mediated by its ability to increase the activity of the JNK signal-trans-

duction pathway *(28)*. JNK can, in turn, specifically phosphorylate c-jun to increase its DNA binding activity.

NF-κB proteins are located in the cytoplasm prior to activation in a complex with IκB inhibitory proteins. Upon cell activation, IκB proteins are phosphorylated by the IκB kinase complexes (IKKs), which targets them for degradation by the S26 proteosome pathway. The NF-κB proteins then translocate to the nucleus where they bind to their cognate recognition sites (reviewed in ref. *29*). CD28 activation appears to lead to both an increase in the level as well as a prolonged presence of certain NF-κB proteins in the nucleus. The level of RelA but in particular, c-Rel increases in response to CD28 *(30,31)*. Unlike, RelA there appears to be little c-Rel stored in the cytoplasm in resting cells. c-Rel appears in the nucleus at late times (>4 h) following activation and its presence is the result of increased expression from the c-Rel gene *(32)*. *c-Rel* gene transcription appears to be controlled at least in part by RelA or other NF-κB proteins and thus may be responding to the increase in these or other transcription factors following CD28 activation. A change in the degradation kinetics of IκB-β has been described in response to CD28 but there is also evidence for increased and sustained degradation of IκB-α *(33,34)*. There has been evidence presented recently that there may be crosstalk between the JNK and the NF-κB activation pathways. It has been shown that the JNK cascade can selectively activate the IKK-β but not the IKK-α pathway that leads to IκB phosphorylation and degradation *(35)*. The fact that the same pathways of AP-1 and NF-κB activation appear to be responsible for sensing the CD28 signal as the TCR signal supports the hypothesis that CD28 simply augments some of the TCR signals.

The region of the IL-2 promoter that is required for and most highly responsive to CD28 activation has been defined and studied in detail (*see* Fig. 2). Not surprisingly, it is a composite site for NF-κB and AP-1 transcription factors and will be referred to here as the CD28 responsive region (CD28RR). The CD28RR contains a nonclassical NF-κB binding site called the CD28RE (or CK-1) as well as an adjacent AP-1 binding site. There have been many studies of the proteins that bind to and mediate the activity of the CD28RR. There appears to be a general consensus that the binding of c-Rel to the CD28RE is important for its activity, although RelA has also been shown to play a role here *(23,31)*. The binding of c-Rel is highly dependent on the presence of the small nuclear architectural factor, HMGI(Y) *(31)*. HMGI(Y) binds to the A/T rich core of the site and although it promotes c-Rel binding, it appears to have little impact on the binding of RelA *(31)*. The basis for this difference is unknown. There have been several studies of the proteins that bind to and activate the AP-1 half of the CD28RR but the results

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                    CK-1                 κB        SP1
GM-CSF 5' CAGGAGATTCCACAGTTCAGGTAGTTCCCCCGCCTCCC 3'

                    CK-1       AP1
IL-2   5' AAAGAAATTCCAAAGAGTCATCAGAAGAGGAAAAATGA 3'

                    CK-1
IL-3   5' ATGGAGGTTCCATGTCAGATAAAGATCCTTCCGACGCC 3'

                    CK-1          AP1
IL-4   5' TAAAATTTTCCAATGTAAACTCATTTTCCCTTGGTTTC 3'
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Fig. 2. Sequences of the CD28 response regions (CD28RRs) that have been identified in the promoters of IL-2, GM-CSF, IL-3, and IL-4. The sequences of the human genes are shown. The boxed areas of the sequence represent regions that are known transcription factor-binding sites. The names given to the boxes are those usually used in the literature. The CK-1 element is the conserved region across all the sequences.

are not consistent. It has been shown that combinations of RelA and c-jun as well as c-Rel and ATF-1/CREB2 could transactivate the CD28RR in transient transfection assays *(36,37)*. However, an analysis of the protein complexes binding to the CD28RR in Jurkat T cells or peripheral blood mononuclear cells (PBMCs) has revealed that c-Rel, c-jun, and c-fos are the dominant proteins present in the inducible complexes *(38)*. Neither of these sites functions well alone and there is a strong synergy in the context of the native promoter. The proteins that bind to these sites do not do so cooperatively, suggesting that the synergy is manifested at the level of recruitment of coactivators or the basal transcriptional machinery.

The GM-CSF and IL-3 promoters contain a highly related CD28RE/CK-1 to that found in the IL-2 gene (*see* Fig. 2). The IL-3 CD28RE-like region has not been well-studied, but the GM-CSF CD28RE/CK-1 has very similar properties to that of the IL-2 CD28RE, having a high affinity for the c-Rel transcription factor, binding HMGI(Y), and requiring adjacent sequences for activity *(31)*. However, the GM-CSF CD28RE is not flanked by an AP-1 binding site and requires an NF-κB/Sp-1 region located just downstream for its activity. This entire region is referred to as the CD28RR for GM-CSF. The NF-κB/SP-1 site of GM-CSF contains a classical NF-κB site that binds RelA/p50 heterodimers in response to TCR activation and does not appear to respond directly to CD28 at least in terms of DNA binding. The Sp1 site located immediately adjacent to the NF-κB site also appears to have a func-

tional role in this region. Why two genes which appear to be coordinately regulated by CD28 have developed distinct modules for this response remains to be determined. Many NF-κB sites, or in particular multiple copies of NF-κB sites, can also respond weakly to CD28 signals, e.g., in the human immunodeficiency virus long terminal repeat (HIV LTR) *(39)*. There are also examples of NFAT/AP-1 composite sites that are responsive to CD28 *(40)*. This raises the question of whether there is a specific CD28 responsive element/region or whether any region containing an NF-κB or AP-1 site can respond to CD28. Given the hypothesis outlined earlier, that the role of CD28 may be simply to augment the TCR signal then the latter may in fact be true. On the other hand, the IL-2 gene in particular may have developed a specialized CD28RR that enables the promoter to sense the CD28 increase in TCR signal, in a very precise manner. Thus combining a low-affinity, specialized NF-κB site with a low-affinity AP-1 site may have generated an element that requires both cognate transcription factors to reach a specific functional threshold. This threshold would only be reached in the appropriate costimulatory environment. It is interesting to note that the G-CSF gene, which is not expressed in T cells, has utilized a highly related CD28RE-like sequence to regulate its response to TNF-α and IL-1 in fibroblasts *(41)*. Here the "CD28RE"/CK-1 operates in synergy with adjacent C/EBP binding sites. The IL-8 and I-CAM-1 promoters also respond with a similar combination of sites to inflammatory stimuli, raising the possibility of a CK-1/C/EBP module as a general mechanism of response to inflammatory stimuli *(42,43)*. Thus, a nonconsensus NF-κB site that cannot operate alone has been usurped by several different responses required for both innate and adaptive immunity.

The cytokines previously described are those that respond to initial T cell activation in naïve cells. It is important to ask if the effector cytokines produced by Th1 and Th2 cells are directly affected by CD28 activation signals. One could envisage a situation where CD28 is only necessary for the initial steps of naive T cell activation and once proliferation is established by the expression of IL-2 and its high-affinity receptor, the CD28 signal becomes redundant, being superseded by IL-2, IL-4, IL-12, or other cytokine-signaling events. Indeed, restimulation of memory T cells is generally thought to be independent of costimulation. There are no clearly defined CD28RR regions in any of the effector cytokine gene promoters. A recent report, however, showed that IL-4 did respond to CD28 activation in Jurkat T cells and that this was manifested at the level of promoter activity *(44)*. A region of the promoter known as P1 was identified as a CD28RR and was shown to bind c-Rel and AP-1 proteins (*see* Fig. 2) *(44)*. Compared

to the level of CD28 response reported for the IL-2 CD28RR, the P1 region of IL-4 responded only weakly to the CD28 signal. Rooney et al. *(40)*, have also described the CD28 activation of an NFAT/AP-1 region in the IL-4 promoter as described earlier. There is no direct evidence on whether CD28 directly affects the activity of other Th2 cytokines such as IL-5, IL-13, or IFN-γ in Th1 cells and this area requires further investigation before any useful conclusion can be drawn. There is, however, experimental evidence that CD28 signaling is necessary for the development of the Th1/Th2 phenotypes in animal models. It has been shown that CD28 is necessary for the development of Th2-mediated, allergic airway responses in mice and that blocking B7.2:CD28 interactions reduced allergen-specific responses in T cells from atopic asthmatics *(45–47)*. The development of autoreactive effector cells in models of autoimmunity has also been shown to respond to CD28 blockade or absence (reviewed in ref. *5*). These effects, however, may not be direct and are consistent with the hypothesis that the major role of CD28 is in the initial production of IL-2 and the priming of T cells for proliferation and differentiation.

4. THE ROLE OF ARCHITECTURAL TRANSCRIPTION FACTORS IN CYTOKINE GENE TRANSCRIPTION

As discussed previously, the promoter/enhancer regions of cytokine genes assemble a complex array of transcription factors in a structure referred to as an enhanceosome. The architecture of this complex depends on DNA structure, protein:protein as well as protein:DNA interactions. The HMGI(Y) family of architectural transcription factors appear to play an important role in the assembly and stability of these complexes (reviewed in ref. *48*). The HMGI(Y) family consists of three members; HMGI, HMGY, and HMGI-C. HMGI and HMGY are the products of alternately spliced mRNAs from the same gene, whereas HMGI-C is the product of a separate gene. All three proteins are approx 100 amino acids in length and are highly related. Each protein contains three conserved DNA-binding domains known as A/T hooks. The DNA-binding domains recognize the minor groove structure of A/T stretches in DNA and can use each A/T hook to bind to adjacent A/T stretches on DNA. HMGI(Y) proteins have been shown to have many possible modes of action. They can alter DNA structure by modulating the natural bends on DNA. HMGI(Y) proteins have been well-documented to modulate the binding of many transcription factor families to DNA. HMGI(Y) has also been shown to interact directly with many transcription factors such as the ATF bZIP proteins. Thus, HMGI(Y) could have a major role in the assembly of enhanceosome complexes. In addition,

HMGI(Y) has been shown to bind to nucleosomes assembled on DNA in vitro and affect the rotational setting of the nucleosome. It has also been shown to antagonize the repressive effect of histone H1 on transcription in vitro. The highest level of HMGI(Y) proteins appears to be associated with actively dividing cells and abnormal levels have also been found in some tumors. The DNA-binding capacity of HMGI(Y) can be affected by phosphorylation of the protein by cdc2 kinase or caesin kinase II, thus leading to speculation about its role in the cell-cycle and signal transduction.

Recent studies have shown that HMGI(Y) may play a major role in the regulation of T cell cytokines. The IL-2 gene promoter is highly A/T rich and it was not surprising to find that HMGI(Y) had many binding sites across the first 300-bp region of the promoter *(24)*. The most important aspect of this was that the HMGI(Y) binding sites were all located within or close to known transcription factor-binding sites. Subsequent experiments showed that HMGI(Y) could modulate the binding of many of the transcription-factor families that are thought to play an important role in the activity of the IL-2 promoter *(24)*. This can be illustrated by an analysis of the effects of HMGI(Y) on the binding of factors to the IL-2 CD28RR *(24,31)*. The CD28RE/NF-κB binding site can bind c-Rel with high efficiency but only in the presence of HMGI(Y), whereas the binding of RelA to this site is much less dependent on HMGI(Y). The CD28RE of IL-2 or GM-CSF can also bind members of the NFAT family of transcription factors and HMGI(Y) either promotes or inhibits the binding of NFATp depending on the relative ratio of the proteins in the in vitro binding assays. Binding of AP-1 to the adjacent site in IL-2 (which does not have an A/T sequence) is also affected by HMGI(Y). The relevance of these in vitro binding studies is borne out by transfection studies in either T cell lines or primary T cells where depleting the level of HMGI(Y) by antisense expression greatly inhibits IL-2 promoter activity *(24)*. Of even greater significance is the fact that reducing HMGI(Y) levels leads to a decrease in the production of IL-2 from the endogenous gene, which in turn leads to reduced proliferation of the primary T cells *(24)*. On the other hand, overexpression of HMGI(Y) in Jurkat T cells or primary T cells leads to increased expression of IL-2 and thus increased proliferation in the primary cells. It has also been shown that the activity of the IL-2 receptor alpha promoter is dependent on HMGI(Y), leading to the conclusion that HMGI(Y) levels plays an important role in T cell proliferation *(49,50)*. Indeed, the levels of HMGI(Y) in Jurkat T cells are increased by stimulation with mitogenic signals *(24)*.

Other cytokines that are coexpressed with IL-2 such as GM-CSF and IL-3 also seem to be positively regulated by HMGI(Y). On the other hand, it has

been shown that HMGI(Y) plays an inhibitory role in the regulation of IL-4 and this may be owing to the fact that HMGI(Y) can inhibit the binding of NFAT proteins to certain sites on the IL-4 promoter *(51)*. Intriguingly, IL-4 signaling has been shown to lead to phosphorylation of HMGI(Y) on casein kinase II consensus sites at the C-terminus *(52)*. This in turn leads to decreased DNA binding and may, in turn, reduce the inhibitory effect of HMGI(Y) on the binding of certain transcription factors such as NFAT to DNA. It is possible to speculate that the phosphorylation of HMGI(Y) by IL-4 is one mechanism by which IL-4 increases its own expression in Th2 type cells. Whether HMGI(Y) plays a distinct role in Th2 compared to Th1 cells has yet to be determined. Its positive transcription-activation role may be limited to the cytokine genes that are activated immediately following T cell activation becuase increases in HMGI(Y) levels are associated with proliferation.

Another family of architectural transcription factors that may play a role in the regulation of cytokine gene transcription are the Sry-like HMG box factors. This family of proteins is very distinct from the HMGI(Y) proteins discussed earlier. They are a large family of proteins including Sry, the many related Sox proteins, and TCF/LEF proteins and are related by the presence of a so-called HMG box that is required for DNA binding. These proteins can have major effects on DNA structure and generate large bends in DNA, thus affecting the structure of protein:DNA complexes and hence transcription (reviewed in ref. *53*). TCF-1 and LEF-1 are highly related proteins that appear to play a major role in T cell differentiation *(53)*. Their role in differentiation parallels the role of other HMG-box proteins in development of other tissues. They have been shown to affect the transcription of genes such as those encoding CD4 and T cell receptor genes whose expression is important in the differentiation of the T cell repertoire in the thymus. Because the expression of some of the cytokines discussed in this chapter is restricted to T cells, it is possible that factors such as TCF/LEF are involved in their T cell-specific expression rather than inducible expression *per se*. A recent detailed accessibility mapping of a region 600 bp upstream of the IL-2 transcription start site has revealed a region between –350 and –600 that is constitutively accessible to DNase I cleavage in unstimulated as well as activated EL-4 T cells *(54)*. In contrast, the more proximal region between –1 and –350 is only accessible in activated cells. It is possible that the upstream region in some way marks the gene for expression in T cells, whereas the proximal region is required for inducible responses. The complexes that bind to a part of the upstream region have been shown to contain TCF/LEF, Oct proteins, and HMGI(Y) *(54)*. This raises the intriguing possibility that TCF/LEF may be involved in the T cell-specific expression of IL-2.

Such a "marking" phenomenon may occur during differentiation of T cells in the thymus. More investigation is, however, required to determine if this possibility is correct.

5. TRANSCRIPTIONAL REGULATION VIA CHROMATIN REMODELING AND MODIFICATION

Most of the information and models described previously comes from an analysis of cytokine promoter/enhancer regions in transient transfection assays in cell lines in culture. These experiments generally deal with short DNA fragments (hundreds of base pairs) linked to reporter genes and transfected into cells in large numbers (hundreds of copies per cell). The chromatin context of these plasmids has not been considered. Cellular genes are normally incorporated into chromatin and this forms an important level of regulatory control of gene transcription. In general, inactive genes are found in condensed chromatin, containing unmodified histones and densely methylated DNA, whereas acetylated histones and demethylated DNA characterize transcriptionally competent genes.

The structural unit of chromatin is the nucleosome, which is composed of 147 bp of DNA wrapped around a histone octamer (reviewed in ref. *55*). The histone proteins are composed of two domains: a central fold, which contributes to the histone core of the nucleosome and is constrained by the DNA; and a flexible amino terminal histone tail extending out of the core, which contains conserved residues that can be postranscriptionally modified by either acetylation or phosphorylation. The histones can be considered general gene repressors, and can regulate gene accessibility by at least two mechanisms. First, transcription factor-binding sites on DNA positioned within the nucleosome core may be inaccessible, preventing binding of transcription factors to these sites. A first step in "opening" silent loci may therefore involve remodeling of the chromatin so that promoter regions become accessible to transcription factors (reviewed in refs. *56,57*). Multi-subunit ATP-dependent remodeling complexes have been found in yeast (the yeast SWI/SNF complex and RSC complex), Drosophila (NURF, CHRAC, and ACF), and mammalian cells (BRG1 and hBRM-associated complexes), which are able to remodel chromatin by destabilizing the nucleosome or repositioning it on the DNA (reviewed in refs. *58–60*). Second, acetylation of the histone tails may also alter the nucleosomal structure. Acetylation of lysine residues neutrilizes positive charges, decreasing the affinity for DNA, thus altering the nucleosomal conformation and increasing accessibility of DNA elements to transcription factors. The histone tails are also proposed to contribute to the formation of higher order chromatin structures, possibly

through contacts with adjacent nucleosomes. In vitro at least, acetylation of nucleosome arrays can disrupt higher-order chromatin structure, which may increase chromatin accessibility. Recently, a range of proteins have been found to contain histone acetyltransferase (HAT) activity including GCN5, p300/CBP, P/CAF, SRC-1, and TAFII250 or histone deacetylase activity (HDACs), including yeast RPD3 and human HDAC1 (reviewed in ref. *60*). Many of the proteins possessing HAT or HDAC activity are proteins that have previously been identified as having a role in transcription. For example several HATS are known transcriptional coactivators (for example, CBP/p300, ACTR, and SRC-1), whereas HDACs are often components of multiprotein complexes that contain proteins involved in transcriptional repression. Epigenetic modification of DNA by methylation is also a hallmark of silent genes, and it is becoming clear that the demethylation of CpG dinucleotides may be an important step in derepressing silent genes.

Chromatin structure may play a role in the regulation of cytokine gene expression, either in controlling the cell-type-specific expression of certain cytokines or in the induction of cytokine genes following T cell activation. Alterations in chromatin structure are frequently detected on the basis of changes in the accessibility to DNase I. DNase I sites have been mapped for a number of cytokine genes and changes in these sites observed in response to activation. For example, DNase I hypersensitivity mapping has identified inducible DNase I hypersensitive (DH) sites 3-kb upstream of the *GM-CSF* gene and 14-kb upstream of the *IL-3* gene *(61,62)*. The IL-3 DH site is inducible only in T cells, suggesting that this site plays a role in T cell-specific expression of IL-3. The GM-CSF DH site is inducible in all cell types expressing GM-CSF. Both of these inducible DH sites contain binding sites for activation-dependent transcription factors. The IL-3 site contains 4 NFAT sites, one of which overlaps with and cooperates with an Oct binding site. The GM-CSF site contains 3 NFAT binding sites linked to AP-1 sites. Similarly, chromatin remodeling of the *IL-2* gene has been detected upon T cell activation *(54)*. In vivo footprinting with DNaseI or restriction enzyme-accessibility studies have revealed inducible accessibility in the promoter region upon activation of T cells only. The fact that in all three genes these inducible hypersensitive sites only occur in cells expressing the cognate genes suggests a functional role for these regions. DNase I footprinting of a more distal region of the *IL-2* gene revealed constitutive DH sites between –300 and –600 in resting EL-4 T cells, but not non-T cells *(54)*. This region has been proposed to play a role in the T cell specificity of IL-2. Protein-DNA complexes containing the HMG proteins HMGI/Y and LEF-I were found to bind in this region. The presence of LEF-1 is particularly interesting because, LEF-1 is thought to be important in T cell development.

Comparison of the chromatin structure of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 in naive T cells and differentiated Th1 and Th2 cells revealed tissue-specific chromatin remodeling *(63)*. Terminally differentiated Th1 or Th2 cell clones showed marked differences in their chromatin configuration on the IL-4 and INF-γ loci as assessed by DNase I accessibilitiy. In Th2 clones, which express IL-4, the IL-4 locus was found to be accessible, whereas the IFN-γ locus displayed a closed configuration. The converse was true in Th1 clones. In agreement with this, naive T cells did not display accessible configurations of either gene, but upon differentiation to either Th1 or Th2 acquired IFN-γ or IL-4 accessible patterns, respectively. Therefore, the differentiation of T cells was associated with the remodeling of genes that confer the effector phenotype of the differentiated cells. In support of this, a second study has shown that three DH sites, occur in the intergenic region between IL-4 and IL-13 and although one of these sites appears in both Th1 and Th2 cells, as well as $CD4^+$ naive T cells, the other two sites appear exclusively in differentiated Th2 cells *(64)*.

These studies then demonstrate that chromatin is disrupted upon activation of T cells, and that the chromatin configuration of a cytokine locus may be involved in regulation of cell-type specificity. These chromatin-remodeling studies suggest that nucleosomes positioned across the cytokine genes in naive cells are remodeled upon activation (e.g., IL-2, GM-CSF, and IL-3) or Th-cell differentiation (e.g., IFN-γ and IL-4). There have been as yet no direct studies of nucleosome positioning on T cell cytokines. A recent study of the IL-12 p40 promoter in macrophages, however, presents a model that may be applicable to many inducible cytokine genes. High-resolution micrococcal-nuclease analysis showed that a positioned nucleosome spans the IL-12 p40 promoter, followed by a linker region and three nucleosomes positioned further upstream *(65)*. Upon activation of macrophages with lipopolysaccharide (LPS) or heat-killed Listeria monocytogenes (HKLM) the promoter positioned nucleosome was selectively remodeled, but the upstream nucleosomes remained in position. Although such studies have not been done in T cells, this study supports the notion that chromatin remodelling contributes to the rapid induction of cytokine genes, by increasing promoter accessibility. Other studies also support the idea that nucleosome remodeling is important in inducible gene transcription. For example, a link between T cell activation and chromatin remodeling has been indicated by studies showing that upon activation, the chromatin-remodeling Swi/Snf complex becomes stably associated with chromatin *(66)*. Similarly a link between chromatin modification and inducible gene expression has been shown by studies of the *IFN*-β gene in which the histones associated with the gene become hyperacetylated upon viral induction in HeLa cells *(67)*.

A link between CpG methylation of mammalian DNA and transcriptional silencing of genes has been proposed for many years, although the methods by which methylation represses transcription are not completely understood. Methylation of CpG dinucleotides may act directly to inhibit binding of transcription factors or the transcription machinery to DNA. Alternatively, methylation may influence the chromatin structure (reviewed in refs. *68–70*). There is now evidence that CpG methylation stimulates histone deacetylation, because complexes have been found that contain methyl-binding proteins and histone deactylases. The model suggested is that the methyl-binding protein MeCP2 recruits the corepressor mSin3A and the deacetylases HDAC1 and HDAC2 forming a repressor complex on methylated DNA that mediates histone deacetylation. Although control of cytokine gene transcription by methylation has not been widely studied, there is growing evidence that it is an important control mechanism in the generation and maintainence of Th1 and Th2 specific gene expression. Demethylation has been correlated with the activity of *IL-3*, *IL-4*, *IL-5*, and *IFN*-γ genes.

The most studied cytokine in this respect is INF-γ. The 108 base pair IFN-γ proximal promoter contains two important regulatory control elements, and the most proximal of these sites is able to reproduce the PMA/ionophore inducible, cyclosporin-sensitive expression of IFN-γ and also appears critical for the Th1-specific expression profile of IFN-γ *(71)*. A CpG dinucleotide within this element is methylated in naive T cells and Th2 clones, but becomes demethylated during Th1 differentiation. Methylation at this site correlates with the inability of naive T cells and Th2 clones to express IFN-γ *(72)*. A further study extended this finding, showing that thymocytes, neonatal T cells, and naive T cells are hypermethylated at this proximal site, whereas adult $CD8^+$ T cells and Th2 cells are hypomethylated *(73)*. This regulatory element has been shown to bind the transcription factors CREB, ATF-2, Jun, and electrophoretic mobility shift assay (EMSA) using oligonucleotides incorporating a methylated CpG dinucleotide at the critical position demonstrated reduced binding of all these factors, although the effect was more pronounced for CREB *(71)*. Although the above data was generated using methyl-sensitive restriction enzyme, bisulfite genomic sequencing of CpG dinucleotides across the IFN-γ promoter has confirmed the link between demethylation of the IFN-γ promoter and gene expression *(74)*. This study also showed that the ability of cells to produce IL-3 can similarly be linked to demethylation of the gene. CpG dinucleotides within the IL-3 promoter are mostly associated with transcription factor-binding sites, and therefore methylation may also affect transcription factor binding to this promoter. A further study has shown that in Th2 cells the IL-4

and IL-13 genes are demethylated. Although in naive T cells, the IL-4 and IL-13 genes are hypermethylated, the differentiation to Th2 cells is characterized by both chromatin remodeling and demethylation of the IL-4/IL-13 locus *(63)*. Therefore, the derepression of silent cytokine genes probably involves the coordinate demethylation and remodeling of the chromatin. This supports the model referred to earlier in which methylation and histone deacetylation are linked through the action of multiprotein complexes.

Recently a link between deregulation of cytokine gene transcription and gene methylation has been demonstrated in T cells. It is well-established that HIV or human T leukemia virus (HTLV) infection of T cells results in disregulated production of cytokines. Mikovits et al. *(75)* have shown that acute infection of T cells with HIV results in upregulation of DNA methyltransferase mRNA expression and activity. They established that this correlated with a general increase in genome methylation, but in particular it resulted in altered methylation of the IFN-γ gene in Th1 cells, via *de novo* methylation at the site that was shown in the aforementioned studies to be critical for transcriptional regulation of this gene. This correlated with decreased IFN-γ mRNA and protein expression.

Finally, it is well-established that differentiated effector Th cells respond far more rapidly and with much higher cytokine production than naive T cells. Although cytokine expression is only transient following initial stimulation, the cells can essentially remember this expression pattern, which is reflected later by the same and more rapid cytokine production following restimulation. This may well be explained in large part by the fact that the chromatin is remodeled and demethylated upon differentiation and that this state is then maintained stably. For example, analysis of the IFN-γ promoter through eight generations has shown that the methylation patterns, once established, can be faithfully inherited in the absence of stimuli *(74)*.

There is now also evidence appearing that links the differentiation of Th cells with the cell cycle. Bird et al. *(76)* have demonstrated that although IL-2 expression is independent of cell cycle, cells must enter S-phase of the first cell cycle after activation before they are able to express IFN-γ and must undergo at least three cell cycles before they express IL-4. Two further studies support this idea, demonstrating that, like IL-4 and IFN-γ, the expression of IL-3, IL-5, and IL-10 are also linked to the cell cycle *(77,78)*. DNA synthesis has long been proposed as an opportunity for remodeling of a gene from an inactive to an active state. DNA synthesis during S phase may then correlate with epigenetic modification of the DNA. Modifications such as acetylation or demethylation may occur following new DNA synthesis, which is carried through to the next cell generation. These studies speculate that one mechanism by which cytokine expression is repressed in naive T

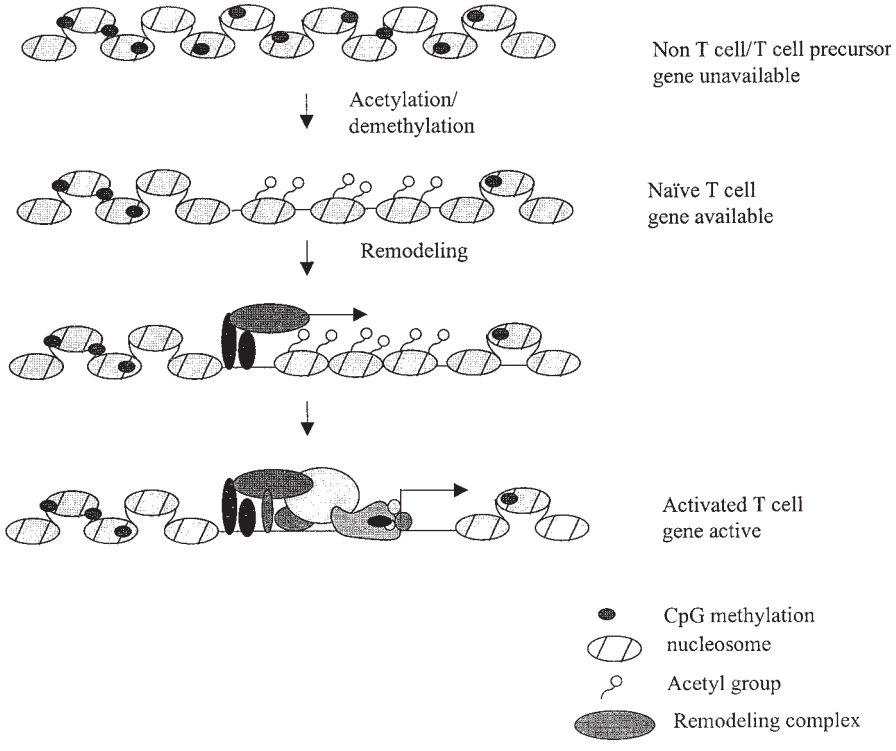

Fig. 3. A hypothetical model of the steps involved in the activation of a cytokine gene in T cells. In a non-T cell or precursor T cell, a cytokine gene may be in inactive chromatin characterized by unacetylated histones and DNA methylation at CpG dinucleotides. The development of a T cell may involve acetylation and/or demethylation of the genes that will respond to T cell activation generating an "available" chromatin configuration in a naive T cell. Similar modifications may occur for Th1 and Th2 expressed cytokines during Th-cell differentiation. Upon T cell activation, transcription factors bind to the promoter/enhancer regions of the gene, recruit chromatin remodeling complexes, and, in turn, allow the formation of an active enhanceosome.

cells is owing to methylation of the genes, and that progression through the cell cycle provides an opportunity for demethylation of these genes to occur.

It is clear that chromatin remodeling and modification is important in the regulation of cytokine genes. It has been shown that the inability of naive T cells to express certain cytokines may be owing to repressive chromatin effects. The differentiation to Th1 or Th2 subtypes involves chromatin remodeling and demethylation of, for example, the *IFN*-γ and *IL-4* genes,

respectively, and once differentiated the open or active chromatin states are stably maintained and inherited, providing a cellular memory. Upon T cell activation, chromatin remodeling occurs, increasing promoter accessibility and allowing rapid induction of cytokine genes. A possible model for these events is described in Fig. 3.

6. CONCLUSION

We have previously described many of the important principles underlying inducible cytokine gene transcription in T cells. It is now necessary to integrate this information into a model that considers the chromatin context of the genes. It will be important to map precisely the nucleosome positions across the control regions of the cytokine genes and to monitor the changes that occur in response to activation. It will also be necessary to reproduce this chromatin structure in vitro in order to understand the molecular mechanisms that govern chromatin remodeling at the cytokine loci. Finally, transgenic-mouse models in which transcription can be analyzed in the context of chromatin in normal T cells will be important. Given the enormous recent development in regard to chromatin remodeling and its relationship to gene transcription, these goals should be achievable.

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