REGULATION OF LYMPHOKINE GENE EXPRESSION IN MURINE T LYMPHOCYTES

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STATEMENT

The research described in this thesis was solely and entirely conducted by the author unless acknowledgement is made in the text. It has not been presented for any other degree.

Honami Naora

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I am indebted to two wonderful parents for their patience, support and fortitude.

ABBREVIATIONS

bp	base pairs
CSF	colony-stimulating factor
DNase I	deoxyribonuclease I
IL	interleukin
kb	kilobase pairs
Th	helper T cell
Tris	tris(hydroxymethyl)aminomethane
RNase A	ribonuclease A

Other abbreviations used are defined in the text.

ABSTRACT

Given the critical role of lymphokines as transient signals which mediate the proliferation, differentiation and function of haemopoietic cells in response to immunological stress, the regulation of the temporal expression of their genes by activated T lymphocytes is central to the coordination of immune responses to provide a "balanced" network of mediators, without chronic and potentially deleterious stimulation of specific target cells.

This thesis describes studies which investigate and compare the mechanisms which regulate the expression of the IL-5 gene and a variety of other different lymphokine genes in murine T lymphocytes. Lymphokine mRNA accumulation patterns were compared in the murine helper T clone D10.G4.1 in response to cellular stimulation by various agents which either activate or inhibit known specific steps in several key T cell activation pathways. Northern blot analysis suggests that the accumulation of the IL-3, IL-4 and GM-CSF mRNAs are predominantly regulated by a cyclosporin A (Cs A)-sensitive Ca^{2+}-dependent pathway emanating from T cell receptor (TCR)/CD3 stimulation. The accumulation of the IL-5, IL-6 and IL-10 mRNAs appear, in contrast, to be largely regulated by Cs A-resistant signalling pathways. Signals mediated by protein kinase C activation may selectively and independently regulate IL-6 and IL-10 mRNA accumulation, and appear to predominantly regulate the accumulation of IL-5 mRNA. The accumulation of the IL-5, IL-6 and IL-10 mRNAs appear to be additionally regulated by mechanisms independent of direct TCR/CD3 stimulation. These include signalling pathways emanating from the CD45 and CD2 cell surface molecules, the IL-1 receptor and adenylate cyclase activation. These studies suggest that the expression of a number of lymphokine genes may be differentially regulated by intracellular pathways emanating from distinct T cell surface events.

Studies conducted in D10.G4.1 cells and also in the transformed murine T cell line EL4.23 suggest that the accumulation of each different lymphokine mRNA species is dynamically and differentially regulated by distinct and specific sets of transcriptional and post-transcriptional control mechanisms. Although no lymphokine mRNAs are detectable in unstimulated T cells by Northern blot analysis, nuclear run-off analysis indicated that the IL-6, IL-10 and GM-CSF genes are transcribed at low levels, while other lymphokine genes appear transcriptionally inactive in the absence of stimuli. The accumulation patterns of IL-5, IL-6 and IL-10 mRNAs appear to be predominantly regulated by changing rates in transcription of their genes in response to Cs A-resistant signals. IL-4 mRNA accumulation appears to be largely regulated at the transcriptional level by Cs A-sensitive Ca^{2+}-dependent signals. In contrast to the IL-4, IL-6 and IL-10 genes, activation of IL-5 gene transcription requires obligatory *de novo* protein synthesis. Transcription of the IL-2 gene appears to involve the coordinate actions of labile Cs A-sensitive activators and labile repressors. The accumulation of IL-3 mRNA, and in particular that of GM-CSF mRNA, appear to largely involve their enhanced stabilization by Cs A-sensitive Ca^{2+}-dependent signals. The accumulation of these two species and those of IL-2, IL-6 and IL-10 mRNAs also appear to be dynamically regulated by a constitutively active labile mechanism which mediates their specific degradation.

While the activation of IL-5 gene transcription in response to T cell activation signals was observed by nuclear run-off analysis, such responsiveness was not observed in transcriptionally competent cell-free systems. This suggests that IL-5 gene transcription is dynamically regulated by higher order chromatin structures. DNase I hypersensitivity analysis was conducted to identify the locations of putative regulatory regions of the IL-5 gene. The region surrounding a hypersensitive site induced just 5' of the

TATA box of the IL-5 gene was identified by subsequent DNase I footprinting and gel shift analyses to contain two discrete elements (mIL-5A and mIL-5B) which independently bind distinct inducible nuclear proteins. The strong correlation between the cell-specificity and signal-dependent modulation of the transcriptional activity of the IL-5 gene, and of protein interactions with these two elements suggests that they may be involved in regulating IL-5 gene transcription. Sequences of mIL-5B appear unique to the IL-5 gene, while sequences of mIL-5A are highly conserved in corresponding locations in the GM-CSF and IL-4 genes. Closely related, but possibly not identical, inducible nuclear proteins appear to interact with the conserved elements of these three genes, and may function to cooperatively link the core promoter machinery with other regulatory elements further upstream.

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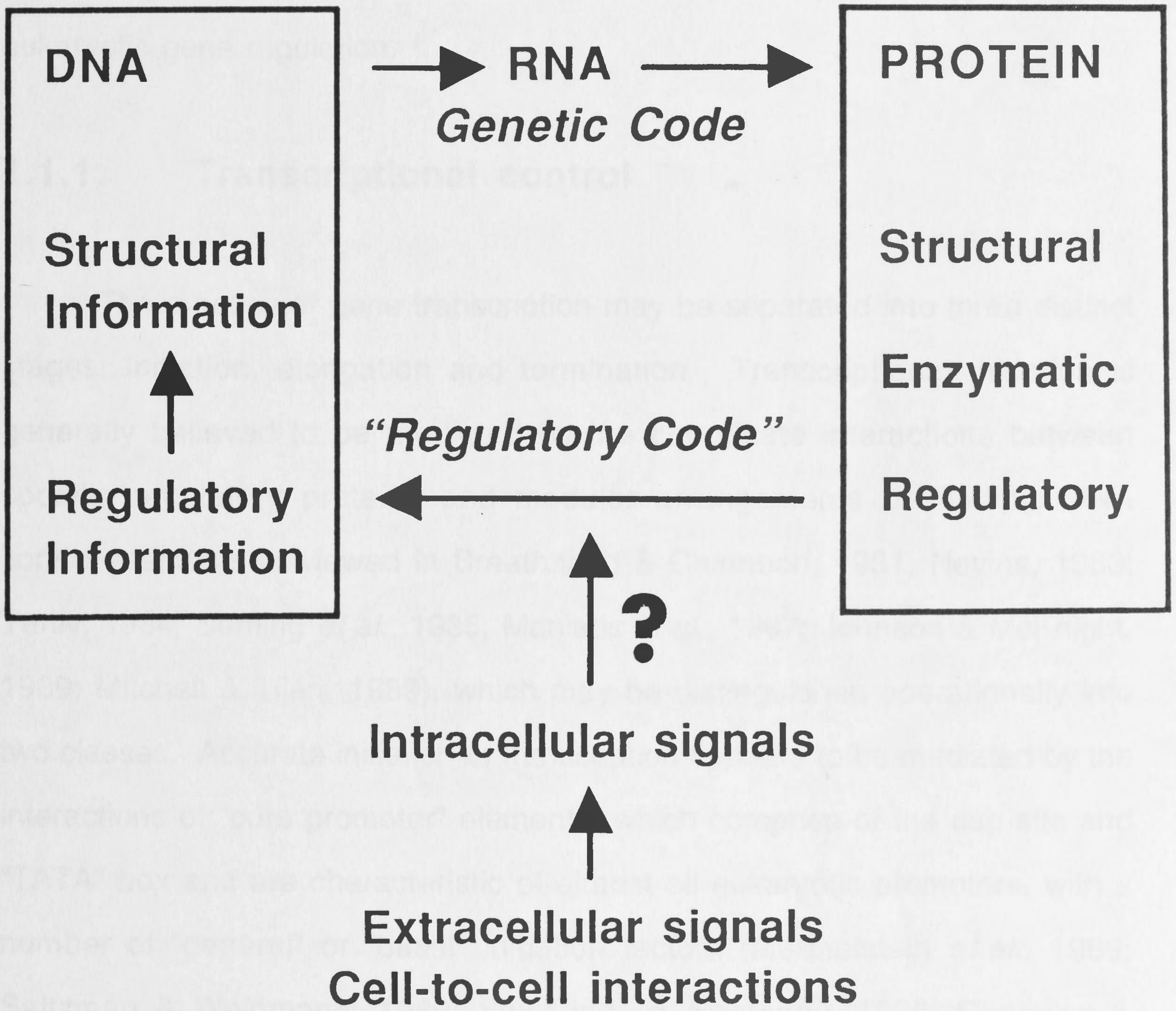
CHAPTER 1: INTRODUCTION

The sequential patterns of cell growth and differentiation, which ultimately manifest in Metazoan form, may be essentially ascribed to the coordinated expression of genetic information in both spatial and temporal dimensions, and its interaction with information external to the genomic micro-environment. This involves a dynamic hierarchy of integrated regulatory mechanisms which, at one level, control expression of any single gene and at another, coordinately modulate expression of networks of genes.

In addition to the structural information encoding functional gene products, nucleic acids contain a multiplicity of regulatory information which determine the parameters within which the structural information is expressed. It is this molecular "program" of regulatory information that converts the linear array of genetic messages contained in the DNA into the three-dimensional Metazoan form. As communication of the regulatory information involves specific proteins which are themselves encoded in the structural information, the whole process of cell growth and differentiation may be envisaged as a self-controlling morphogenetic "information cycle" which, as will be elaborated upon later, is dynamically influenced by the cellular environment and inter-cellular communication (Fig. 1.1.).

While the early period of molecular biology has largely focused upon elucidating the genetic code which deciphers the structural information, a fundamental objective in current molecular biology is the elucidation of the putative "regulatory code", namely, mechanisms which decipher and implement the regulatory information.

Fig. 1.1. Schematic representation of the "information cycle" illustrating the nexus between structural and regulatory information encoded in the DNA and information external to the genomic micro-environment.


DNA
RNA
PROTEIN
Genetic Code
Structural
Information
Regulatory
Information
"Regulatory Code"
Structural
Enzymatic
Regulatory
?
Intracellular signals
Extracellular signals
Cell-to-cell interactions

1.1. REGULATION OF EUKARYOTIC GENE EXPRESSION

Eukaryotic gene expression may be regulated at several levels including organisation of chromatin structure and transcription, processing and export of mRNA from the nucleus, translation and post-translational processing. Of these, transcriptional control appears to be a major mode of eukaryotic gene regulation.

1.1.1. Transcriptional control

The process of gene transcription may be separated into three distinct stages: initiation, elongation and termination. Transcriptional initiation is generally believed to be mediated by the coordinate interactions between specific regulatory proteins and modular arrangements of multiple DNA control elements (reviewed in Breathnach & Chambon, 1981; Nevins, 1983; Yaniv, 1984; Serfling *et al.,* 1985; Maniatis *et al.,* 1987; Johnson & McKnight, 1989; Mitchell & Tijan, 1989), which may be distinguished operationally into two classes. Accurate initiation of transcription appears to be mediated by the interactions of "core promoter" elements, which comprise of the cap site and "TATA" box and are characteristic of almost all eukaryotic promoters, with a number of "general" or "basal" initiation factors (Mermelstein *et al.,* 1989; Saltzman & Weinmann, 1989; Sawadogo & Sentenac, 1990; Conaway & Conaway, 1991). Selective modulation of the rate or efficiency of transcription appears, however, to be mediated by the interactions of sequence-specific regulatory factors with upstream promoter elements and enhancers, the latter which are characterized by their ability to modulate transcriptional activity in an orientation- and distance- independent manner relative to the "TATA" box (Khoury & Gruss, 1983; Serfling *et al.,* 1985; Atchison, 1988; Muller *et al.,* 1988). The activity and/or expression of many of these specific *trans-*

activating regulatory factors, as will be subsequently discussed, appear to be differentially modulated in specific cell types and/or in response to different developmental or environmental signals.

Transcriptional "programs" for modulating inducible, spatial or temporal expression patterns of individual, or subsets of, eukaryotic genes may therefore be "conferred" by the particular sequence, unique composition and spatial organisation of upstream promoter elements and enhancers constituting the complex set utilized by a given gene. Such a molecular "program" appears to be "implemented" by the interaction of such *cis*-acting elements with sequence-specific *trans*-activating factors. These factors are believed to associate with the general or basal transcriptional machinery at the "core promoter", either directly or via interactions with certain adaptor proteins or "co-activators", to assemble preinitiation complexes (Ptashne, 1986; Atchison, 1988; Muller *et al.,* 1988; Marriott & Brady, 1989; Saltzman & Weinmann, 1989; Sawadogo & Sentenac, 1990; Conaway & Conaway, 1991; Roeder, 1991). Under this model, transcriptional initiation is a complex mechanism which requires both specific DNA-protein and protein-protein interactions. This model has been supported by a number of structure/function "domain-swapping" studies which demonstrate (i) that DNA-binding is essential but alone insufficient to mediate transcriptional activation and (ii) that DNA-binding and activation properties of a transcription factor reside independently in spatially and functionally distinct domains (Hope & Struhl, 1986; Keegan *et al.,* 1986). These domains often contain distinct structural motifs which have been found to be common in many eukaryotic transcription factors (reviewed in Ptashne, 1988; Brennan & Matthews, 1989; Struhl, 1989; Berg, 1990; Harrison & Aggarwal, 1990; Latchman, 1990).

Transcriptional regulation of eukaryotic gene expression may also be mediated at the level of elongation. One well-characterized example is the rapid down-regulation of c-*myc* mRNA biogenesis during differentiation of promyelocytic leukemia cells which appears to be mediated by mechanisms that prematurely attenuate transcription within the first exon of the c-*myc* gene (Bentley & Groudine, 1986;1988; Eick & Bornkamm, 1986).

1.1.2. Chromatin structure and DNA methylation

The coordinated interactions between *cis*-acting elements and *trans*-activating factors which mediate transcriptional initiation may be modulated by the synthesis and/or activity of the sequence-specific transcription factors (discussed in section 1.1.4.). A large body of structural and functional evidence over the past decade suggests, however, that the transcriptional regulation of gene expression may ultimately be controlled by the packaging of DNA with histones into nucleosomes and higher order chromatin structures, an intrinsically dynamic phenomenon specific to eukaryotes (reviewed in Reeves, 1984; Eissenberg *et al.,* 1985; Pederson *et al.,* 1986; Hansen & Ausio, 1992). Studies using *in vitro* chromatin assembly systems have demonstrated that assembly of nucleosomes over promoter regions may render a DNA template refractory to transcription (Knezetic & Luse, 1986), while formation of transcriptional preinitiation complexes on DNA templates prior to or during nucleosome assembly may alleviate promoter repression by nucleosomes (Matsui, 1987; Workman & Roeder, 1987). Depletion of nucleosomes has also been shown *in vivo* to activate normally repressed promoters (Han & Grunstein, 1988), suggesting that histones may play a fundamental role in the repression of basal level transcription.

The high degree of sensitivity of transcriptionally active chromatin to non-specific nucleases such as DNase I has been generally attributed to local displacement of nucleosomes (reviewed in Eissenberg *et al.,* 1985; Yaniv & Cereghini, 1986; Elgin, 1988; Gross & Garrard, 1988; Grunstein, 1990). Such "hypersensitive" sites are often induced in response to specific signals or in specific tissues, and are frequently associated with protein-binding regulatory regions of genes which are responsive to such spatial, environmental or developmental cues (Wu, 1980; Zaret & Yamamoto, 1984; Siebenlist *et al.,* 1986). Such strong correlation between transcriptional activity and local disruptions in chromatin structure has supported the notion that chromatin structure is dynamic and may play a fundamental role in regulating the accessibility of *cis*-acting elements to *trans*-activating factors. While the underlying mechanisms of active chromatin structure are as yet unclear, one model suggests that certain regulatory factors may activate repressed promoters by directly disrupting existing chromatin structure (Workman & Roeder, 1987; Grunstein, 1990; Pham *et al.,* 1991). Given, however, that alterations in chromatin structure may be a requirement for, as well as a consequence of, the process of establishing transcriptional competence, the relationship between these two inextricably linked events may not necessarily be a simple issue of cause and effect.

While a direct correlation between transcriptional activity and nuclease hypersensitivity has been demonstrated, an inverse correlation has been shown between transcriptional activity and the presence of methylated cytosines in CpG dinucleotides in regulatory regions of higher eukaryotic DNA. Methylation may, furthermore, suppress formation of nuclease hypersensitive sites, while hypomethylation appears to create a permissive environment for generating hypersensitivity (reviewed in Doerfler, 1983; Bird, 1984; Gross & Garrard, 1988; Dynan, 1989; Lewis & Bird, 1991). Whether

under-methylation is a cause or consequence of transcriptional activation is a subject of some dispute. Given that DNA methylation potentially disrupts DNA-protein interactions, methylation may be an integral component of a general gene silencing system which could influence the linear and spatial organisation of chromatin (Cedar, 1988; Watt & Molloy, 1988).

1.1.3. Post-transcriptional and translational regulation

An inherent consequence of two fundamental differences that exist between the organisation of prokaryotic and eukaryotic genetic information is increased complexity in the modes by which eukaryotic gene expression is regulated. The first difference is the "split" organisation of the majority of eukaryotic genes into exon and intron regions, although a few prokaryotic genes appear to possess a similar organisation (Belfort, 1989). The second difference is the segregation of eukaryotic genetic material in the nucleus and consequent spatial separation of transcription from translation. The post-transcriptional processing of the primary transcript, which involves stability of the intranuclear RNA precursor, splicing of introns, 5' capping, 3' polyadenylation and the subsequent transport of the mature transcript into the cytoplasm, potentiate distinct levels at which eukaryotic gene expression may be regulated (reviewed in Breathnach & Chambon, 1981; Nevins, 1983). Flexibility in the selection of splice sites forms the basis for alternative splicing of the primary transcript and allows plasticity of gene expression without affecting the transcriptional event. Different mRNA species and hence related, but structurally and functionally distinct protein isoforms, may be generated in different tissues and/or in response to different developmental and environmental signals (reviewed in Breitbart *et al.*, 1987; Smith *et al.*, 1989).

Eukaryotic mRNAs display heterogenous cytoplasmic stability in a wide variety of cell types and also in response to different signals. One example of a relatively stable species is β-globin mRNA which has a half-life of greater than 17 h (Lowenhaupt & Lingrel, 1978). Many inducible eukaryotic genes associated with regulation of cell growth and differentiation, including oncogenes (Dani *et al.,* 1984; Mitchell *et al.,* 1985; Rabbitts *et al.,* 1985) and cytokines (Raj & Pitha, 1981; Lindsten *et al.,* 1989; Yamato *et al.,* 1989), encode mRNAs with short half-lives of between 0.5 to 2 h, which may be modulated in response to specific cellular signals in specific cell types. The rates and selectivity of mRNA degradation are therefore variable and regulation of these processes potentiate control points of gene expression (reviewed in Brawerman, 1987;1989; Raghow, 1987; Hentze, 1991).

Poly(A) addition to non-adenylated mRNAs and poly(A) elongation of adenylated mRNAs have been shown to enhance stabilization of these mRNAs, suggesting that the rate of mRNA turnover may be influenced by poly(A) metabolism (Green & Dove, 1988; Zingg *et al.,* 1988). The poly(A) tail, the degradation of which appears to precede that of the mRNA body (Brewer & Ross, 1988; Wilson & Treisman, 1988; Swartwout & Kinniburgh, 1989), appears to be protected from nuclease attack by virtue of its association with a specific poly(A) binding protein (PABP) into a nucleosome-like ribonucleoprotein complex (Baer & Kornberg, 1983; Bernstein & Ross, 1989; Bernstein *et al.,* 1989). The association of poly(A) tails of most mRNAs with PABP, whose structure is highly conserved during evolution (Adam *et al.,* 1986; Sachs *et al.,* 1986; Grange *et al.,* 1987) is, however, insufficient to explain the heterogeneity of cytoplasmic mRNA stability. The selectivity of mRNA degradation has been postulated to be mediated by interactions between certain sequences, often found in the 3'-untranslated regions (3'-UTRs) of many short-lived mRNAs, with specific cytoplasmic factors. These

interactions are believed to destabilize the association of PABP with the poly(A) tail, thus enhancing degradation of the poly(A) tail and ultimately the mRNA body. (Bernstein & Ross, 1989; Brewer & Ross, 1989; Bohjanen *et al.*, 1991; Hentze, 1991).

Control at the translational level may be functions of the efficiency of ribosome binding and of interactions between ribosomes, initiation factors and tRNA. Several recent lines of evidence implicate a regulatory role of certain 3'-untranslated sequences in the initiation of translational activation (Ch'ng *et al.*, 1990; Jackson & Standart, 1990). Post-translational modification potentiates the generation of related, but structurally and functionally distinct, proteins without affecting the translational event, and plays an important role in modulating the activity of transcription factors as discussed below.

1.1.4. Regulation of the "regulators"

The mediation of highly regulated spatial, temporal or inducible eukaryotic gene expression by sequence-specific *trans*-activating factors inherently necessitates a second "tier" of regulatory mechanisms to ensure that such factors are active only in the particular tissue and/or become active only in response to the particular developmental or environmental signal. Observations that transcriptional activation of certain genes requires *de novo* protein synthesis has suggested two possible mechanisms by which such regulatory factors may themselves be regulated, namely, (i) control of the synthesis of the factor so that it is expressed only when required and (ii) control of the activity so that a pre-existing factor becomes activated only when required (reviewed in Berk, 1989; Johnson & McKnight, 1989; Mitchell & Tijan, 1989).

Control of the synthesis of a transcription factor may be mediated by regulating transcription of the gene encoding the factor. Expression of the gene encoding the liver-specific factor C/EBP, for example, appears to be regulated by tissue-specific transcriptional control mechanisms (Xanthopoulos *et al.,* 1989;1991). Control of synthesis may also be exerted at the level of RNA splicing. One example is the thyroid hormone gene, where alternative splicing yield two species, one of which lacks the hormone-binding domain. This inactive form acts as a repressor which binds to the same DNA motif as the active repressor but cannot activate transcription in response to hormone (Koenig *et al.,* 1989). Translational efficiency of mRNA encoding the yeast factor GCN4 may be modulated by differential usage of translational initiation codons in response to specific signals (Fink, 1986).

Two well-characterized post-translational mechanisms which modulate the activity of pre-existing regulatory factors include phosphorylation and ligand-induced conformational changes. Interconversion of regulatory proteins between phosphorylated and dephosphorylated forms is a major versatile mechanism for reversibly modulating their activities in response to alterations in cellular biochemistry, and is due to a dynamic equilibrium resulting from the actions of kinases and phosphatases which often demonstrate pleiotropy and broad specificity (reviewed in Cohen, 1985). Phosphorylation appears, for example, to stimulate the DNA-binding ability of serum response factor (Prywes *et al.,* 1988) and the transcriptional activation potential of the cAMP-responsive element binding protein (CREB) (Yamamoto *et al.,* 1988).

Several transcription factors appear to be associated in the cytoplasm with specific inhibitory proteins which dissociate from the factor in response to particular signals. This dissociation induces a conformational change which

converts the factor into an active form. Addition of hormone, for example, releases certain steroid receptors from specific inhibitory proteins, enabling the receptors to bind DNA and activate transcription (Sanchez *et al.,* 1987; Howard & Distelhorst, 1988). Other examples include the "unmasking" of the activation domain of the yeast factor GAL4 by dissociation of an inhibitory factor in response to galactose (Johnston *et al.,* 1987) and the "unmasking" of the nuclear translocation signal of the glucocorticoid receptor by dissociation of an inhibitory factor in response to hormone (Picard & Yamamoto, 1987). The complex mechanisms which modulate the activity of the pleiotropic factor NF-κB, which is involved in regulating inducible expression of a variety of viral and cellular genes (reviewed in Lenardo & Baltimore, 1989), have been extensively investigated over recent years. NF-κB appears present in an inactive form in the cytoplasm, associated with an inhibitory protein, IκB (Baeuerle & Baltimore, 1988a,b). Following cellular activation with a variety of stimuli, including mitogens, cytokines, viral *trans*-activators or double-stranded RNA (Sen & Baltimore, 1986, Lowenthal *et al.,* 1989; Osborn *et al.,* 1989; Visvanathan & Goodbourn, 1989), the inhibitory IκB protein becomes phosphorylated and dissociates from NF-κB. This enables the latter to translocate to the nucleus where it interacts with its DNA recognition sequence to mediate gene transcription (Baeuerle & Baltimore, 1988a,b; Ghosh & Baltimore, 1990).

1.1.5. Integration of gene regulation and the "macro-environment"

The genomic DNA sequence plays a vital determinative role in the regulation of eukaryotic gene expression. All levels of control described above involve specific nucleotide sequences, either 5' and/or 3' to (and sometimes within) a gene (transcriptional control), at boundaries of introns (splicing) or at specific sites within the mature transcript (mRNA stability and

translational control). The regulatory functions of these specific nucleotide sequences in the expression of a given gene product may be mediated by other gene products, for example, transcription factors, initiation and elongation factors, small ribonucleoprotein particles, poly(A) binding protein or specific mRNA stabilizing/destabilizing factors. The expression of any given group of genes, and alterations in cellular biochemistry that result, are thus dependent on prior expression of other groups of genes. The relationship between structural and regulatory information depicted earlier in the "information cycle" (Fig. 1.1.), is therefore not only dynamic, but also hierarchical.

While the sequential patterns of cell growth and differentiation may be viewed quite validly, at the molecular level, in terms of a genetic program, such patterns may ultimately be influenced by interactions between the genetic information and the outer environment. The mediation of spatial, temporal or inducible gene expression, which give rise to changing patterns of cell growth and differentiation and morphogenesis, inherently involves (i) the recognition of an external cue at the cell surface, which may include those generated as a consequence of cell-to-cell interactions, (ii) transduction of this signal into the cytoplasm and subsequently to the nucleus, and (iii) modulation of gene expression in response to the transduced signal. The process of cell growth and differentiation may be, therefore, more broadly (and correctly) envisaged as involving not only a dynamic hierarchy of interactions between structural and regulatory infomation, but also dynamic interactions between regulatory information and information external to the genomic micro-environment (Fig. 1.1.).

1.2. REGULATION OF THE "CYTOKINE NETWORK'

Investigating the dynamic molecular mechanisms which integrate "inter-", "extra-" and "intra-" cellular communication with the genetic programs which control the plasticity and specificity of eukaryotic cellular growth and differentiation requires a model experimental system which is amenable to genetic analysis, which demonstrates sufficient, but not overwhelming, complexity of gene expression, and for which variations in gene expression may be assayed. In this regard, the T lymphocyte, which expresses a battery of transient mediators of haemopoietic cell proliferation, differentiation and function in response to antigen, presents a challenging, yet ideal, multi-dimensional model system.

1.2.1. Cytokines as pleiotropic mediators of haemopoiesis

Blood cell growth and differentiation is a dynamic process which is maintained continuously in adults by the self-renewal of pluripotent stem cells and their subsequent proliferation and differentiation through the various myeloid and lymphoid cell lineages. Such "constitutive" haemopoiesis appears to be governed by a developmental program that proceeds in the absence of immunological stress in the bone marrow micro-environment, and may be differentiated, both temporally and spatially, from haemopoiesis induced in response to cellular recognition of antigen. The proliferation and differentiation of all blood cell types involved in "inducible" haemopoiesis is regulated by numerous soluble glycoprotein factors, collectively termed cytokines. These include colony-stimulating factors (CSFs), interleukins (ILs), interferons (IFNs), tumour necrosis factor (TNF) and transforming growth factor (TGF). A number of these factors also play a major role in regulating "constitutive" haemopoiesis, along with complex cell-cell interactions

(reviewed in Clark & Kamen, 1987; Balkwill & Burke, 1989; Nicola, 1989; Arai *et al.*, 1990; Whetton, 1990; Metcalf, 1992).

Cytokines are encoded by single copy genes which demonstrate considerable homology between human and mouse at the nucleotide and amino acid levels. Despite the overall poor homology between different cytokine genes, these genes appear to share similar structural (i.e. exon/intron) organisation, and many appear to be "clustered" within certain chromosomal regions. Such features have given rise to considerable speculation as to their evolution by ancestral gene duplication (reviewed in Arai *et al.*, 1990). The IL-3 and GM-CSF genes, for example, are located only 9 kb and 14 kb apart on human chromosome 5 and mouse chromosome 11 respectively (Yang *et al.*, 1988; Lee & Young, 1989), to which the IL-4, IL-5, M-CSF and M-CSF receptor genes have also been mapped (Le Beau *et al.*, 1986; Lee *et al.*, 1989; Chandrasekharappa *et al.*, 1990).

While the T lymphocyte is a major source of cytokines, other haemopoietic and non-haemopoietic cells, including B cells, macrophages, fibroblasts, mast and endothelial cells also express overlapping, as well as distinct, sets of factors. Apart from the complexity of their expression patterns, cytokines are characterized by the specificity and pleiotropy of their biological actions, for example:

(1) a single cytokine may interact with more than one cell type
(2) a single cell type may interact with more than one cytokine
(3) a single cytokine may possess multiple biological activities
(4) many cytokines possess overlapping biological activities
(5) the biological activity of one cytokine may be modulated by that of another cytokine.

Such complex patterns of cytokine expression and activity have given rise to the notion of haemopoiesis as a dynamic system of "inter-" cellular communication coordinated by an integrated "cytokine network". Regulation of both the activity and expression of this "cytokine network" is therefore critical for the correct implementation of the genetic programs of proliferation and differentiation of target cells. Key determinants of the specificity and pleiotropy of cytokine activity may involve its interaction with specific receptors, which are expressed on the surfaces of target cells, and the nature of the coupling between the receptor and signal transduction pathways of the target cell (reviewed in Nicola, 1989; Arai *et al.,* 1990).

Central to the initiation and coordination of "inducible" haemopoiesis is the activation of quiescent helper T cells by antigen and the consequent expression of a battery of lymphokine genes. These processes must be tightly regulated to provide a balanced network of mediators at the appropriate time, without the chronic and potentially deleterious stimulation of specific target cells which may contribute to the development of numerous disorders. Several lines of clinical evidence, for example, have suggested a link between the enhanced expression of the gene encoding IL-5, a lymphokine which stimulates growth, differentiation and activation of eosinophils (Campbell *et al.,* 1987; 1988; Sanderson *et al.,* 1988), with the cell-specific nature of the hypereosinophilic syndrome and the eosinophilia associated with helminth infections and allergic disorders (Coffman *et al.,* 1988; Limaye *et al.,* 1989; Owen *et al.,* 1989; Yamaguchi *et al.,* 1990).

1.2.2. Activation of helper T cells

Central to the process of T cell activation is the specific recognition by the T cell receptor (TCR)/CD3 complex of antigen presented by B cells and

macrophages in association with major histocompatibility complex molecules. This interaction rapidly triggers the activation of certain protein tyrosine kinases (PTKs) associated with CD4 and the TCR/CD3 complex, which couples the latter to phospholipase C activation and consequent hydrolysis of phosphatidylinositol 4,5- bisphosphate (PIP_2) (Samelson *et al.,* 1986; June *et al.,* 1990a; Mustelin *et al.,* 1990; Klausner & Samelson, 1991). The hydrolytic products, diacylglycerol and inositol triphosphate, respectively activate protein kinase C and induce elevation in levels of intracellular calcium ($Ca^{2+}{}_i$) (Nishizuka, 1984; Imboden *et al.,* 1985; Imboden & Stobo, 1985; Weiss *et al.,* 1986; Berridge, 1987). $Ca^{2+}{}_i$, as well as protein kinase C, may also modulate the phosphorylation state of numerous intracellular proteins by the activation of Ca^{2+}/calmodulin- dependent protein kinases and phosphatases (reviewed in Cohen, 1985).

A second signal provided by IL-1, a product of activated monocytes, is generally believed to be required, in addition to those mediated by antigenic stimulation of the TCR/CD3 complex, to fully activate the T cell *in vivo* (Mizel, 1982). Several lines of evidence suggest that the activation pathway mediated via the IL-1 receptor is independent of the PIP_2 signal transduction pathway and may involve adenylate cyclase-mediated elevation of intracellular cyclic AMP levels and consequent protein kinase A activation (Abraham *et al.,* 1987; Shirakawa *et al.,* 1988; Chedid *et al.,* 1989; Chedid & Mizel, 1990; Macchia *et al.,* 1990; Dornand *et al.,* 1992). Several other cell surface molecules appear to be involved in mediating T cell activation. These include “accessory molecules”, such as CD28, which initiate and/or regulate signal transduction pathways that are distinct and independent from those mediated via the TCR/CD3 complex (June *et al.,* 1987;1989;1990b), or “co-receptors”, such as CD4, which appear to augment TCR/CD3-dependent signalling (Fleischer & Schrezenmeier, 1988; Janeway, 1989; Mustelin &

Altman, 1989; Rudd, 1990). CD45, a phosphotyrosine phosphatase abundantly expressed on the surfaces of most T cells, appears to play a critical role in coupling the TCR/CD3 complex with the PIP_2 signal transduction pathway by activating, by tyrosine dephosphorylation, certain TCR/CD3- and CD4- associated PTKs (Mustelin *et al.,* 1989;1992; Koretzy *et al.,* 1990; Ashwell, 1991; Klausner & Samelson, 1991).

Mechanisms involving the subsequent transduction of intracellular signals downstream of these early membrane events have, as yet, eluded discovery, but may presumably involve phosphorylation (and dephosphorylation) of key regulatory proteins. This problem has been addressed in recent years by a "backwards" approach. The general scheme of this approach has involved identification and characterization of DNA sequences which may be involved in regulating the inducible expression of specific lymphokine genes, and of the specific proteins which interact with these control regions and which may be potential intracellular targets for signals emanating from the early membrane events. This approach has been greatly facilitated by the establishment of stable murine and human transformed T cell lines and the derivation of antigen-specific murine helper T cell clones, which have been classified into two major subsets based on their heterogenous patterns of lymphokine secretion after mitogenic stimulation. While both subsets express IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α, Th1 clones selectively express IL-2, IFN-γ and lymphotoxin and Th2 clones selectively express IL-4, IL-5, IL-6 and IL-10 (reviewed in Mosmann & Coffman, 1989a,b).

1.2.3. Regulation of lymphokine gene expression

In keeping with the role of lymphokines as transient signals which mediate the proliferation, differentiation and function of haemopoietic cells in response to immunological stress, it might be expected that their genes should only be expressed as long as the T cell is in contact with the antigen, and that their mRNAs should be short-lived so that they are degraded soon after removal of the antigenic stimulus. Lymphokine mRNAs are usually undetectable in quiescent T cells and reach maximum steady state levels after several hours of stimulation (reviewed in Taniguchi, 1988; Arai *et al.,* 1990). The mechanisms which regulate the kinetics of induction and decay of mRNA levels appear, however, to be unique to each lymphokine gene. The IL-2 gene, for example, appears to be regulated by specific sets of transcriptional and post-transcriptional mechanisms (Efrat & Kaempfer, 1984; Shaw *et al.,* 1987; 1988a; Ohmura & Onoue, 1990). GM-CSF gene expression appears, however, to be predominantly regulated at the post-transcriptional level in a variety of T and non-T cell models (Thorens *et al.,* 1987; Bickel *et al.,* 1990; Wodnar-Filipowicz & Moroni, 1990; Akahane & Plutznik, 1992; Iwai *et al.,* 1992). These mechanisms appear to vary between different cell systems and may be differentially modulated by different signalling pathways. The transcription of the IL-2 and GM-CSF genes, for example, may be activated in response to signals mediated via the TCR/CD3 complex, while their mRNAs appear to be stabilized by CD28-mediated signals (Lindsten *et al.,* 1989).

In recent years, a number of DNA elements have been identified in the proximal 5'-flanking regions of several lymphokine genes which appear to specifically interact with numerous constitutive or inducible DNA-binding nuclear proteins and regulate the transcription of these genes in response to T cell activation signals. The transcriptional activation of the IL-2 gene, for

example, appears to involve cooperative interactions between numerous transcription factors, which interact with their respective recognition elements located within a 300 bp region 5' of the TATA box. This proposal has been supported by the dramatic alterations in chromatin structure observed within this region which parallel transcriptional activation of the IL-2 gene (Siebenlist *et al.,* 1986; Brunvand *et al.,* 1988; Durand *et al.,* 1988). These *trans*-activators include ubiquitous factors such as NF-κB, Oct-1 and AP-1 (Emmel *et al.,* 1989; Hoyos *et al.,* 1989; Serfling *et al.,* 1989; Shibuya *et al.,* 1989; Kamps *et al.,* 1990), which regulate the inducible expression of numerous genes in a variety of cell types. Regulation of IL-2 gene transcription also involves more specific factors such as NF-AT, which is activated when the Ca^{2+}-dependent signal generated by antigenic stimulation induces a pre-existing T cell-specific cytoplasmic subunit to translocate to the nucleus and associate with a newly synthesized nuclear subunit (Shaw *et al.,* 1988b; Flanagan *et al.,* 1991; Clipstone & Crabtree, 1992).

Post-transcriptional control of lymphokine gene expression appears to be largely mediated at the level of cytoplasmic mRNA stability. This may involve certain AU-rich sequences which are present in the 3'-UTRs of many labile mRNAs (Caput *et al.,* 1986) and appear to bind specific cytoplasmic factors (Malter, 1989; Bohjanen *et al.,* 1991; 1992; Vakapoulou *et al.,* 1991). Insertion of these sequences from the 3'-UTR of the GM-CSF mRNA into the 3'-UTR of the β-globin mRNA has been demonstrated to destabilize the normally stable β-globin mRNA (Shaw & Kamen, 1986). The instability conferred by such AU-rich sequences in the GM-CSF mRNA has been shown to be alleviated by cellular treatment with protein synthesis inhibitors and cellular activators (Shaw & Kamen, 1986; Akashi *et al.,* 1991). A recent report suggests, however, that other elements, apart from the conserved AU-rich sequences, may also regulate GM-CSF mRNA stability (Iwai *et al.,*

1991). These studies suggest that the regulation of lymphokine gene expression at the post-transcriptional level, as well as at the transcriptional level, may involve the interactions of multiple *cis*-acting elements with specific *trans*-acting factors, whose activities and/or syntheses may be modulated by specific activation signals.

1.3. SCOPE OF THIS THESIS

If alterations in patterns of cellular growth and differentiation may be manifestations of interactions between information external to the genomic micro-environment and the dynamic hierarchy of interactions between structural and regulatory genetic information (section 1.1.5.), understanding the molecular bases of such processes involves several fundamental questions including:

(1) how is the expression of an individual gene regulated?

(2) how are regulatory mechanisms of groups of genes integrated?

(3) how do these coordinated regulatory networks interact with the external information to modulate the parameters within which the structural information is expressed?

In respect of understanding the molecular basis of T cell activation which forms the fundamental core of "inducible" haemopoiesis, numerous studies over recent years have provided considerable insight into the molecular mechanisms which regulate expression of specific lymphokine genes. Many of these studies have, however, restricted their attention to the regulation of expression of an individual lymphokine gene, in response to activation of a specific signalling pathway in a particular cell system. Comparison of the molecular mechanisms which control the expression of different lymphokine genes, and which may be modulated by different signal

transduction pathways have been limited. Understanding how the molecular mechanisms which regulate expression of one lymphokine gene integrate with, and are coordinated with respect to, those which regulate expression of other lymphokine genes, is therefore essential in order to extend our understanding of how lymphokines, as transient mediators, coordinate "inducible" haemopoiesis as a network.

This thesis describes the initial phase of a long-term project aimed at investigating the molecular basis of T cell activation, in particular the link between early events at the cell membrane and the consequent modulation of genetic events in the nucleus. The bulk of this thesis concerns two major molecular studies:

(1) a comparative investigation of the involvement of, and inter-relationships between, multiple signal transduction mechanisms in the induction of expression of a battery of lymphokine genes in activated murine T lymphocytes, and,

(2) a comparative investigation of the modes by which the temporality of expression of different lymphokine genes may be regulated in response to activation of these multiple signalling pathways.

The second part of this thesis focuses upon investigating the as yet largely uncharacterized molecular mechanisms which regulate expression of the murine gene encoding IL-5, a lymphokine which promotes B cell growth and stimulates growth, differentiation and activation of eosinophils (Kinashi *et al.*, 1986; Campbell *et al.*, 1987; 1988; Sanderson *et al.*, 1988). IL-5 has been the subject of particular interest in the laboratory in recent years, particularly given the linkage between the cell-specific nature of eosinophilia and IL-5 gene expression (Coffman *et al.*, 1989; Limaye *et al.*, 1989; Owen *et al.*, 1989; Yamaguchi *et al.*, 1990; Sanderson, 1992). Such clinical

observations imply the possible existence of specific molecular "programs" which may be unique to the regulation of expression of the IL-5 gene, but which must ultimately be integrated with those which regulate other lymphokine genes.

CHAPTER 2:

DIFFERENTIAL INDUCTION OF LYMPHOKINE GENE EXPRESSION IN ACTIVATED MURINE T LYMPHOCYTES: INVOLVEMENT AND INTER-RELATIONSHIPS OF MULTIPLE SIGNALLING PATHWAYS

2.1. INTRODUCTION

Activation of quiescent T lymphocytes is a complex process, involving the initial interaction of certain cell surface molecules with their specific ligands and the activation of multiple intracellular second messanger pathways, ultimately leading to the induction of lymphokine gene expression and specific processing of gene products.

Central to the process of T cell activation is the specific recognition by the T cell receptor (TCR)/CD3 complex of antigen presented by accessory cells in association with major histocompatibility complex molecules. The conformational alterations of the TCR/CD3 complex induced by antigen presentation rapidly triggers a dynamic interplay between CD45, a phosphotyrosine phosphatase, and protein tyrosine kinases (PTKs) associated with CD4 and the TCR/CD3 complex. This interplay couples the TCR/CD3 complex to phospholipase C activation and consequent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Samelson *et al.*, 1986; Fleischer & Schrezenmeier, 1988; Mustelin *et al.*, 1989;1990;1992; Mustelin & Altman, 1989; June *et al.*, 1990a; Koretzy *et al.*, 1990; Rudd, 1990; Ashwell, 1991; Klausner & Samelson, 1991). The regulatory role of G proteins in these early TCR/CD3-mediated signal transduction events remains, as yet, ambiguous (Imboden *et al.*, 1986; June *et al.*, 1990a). The products of PIP_2 hydrolysis, namely, diacylglycerol and inositol triphosphate

respectively activate protein kinase C (PKC) and induce elevation in levels of intracellular calcium ($[Ca^{2+}]_i$). These processes may be mimicked by cellular treatment with mitogenic lectins, monoclonal antibodies directed against the TCR or CD3, or by the combined actions of phorbol esters, which activate PKC, and calcium ionophores, which raise $[Ca^{2+}]_i$ (Nishizuka, 1984; Imboden *et al.,* 1985; Imboden & Stobo, 1985; Weiss *et al.,* 1986; Berridge, 1987).

Activation of T lymphocytes also appears to involve several other cell surface molecules. CD28 appears to be involved in mediating T cell activation as an "accessory molecule", which initiates and/or regulates signal transduction pathways that are distinct and independent from those mediated by antigenic stimulation of the TCR/CD3 complex (Hara *et al.,* 1985; Moretta *et al.,* 1985; June *et al.,* 1987; 1989). Early proposals of CD2 as a classical "accessory molecule" are somewhat controversial in the light of recent reports which demonstrate the TCR/CD3-dependence of CD2 signalling and the physical association between these two receptors and CD45 in activated T cells (Meuer *et al.,* 1984; Breitmeyer *et al.,* 1987; Bockenstedt *et al.,* 1988; Holter *et al.,* 1988; Brown *et al.,* 1989; Schraven *et al.,* 1990). CD2, like the TCR/CD3 complex, appears to be coupled to the PIP_2 pathway by a mechanism involving CD45-mediated modulation of PTK activity (Pantaleo *et al.,* 1987; Danialien *et al.,* 1991; Koretzy *et al.,* 1991).

The "antigen-independent" signal provided by IL-1, a product of activated monocytes, is generally believed to be required, in addition to signals mediated by antigenic stimulation of the TCR/CD3 complex, to fully activate the T cell *in vivo* (Mizel, 1982). Reports of the nature of the IL-1 signal transduction pathway are varied. Several studies have demonstated that the IL-1 signal requirements for optimal T cell activation may be replaced by phorbol ester treatment (de Vries *et al.,* 1982; Zlotnik & Daine, 1986).

Other lines of evidence indicate, however, that the IL-1 signal transduction pathway may be independent of PKC activity and $[Ca^{2+}]_i$ elevation (Abraham *et al.,* 1987; Macchia *et al.,* 1990; Dornand *et al.,* 1992), and may involve adenylate cyclase-mediated elevation in levels of intracellular cyclic AMP ($[cAMP]_i$) and consequent protein kinase A (PKA) activation (Shirakawa *et al.,* 1988; Chedid *et al.,* 1989; Chedid & Mizel, 1990).

Mechanisms involving the subsequent transduction of signals downstream of these early events are, as yet, poorly understood, but presumably may involve modulation of the phosphorylation states of key intracellular regulatory factors. The possibility that intracellular signals emanating from the different early membrane events may independently modulate the expression of different lymphokine genes has been raised by several reports which demonstrate that expression of certain lymphokine genes may be selectively inhibited by the immunosuppressive agent cyclosporin A (Cs A) (June *et al.,* 1987; Bickel *et al.,* 1988; Bohjanen *et al.,* 1990). Cs A has recently been demonstrated to block a pathway involving the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Clipstone & Crabtree, 1992; Fruman *et al.,* 1992; O'Keefe *et al.,* 1992).

In the present study, the modulation of expression of a variety of different lymphokine genes by several key signalling pathways was investigated in the antigen-specific murine T helper Type 2 (Th2) clone D10.G4.1 (Kaye *et al.,* 1983) as a model experimental system. Lymphokine mRNA levels were compared in response to cellular stimulation by mitogenic lectin, IL-1, Ca^{2+} ionophore, activators of PKC and adenylate cyclase and a monoclonal antibody directed against specific antigen recognition sites of the TCR. The effects of Cs A and inhibitors of specific protein kinases and of protein synthesis upon the induction of different lymphokine mRNAs were

examined to “dissect” the signalling pathways leading to expression of these various genes. Furthermore, given recent suggestions of the physical and functional inter-relationships between CD2, CD45 and the TCR/CD3 complex, the modulation of lymphokine gene expression by CD2 and CD45 stimulation was also investigated.

2.2. MATERIALS AND METHODS

2.2.1. Reagents, cell lines and cDNA clones

(1) Reagents

Phorbol 12-myristate 13-acetate (Sigma), forskolin (Calbiochem), A23187 (Boehringer), Calphostin C (Calbiochem) and H-89 (Calbiochem) were dissolved in DMSO (dimethylsulfoxide) at 1 mg/ml, 10 mM, 10 mg/ml, 2 mM and 10 mM respectively and diluted with H_2O prior to use. Cs A was a gift from Dr. C. Simeonevic (JCSMR) and was dissolved in ethanol at 1 mg/ml and diluted with H_2O prior to use. Cycloheximide (Sigma) was dissolved in H_2O at 10 mg/ml. Concanavalin A (Pharmacia) and recombinant IL-1α (Promega) were dissolved in PBS (phosphate-buffered saline) at 1 mg/ml and 10 μg/ml respectively. 3D3 monoclonal antibody was provided by Dr. P. Hodgkin (JCSMR). Monoclonal antibodies (mAbs) directed against CD2 and CD45 were provided by Dr. J. Altin (JCSMR).

(2) Cell Lines

D10.G4.1 is a conalbumin-specific Th2 clone derived from AKR/J mice (Kaye *et al.,* 1983) and was obtained from DNAX. D10.G4.1 cells were allo-stimulated at 5 x 10^5 cells/ml every 10 days with a 10-fold excess of irradiated C57Bl/6 spleen cells, and maintained in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (CSL), 1 mM sodium pyruvate, 2 mM glutamine, 0.05 mM β-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin and recombinant IL-2 (50 units/ml).

Recombinant IL-2 was obtained from culture supernatants of C127 mammary tumour cells stably transformed with the murine IL-2 cDNA cloned into a modified bovine papilloma virus-based expression vector (Harasuyama

& Melchers, 1988). This cell line was obtained from Dr. F. Melchers (Basel Institute for Immunology, Basel) and maintained in IMDM medium (GIBCO) supplemented with 5% fetal calf serum (CSL), 2 mM glutamine, 0.05 mM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin, and selected with G418 (1 mg/ml) (Geneticin, Sigma). After antibiotic selection for a minimum of 2 weeks, cells were washed in HBSS (Hanks Balanced Salt Solution) and cultured in G418-free medium for 2 days at 1 x 10^5 cells/ml. The biological activity of IL-2 in the conditioned medium was assayed by Mrs. S. Chin (JCSMR) by measuring IL-2-dependent proliferation of HT-2 cells.

(3) cDNA clones

The murine IL-3 and IL-5 cDNA probes were 588 bp and 600 bp *Eco* RI fragments respectively derived from pILM21 (Fung *et al.,* 1984) and pEDFM-15 (Campbell *et al.,* 1988). The murine GM-CSF cDNA probe was a 700 bp *Eco* RI/*Hind* III fragment derived from pGM38 (Gough *et al.,* 1984) and provided by Dr. A. Dunn (Ludwig Institute for Cancer Research, Melbourne). The murine IL-4, IL-6 and IL-10 cDNA probes were 373 bp *Rsa* I, 945 bp *Nla* IV/*Ssp* I and 594 bp *Sac* I fragments respectively derived from pCD IL-4 (Lee *et al.,* 1986), pCD IL-6 (Chiu *et al.,* 1988) and pCD IL-10 (Moore *et al.,* 1990). These plasmids were provided by DNAX. The *Nla* IV/*Ssp* I fragment derived from pCD IL-6 was recloned into pBCB07 and provided by Dr. I. Ramshaw (JCSMR). The β-actin cDNA probe, used as a control for constitutive gene expression, was a 2.1 kb *Xho* I fragment derived from pHFβA-1 (Gunning *et al.,* 1983) and provided by Dr. P. Gunning (Children's Hospital & Medical Research Foundation, Sydney).

2.2.2. Preparation and analysis of plasmid DNA

(1) Small scale plasmid DNA isolation

Plasmid DNA was isolated on a small scale according to the alkaline lysis method modified from Maniatis *et al.* (1989). Bacterial cells, harvested from a 1 ml overnight culture were lysed in 100 μl of 50 mM glucose; 25 mM Tris-Cl [pH 8.0]; 10 mM EDTA (ethylene diaminetetraacetic acid) and 4 mg/ml lysozyme for 5 min at room temperature. 200 μl of 0.2 M NaOH; 1.0% SDS (sodium dodecyl sulphate) was added and the suspension incubated on ice for 5 min. 150 μl of 5 M potassium acetate [pH 4.6] was added and the suspension incubated on ice for 15 min. Following centrifugation at 12,000 x g for 5 min at 4°C, the supernatant was extracted with phenol:chloroform:isoamylalcohol (25:24:1). Plasmid DNA was precipitated by addition of an equal volume of isopropanol, and recovered by centrifugation at 12,000 x g for 10 min at 4°C. The DNA pellet was washed in 70% ethanol, vacuum-dried and resuspended in 10 mM Tris-Cl [pH 8.0]; 1 mM EDTA containing 20 μg/ml RNase A (Boehringer).

(2) Large scale plasmid DNA isolation

Plasmid DNA was isolated on a large scale according to the rapid boiling method modified from Maniatis *et al.* (1989). Bacterial cells were harvested from a 500 ml overnight culture, amplified in the presence of chloroamphenicol (170 μg/ml), and lysed in 10 ml STET (100 mM NaCl; 10 mM Tris-Cl [pH 8.0]; 1 mM EDTA; 5% Triton X-100) containing 2 mg/ml lysozyme (Sigma). The suspension was heated until boiling, immersed in boiling water for 40 sec and cooled in ice-cold water. The suspension was centrifuged at 25,000 rpm for 30 min at 18°C in an SW41 rotor. Nucleic acids were precipitated by the addition to the supernatant of 0.1 volume of 3 M sodium acetate [pH 5.2] and an equal volume of isopropanol, and

recovered by centrifugation at 15,000 x g for 20 min at 4°C. The pellet was vacuum-dried and resuspended in 10 mM Tris-Cl [pH 8.0]; 0.1 mM EDTA with cesium chloride and ethidium bromide at 1 g/ml and 800 μg/ml, respectively. The suspension was centrifuged in Quick Seal tubes (Beckman) at 42,000 rpm for 40 h at 18°C in a Ti75 rotor. The plasmid band was withdrawn, repeatedly extracted with water-saturated isopropanol and dialyzed overnight at 4°C against 1000 volumes of 10 mM Tris-Cl [pH 8.0]; 0.1 mM EDTA.

(3) Restriction digestion and agarose gel electrophoresis of DNA

Restriction enzyme digestion of DNA was carried out under conditions specified by the manufacturers. Reactions were terminated by addition of bromophenol blue, xylene cyanol and Ficoll (Type 400; Pharmacia) to 0.05%, 0.05% and 3% respectively. Samples were heated at 65°C for 5 min and cooled on ice prior to electrophoresis on 0.8 to 2.0% agarose gels in 40 mM Tris-acetate [pH 7.8]; 2 mM EDTA containing 0.5 μg/ml ethidium bromide.

(4) Isolation of DNA fragments

Electrophoresis of digested DNA was carried out as described above using low melting temperature agarose gels (SeaPlaque). The DNA fragment(s) of interest was excised from the gel, melted at 65°C, extracted with phenol and precipitated by addition of sodium acetate [pH 5.2] and ethanol to 0.3 M and 70% respectively.

(5) Ligations and transformations

1 μg/ml of digested vector DNA and 8 μg/ml of insert DNA were incubated in 50 mM Tris-Cl [pH 7.4]; 10 mM $MgCl_2$; 1 mM DTT (dithiothreitol); 1 mM ATP; 100 μg/ml BSA (bovine serum albumin) and

1 mM spermidine with 5 units of T4 DNA Ligase (Pharmacia) overnight at 4°C. Preparation and transformation of competent bacterial cells were carried out according to Chung *et al.* (1989). An early log phase culture of host cells in LB was added to an equal volume of 2 x TSS (1 x TSS: LB with 10% (wt/vol) PEG (polyethylene glycol) 3350; 5% (vol/vol) DMSO; 50 mM $MgSO_4$ at a final pH of 6.5). A 100 µl aliquot of cells was incubated with 100 pg of plasmid DNA at 4°C for 30 min. 0.9 ml LB was added, cells grown at 37°C for 1 h and aliquots plated onto LB plates containing the appropriate antibiotic(s).

2.2.3 Preparation and Northern blot analysis of RNA

Solutions used in RNA preparations (excluding any containing Tris) were treated overnight with 0.1% (vol/vol) DEPC (diethylpyrocarbonate) (Sigma) and then autoclaved.

(1) Preparation of Total RNA

Total cellular RNA was prepared according to Chomczynski & Sacchi (1987) with modifications. 1 to 2 x 10^7 tissue culture cells were washed with PBS and lysed in 500 µl of 4 M guanidinium isothiocyanate and 25 mM sodium citrate [pH 7.0] containing 0.2 M sodium acetate [pH 4.0] and 0.72% (vol/vol) β-mercaptoethanol. The suspension was extracted with 500 µl phenol and 100 µl chloroform:isoamylalcohol (24:1), incubated on ice for 20 min and centrifuged at 12,000 x g for 20 min at 4°C. An equal volume of isopropanol was added to the supernatant. Following precipitation at -20°C, RNA was recovered by centrifugation at 12,000 x g for 20 min at 4°C, washed in 70% ethanol, vacuum-dried and resuspended in DEPC-treated H_2O.

(2) Formaldehyde gel electrophoresis of RNA

20 μg of RNA sample was heated at 65°C for 15 min in a mixture containing 2.2 M formaldehyde, 50% deionized formamide (vol/vol); 1 x MOPS electrophoresis buffer (20 mM MOPS (3-[*N*-morpholino]-propane-sulfonic acid) [pH 7.0]; 5 mM sodium acetate; 1 mM EDTA) and 1 x RNA loading buffer (10% glycerol, 0.2 mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol). RNA was electrophoresed on 1.2% agarose gels containing 1 x MOPS electrophoresis buffer and 2.2 M formaldehyde.

(3) Northern transfer of RNA

RNA was transferred to nitrocellulose membrane (BA85, Schleicher & Schuell) according to Maniatis *et al.*(1989) with modifications. Following electrophoresis, the gel was soaked in several changes of DEPC-treated H_2O and then in 2 changes of 10 x SSC (1 x SSC: 150 mM NaCl; 15 mM sodium citrate [pH 7.0]) for 15 min. The gel was placed on a wick prepared from 3 sheets of Whatman 3MM paper soaked in 20 x SSC on a horizontal glass plate and smoothed to remove air bubbles. A nitrocellulose membrane, cut to the same dimensions as the gel and pre-soaked in 2 x SSC, was placed on the gel, followed by a piece of 3MM paper saturated in 2 x SSC. Two pieces of dry 3MM paper and a 5 cm thickness of paper towels were stacked on the gel, followed by a glass plate and weight. After overnight transfer, the filter was baked for 2 h at 80°C under vacuum.

(4) Prehybridization, hybridization and washing conditions

The nitrocellulose fliter containing immobilized RNA was prehybridized at 42°C for 2-16 h in hydridization solution containing 50% formamide (vol/vol); 25 mM KH_2PO_4 [pH 7.4]; 5 x SSC; 5 x Denhardt's solution (0.1% BSA; 0.1% Ficoll (Type 400); 0.1% polyvinylpyrrolidone) and 200 μg/ml sonicated and denatured salmon sperm DNA. Fresh hybridization

solution was added prior to addition of the ^{32}P-labeled probe (~2 x 10^6 cpm/ml) and hybridization allowed to proceed for 16-24 h at 42°C. Following hybridization, the filter was washed successively in 1 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS at 65°C for 30 min, and exposed to Kodak XAR-5 film at -70°C with intensifying screens. Filters were stripped of hybridizing material by washing at 70°C in 0.1% SDS and exposed to XAR-5 film at -70°C to check completeness of removal. Filters were re-hybridized with different probes as described above. All Northern blot analyses were conducted in two independent experiments, one filter being successively hybridized with various probes in the opposite order of sequence as a second duplicate filter.

(5) Synthesis of ^{32}P-labeled DNA probes by random priming

100-200 ng of linearized template DNA and 1 pmole of synthetic random decamers were boiled for 5 min, snap-chilled on ice and incubated in a total volume of 50 µl containing 20 mM Tris-Cl [pH 7.5]; 10 mM $MgCl_2$; 10 mM DTT; 50 µg/ml BSA; 100 µCi [α-^{32}P] dATP (3000 Ci/mmole, Amersham) and 40 µM each of dGTP, dCTP and dTTP with 5 units of DNA Polymerase (Klenow) (Pharmacia) for 2-4 h at room temperature. The reaction was terminated by addition of EDTA to 20 mM and unincorporated labeled nucleotides were removed by chromatography on a Sephadex G-50 Nick column (Pharmacia) in 10 mM Tris-Cl [pH 7.9]; 1 mM EDTA. The probe was denatured by boiling for 5 min prior to addition to the hybridization solution. ^{32}P-labeled DNA probes were also synthesized using the Prime-a-Gene Labeling System (Promega).

(6) Quantitation of Hybridization Signals

The relative intensities of hybridization signals on Northern blots were quantitated using the PhosphoImager (Molecular Dynamics).

2.3. RESULTS

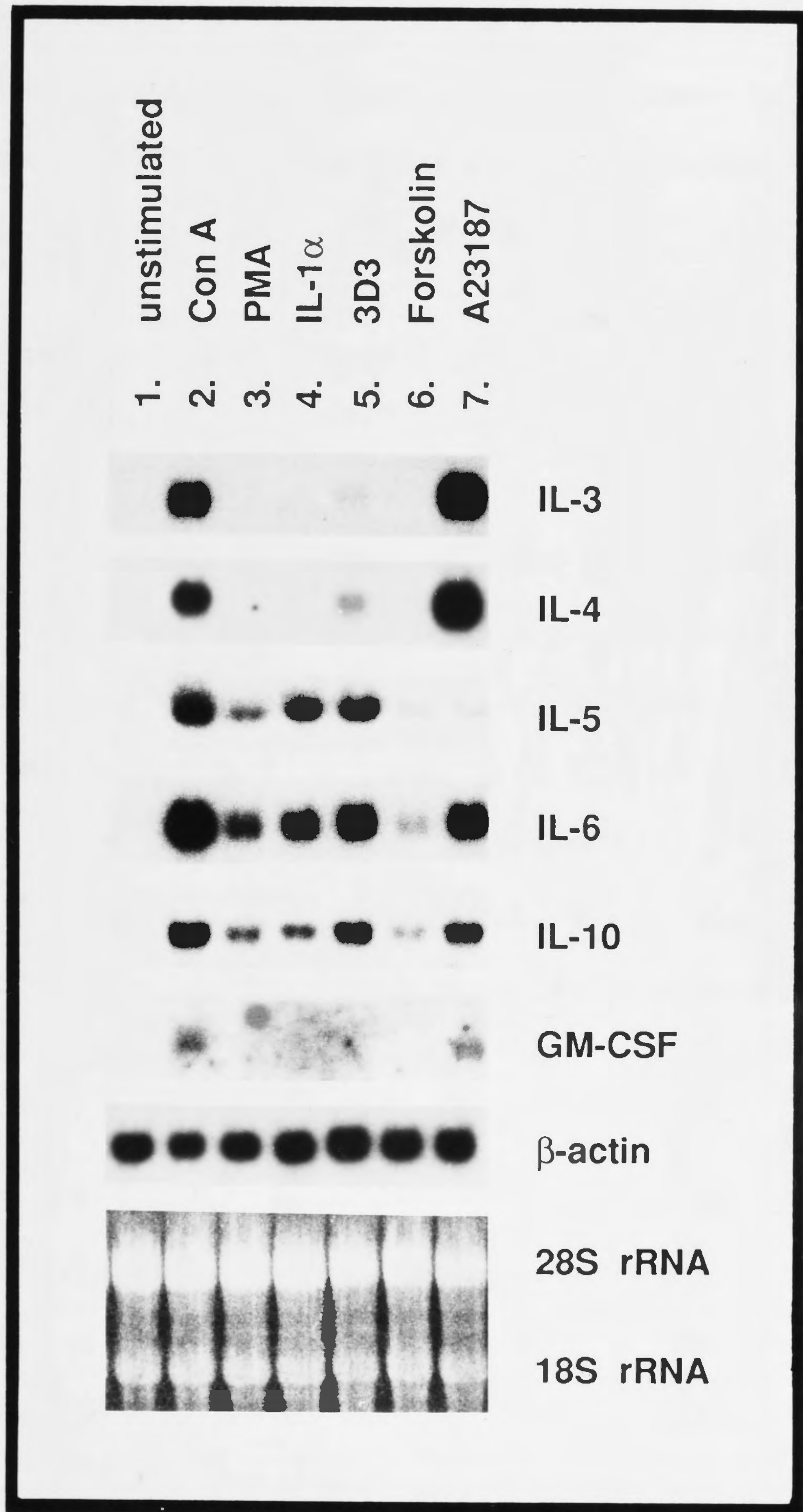
2.3.1. Induction of lymphokine gene expression by lectin, antigen recognition site-specific antibody and IL-1 stimulation, PKC and adenylate cyclase activation and Ca^{2+} mobilization

The differential induction of lymphokine gene expression was investigated by Northern blot analysis of total RNA, prepared from D10.G4.1 cells stimulated in culture with a variety of agents. These included the mitogenic lectin concanavalin A (Con A), the phorbol ester phorbol 12-myristate 13-acetate (PMA), an activator of PKC (Nishizuka, 1984), recombinant IL-1α, the antigen recognition site-specific antibody 3D3 (Kaye *et al.,* 1983), forskolin, a specific activator of adenylate cyclase (Seamon & Daly, 1983) or the calcium ionophore A23187.

As shown in Fig. 2.1., no lymphokine mRNAs were detected in unstimulated D10.G4.1 cells (Lane 1). IL-3, IL-4, IL-5, IL-6, IL-10 and GM-CSF mRNAs were detected after stimulation with either Con A, 3D3 or A23187 (Lanes 2,5,7). Stimulation with A23187 induced levels of IL-3, IL-4, IL-6, IL-10 and GM-CSF mRNAs comparable to those optimally induced by Con A. Only low levels of IL-5 mRNA, however, accumulated in response to A23187 stimulation (Lane 7). This suggests that signals other than Ca^{2+} mobilization may be required to fully potentiate IL-5 gene expression. 3D3 stimulation induced levels of IL-5, IL-6 and IL-10 mRNAs comparable to levels induced by Con A. Low levels of IL-3 and IL-4 mRNAs and a barely detectable level of GM-CSF mRNA were induced by 3D3 compared to levels induced by Con A (Lanes 2,5).

Fig. 2.1. **Induction of lymphokine gene expression in D10.G4.1 cells by lectin, antigen recognition site-specific antibody or IL-1 stimulation, PKC or adenylate cyclase activation or elevation in $[Ca^{2+}]_i$.**

Total cellular RNA was prepared from unstimulated D10.G4.1 cells (Lane 1) or D10.G4.1 cells stimulated for 3 h at 2 x 10^6 cells/ml with either Con A (5 μg/ml), PMA (10 ng/ml), IL-1α (1 ng/ml), 3D3 (1:1000 dilution), forskolin (20 μM) or A23187 (1 μg/ml) (Lanes 2-7). 20 μg of RNA was used to prepare Northern blots which were hybridized with the indicated ^{32}P-labeled murine lymphokine cDNA probes as described in Materials and Methods. Northern blots were also hybridized with a ^{32}P-labeled β-actin cDNA probe as a control for constitutive mRNA accumulation. Equalization of RNA content was confirmed by visualization of 28S and 18S ribosomal RNAs (rRNA) on ethidium bromide-stained RNA gels. The results shown are representative of two independent experiments.



1. unstimulated
2. Con A
3. PMA
4. IL-1α
5. 3D3
6. Forskolin
7. A23187
IL-3
IL-4
IL-5
IL-6
IL-10
GM-CSF
β-actin
28S rRNA
18S rRNA

As shown in Fig. 2.1., IL-5, IL-6 and IL-10 mRNAs, but not IL-3, IL-4 nor GM-CSF mRNAs, were induced in D10.G4.1 cells in response to stimulation with either PMA, IL-1α or forskolin (Lanes 3,4,6). These observations suggest that the expression of the IL-5, IL-6 and IL-10 genes may be selectively and independently regulated by either the PKC-, IL-1- or cAMP-dependent signalling pathways. Futhermore, these results suggest that the IL-1 signal transduction pathway may share a mechanism common to both the PKC- and cAMP-dependent pathways in D10.G4.1 cells.

2.3.2. Effect of protein synthesis inhibition upon induction of lymphokine mRNAs

D10.G4.1 cells were stimulated with the various activators in the continual presence of cycloheximide (CHX), a commonly used protein synthesis inhibitor. As shown in Fig. 2.2., the induction of IL-5 mRNA by either Con A, PMA, forskolin, A23187, IL-1α or 3D3 was inhibited by CHX when present from the outset of stimulation by these agents (Lanes 2,7,12,17,23,28). This suggests that the induction of IL-5 gene expression by a number of different signalling pathways requires obligatory *de novo* protein synthesis.

The effect of CHX upon the induction of the other lymphokine mRNAs differed, however, depending on the mode of stimulation. The addition of CHX at the outset of stimulation with either Con A or A23187 inhibited the induction of GM-CSF mRNA and partially inhibited levels of IL-4 mRNA (Fig. 2.2., Lanes 2,17). The induction of IL-3 mRNA by Con A or A23187 was, in contrast, not significantly affected by CHX. These observations suggest that the induction of GM-CSF and IL-3 mRNAs by the Ca^{2+} signal may, respectively, involve newly synthesized and pre-existing factors.

Maximal induction of IL-4 mRNA in response to the Ca^{2+} signal may require protein synthesis.

Although stimulation with IL-1α did not induce GM-CSF and IL-4 mRNAs, the addition of CHX at the outset of IL-1α stimulation induced significant levels of these two species (Fig. 2.2., Lanes 22,23). The accumulation of IL-3 mRNA, which is not responsive to IL-1α stimulation alone, was not induced by CHX under the same conditions. CHX did not induce the GM-CSF, IL-4 or IL-3 mRNAs in cells stimulated with either PMA or forskolin which alone, like IL-1α, were unable to induce these mRNAs (Lanes 6,7,11,12). These observations suggest that a labile mechanism may selectively suppress the induction of GM-CSF and IL-4 mRNAs in IL-1-stimulated cells.

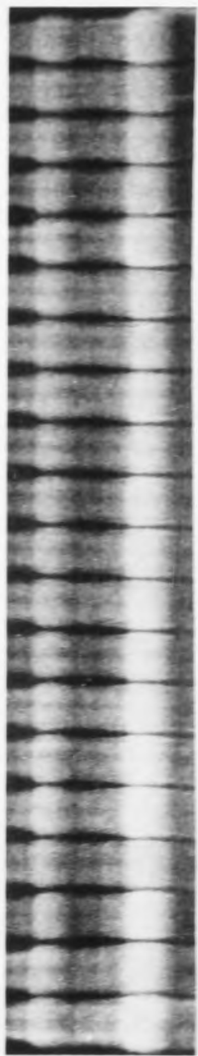
Levels of IL-6 mRNAs induced by PMA, IL-1α or 3D3 were noticeably enhanced by CHX, while levels induced by Con A, forskolin or A23187 were not significantly affected (Fig. 2.2.). Levels of IL-10 mRNA induced by PMA, forskolin or A23187 were partially inhibited by CHX, while reduction in levels induced by either Con A, IL-1α or 3D3 were not noticeable. The addition of CHX at the outset of 3D3 stimulation led to small increases in levels of GM-CSF, IL-3 and IL-4 mRNAs.

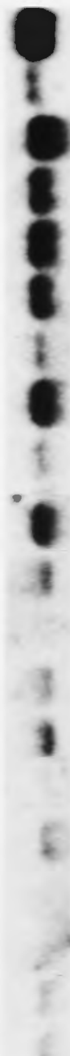
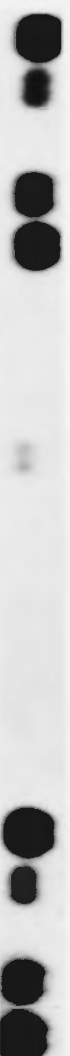
2.3.3. Effect of cyclosporin A upon the induction of lymphokine mRNAs

D10.G4.1 cells were stimulated with the various activators in the continual presence of Cs A from the outset of stimulation. As shown in Fig. 2.2., the induction of the IL-3, IL-4 and GM-CSF mRNAs by either Con A, 3D3 or A23187 was completely abolished by Cs A (Lanes 3,29,18). This suggests that signals mediated by a Cs A-sensitive Ca^{2+}-dependent

Fig. 2.2. Effect of cyclosporin A, inhibition of protein synthesis and inhibition of protein kinase activity upon induction of lymphokine gene expression by different signalling pathways.

Total cellular RNA was prepared from D10.G4.1 cells stimulated for 3 h with Con A (Lanes 1-5, 21), PMA (Lanes 6-10), forskolin (Lanes 11-15), A23187 (Lanes 16-20), IL-1α (Lanes 22-26) or 3D3 (Lanes 27-31) in the absence or in the continual presence, from the outset of stimulation, of CHX (20 μg/ml), Cs A (1 μg/ml), Cal C (0.5 μM) or H-89 (30 μM). Exposure of D10.G4.1 cells to the inhibitors under these conditions was observed not to exert any cytotoxic effects. Cells were stimulated with the activators under the conditions previously indicated in Fig. 2.1., and mRNA accumulation analysed by Northern blot analysis as previously described.



1. Con A
2. Con A + CHX
3. Con A + Cs A
4. Con A + Cal C
5. Con A + H-89
6. PMA
7. PMA + CHX
8. PMA + Cs A
9. PMA + Cal C
10. PMA + H-89
11. Forskolin
12. Forskolin + CHX
13. Forskolin + Cs A
14. Forskolin + Cal C
15. Forskolin + H-89
16. A23187
17. A23187 + CHX
18. A23187 + Cs A
19. A23187 + Cal C
20. A23187 + H-89
21. Con A
22. IL-1α
23. IL-1α + CHX
24. IL-1α + Cs A
25. IL-1α + Cal C
26. IL-1α + H-89
27. 3D3
28. 3D3 + CHX
29. 3D3 + Cs A
30. 3D3 + Cal C
31. 3D3 + H-89
18S rRNA
28S rRNA
β-actin
GM-CSF
IL-10
IL-6
IL-5
IL-4
IL-3

pathway are obligatory for the expression of the IL-3, IL-4 and GM-CSF genes in D10.G4.1 cells.

In contrast to the IL-3, IL-4 and GM-CSF mRNAs, the induction of the IL-5, IL-6 and IL-10 mRNAs by either Con A or 3D3 was relatively resistant to Cs A (Fig. 2.2., Lanes 3,29). The selective induction of the IL-5, IL-6 and IL-10 mRNAs by either PMA, forskolin or IL-1α was almost completely resistant to Cs A (Lanes 8,13,24). These observations suggest that the expression of the IL-5, IL-6 and IL-10 genes is predominantly regulated by Cs A-resistant pathways in D10.G4.1 cells.

As shown in Fig. 2.2., the induction of the IL-5, IL-6 and IL-10 mRNAs by A23187 was partially inhibited by Cs A (Lane 18). This suggests that the Ca^{2+} signal may regulate their accumulation via Cs A-sensitive and Cs A-resistant pathways. IL-5 mRNA, in contrast to the IL-6 and IL-10 mRNAs, was only weakly induced by A23187 (Lane 16). Furthermore, the induction of IL-5 mRNA by either Con A or 3D3 demonstrated greater resistance to Cs A than the induction of IL-6 and IL-10 mRNAs (Lanes 3,29). These observations suggest that Cs A-resistant, Ca^{2+}-independent pathways play a predominant role in regulating IL-5 gene expression.

2.3.4. Effect of protein kinase inhibitors upon induction of lymphokine mRNAs

D10.G4.1 cells were stimulated with the various activators in the presence, from the outset, of the protein kinase (PK) inhibitors Calphostin C (Cal C) and H-89 which are reported to demonstrate high specificity for PKC and PKA respectively (Kobayashi *et al.,* 1989; Chijiwa *et al.,* 1990). Cal C interacts with the diacylglycerol/phorbol ester-binding regulatory domain of PKC. Other more commonly used PKC inhibitors such as staurosporine or

H-7 interact with the nucleotide-binding catalytic domain and therefore may not demonstrate a high degree of specificity over other PKs which also contain similar domains (Ruegg & Burgess, 1989; Tamaoki, 1991). Cal C inhibited PMA-induced IL-5 mRNA levels by ~70% and H-89 reduced forskolin-induced levels by ~80% as determined by quantitation of the relative intensities of hybridization signals using PhosphoImager analysis. Conversely, the inhibitory effects of Cal C upon forskolin-induced IL-5 mRNA levels and H-89 upon PMA-induced levels were significantly smaller (~5%). These observations suggest that these inhibitors demonstrate reasonably high specificity for their respective known cellular targets. Cal C nor H-89, furthermore, did not significantly affect A23187-induced mRNA accumulation (Lanes 19,20), suggesting that the Ca^{2+} signal may independent of PKC and PKA activity.

Although PMA stimulation alone appeared insufficient to induce GM-CSF mRNA (Fig. 2.2., Lane 6), Cal C significantly inhibited Con A- and 3D3- induced accumulation of GM-CSF mRNA (Lanes 4,30). This suggests that PKC activation may be required, along with other obligatory signals, to fully potentiate GM-CSF gene expression. Levels of IL-3 and IL-4 mRNAs induced by Con A and levels of IL-4 mRNA induced by 3D3 were, however, relatively resistant to Cal C, while 3D3-induced IL-3 mRNA levels were slightly reduced. H-89 did not significantly inhibit Con A- or 3D3- induced expression of any of the six lymphokine genes (Lanes 5,31). Although PMA or IL-1α alone were unable to induce the IL-3, IL-4 and GM-CSF mRNAs, low levels of IL-3 and IL-4 mRNAs, but not GM-CSF mRNA, were detected in PMA- or IL-1α- stimulated cells which were co-treated with Cal C (Lanes 9,25). Co-treatment with H-89, however, did not enhance accumulation of these mRNAs (Lanes 10,26). These observations give rise to the possibility that PKC signals may negatively modulate the expression of the IL-3 and IL-4

genes. While it cannot be discounted that Cal C may possibly influence other cellular activities apart from PKC inhibition, the observations suggest that the GM-CSF gene is differentially regulated from the IL-3 and IL-4 genes.

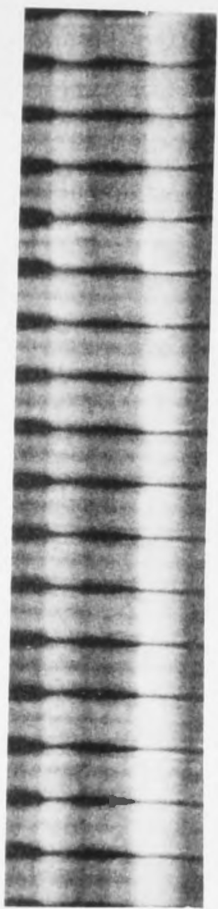
Cal C significantly inhibited accumulation of IL-5 mRNA levels induced by Con A, PMA or 3D3, while IL-6 mRNA levels were inhibited by Cal C to a lesser degree (Fig. 2.2., Lanes 4,9,30). The induction of IL-10 mRNA by these activators was relatively resistant to Cal C. Levels of IL-5 and IL-6 mRNAs induced by IL-1α were partially inhibited by either Cal C or H-89 (Lanes 25,26), suggesting that the IL-1 signal transduction pathway may involve both PKC and PKA activation. The induction of IL-10 mRNA by IL-1α was, however, relatively unaffected by the two inhibitors. This gives rise to the possibility that other kinases may be involved in the IL-1 signal transduction pathway. Notwithstanding other possible actions of these inhibitors, the observed differences in responses to these inhibitory agents suggest that the IL-10 gene may be differentially regulated from the IL-5 and IL-6 genes.

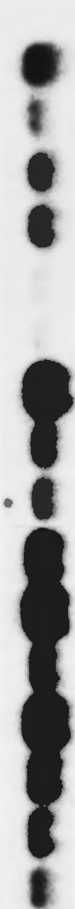
2.3.5. Modulation of lymphokine gene expression patterns by synergy between signalling pathways

To further examine the signal requirements for the expression of different lymphokine genes, the effects of co-stimulation with different combinations of activators upon induction of their mRNAs were investigated. Although the IL-5, IL-6 and IL-10 mRNAs are coordinately induced in response to either PMA or IL-1α, co-stimulation with these two agents, either together or in combination with other activators, differentially modulated their accumulation patterns. As shown in Fig. 2.3., co-stimulation with PMA and IL-1α induced significant levels of IL-5 mRNA (Lane 8), the hybridization signal of which was over two-fold the sum of IL-5 mRNA hybridization signals induced by PMA and IL-1α alone (Lanes 3,4). Such synergistic increases in

Fig.2.3. **Modulation of lymphokine gene expression patterns by co-activation of different signalling pathways.**

Total cellular RNA was prepared from unstimulated D10.G4.1 cells (Lane 1) or cells stimulated for 3 h with either Con A, PMA, IL-1α, 3D3, forskolin or A23187 (Lanes 2-7). D10.G4.1 cells were also stimulated for 3 h with different combinations of activators (Lanes 8-17). Cells were stimulated with the various activators under the conditions previously indicated in Fig. 2.1., and mRNA accumulation analysed by Northern blot analysis as previously described.





18S rRNA
28S rRNA
β-actin
GM-CSF
IL-10
IL-6
IL-5
IL-4
IL-3
1. unstimulated
2. Con A
3. PMA
4. IL-1α
5. 3D3
6. Forskolin
7. A23187
8. PMA + IL-1α
9. PMA + 3D3
10. PMA + Forskolin
11. PMA + A23187
12. IL-1α + 3D3
13. IL-1α + Forskolin
14. IL-1α + A23187
15. 3D3 + Forskolin
16. 3D3 + A23187
17. Forskolin + A23187

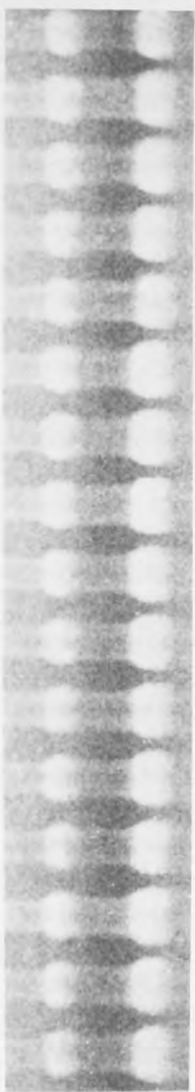
IL-6 and IL-10 mRNA levels were not observed. IL-1α in combination with 3D3 also induced an approximately two-fold synergistic increase in IL-5 mRNA levels, but not IL-6 nor IL-10 mRNA levels (Lane 12). Co-stimulation with PMA and A23187 induced synergistic increases in IL-5, IL-6 and GM-CSF mRNA levels (Lane 11). This observation provides further evidence that PKC activation, although alone insufficient to induce the GM-CSF gene, may be required to fully potentiate its expression. Secondly, these results suggest that the IL-5, IL-6 and GM-CSF genes may be differentially regulated from the IL-3, IL-4 and IL-10 genes by their requirement for both the PKC and Ca^{2+} signals for optimal induction. IL-1α also synergized with A23187 in the induction of the IL-5, IL-6 and GM-CSF mRNAs (Lane 14). This suggests that PKC activation may be involved in the IL-1 signalling pathway. Co-stimulation with other combinations of activators induced only additive increases in lymphokine mRNA levels.

D10.G4.1 cells were also stimulated with different combinations of activators in the presence of either Cal C or H-89 to further dissect the signalling requirements for induction of the various lymphokine genes. As shown in Fig. 2.4., the synergy between PMA and IL-1α in the induction of IL-5 mRNA was dramatically inhibited by Cal C (Lane 6). The induction of IL-5 mRNA by A23187 in combination with either PMA or 3D3, or by IL-1α in combination with either A23187 or 3D3, was also significantly inhibited by Cal C (Lanes 3,9,12,15). These observations suggest that the PKC signal may play a predominant role in inducing expression of the IL-5 gene.

As shown in Fig. 2.4., the synergy between A23187 and either PMA or IL-1α in the induction of IL-5, IL-6 and GM-CSF mRNAs was significantly inhibited by Cal C (Lanes 3,12). This suggests that the IL-1 signal transduction pathway may involve PKC activation. The synergy between

Fig. 2.4. **Effect of protein kinase inhibitors upon lymphokine gene expression patterns induced by co-activation of different signalling pathways.**

Total cellular RNA was prepared from D10.G4.1 cells stimulated for 3 h with either Con A (Lane 1) or different combinations of PMA, IL-1α, 3D3 or A23187 in the absence or in the continual presence, from the outset of stimulation, of either Cal C or H-89, and analysed by Northern blot analysis. Cells were treated with the various activators and inhibitors at the concentrations previously indicated in Figs. 2.1. and 2.2.



18S rRNA
28S rRNA
β-actin
GM-CSF
IL-10
IL-6
IL-5
IL-4
IL-3
1. Con A
2. PMA + A23187
3. PMA + A23187 + Cal C
4. PMA + A23187 + H-89
5. PMA + IL-1α
6. PMA + IL-1α + Cal C
7. PMA + IL-1α + H-89
8. 3D3 + A23187
9. 3D3 + A23187 + Cal C
10. 3D3 + A23187 + H-89
11. IL-1α + A23187
12. IL-1α + A23187 + Cal C
13. IL-1α + A23187 + H-89
14. IL-1α + 3D3
15. IL-1α + 3D3 + Cal C
16. IL-1α + 3D3 + H-89

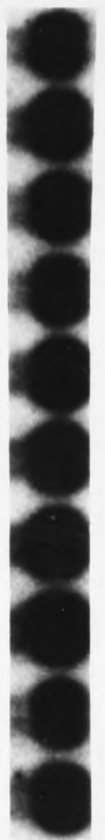
A23187 and IL-1α in the induction of IL-5 and IL-6 mRNAs was also significantly inhibited by H-89 (Lane 13). This suggests that PKA activation may also be involved in the IL-1 signal transduction pathway. Synergy between A23187 and IL-1α in the induction of GM-CSF mRNA was, however, only slightly inhibited by H-89. This is consistent with previous observations indicating that PKA-dependent pathways may not be involved in regulating the induction of GM-CSF mRNA (sections 2.3.1., 2.3.4.). The PKC component of the IL-1 pathway appears to play a greater role than the PKA component in the induction of IL-5 mRNA, as the synergy between IL-1α and either PMA or 3D3 was not significantly inhibited by H-89 (Lanes 7,16).

2.3.6. Modulation of lymphokine gene expression by CD45 and CD2

The possibility that signals mediated by the CD45 and CD2 molecules may modulate the expression of different lymphokine genes, independently of direct TCR/CD3 stimulation, was investigated by examining lymphokine mRNA levels in D10.G4.1 cells stimulated with either 3D3, anti-CD45 mAb or mAbs directed against two putatively different CD2 isotypes. These were tentatively termed CD2(1) and CD2(2), the former being distinguished by its ability to co-precipitate with CD45 (J. Altin, pers. comm.). As shown in Fig. 2.5., stimulation of D10.G4.1 cells with either anti-CD45, anti-CD2(1) or anti-CD2(2) induced low levels of IL-5 mRNA and moderate levels of IL-6 and IL-10 mRNAs. Co-stimulation with the two anti-CD2 mAbs induced additive increases in the levels of these three species. In contrast, the IL-3, IL-4 and GM-CSF mRNAs were not induced in response to stimulation with anti-CD45 or either of the two anti-CD2 mAbs. Given that the IL-5, IL-6 and IL-10 mRNAs may also be selectively induced by the IL-1-, PKC- or cAMP- dependent pathways (section 2.3.1.), these observations suggest that some common

Fig. 2.5. Modulation of lymphokine gene expression by CD45 or CD2 stimulation.

Total cellular RNA was prepared from D10.G4.1 cells stimulated for 3 h with either 3D3 (1:1000 dilution), anti-CD45 mAb (5 μg/ml) or two different anti-CD2 mAbs ((1) and (2)) (5 μg/ml), or co-stimulated with different combinations of these mAbs, and analysed by Northern blot analysis as previously described. Concentrations of the mAbs used were those which demonstrated maximal binding to their respective ligands (J. Altin, pers. comm.).



1. 3D3
2. CD2(1)
3. CD2(2)
4. CD45
5. CD2(1) + (2)
6. 3D3 + CD2(1)
7. 3D3 + CD2(2)
8. 3D3 + CD45
9. 3D3 + CD2(1) + (2)
10. 3D3 + CD2(1) + (2) + CD45
18S rRNA
28S rRNA
β-actin
GM-CSF
IL-10
IL-6
IL-5
IL-4
IL-3

mechanism may exist between these activation pathways and those mediated by CD45 and CD2.

To investigate whether the CD45 and CD2 molecules may modulate the expression of the lymphokine genes induced by TCR/CD3 stimulation, lymphokine mRNA levels were examined between D10.G4.1 cells stimulated with 3D3 alone, or with 3D3 in combination with anti-CD45 and/or either one or both of the two anti-CD2 mAbs. At the concentrations used in this study, no interference between these mAbs in their binding to their respective ligands could be detected (J. Altin, pers. comm.). As shown in Fig. 2.5., stimulation with either or both of the two anti-CD2 mAbs did not appear to significantly affect the levels of all six lymphokine mRNAs induced by 3D3 stimulation. Levels of all six lymphokine mRNAs induced by 3D3 were, however, reduced when D10.G4.1 cells were co-stimulated with anti-CD45 (Lanes 8,10). This negative effect of anti-CD45 upon 3D3-induced gene expression appeared to differ between lymphokine genes. CD45 stimulation substantially inhibited 3D3-induced levels of IL-3, IL-4 and GM-CSF mRNAs, while inhibition of IL-5, IL-6 and IL-10 mRNA levels was less marked. These observations suggest that CD45 stimulation may (1) negatively modulate lymphokine gene expression induced in response to TCR/CD3 stimulation, and (2) also mediate positive signals to a distinct group of lymphokine genes, independently of direct TCR/CD3 stimulation.

2.4. DISCUSSION

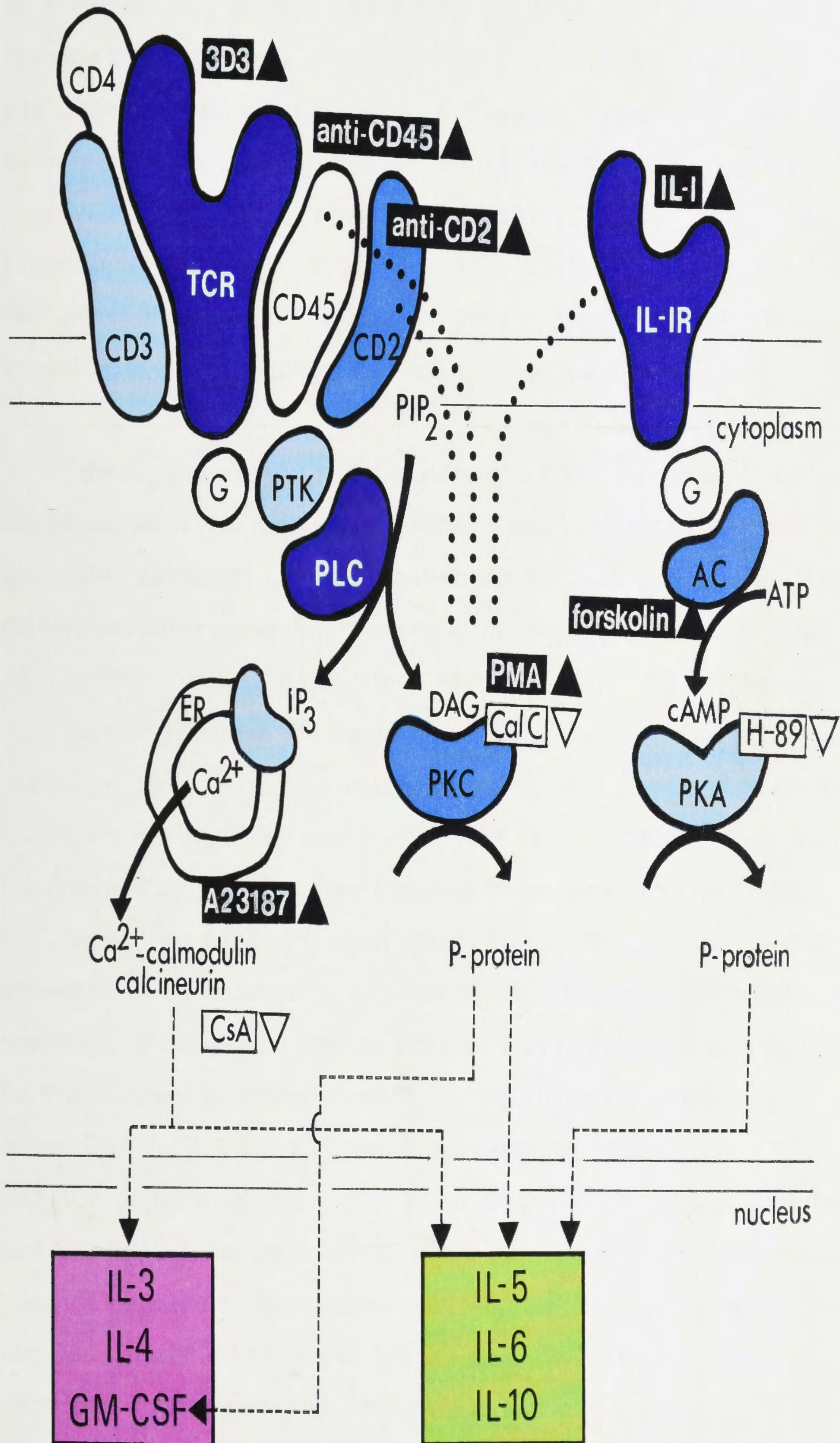
A number of recent studies in T cell clones have demonstrated the inducibility of expression of a variety of lymphokine genes in response to TCR/CD3 stimulation. Studies described in this chapter suggest that the intracellular signalling pathways emanating from the TCR/CD3 complex may differentially regulate the expression of a number of lymphokine genes in the murine Th2 clone D10.G4.1. The studies also reveal that the expression of these lymphokine genes may be differentially and independently regulated by intracellular signalling pathways emanating from the IL-1 receptor and the CD45 and CD2 cell surface molecules.

2.4.1. Coordinate and differential regulation by the two TCR/CD3-mediated second messanger pathways

Studies described in section 2.3.1. indicated that the GM-CSF, IL-3, IL-4, IL-5, IL-6 and IL-10 genes are coordinately expressed in response to cellular stimulation by the lectin Con A, the antigen recognition site-specific antibody 3D3 or the calcium ionophore A23187. The expression of the IL-5, IL-6 and IL-10 genes could, however, be selectively and independently induced by the phorbol ester PMA. This suggests that the two parallel second messanger pathways activated by TCR/CD3-mediated PIP_2 hydrolysis may differentially regulate the expression of two distinct groups of lymphokine genes (Fig. 2.6.). While either PKC activation or Ca^{2+} mobilization may independently induce the expression of the IL-5, IL-6 and IL-10 genes, the Ca^{2+} signal appears obligatory for induction of GM-CSF, IL-3 and IL-4 gene expression. Furthermore, the Ca^{2+} signal appears to be mediated via distinct pathways which also differentially modulate the expression of these two groups of genes. Expression of the GM-CSF, IL-3 and IL-4 genes appears to

Fig. 2.6. Schematic representation of the inter-relationships between different signal transduction mechanisms which regulate the expression of different lymphokine genes in D10.G4.1 cells.

The postulated linkage between different signal transduction pathways and the regulation of expression of individual, or distinct groups of, lymphokine genes are indicated by broken lines. The signalling pathways which are proposed to emanate from the IL-1 receptor, CD2 or CD45 molecules are indicated by dotted lines. Agents used in this study which either activate ▲ or inhibit ▽ distinct steps in these multiple activation pathways are denoted close to their sites of action. "P-protein" refers to intracellular proteins phosphorylated by protein kinases. Abbreviations include: IL-1R: IL-1 receptor; G: G protein; PLC: phospholipase C; AC: adenylate cyclase; DAG: diacylglycerol; IP_3: inositol triphosphate; ER: endoplasmic reticulum. Other abbreviations are refered to in the text.



3D3
CD4
anti-CD45
IL-1
anti-CD2
TCR
CD45
IL-1R
CD3
CD2
PIP2
cytoplasm
G
PTK
G
PLC
AC
ATP
forskolin
PMA
IP3
DAG
ER
Cal C
cAMP
H-89
Ca2+
PKC
PKA
A23187
Ca2+-calmodulin
calcineurin
P-protein
P-protein
CsA
nucleus
IL-3
IL-4
GM-CSF
IL-5
IL-6
IL-10

be predominantly regulated by a Cs A-sensitive Ca^{2+}-dependent pathway (section 2.3.3.). Such a pathway may involve the Ca^{2+}/calmodulin-dependent phosphatase calcineurin, whose activity has recently been demonstrated to be inhibited by a complex formed between Cs A and its intracellular target cyclophilin (Takahashi *et al.*, 1989; Liu *et al.*, 1991; Clipstone & Crabtree, 1992; Fruman *et al.*, 1992; O'Keefe *et al.*, 1992). Regulation of the IL-5, IL-6 and IL-10 genes by the Ca^{2+} signal may, however, involve both Cs A-sensitive and Cs A-resistant pathways.

Synergy between phorbol esters and Ca^{2+} ionophores in inducing T cell proliferation and lymphokine synthesis has been extensively reported and generally ascribed to the requirement of activating both PKC and Ca^{2+}/calmodulin-dependent kinases in order to fully trigger T cells (Truneh *et al.*, 1985; Kumagai *et al.*, 1987; Macchia *et al.*, 1990). Several reports have further extended the basis of this synergy to involve enhanced PKC activation by positive cooperative binding of Ca^{2+} and diacylglycerol analogues to PKC (May *et al.*, 1985; Wolf *et al.*, 1985a,b). This "two-signal requirement" appeared to differ between lymphokine genes in D10.G4.1 cells. PKC activation, although alone insufficient to induce the GM-CSF gene, appeared to be required in addition to the obligatory Ca^{2+} signal to fully potentiate its expression (section 2.3.5.). This requirement was not observed for the IL-3 and IL-4 genes. PMA and A23187 also synergized for optimal induction of the IL-5 and IL-6 genes, but not the IL-10 gene. The IL-5, IL-6 and GM-CSF genes therefore appear to be differentially regulated from the IL-3, IL-4 and IL-10 genes by their "two signal requirement". Furthermore, the IL-5 gene appears to be differentially regulated from other genes by the obligatory requirement of the PKC signal for its inducibility. The inducibility of the IL-5 gene in response to the Ca^{2+} signal alone was significantly weaker than observed for the other lymphokine genes, and the synergy between PMA and

A23187 in the induction of IL-5 mRNA was severely inhibited by the PKC inhibitor Cal C (section 2.3.5.).

The absence of synergy between PMA and A23187 in the induction of the IL-3, IL-4 and IL-10 genes appears to correlate with the slightly stimulatory effect of Cal C upon accumulation of their mRNAs (section 2.3.4.). This may suggest that PKC signals may negatively modulate IL-3 and IL-4 gene expression, and coordinately exert positive and negative influences upon IL-10 gene expression. Such pleiotropic modulation may be possibly mediated by different PKC isoenzymes (Nishizuka, 1988; Huang *et al.,* 1989). Although the observations raise the possibility that suppression of PKC activity may slightly enhance expression of these genes, the converse implication, namely that PKC activation may inhibit their induction by the Ca^{2+} signal, was not observed (section 2.3.5.). One explanation may be that the weak negative effect of the PKC signal may be overcome by the stronger positive effect of the Ca^{2+} signal. The possibility that Cal C may influence other cellular activities cannot, however, be discounted. Future investigations of the regulatory role of PKs in modulating lymphokine gene expression may require comparison between several other chemical PK inhibitors, and the use of T cells transfected with cDNAs encoding specific protein inhibitors of PKs (Chedid & Mizel, 1990) or T cell mutants deficient in a specific PK signal transduction pathway (Peyron *et al.,* 1991a).

2.4.2. Regulation of lymphokine gene expression by the IL-1 signalling pathway

The results of the current study indicate that the IL-5, IL-6 and IL-10 genes may also be selectively and independently induced in response to stimulation of IL-1 receptor or activation of adenylate cyclase, as well as PKC activation (section 2.3.1.). It was observed that IL-1α can replace PMA in

synergizing with A23187 for optimal induction of the GM-CSF, IL-5 and IL-6 genes and that this synergy is significantly inhibited by Cal C (sections 2.3.4., 2.3.5.). Synergy between IL-1 and A23187 in the induction of the IL-5 and IL-6 genes, which are responsive to cAMP, was also inhibited by H-89, a PKA inhibitor. These observations suggest that the IL-1 signal transduction pathway may share a mechanism common to both the PKC- and PKA- dependent pathways in D10.G4.1 cells (Fig. 2.6.). The PKC component of the IL-1 signal transduction pathway appears, however, to play a greater role than PKA in the regulation of these genes, in particular that of IL-5. Synergy between PMA and IL-1α was observed only in the induction of IL-5 mRNA and was severely inhibited by Cal C. This provides further evidence that the IL-5 gene may be differentially regulated from the other lymphokine genes by the obligatory requirement of the PKC signal for its induction.

Reports of the nature of the IL-1 signal transduction pathway are varied. The possibility that the interaction of IL-1 with its receptor may modulate PKC activity has been implied by several studies which demonstrate that the IL-1 signal requirement may be replaced by phorbol ester stimulation (de Vries *et al.,* 1982; Zlotnik & Daine, 1986). Other lines of evidence have, however, indicated that the IL-1 signal transduction pathway may be independent of PKC activation (Macchia *et al.,* 1990; Dornand *et al.,* 1992) and may involve adenylate cyclase-mediated elevation in $[cAMP]_i$ and consequent PKA activation (Shirakawa *et al.,* 1988; Chedid *et al.,* 1989; Chedid & Mizel, 1990). A recent study in D10.A cells, a sub-line of the parental clone used in the present study, demonstrated that IL-1 stimulation may induce transient increases in both cAMP and diacylglycerol, PKC activation and phosphorylation of an intracellular target for PKC (Munoz *et al.,* 1990).

Results of this study are also consistent with reports indicating that the IL-1 signal transduction pathway is independent of Ca^{2+} mobilization (Abraham *et al.,* 1987). The IL-3, IL-4 and GM-CSF genes, which require the Ca^{2+} signal for induction, were not inducible by IL-1α stimulation alone (section 2.3.1.). IL-1 has been demonstrated to stimulate diacylglycerol production from phosphatidylcholine in the absence of PIP_2 hydrolysis in T cells (Rosoff *et al.,* 1988). The IL-1 signal transduction pathway in D10.G4.1 cells may involve PKC (and PKA) activation without concommitant Ca^{2+} mobilization (Fig. 2.6.). The possibility that the IL-1 signal transduction pathway may involve other kinases as well as PKC and PKA cannot, however, be discounted. Studies described in section 2.3.2. suggest that a labile mechanism selectively suppresses the induction of the GM-CSF and IL-4 mRNAs in D10.G4.1 cells stimulated with IL-1α, but not with activators of PKC or adenylate cyclase. Furthermore, in contrast to the IL-5 and IL-6 mRNAs, the induction of IL-10 mRNA by IL-1α was relatively unaffected by either Cal C or H-89. These observations suggest that the intracellular pathways emanating from the IL-1 receptor in D10.G4.1 cells may involve other kinases apart from PKC and PKA. Furthermore, these different signal transduction pathways appear to differentially regulate the expression of a number of lymphokine genes. The use of signal transduction mutants will be of potentially enormous value in future studies in defining the components of the IL-1 signal transduction pathway and their roles in regulating the expression of different lymphokine genes.

The antagonistic relationship between the PKC and PKA pathways has been extensively reported in a number of cell types (Nishizuka, 1986), although positive cross-talk between these two signalling pathways has been observed (Yoshimasa *et al.,* 1987). Elevation of $[cAMP]_i$ in T cells has been reported to inhibit TCR/CD3- or CD2- mediated PIP_2 hydrolysis, IL-2 and IL-2

receptor gene expression and T cell proliferation (Novogrodsky *et al.*, 1983; Mary *et al.*, 1987; Patel *et al.*, 1987; Bismuth *et al.*, 1988; Lerner *et al.*, 1988; Rincon *et al.*, 1988). $[cAMP]_i$ elevation has been induced in these T cell models by various means including permeant cAMP analogues, direct stimulation of adenylate cyclase by forskolin, activation of the adenylate cyclase stimulatory G protein by cholera toxin, inhibition of phosphodiesterase which degrades intracellular cAMP or by use of physiological agonists such as prostaglandin E_2. The role of cAMP in IL-1 signal transduction has been argued against on the basis that activators of $[cAMP]_i$ elevation have been sometimes unable to mimic the action of IL-1 in several systems and are inhibitory to T cell activation (O'Neill *et al.*, 1990). This notion has been countered by the argument that IL-1 induces small transient increases in $[cAMP]_i$ which are rapidly turned over by phosphodiesterase, while mimicking agents, such as those described above, may dramatically induce high, often non-physiological cAMP levels in a sustained fashion which may be ultimately inhibitory (Mizel, 1990). This is analogous to the negative feedback regulation mediated by sustained PKC activation induced by prolonged PMA stimulation (Cantrell *et al.*, 1985; Bijsterbosch & Klaus, 1987). Negative modulation of lymphokine gene expression patterns by co-stimulation with forskolin was, however, not observed in this study (section 2.3.5.). Conversely, increases in $[Ca^{2+}]_i$ have been reported to attenuate cAMP-mediated signals via the action of Ca^{2+}/calmodulin-dependent phosphatases and phosphodiesterases (Cohen, 1985). Negative modulation of forskolin-induced lymphokine gene expression by $[Ca^{2+}]_i$ elevation also was not observed.

2.4.3. Regulation of lymphokine gene expression by the CD45 and CD2 cell surface molecules

The results of the current study indicate that stimulation of either the CD45 or CD2 cell surface molecules may selectively induce expression of the

IL-5, IL-6 and IL-10 genes, but not the IL-3, IL-4 nor GM-CSF genes, in D10.G4.1 cells. The ability of either CD45 or CD2 to induce the expression of a specific group of lymphokine genes, independently of one another and of direct TCR/CD3 stimulation, is a novel and controversial finding in the light of recent studies which propose the existence of dynamic physical and functional inter-relationships between these cell surface molecules.

Firstly, the existence of a functional inter-relationship between CD45, CD2 and the TCR/CD3 complex has been implicated by several cross-linking studies which demonstrate physical association between these molecules on the surfaces of activated T lymphocytes (Brown *et al.,* 1989; Schraven *et al.,* 1990; Volarevic *et al.,* 1990). Secondly, more recent lines of evidence have implicated the role of CD45 in T cell activation as that of an integral intermediary in a linear progression of signalling events emanating from stimulation of the TCR/CD3 complex and also CD2. CD45 has recently been demonstrated to activate, by tyrosine dephosphorylation, two PTKs of the *src* family, namely $p59^{fyn}$ and $p56^{lck}$, which are respectively associated with the TCR/CD3 complex and CD4 (Veillette *et al.,* 1988; Samelson *et al.,* 1990; Mustelin *et al.,* 1989;1992). These PTKs are highly expressed in T cells and are active during, and appear critical for, TCR/CD3-mediated T cell activation (Marth *et al.,* 1985;1989; Cooke *et al.,* 1991). These two PTKs have been proposed to be likely candidates to phosphorylate, among other substrates, the signal transducing ζ chain of CD3 (Mustelin & Altman, 1989; Irving & Weiss, 1991; Koretzy *et al.,* 1991; Klausner & Samelson, 1991). This tyrosine phosphorylation event appears critical for coupling the TCR/CD3 complex to phospholipase C activation and consequent PIP_2 hydrolysis (June *et al.,* 1990a; Koretzy *et al.,* 1990; Mustelin *et al.,* 1990; Peyron *et al.,* 1991b; Strauss & Weiss, 1992).

CD2, like the TCR/CD3 complex, also appears to be coupled to the PIP_2 pathway by a similar mechanism involving CD45-mediated modulation of PTK activity, possibly that of $p56^{lck}$ (Pantaleo *et al.*, 1987; Danielian *et al.*, 1991; Koretzy *et al.*, 1991). The existence of a direct functional interrelationship between CD2 and the TCR/CD3 complex is controversial. While early studies proposed that CD2 regulates an alternate "antigen-independent" pathway (Meuer *et al.*, 1984), recent reports have demonstrated that CD2 signalling is dependent upon TCR/CD3 expression and function (Breitmeyer *et al.*, 1987; Bockenstedt *et al.*, 1988; Holter *et al.*, 1988; Moingeon *et al.*, 1992).

The ability of either CD45 or CD2 to selectively regulate the expression of a specific group of lymphokine genes, independently of one another and of direct TCR/CD3 stimulation, suggests that pathways mediated via either of these two cell surface molecules may not involve PIP_2 hydrolysis. Observations described in section 2.3.6. indicated that stimulation of either CD45 or CD2 could selectively induce the expression of the IL-5, IL-6 and IL-10 genes, which are also selectively inducible by PMA. Stimulation of CD45 or CD2 did not, however, induce the expression of the IL-3, IL-4 or GM-CSF genes which are predominantly regulated by the Ca^{2+} signal. This suggests that the signal transduction pathways emanating from either the CD45 or CD2 molecules may involve PKC activation without concommitant Ca^{2+} mobilization. PKC activation may be possibly induced by diacylglycerol derived from sources other than PIP_2, such as phosphatidylcholine (Rosoff *et al.*, 1988). Given that CD2, unlike CD45, does not appear to possess intrinsic tyrosine phosphatase activity, activation of their putatively common downstream target(s) may be converged upon by different upstream events independently mediated by these two cell surface molecules.

The observation that co-stimulation with anti-CD45 mAb inhibits 3D3-induced expression of all six lymphokine genes (section 2.3.6.) appears, at first instance, difficult to reconcile with the critical role that CD45 plays in regulating TCR/CD3- (and CD2-) mediated signal transduction. Immunofluorescent binding studies appear to negate the possibility that the observed negative effects of anti-CD45 mAb, under the conditions used in this study, may be due to its steric hinderance of interactions between 3D3 (and CD2 mAb) and their respective targets (J. Altin, unpublished results). Early observations of negative modulation by anti-CD4 and anti-CD8 mAbs of TCR/CD3-mediated responses have been explained by a similar proposal (Emmrich *et al.,* 1987; Jonsson *et al.,* 1989). Several recent lines of evidence have demonstrated, however, that co-stimulation with anti-CD45 mAb may inhibit TCR/CD3- or CD2- mediated PIP_2 hydrolysis and Ca^{2+} mobilization (Ledbetter *et al.,* 1988; 1991; Kiener & Mittler, 1989; Goldman *et al.,* 1992; Turka *et al.,* 1992). The tyrosine phosphatase activity of CD45 may conversely be negatively regulated by elevation in $[Ca^{2+}]_i$ (Ostergaard & Trowbridge, 1991). These observations together suggest that the functional inter-relationship between CD45 and Ca^{2+} mobilization is one of mutual co-modulation and may not necessarily be inconsistent with the role that CD45 plays in regulating the PIP_2 signal transduction pathway. The negative modulation by CD45 stimulation upon the PIP_2 signalling pathway observed in these previous studies, and upon 3D3-induced expression of all six lymphokine genes observed in the current study, may be attributable to some form of negative feedback regulation. The weaker inhibitory effect exerted by anti-CD45 mAb upon 3D3-induced expression of the IL-5, IL-6 and IL-10 genes may be attributable to their expression being selectively and positively modulated by activation signals emanating from CD45, independently of, and notwithstanding, its negative modulation of TCR/CD3-mediated activation.

Given the dynamic nature of the physical and functional inter-relationships that appear to exist between the TCR/CD3 complex, CD45, CD2 and CD4 molecules, the ability of CD45 to activate various pathways which may be inter-dependent or independent of those mediated via the TCR/CD3 complex or CD2 raises several intriguing questions. These include whether the triggering of these different pathways by CD45 is due to its phosphatase activity *per se* or to some other, yet unknown, feature of this complex molecule, and whether these various pathways may be activated by different CD45 isoforms.

This study provides evidence that lymphokine genes may be differentially regulated by (i) the two parallel second messanger pathways activated by stimulation of the TCR/CD3 complex, and (ii) signalling pathways activated by other cell surface molecules such as CD2, CD45 and the IL-1 receptor, which may involve mechanisms distinct from those emanating from TCR/CD3 stimulation. Certain intracellular pathways may induce the synthesis of specific factors which regulate the expression of specific lymphokine genes such as those of IL-5 and GM-CSF. The regulation of other lymphokine genes such as those of IL-3 or IL-6 appear, however, to involve pre-existing factors which are presumably activated by modification, for example, by phosphorylation or dephosphorylation, in response to specific signals. These factors may be involved in regulating the expression of the different lymphokine genes at either the transcriptional or post-transcriptional levels. Investigations of the molecular mechanisms by which T cell activation signals may regulate expression of different lymphokine genes are discussed in the following chapter.

CHAPTER 3:

DIFFERENTIAL REGULATION OF LYMPHOKINE GENE EXPRESSION BY MULTIPLE TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL MECHANISMS

3.1. INTRODUCTION

Regulation of the temporality of lymphokine gene expression is critical to the role of lymphokines as transient signals which mediate the proliferation, differentiation and function of haemopoietic cells in response to immunological stress. It might therefore be expected that lymphokine genes should only be expressed as long as the T cell is in contact with the antigen and that their expression should cease soon after removal of the antigenic stimulus.

The temporal expression of the lymphokine genes studied to date appear to be regulated by multiple, different sets of transcriptional and post-transcriptional mechanisms. These mechanisms may vary depending on the specific cell type and stimulus. Increases in GM-CSF gene transcription, for example, have been observed upon stimulation of human fibroblasts with phorbol esters or TNF-α (Koeffler *et al.,* 1988), murine T cell lines with lectin or specific antigen (James & Kazenwadel, 1989; Brorson *et al.,* 1991) or endothelial cells with IL-1 (Kaushansky, 1989). GM-CSF gene expression appears, however, to be regulated at the post-transcriptional level in B cells stimulated with IL-1 (Akahane & Plutznik, 1992), certain murine T cell lines stimulated with phorbol esters (Bickel *et al.,* 1990; Iwai *et al.,* 1992) or mouse macrophages stimulated with lipopolysaccharide (Thorens *et al.,* 1987) and

at both the transcriptional and post-transcriptional levels in mast cells stimulated with Ca^{2+} ionophores (Wodnar-Filipowicz & Moroni, 1990).

Lymphokine-specific mRNAs, like many which encode inducible regulators of cellular growth and differentiation, including other cytokines (Raj & Pitha, 1981; Yamato *et al.,* 1989) and oncogenes (Dani *et al.,* 1984; Mitchell *et al.,* 1985; Rabbitts *et al.,* 1985), are relatively short-lived. Regulation of mRNA stability appears to be a predominant mode of post-transcriptional control of the lymphokine genes studied to date. Apart from activating transcription of their respective genes, T cell activation signals also appear to stabilize IL-2 and IL-3 mRNAs (Shaw *et al.,* 1987; 1988a; Ohmura & Onoue, 1990; Ryan *et al.,* 1991). Stabilization of GM-CSF mRNA by cellular signals has been extensively reported in a variety of cell types which express its gene (Thorens *et al.,* 1987; Koeffler *et al.,* 1988; Bickel *et al.,* 1990; Wodnar-Filipowicz & Moroni, 1990; Akahane & Plutznik, 1992; Iwai *et al.,* 1992). Several studies have reported that cellular treatment with protein synthesis inhibitors may enhance the stabilization of certain cytokine and oncogene mRNAs. This phenomenon, often referred to as "superinduction", has generally been ascribed to the suppression by protein synthesis inhibitors of a labile mechanism which mediates the degradation of these specific mRNAs (Dani *et al.,* 1984; Mitchell *et al.,* 1985; Shaw & Kamen, 1986; Shaw *et al.,* 1987; 1988a; Brewer & Ross, 1989).

While previous reports have provided considerable insight into the transcriptional and post-transcriptional mechanisms which regulate the expression of several specific lymphokine genes, these studies have, in general, focused their attention upon regulation of an individual gene in isolation and have been conducted in a variety of different cell systems. Comparison of regulatory mechanisms which control the expression of

different lymphokine genes in the same cell system has been limited. The current study sought to be a comparative investigation of the molecular bases of regulation of a number of different lymphokine genes, in particular that of IL-5, whose regulatory mechanisms have, to date, not been extensively characterized. Furthermore, previous studies have largely investigated regulatory mechanisms in transformed cell lines. In the present study, mechanisms which regulate the expression of a variety of lymphokine genes were investigated in the murine Th2 clone D10.G4.1. Regulatory mechanisms were also compared in the transformed murine T cell line EL4.23, variants of which have been used in several previous studies (Shaw *et al.,* 1987; 1988a; Bickel *et al.,* 1990; Iwai *et al.,* 1991; 1992).

Several recent lines of evidence suggest that different stimuli may modulate the expression of lymphokine genes at different levels in a given cell type. CD3 stimulation, for example, appears to activate transcription of several lymphokine genes in human peripheral blood T cells, while CD28-mediated signals appear to stabilize their mRNAs (Lindsten *et al.,* 1989). Studies described in Chapter 2 suggested that a number of intracellular signalling pathways emanating from various cell surface-mediated events may differentially regulate the accumulation of lymphokine mRNAs in D10.G4.1 cells. The possibility that these different signalling pathways may regulate the expression of different lymphokine genes at either the transcriptional and/or post-transcriptional levels in D10.G4.1 cells was also investigated in the current study.

The molecular bases of lymphokine gene regulation were investigated by two techniques, (i) Northern blot analysis, to measure steady state levels of mRNA accumulation, and (ii) nuclear run-off transcription analysis, which involves measurement of nascent RNA initiated *in vivo* and elongated *in vitro*

in isolated nuclei, to assess the relative contribution of transcriptional and post-transcriptional mechanisms to mRNA accumulation (reviewed in Marzluff & Huang, 1985).

3.2. MATERIALS AND METHODS

Isolation and Northern blot analysis of total cellular RNA has been previously described in section 2.2.3. Each Northern blot analysis was conducted in two independent experiments, one filter being successively hybridized with various probes in the opposite order of sequence as a second duplicate filter. The relative intensities of hybridization signals were quantitated using the PhosphoImager (Molecular Dynamics).

3.2.1. Cell lines, reagents and cDNA clones

(1) Cell Lines and reagents

The source and maintenance of the murine Th2 clone D10.G4.1 (Kaye *et al.*, 1983) has been previously described in section 2.2.1.(2). EL4.23 is a sub-line of the murine T lymphoma cell line EL4 (Farrar *et al.*, 1980) and was provided by Dr. C. Sanderson (National Institute for Medical Research, Mill Hill, London). EL4.23 cells were maintained in RPMI 1640 medium (GIBCO), supplemented with 10% fetal calf serum (Cytosystems), 1 mM sodium pyruvate, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Actinomycin D and α-methyl mannoside were obtained from Sigma and were dissolved in H_2O at 200 μg/ml and 1.0 M respectively. The sources and preparation of other reagents used to treat tissue culture cells in this study have been previously described in section 2.2.1.(1).

(2) cDNA clones

The sources and details of the murine IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF and β-actin cDNA probes used for Northern blot analysis have been previously described in section 2.2.1.(3). The murine IL-2 cDNA probe was a 563 bp *Sac* I/*Acc* I fragment derived from pCD IL-2 (Yokota *et al.*, 1985)

and was provided by DNAX. As a positive control for constitutive gene expression, in addition to the β-actin cDNA, a 1040 bp *Dra* I/*Bam* HI fragment, containing the three coding units of the human ubiquitin gene UbB (Baker & Board, 1987), was used as a probe. This fragment was derived from pRB130 which was provided by Dr. R. Baker (JCSMR). Sequences used as unlabeled targets in nuclear run-off transcription analysis were also derived from these plasmids. As these cDNAs were originally cloned into various different vectors, cDNA sequences, indicated in the figures, were individually sub-cloned by the present investigator into the same vector, pBluescript SK- (Stratagene), to enable comparison between different nuclear run-off transcripts of any potential non-specific hybridization to vector sequences. Subcloning, isolation, restriction digestion and agarose gel electrophoresis of plasmid DNA has been previously described in section 2.2.2.

3.2.2. *In vitro* nuclear run-off transcription analysis

(1) Isolation of Nuclei

Nuclei were isolated from unstimulated or stimulated cells according to Greenberg & Ziff (1984) with modifications. 5×10^7 cells were harvested, washed in ice-cold PBS and lysed in 5 ml ice-cold lysis buffer (10 mM Tris-Cl [pH 7.4]; 10 mM NaCl; 3 mM $MgCl_2$; 0.2% Nonidet P-40). Following centrifugation at 800 x g for 5 min at 4°C, the nuclei were resuspended in 100 µl nuclear storage buffer (40% glycerol; 50 mM Tris-Cl [pH 8.0]; 5 mM $MgCl_2$; 0.1 mM EDTA) and frozen in liquid nitrogen until use.

(2) Transcription reactions and isolation of transcripts

Thawed nuclei were incubated for 30 min at 30°C in a total volume of 200 µl containing 20% glycerol; 30 mM Tris-Cl [pH 8.0]; 5 mM $MgCl_2$; 150 mM KCl; 2 mM $MnCl_2$; 1 mM DTT; 0.5 mM each of ATP and CTP;

50 μCi each of [α-^{32}P] UTP and [α-^{32}P] GTP (3000 Ci/mmole, Amersham) and 40 units of RNasin (Promega). Transcripts were isolated by the modified single-step guanidinium isothiocyanate method (Chomczynski & Sacchi, 1987) previously described in section 2.2.3.(1).

(3) Southern transfer of DNA

cDNA plasmids were digested with the appropriate restriction enzymes, electrophoresed on 2.0% agarose gels and transferred on to nitrocellulose (BA85, Schleicher & Schuell) or nylon (Hybond N+, Amersham) membranes according to Maniatis *et al.* (1989) with modifications. Following electrophoresis, the gel was treated in 0.25 M HCl for 5 min, rinsed in H_2O and denatured in 1.5 M NaCl; 0.5 M NaOH for 30 min with shaking. The gel was subsequently rinsed with H_2O and neutralized in 1.5 M NaCl; 0.5 M Tris-Cl [pH 7.2]; 1 mM EDTA for 30 min with shaking. Procedures for capillary blotting and fixation of nucleic acids to membranes have been previously described (section 2.2.3.(3)).

(4) Hybridization of ^{32}P-labeled run-off transcripts

Digested cDNA plasmids, immobilized on filters, were hybridized to the run-off products according to Linial *et al.* (1985) with modifications. Filters were pre-hybridized at 65°C for 2 to 16 h in 1 ml hybridization solution (10 mM Tris-Cl [pH 7.4]; 0.2% SDS; 10 mM EDTA; 0.3 M NaCl; 1 x Denhardt's solution and 200 μg/ml denatured yeast RNA). Fresh hybridization solution was added prior to addition of the ^{32}P-labeled run-off products and hybridization allowed to proceed for 36 h at 65°C. Following hybridization, the filter(s) was washed as previously described for Northern blot analysis (section 2.2.3.(4)) or, alternatively, were washed in 2 x SSC at 65°C for 1 h, then incubated at 37°C in 2 x SSC containing 20 μg/ml RNase A for 30 min and subsequently washed in 2 x SSC at 37°C for 1 h.

Each nuclear run-off transcription analysis was repeated in three independent experiments, using new filters containing immobilized cDNA for each experiment.

3.3. RESULTS

3.3.1. Kinetics of lymphokine mRNA accumulation and gene transcription in D10.G4.1 cells.

Studies described in Chapter 2 demonstrated that the GM-CSF, IL-3, IL-4, IL-5, IL-6 and IL-10 mRNAs are coordinately and optimally induced in D10.G4.1 cells in response to Con A stimulation. The kinetics of steady state accumulation of these lymphokine mRNAs in D10.G4.1. cells in response to Con A were investigated by Northern blot analysis. As shown in Fig. 3.1., the levels of IL-3, IL-4 and IL-5 mRNAs rose rapidly to peak after 6 h stimulation. The low levels of GM-CSF mRNA accumulation peaked earlier at 3 h. IL-3, IL-4 and GM-CSF mRNA accumulation declined after 9 h, while the decline in IL-5 mRNA levels was significantly slower. The levels of IL-6 and IL-10 mRNAs rose rapidly within 1.5 h of Con A stimulation, and were maintained for approximately 6 h before slowly declining. Significant levels of IL-5, IL-6 and IL-10 mRNAs were still detectable after 15 h stimulation.

To determine whether the changing patterns of accumulation of these lymphokine mRNAs are attributable to alterations in the transcriptional activity of their respective genes, nuclear run-off assays were performed. While none of the six lymphokine mRNAs were detected by Northern blot analysis in unstimulated D10.G4.1 cells (Fig. 3.1., Lane a), low levels of GM-CSF and IL-6 gene transcription and a significant level of IL-10 gene transcription were detected in unstimulated D10.G4.1 cells by nuclear run-off analysis (Fig. 3.2.(a)). Transcription of the IL-3, IL-4 and IL-5 genes was not observed in unstimulated D10.G4.1 cells. Following Con A stimulation, transcription of the IL-4, IL-5, IL-6 and IL-10 genes increased and then declined with kinetics broadly similar to their respective steady state mRNA accumulation patterns

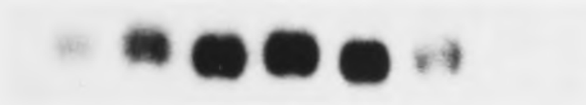
Fig. 3.1. Northern blot analysis of the kinetics of steady state lymphokine mRNA accumulation in D10.G4.1 cells.

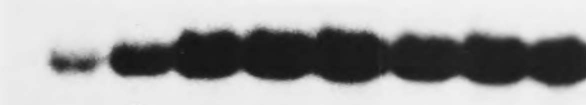
Total cellular RNA was prepared from unstimulated D10.G4.1 cells (Lane a) or cells stimulated at 2×10^6 cells/ml with Con A (5 μg/ml) for the variable periods of time indicated (Lanes b-i). 20 μg of RNA was used to prepare Northern blots which were hybridized with the indicated ^{32}P-labeled murine lymphokine cDNA probes as described in Materials and Methods. Northern blots were also hybridized with ^{32}P-labeled β-actin and ubiquitin cDNA probes as controls for constitutive mRNA accumulation. Equalization of RNA content was confirmed by visualization of 28S and 18S ribosomal RNAs (rRNA) on ethidium bromide-stained RNA gels. The results shown are representative of two independent experiments.

Period of Con A stimulation (hours)

a. 0
b. 1.5
c. 3.0
d. 4.5
e. 6.0
f. 7.5
g. 9.0
h. 12.0
i. 15.0

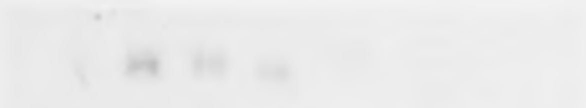
IL-3


IL-4


IL-5

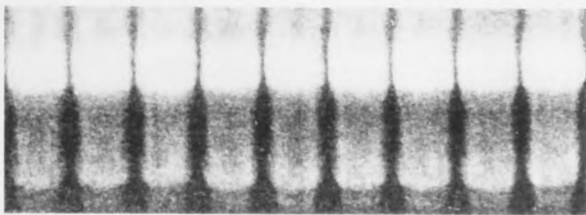
IL-6

IL-10


GM-CSF

β-actin

Ubiquitin

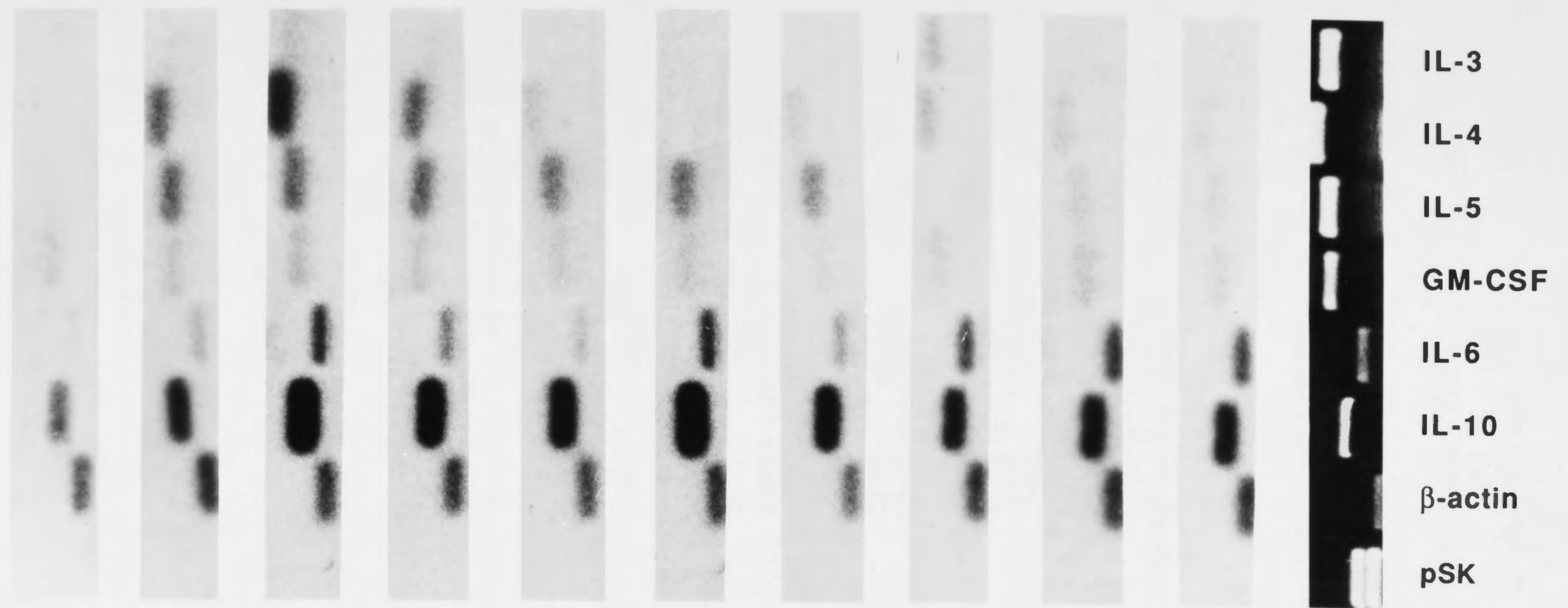

28S rRNA

18S rRNA

<u>Fig. 3.2.</u> Nuclear run-off analysis of the transcriptional activity of lymphokine genes in D10.G.4.1 cells.

Nuclei were isolated from unstimulated D10.G4.1 cells (Lane a) or cells stimulated with Con A (5 μg/ml) for the variable periods of time indicated (Lanes b-e). Nuclei were also isolated from D10.G4.1 cells which were stimulated for 6 h with Con A in the presence, from the outset of stimulation, of Cs A (1 μg/ml) (Lane f) or CHX (20 μg/ml) (Lane h). D10.G4.1 cells were also stimulated for 6 h with Con A and subsequently incubated for a further 3 h with the addition of Cs A and/or CHX (Lanes g,i,j) in the continuing presence of Con A. Nascent RNA, initiated *in vivo*, were ^{32}P-labeled by elongation in isolated nuclei *in vitro*, extracted and hybridized to approximately 500 ng of the indicated cDNA sequences. The IL-3, IL-4, IL-5, GM-CSF, IL-6, IL-10 and β-actin target sequences were, respectively, 280 bp *Bam* HI/*Nco* I, 146 bp *Rsa* I/*Sac* I, 304 bp *Sty* I, 334 bp *Hae* III, 945 bp *Nla* IV/*Ssp* I, 594 bp *Sac* I and 2.1 bk *Xho* I fragments, which were derived from the original plasmids described in section 2.2.1.(3) and sub-cloned into the pBluescript SK vector (pSK). These cDNA target sequences were isolated by restriction digestion and agarose gel electrophoresis, as shown, and immobilized on filters. *Pvu* I-digested pSK DNA (1.91 kb and 1.05 kb) was also included as a negative control. The results shown are representative of three independent experiments.

Although hybridization of the run-off transcripts to their respective cDNA targets was conducted under the stringent conditions described in section 3.2.2.(4), data-base searches of the cDNA target sequences were conducted to additionally ensure that the hybridization signals were not attributable to repeated sequences or homology with abundant species such as ribosomal RNA.



Time (hours)	a	b	c	d	e	f	g	h	i	j
Addition of Con A	-	0	0	0	0	0	0	0	0	0
Addition of Cs A	-	-	-	-	-	0	6	-	-	6
Addition of CHX	-	-	-	-	-	-	-	0	6	6
Cell Harvest	0	3	6	9	12	6	9	6	9	9

(Fig. 3.2.(b-e)). Only modest increases in transcription of the IL-3 and GM-CSF genes in response to Con A stimulation were observed. IL-3 and GM-CSF mRNAs remained, however, detectable by Northern blot analysis at times when IL-3 gene transcription became undetectable and the rate of GM-CSF gene transcription had declined to the level observed in unstimulated cells. These observations raise the possibility that mRNA stabilization may be involved in regulating the accumulation of the IL-3 and GM-CSF mRNAs in D10.G4.1 cells.

3.3.2. Kinetics of lymphokine mRNA accumulation and gene transcription in EL4.23 cells

The kinetics of steady state lymphokine mRNA accumulation were also investigated in the transformed T cell line EL4.23, in which T cell activation signals may be replaced by phorbol ester treatment (Farrar *et al.,* 1980). Preliminary experiments indicated that a number of lymphokine mRNAs, including those of IL-2, IL-3, IL-5, IL-10 and GM-CSF, are coordinately and optimally induced in response to PMA stimulation, while their accumulation in response to other activators such as Con A or A23187 was barely detectable. The kinetics of lymphokine mRNA accumulation in EL4.23 cells were slower compared to those observed in D10.G4.1 cells. As shown in Fig. 3.3., the levels of IL-2, GM-CSF and IL-5 mRNAs rose steadily to peak after 8 to 12 h PMA stimulation. IL-2 and GM-CSF mRNA accumulation declined after 16 h while significant levels of IL-5 mRNA were still detectable after 20 h. Only very low levels of IL-3 and IL-10 mRNAs were detectable after 12 h stimulation, which were maintained for approximately 4 h before declining.

While none of the lymphokine mRNAs were detectable in unstimulated EL4.23 cells (Fig. 3.3., Lane a), a significant level of GM-CSF gene transcription and a low level of IL-10 gene transcription were detectable in

Fig. 3.3. Northern blot analysis of the kinetics of steady state lymphokine mRNA accumulation in EL4.23 cells

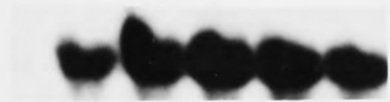
Total cellular RNA was prepared from unstimulated EL4.23 cells (Lane a) or cells stimulated at 2×10^6 cells/ml with PMA (50 ng/ml) for the variable periods of time indicated (Lanes b-f). 20 µg of RNA was used to prepare Northern blots which were hybridized with the indicated ^{32}P-labeled cDNA probes.

Period of PMA stimulation (hours)

a. 0
b. 4
c. 8
d. 12
e. 16
f. 20

IL-2

IL-3


IL-5

IL-10

GM-CSF

β-actin

Ubiquitin

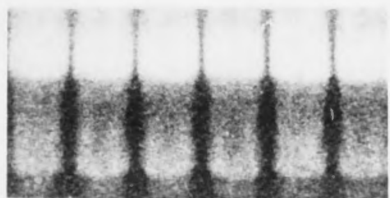
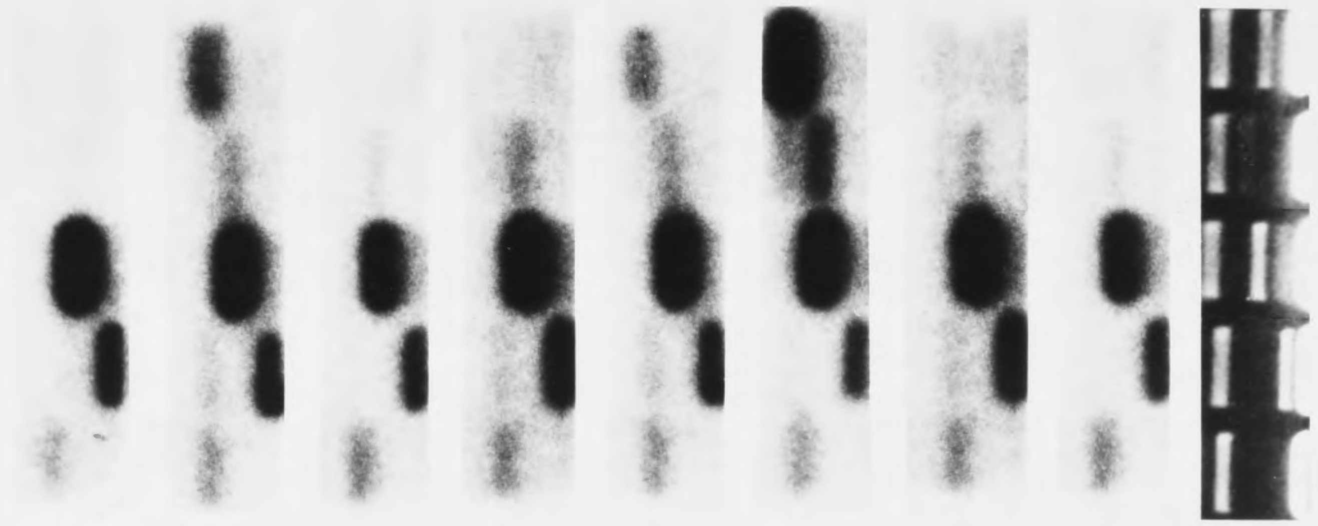

28S rRNA
18S rRNA

Fig. 3.4. Nuclear run-off analysis of the transcriptional activity of lymphokine genes in EL4.23 cells.

Nuclei were isolated from unstimulated EL4.23 cells (Lane a) or cells stimulated with PMA (50 ng/ml) for either 12 or 20 h (Lanes b,e). Nuclei were also isolated from EL4.23 cells which were stimulated for 12 h with PMA in the presence, from the outset, of CHX (20 μg/ml) (Lane c) or Cs A (1 μg/ml) (Lane d), or stimulated for 12 h with PMA and subsequently incubated for a further 8 h with addition of CHX and/or Cs A (Lanes f,g,h) in continuing presence of PMA. Nascent RNA, initiated *in vivo*, were ^{32}P-labeled by elongation in isolated nuclei *in vitro*, extracted and hybridized to approximately 500 ng of the indicated sequences. The IL-2, IL-6, IL-3, IL-10, IL-4, Ubiquitin, IL-5, β-actin and GM-CSF target sequences were, respectively, 251 bp *Bgl* II/*Rsa* I, 945 bp *Nla* IV/*Ssp* I, 280 bp *Bam* HI/*Nco* I, 594 bp *Sac* I, 242 bp *Rsa* I/*Sac* I, 1.04 kb *Dra* I/*Bam* HI, 304 bp *Sty* I, 2.1 kb *Xho* I and 334 bp *Hae* III fragments, which were derived from the original plasmids described in sections 2.2.1.(3) and 3.2.1.(2) and sub-cloned into the pSK vector. Restriction digests of plasmids containing (a) IL-2 and IL-6, (b) IL-3 and IL-10, (c) IL-4 and Ubiquitin, (4) IL-5 and β-actin and (5) GM-CSF sequences were electrophoresed on agarose gels, as shown, and immobilized on filters.



Time (hours)	a	b	c	d	e	f	g	h
Addition of PMA	-	0	0	0	0	0	0	0
Addition of Cs A	-	-	-	0	-	-	12	12
Addition of CHX	-	-	0	-	-	12	-	12
Cell Harvest	0	12	12	12	20	20	20	20

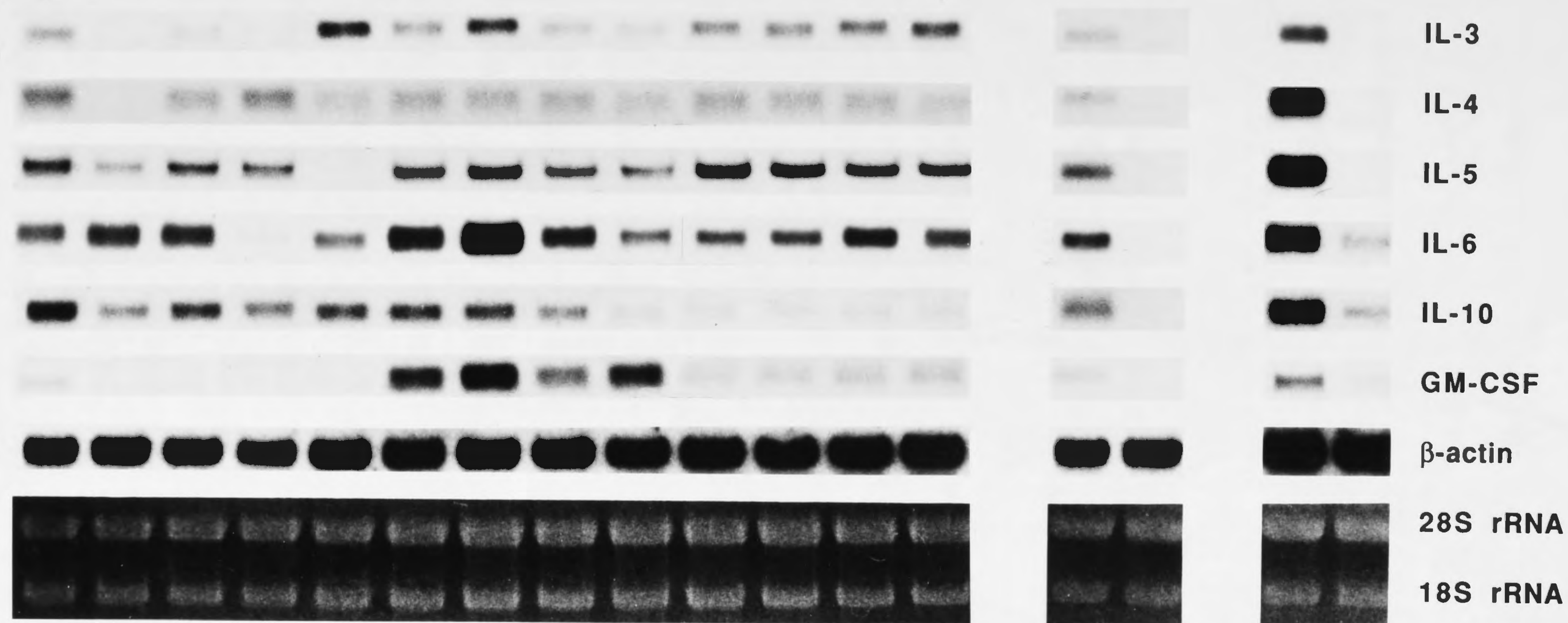
unstimulated cells (Fig. 3.4.(a)). Transcription of the IL-2, IL-3 and IL-5 genes was not detectable in unstimulated EL4.23 cells. Following PMA stimulation, transcription of the IL-2, IL-5 and IL-10 genes increased and then declined with kinetics broadly similar to their respective steady state mRNA accumulation patterns (Fig. 3.4.(b,e)). The rate of transcription of the GM-CSF gene, however, did not alter upon stimulation, while transcription of the IL-3 gene was only just detectable in stimulated cells. These observations suggest that mRNA stabilization may be involved in regulating the accumulation of GM-CSF and IL-3 mRNAs in EL4.23 cells, as well as in D10.G4.1 cells. IL-4 and IL-6 mRNAs, and transcription of their respective genes, were not detectable in unstimulated or stimulated EL4.23 cells (Fig. 3.4.).

3.3.3. The signalling requirement of lymphokine mRNA accumulation in D10.G4.1 cells

In keeping with the role of lymphokines as transient mediators, it might be expected that their genes should only be expressed as long as the T cell remains in contact with the stimulus, and that their mRNAs should disappear soon after removal of the stimulus. To investigate this possibility in D10.G4.1 cells, lymphokine mRNA levels were compared between D10.G4.1 cells which were continually stimulated with Con A for 9 h, and cells which were washed after 6 h stimulation with Con A and subsequently re-cultured for 3 h in Con A-free medium containing α-methyl mannoside to inhibit any residual Con A from binding. As shown in Fig. 3.5, the removal of Con A led to rapid declines in the levels of GM-CSF, IL-3 and IL-6 mRNAs, while a small decline in IL-10 mRNA levels was observed (Lanes a,d). In view of the finding that increases in the rates of GM-CSF and IL-3 gene transcription may not fully account for the accumulation of their mRNAs (section 3.3.1.), these observations provide further evidence that their accumulation in D10.G4.1

Fig. 3.5. **Northern blot analysis of the suppression, super-induction and stability of lymphokine mRNAs in D10.G4.1 cells**

D10.G4.1 cells were stimulated for 9 h with Con A (5 μg/ml) in the absence or presence, from the outset, of Cs A (1 μg/ml) or CHX (20 μg/ml) (Lanes a,b,e). D10.G4.1 cells were also stimulated for 6 h with Con A and subsequently incubated for a further 3 to 6 h with the addition of Cs A, CHX or Act D (10 μg/ml) in the continuing presence of Con A (Lanes c,f,g,h,j,l), or in the absence of Con A (Lanes d,i,k,m). In the latter case, D10.G4.1 cells were washed twice in warm medium after stimulation with Con A for 6 h and subsequently re-cultured in Con A-free medium containing 0.1 M α-methyl mannoside to inhibit residual Con A from binding. D10.G4.1 cells were also stimulated for 3 h with Con A in the absence or presence, from the outset, of Act D (Lanes n,o), or stimulated for 9 h with either Con A or CHX (Lanes p,q). Treatment of D10.G4.1 cells with the inhibitory agents under the conditions described above was not observed to exert cytotoxic effects. 20 μg of total cellular RNA, extracted from D10.G4.1 cells, treated in the indicated manner, was used to prepare Northern blots which were hybridized with the indicated ^{32}P-labeled cDNA probes. Northern blots shown in Lanes p and q were subjected to a period of autoradiographic exposure of approximately three times the length of blots shown in other lanes.



Time (hours)	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q
Addition of Con A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
Removal of Con A	-	-	-	6	-	-	-	-	6	-	6	-	6	-	-	-	-
Addition of Cs A	-	0	6	-	-	-	-	6	-	-	-	-	-	-	-	-	-
Addition of CHX	-	-	-	-	0	6	6	6	6	-	-	6	6	-	-	-	0
Addition of Act D	-	-	-	-	-	-	-	-	-	6	6	6	6	-	0	-	-
Cell Harvest	9	9	9	9	9	9	12	9	9	9	9	9	9	3	3	9	9

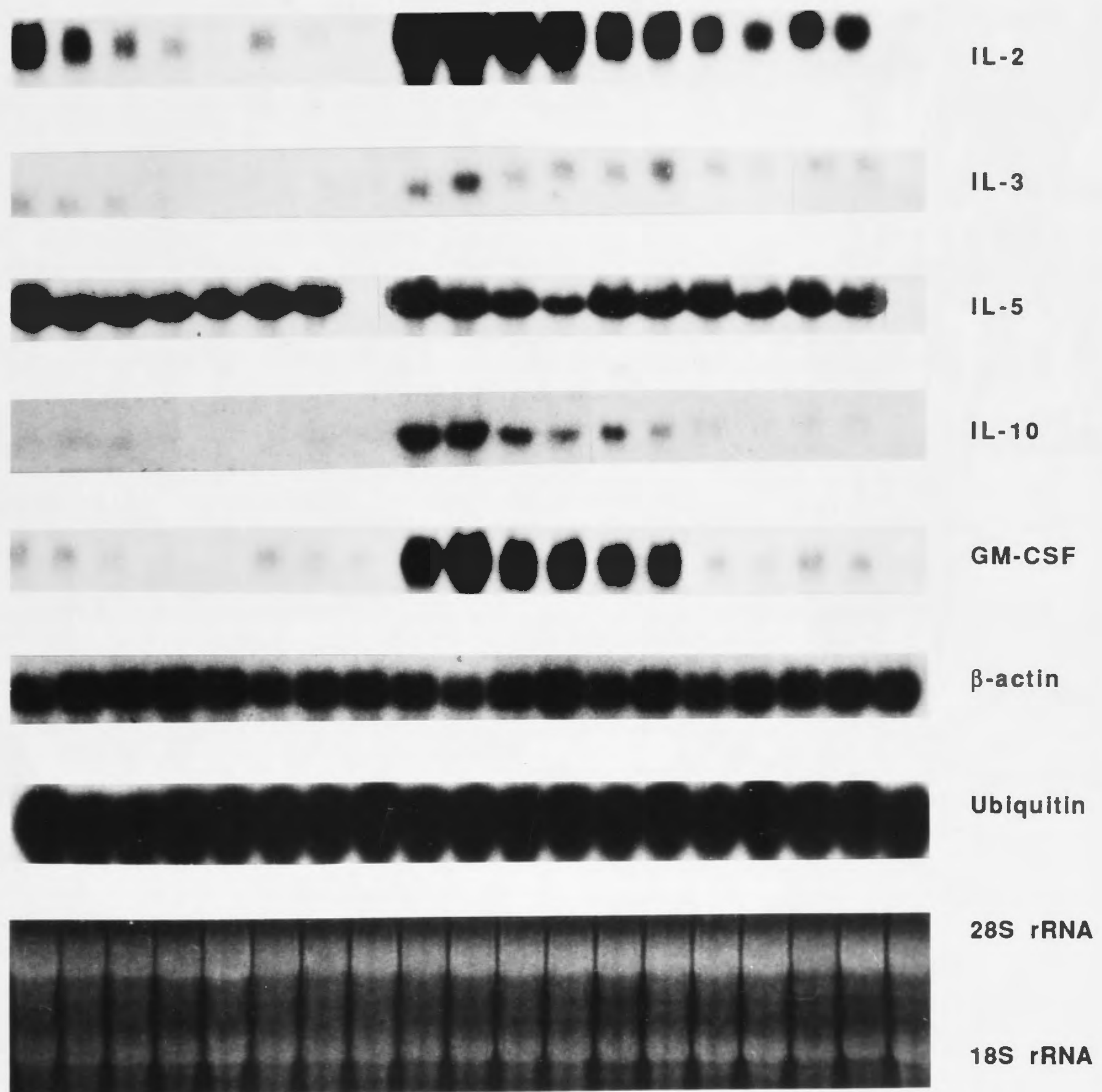
cells may largely involve their stabilization by the Con A-mediated signal. The observed decline in levels of IL-6 and IL-10 mRNAs may, however, be attributable either to their decreased stability and/or to decreases in the transcriptional activity of their genes. In contrast to the other lymphokine mRNAs, the levels of IL-4 and IL-5 mRNAs did not significantly decline when Con A was removed (Lanes a,d).

3.3.4. The signalling requirement of lymphokine mRNA accumulation in EL4.23 cells

The signalling requirement of lymphokine mRNA accumulation was investigated in EL4.23 cells by comparing lymphokine mRNA accumulation patterns between cells which were continually stimulated with PMA for 16 to 20 h (Fig. 3.6., Lanes a,b) and cells which were washed after 12 h stimulation with PMA and subsequently re-cultured for 4 to 8 h in PMA-free medium (Fig. 3.6., Lanes c,d). Levels of IL-2, IL-3, IL-10 and GM-CSF mRNAs noticeably declined after the removal of PMA. In view of the findings that the rate of GM-CSF gene transcription does not significantly change in response to PMA, and that increases in the rate of IL-3 gene transcription may not fully account for accumulation of its mRNA (section 3.3.2.), these observations provide further evidence that the accumulation of IL-3 and GM-CSF mRNAs in EL4.23 cells, as well as in D10.G4.1 cells, may largely involve their stabilization by cellular signals. The decline in IL-2 and IL-10 mRNAs observed upon removal of PMA may, however, be attributed either to their decreased stability and/or to decreases in the transcriptional activity of their genes. In contrast to the other lymphokine mRNAs, IL-5 mRNA levels were not significantly reduced when PMA was removed.

Fig. 3.6. **Northern blot analysis of the suppression, superinduction and stability of lymphokine mRNAs in EL4.23 cells**

EL4.23 cells were stimulated for 16 or 20 h with PMA (50 ng/ml) in the absence or presence, from the outset, of Cs A (1 μg/ml) or CHX (20 μg/ml) (Lanes a,b,e,h). EL4.23 cells were also stimulated for 12 h with PMA and subsequently incubated for a further 4 to 8 h with the addition of Cs A, CHX or Act D (10 μg/ml/) in the continuing presence of PMA (Lanes f,g,i,j,m,n,o,q) or in the absence of PMA (Lanes c,d,k,l,p,r). In the latter case, EL4.23 cells were washed twice in warm medium after stimulation with PMA for 12 h and re-cultured in PMA- free medium. EL4.23 cells were also treated with CHX for 16 h (Lane s). Treatment of EL4.23 cells with the inhibitory agents under the conditions described above was not observed to exert cytotoxic effects. 20 μg of total cellular RNA, extracted from EL4.23 cells treated in the indicated manner, was used to prepare Northern blots which were hybridized with the indicated ^{32}P-labeled cDNA probes.



Time (hours)	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s
Addition of PMA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
Removal of PMA	-	-	12	12	-	-	-	-	-	-	12	12	-	-	-	12	-	12	-
Addition of Cs A	-	-	-	-	0	12	12	-	-	-	-	-	12	12	-	-	-	-	-
Addition of CHX	-	-	-	-	-	-	-	0	12	12	12	12	12	12	-	-	12	12	0
Addition of Act D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	12	12	12	-
Cell harvest	16	20	16	20	16	16	20	16	16	20	16	20	16	20	16	16	16	16	16

3.3.5. The differential Cs A-sensitive signalling requirement of lymphokine mRNA accumulation in D10.G4.1 cells

As previously observed in Chapter 2, Cs A completely inhibited the accumulation of the IL-3, IL-4 and GM-CSF mRNAs in D10.G4.1 cells when present from the outset of Con A stimulation (Fig. 3.5., Lanes a,b). Nuclear run-off analysis indicated that Cs A inhibits the accumulation of these mRNAs at the transcriptional level (Fig. 3.2.(c,f)). The induction of the IL-5, IL-6 and IL-10 mRNAs in response to Con A stimulation was, in contrast, relatively resistant to Cs A (Fig. 3.5., Lanes a,b)), and transcription of their respective genes was not significantly affected by Cs A (Fig. 3.2.(c,f)). These observations suggest that the transcription of the IL-3, IL-4 and GM-CSF genes, but not the IL-5, IL-6 and IL-10 genes, is activated by signals mediated via a Cs A-sensitive pathway.

Although signals mediated via a Cs A-sensitive pathway appear to initially activate IL-3 and IL-4 gene transcription and initially enhance GM-CSF gene transcription in D10.G4.1 cells, the ongoing expression of these genes may require continual signalling via this pathway. To investigate this possibility, lymphokine mRNA accumulation patterns were compared between D10.G4.1 cells which were continually stimulated for 9 h with Con A and cells which were stimulated with Con A for 6 h and then exposed to Cs A for a subsequent 3 h in the continuing presence of Con A. As shown in Fig. 3.5., the addition of Cs A after 6h Con A stimulation significantly reduced the accumulation of IL-3 and GM-CSF mRNAs (Lanes a,c). A similar reduction in levels of these mRNAs was also observed when Con A was removed (Lane d). The small declines in the rates of IL-3 and GM-CSF gene transcription, which were observed when Cs A was added after 6 h Con A stimulation, appeared insufficient to fully account for the reduced

accumulation of their mRNAs (Fig. 3.2.(d,g)). These observations suggest that signals mediated via a Cs A-sensitive pathway not only initially activate IL-3 gene transcription and enhance GM-CSF gene transcription, but may also enhance the stabilization of their mRNAs. The addition of Cs A during the course of Con A stimulation, in contrast, only slightly reduced the accumulation of IL-4 mRNA (Fig. 3.5., Lane c). A significant decrease in IL-4 gene transcription was, however, observed in corresponding nuclear run-off assays (Fig. 3.2.(d,g)). This observation suggests firstly that continual signalling via a Cs A-sensitive pathway is required for ongoing IL-4 gene transcription, as well as initial activation. Secondly, this observation suggests that the IL-4 mRNA is relatively stable, notwithstanding the cessation of continual signalling and the consequent abrogation of ongoing transcription of its gene. The addition of Cs A during the course of Con A stimulation did not significantly affect the transcription of the IL-5, IL-6 or IL-10 genes nor the accumulation of their respective mRNAs (Fig. 3.2.(d,g); Fig. 3.5., Lanes a,c)).

3.3.6. The differential Cs A-sensitive signalling requirement of lymphokine mRNA accumulation in EL4.23 cells

The effect of Cs A upon the initial activation of lymphokine gene expression in EL4.23 cells was investigated by examining the effect of the presence of Cs A, from the outset of PMA stimulation, upon the transcriptional activation of the different lymphokine genes and the induction of their mRNAs. The effect of Cs A upon ongoing lymphokine gene expression, once induction was under way, was investigated by comparing gene transcription and mRNA accumulation patterns between cells continually stimulated with PMA for 16 to 20 h, and cells stimulated with PMA for 12 h and then exposed to Cs A for a subsequent 4 to 8 h in the continual presence of PMA. As shown in Fig. 3.6., the initial induction of IL-5 mRNA (Lanes a,e) and its

subsequent accumulation (Lanes a,b,f,g) were relatively resistant to Cs A. As shown in Fig. 3.4., the initial activation of, and ongoing, IL-5 gene transcription was also resistant to Cs A (Fig. 3.4.(b,d,e,g). This suggests IL-5 gene transcription is regulated by a factor(s) which is responsive to signals mediated via a Cs A-resistant pathway in EL4.23 cells, as well as in D10.G4.1 cells (section 3.3.5.).

Cs A inhibited the induction of the IL-2, IL-3, IL-10 and GM-CSF mRNAs when present from the outset of PMA stimulation (Fig. 3.6., Lanes a,e). The addition of Cs A after 12 h PMA stimulation led to a reduction in the levels of IL-3, IL-10 and GM-CSF mRNAs, and a significant decrease in IL-2 mRNA levels (Fig. 3.6., Lanes a,b,f,g). Nuclear run-off analysis indicated, however, that Cs A abrogates activation of the IL-2 gene transcription, but does not significantly affect transcriptional activities of the IL-10 and GM-CSF genes when present from the outset of PMA stimulation (Fig. 3.4. (b,d)). The addition of Cs A after 12 h PMA stimulation led to a significant reduction in the transcriptional activity of the IL-2 gene, but did not noticeably affect the transcription rates of the IL-10 and GM-CSF genes (Fig. 3.4.(e,g)). The effect of Cs A upon transcription of the IL-3 gene was unable to be determined given the weakness of its activity.

These observations suggest, firstly, that IL-2 gene transcription is induced in EL4.23 cells by a factor(s) which is responsive to signals mediated via a Cs A-sensitive pathway, and which requires continual signalling via this pathway to mediate ongoing transcription. Activation of IL-10 gene transcription appears to be regulated by a factor(s) which is largely responsive to Cs A-resistant signals. The sensitivity of IL-10 mRNA accumulation to Cs A suggests, however, that signals mediated via a Cs A-sensitive pathway may also regulate its expression. Accumulation of GM-CSF mRNA in EL4.23

cells does not appear to involve increases in transcriptional activity, but rather enhanced stabilization of its mRNA mediated by a Cs A-sensitive signalling pathway.

3.3.7. The role of signalling in regulating lymphokine mRNA stability in D10.G4.1 cells

Observations described above suggest that the patterns of accumulation of the IL-4, IL-5, IL-6 and IL-10 mRNAs in D10.G4.1 cells in response to Con A-mediated signals may be attributed, to a large degree, to changing rates in transcription of their respective genes. Mechanisms other than transcription appear to play a significant role in the regulation of GM-CSF and IL-3 mRNA accumulation, and may involve stabilization of their mRNAs. To investigate the stability of lymphokine mRNAs, their accumulation was examined in the absence of ongoing transcription by cellular treatment with actinomycin D (Act D), a non-specific inhibitor of transcription. As evidence of the effectiveness of Act D in blocking transcription, Act D inhibited induction of all six lymphokine mRNAs when added at the outset of stimulation (Fig. 3.5., Lanes n,o).

To investigate the stability of lymphokine mRNAs in D10.G4.1 cells, their accumulation patterns were compared between cells which were stimulated with Con A for 9 h, and cells which were stimulated with Con A for 6 h and subsequently exposed to Act D for another 3 h in the continual presence of Con A. As shown in Fig. 3.5., the levels of IL-4 and IL-5 mRNAs did not significantly decline in the presence of Act D (Lanes a,j), even though Act D was added at a time when their respective genes are maximally transcribed (Fig. 3.2.(c)). This suggests that their mRNAs are relatively stable in the absence of ongoing transcription. IL-6 mRNA levels were slightly reduced in the presence of Act D, while the decline in IL-10 mRNA levels was

more marked (Fig. 3.5., Lanes a,j). The levels of IL-3 and GM-CSF mRNAs did not significantly decline in the presence of Act D. Together with previous observations demonstrating the relatively weak transcriptional inducibility of the IL-3 and GM-CSF genes in response to Con A (Section 3.3.1.), this suggests that stabilization of their mRNAs plays a predominant role in the regulating their expression.

To further investigate the mediation of lymphokine mRNA stabilization by T cell activation signals, lymphokine mRNA levels were compared between D10.G4.1 cells which were stimulated for 6 h with Con A, and exposed to Act D for a subsequent 3 h either in the absence or continual presence of Con A. As shown in Fig. 3.5., the levels of IL-4 and IL-5 mRNAs in the presence of Act D did not significantly differ whether Con A was removed or remained present (Lanes j,k). This suggests that these two species are relatively stable in the absence of ongoing transcription, as well as in the absence of continual signalling (section 3.3.3.). The levels of the other lymphokine mRNAs in the presence of Act D did not significantly differ whether Con A was removed or remained present. It was previously observed, however, that, in the absence of Act D, accumulation of IL-3, IL-6 and GM-CSF mRNAs declined rapidly upon removal of Con A (Lane d). Act D treatment alone therefore appears to stabilize these mRNAs. These observations together imply the existence of two possible mechanisms which may coordinately regulate the stability of the IL-3, IL-6 and GM-CSF mRNAs in D10.G4.1 cells. One mechanism may involve enhancement of stability of these specific mRNAs by the Con A-mediated signal. Stabilization of these mRNAs by Act D may be possibly due its suppression of a labile mechanism which mediates their degradation.

3.3.8. The role of signalling in regulating lymphokine mRNA stability in EL4.23 cells

Observations described above suggest that the patterns of accumulation of the IL-2, IL-5 and IL-10 mRNAs in EL4.23 cells in response to PMA-mediated signals may be attributable, to a large degree, to changing rates in transcription of their respective genes. Mechanisms other than transcription appear to play a significant role in the regulation of GM-CSF and IL-3 mRNA accumulation, and may involve stabilization of their mRNAs. To examine the stability of lymphokine mRNAs in EL4.23 cells, their accumulation patterns were compared between cells which were stimulated with PMA for 16 h, and cells which were stimulated with PMA for 12 h and exposed to Act D for a subsequent 4 h in the continual presence of PMA. As shown in Fig. 3.6., the levels of IL-2, IL-5 and IL-10 mRNA did not significantly decline in the presence of Act D (Lanes a,o), even though Act D was added at a time when their respective genes are maximally transcribed (Fig. 3.4.(b)). This suggests that their mRNAs are relatively stable in the absence of ongoing transcription. The levels of IL-3 and GM-CSF mRNA were also not significantly affected by the presence of Act D (Fig. 3.6., Lanes a,o).

To investigate the mediation of lymphokine mRNA stabilization by PMA signals in EL4.23 cells, lymphokine mRNA levels were compared between cells which were stimulated for 12 h with PMA and exposed for a subsequent 4 h to Act D, either in the absence or continual presence of PMA. As shown in Fig. 3.6., the levels of IL-5 mRNA in the presence of Act D did not significantly differ whether PMA was removed or remained present (Lanes o,p). This provides further evidence that the IL-5 mRNA is relatively stable in the absence of ongoing transcription or continual signalling. The levels of the IL-2, IL-3, IL-10 and GM-CSF mRNA were marginally higher

when PMA was present rather than absent during the course of exposure to Act D (Lanes o,p), suggesting that PMA signals may stabilize these mRNAs. The levels of these mRNAs did not, however, significantly decline in the presence of Act D alone, even when PMA was removed. This suggests that Act D itself may stabilize these mRNAs possibly, as previously described in section 3.3.7., by suppressing the activity of a specific degradative mechanism.

3.3.9. Requirement of *de novo* protein synthesis for induction of lymphokine gene expression in D10.G4.1 cells

As previously described in section 1.1.4., cellular signals may activate the expression of eukaryotic genes by either inducing the synthesis of factors which regulate their transcription, or by presumably modifying, and thus activating, pre-existing factors. The mechanisms by which T cell activation signals activate transcription of different lymphokine genes in D10.G4.1 cells were investigated by examining mRNA accumulation and gene transcription patterns when the protein synthesis inhibitor CHX was added at the outset of Con A stimulation. As shown in Fig. 3.5., CHX completely inhibited the induction of IL-5 mRNA when present from the outset of Con A stimulation (Lanes a,e). Nuclear run-off assays demonstrated that CHX completely inhibits the activation of IL-5 gene transcription in response to Con A (Fig. 3.2.(c,h)). This suggests that a factor(s) (or subunit of a factor), which is obligatory for the induction of IL-5 gene transcription, is synthesized *de novo* in response to Con A-mediated signals. CHX, in contrast, did not significantly affect the transcriptional activity of the IL-6 gene nor accumulation of its mRNA. This suggests that enhancement of IL-6 gene transcription is largely mediated by pre-existing factors whose activity is induced in response to Con A-mediated signals.

The presence of CHX from the outset of Con A stimulation inhibited the induction of GM-CSF mRNA (Fig. 3.5., Lanes a,e) and reduced the rate of its gene transcription to that observed in unstimulated D10.G4.1 cells (Fig. 3.2.(c,h)). This suggests that factors synthesized *de novo* in response to Con A stimulation enhance the transcriptional activity of the GM-CSF gene. CHX significantly reduced the rate of IL-4 gene transcription, and partially decreased the transcriptional activity of the IL-10 gene (Fig. 3.2.(c,h)). IL-4 mRNA levels induced by Con A were partially inhibited by CHX, while the inhibitory effect of CHX upon IL-10 mRNA accumulation was less marked (Fig. 3.5., Lanes a,e). These observations suggest that maximal transcriptional activation of the IL-4 and IL-10 genes in response to Con A stimulation requires protein synthesis. The accumulation of IL-3 mRNA, in contrast, was noticeably elevated when CHX was present from the outset of Con A stimulation (Fig. 3.5., Lanes a,e). This increase in IL-3 mRNA levels was not accompanied by any significant change in the transcriptional activity of the IL-3 gene (Fig. 3.2.(c,h)). This observation suggests, firstly, that transcriptional activation of IL-3 gene expression involves pre-existing factors and, secondly, that CHX may stabilize IL-3 mRNA.

3.3.10. Requirement of *de novo* protein synthesis for induction of lymphokine gene expression in EL4.23 cells

The requirement of *de novo* protein synthesis for the induction of lymphokine gene expression was investigated in EL4.23 cells by examining the effect of CHX, when present from the outset of PMA stimulation, upon lymphokine mRNA accumulation and gene transcription. As shown in Fig. 3.6., CHX severely inhibited the accumulation of IL-2 and IL-5 mRNAs and partially inhibited the induction of IL-10 mRNAs (Lanes a,h). Nuclear run-off assays demonstrated that CHX virtually abolishes the transcriptional

activation of the IL-2 and IL-5 genes, and partially inhibits the activation of IL-10 gene transcription (Fig. 3.4.(b,c)). This suggests that factors which are obligatory for the activation of IL-2 and IL-5 gene transcription are synthesized *de novo* in response to PMA stimulation. Protein synthesis also appears to be required to fully potentiate IL-10 gene transcription. CHX also inhibited the induction of IL-3 mRNA (Fig. 3.6., Lane h). The effect of CHX upon the activation of IL-3 gene transcription was unable to be determined given the weakness of its activity. The presence of CHX from the outset of PMA stimulation did not significantly affect GM-CSF mRNA accumulation nor the rate of GM-CSF gene transcription.

3.3.11. "Superinduction" of specific lymphokine mRNAs by suppression of protein synthesis in D10.G4.1 cells

As previously described in section 3.1., several studies have reported that the accumulation of certain lymphokine mRNAs may be enhanced in response to cellular treatment with protein synthesis inhibitors, once their induction was under way. The nature and specificity of this phenomenon, generally referred to as "superinduction", was investigated in D10.G4.1 cells by examining mRNA accumulation and gene transcription patterns of different lymphokines in cells which were stimulated with Con A for 6 h and then exposed to CHX for a subsequent 3 to 6 h in the continual presence of Con A. As shown in Fig. 3.5., the addition of CHX after 6 h Con A stimulation led to significant increases in levels of IL-3 and IL-6 mRNAs, and a particularly dramatic increase in GM-CSF mRNA levels (Lanes a,f,g). The levels of GM-CSF mRNA induced by CHX remained high at times when steady state levels in the absence of CHX were almost undetectable (Fig. 3.1.). The addition of CHX, however, did not noticeably affect the transcriptional activity of the IL-3, IL-6 and GM-CSF genes (Fig. 3.2.(d,i)).

These observations suggest that the "superinduction" of their mRNAs by CHX may be attributable to decreases in the rate of their degradation. This may presumably involve the suppression by CHX of a labile factor(s) which mediates their specific degradation.

In contrast to the GM-CSF, IL-3 and IL-6 genes, significant decreases in the rates of IL-4 and IL-5 gene transcription were observed when CHX was added after 6 h Con A stimulation (Fig. 3.2.(d,i)). This suggests that ongoing IL-4 and IL-5 gene transcription requires continual synthesis of their regulatory factors. The addition of CHX after 6 h Con A stimulation did not, however, significantly affect the accumulation of IL-5 mRNA and only slightly reduced the levels of IL-4 mRNA accumulation (Fig. 3.5., Lanes a,f,g). This supports previous observations which suggest that the IL-4 and IL-5 mRNAs are relatively stable, compared to those of other lymphokines, notwithstanding the abrogation of ongoing transcription of their genes (section 3.3.7.). IL-10 gene expression was slightly inhibited by the addition of CHX, an observation which appears consistent with the partial requirement of protein synthesis for potentiating its transcriptional activity.

To verify that CHX enhances the stability of specific lymphokine mRNAs in D10.G4.1 cells, accumulation patterns were compared between cells which were stimulated with Con A for 6 h and subsequently exposed to Act D for 3 h either in the absence or presence of CHX. As shown in Fig. 3.5., the levels of IL-4, IL-5 and IL-10 mRNAs in the absence of ongoing transcription were not noticeably affected by CHX (Lanes j,l). The levels of IL-3, IL-6 and GM-CSF mRNAs were, however, noticeably higher in the presence of Act D and CHX, than in the presence of Act D alone. The levels of IL-3, IL-6 and GM-CSF mRNAs in the presence of Act D and CHX did not noticeably differ whether Con A was continually present or absent (Lanes l,m). These

observations provide further evidence that CHX alone enhances the stabilization of these specific mRNAs. As previously described in section 3.3.7., the stabilization of IL-3, IL-6 and GM-CSF mRNAs by Act D alone may involve suppression by Act D of the expression of a putative labile factor which mediates their specific degradation. The combination of Act D and CHX may be presumably more effective in inhibiting such a factor by blocking its synthesis at both the transcriptional and translational levels. Act D treatment alone may inhibit transcription of the gene encoding this putative factor, but will not prevent the conversion of its steady state translatable mRNAs into functional proteins.

Treatment of D10.G4.1 cells with CHX alone induced low levels of IL-6, GM-CSF and IL-10 mRNAs which were detectable after prolonged autoradiographic exposure (Lane q). This induction was not attributable to increases in transcription (data not shown). Other lymphokine mRNAs were, however, not induced by CHX alone. These observations suggest that CHX may stabilize the low levels of IL-6, GM-CSF and IL-10 transcripts which are constitutively expressed in unstimulated D10.G4.1 cells (Fig. 3.2.(a)). The labile mechanism which degrades specific lymphokine mRNAs may therefore be constitutively active. Although CHX alone appears to stabilize IL-10 transcripts in unstimulated D10.G4.1 cells, CHX did not appear to noticeably enhance IL-10 mRNA levels, once their induction in response to Con A was under way (Fig. 3.5., Lanes f,g). This may be due to the inhibition by CHX of protein synthesis which appears to be required for maximizing the rate of IL-10 gene transcription in response to Con A stimulation (section 3.3.9.).

As shown in Fig. 3.5., the level of “superinduction” of IL-3 mRNA by CHX was significantly reduced in the presence of Cs A (Lane h). The small decline in the rate of IL-3 gene transcription which was observed when Cs A

was present during the course of CHX treatment appeared insufficient to account for the decline in IL-3 mRNA levels (Fig. 3.2.(j)). This provides further evidence that Cs A-sensitive signals are required for the stabilization of IL-3 mRNA. The level of "superinduction" of GM-CSF mRNA by CHX was, however, only slightly reduced in the presence of Cs A (Fig. 3.5., Lanes f,h). This suggests that Cs A-sensitive signals may stabilize IL-3 mRNA more efficiently than GM-CSF mRNA, and may account for the low steady state levels of GM-CSF mRNA, compared to those of IL-3 mRNA, which accumulate in response to Con A stimulation (Fig. 3.1.).

The levels of "superinduction" of IL-6 mRNA by CHX was, in contrast to the IL-3 and GM-CSF mRNAs, not noticeably affected by Cs A (Fig. 3.5., Lanes f,h). The absence of the Con A stimulus during the course of CHX treatment, however, significantly reduced the level of IL-6 mRNA "superinduction" (Lanes f,i). This suggests that IL-6 mRNA may be stabilized by Cs A-resistant signals. Given that Cs A-resistant signals also appear to regulate IL-6 gene expression at the transcriptional level (section 3.3.5.), the observed reduction in levels of IL-6 mRNA "superinduction" by the abrogation of continual signalling may not only be attributable to a reduction in mRNA stability, but also to a reduction in transcriptional activity.

3.3.12. "Superinduction" of specific lymphokine mRNAs by suppression of protein synthesis in EL4.23 cells

The nature and specificity of mRNA "superinduction" was investigated in EL4.23 cells by comparing mRNA accumulation and gene transcription patterns of different lymphokines between cells which were continually stimulated with PMA for 16 to 20 h, and cells which were stimulated with PMA for 12 h and then exposed to CHX for a subsequent 4 to 8 h in the continual

presence of PMA. As shown in Fig. 3.6., the addition of CHX after 12 h PMA stimulation led to dramatic increases in levels of GM-CSF mRNA (Lanes a,b,i,j). Quantitation of the relative intensities of the hybridization signals by PhosphoImager analysis detected an almost 40-fold increase in GM-CSF mRNA levels after 8 h exposure to CHX. The addition of CHX, however, did not noticeably affect the rate of GM-CSF gene transcription (Fig. 3.4.(e,f)). These observations suggest that the "superinduction" of GM-CSF mRNA by CHX may be attributable to decreases in the rate of its degradation. This may presumably involve the suppression by CHX of a labile factor(s) which mediates its specific degradation.

Given the requirement of signalling for the stabilization of GM-CSF mRNA in EL4.23 cells (section 3.3.8.), the effect of signalling abrogation upon its "superinduction" by CHX was investigated. As shown in Fig. 3.6., the levels of GM-CSF mRNA were slightly reduced when Cs A was present during the course of CHX treatment (Lanes i,j,m,n), but remained nevertheless signficantly high. These observations suggest that the efficiency of GM-CSF mRNA stabilization by Cs A-sensitive signals may be only slightly higher than the efficiency of its degradation by a labile mechanism. This may account for the low steady state levels of GM-CSF mRNA which accumulate in EL4.23 cells in response to PMA, as well as in Con A-stimulated D10.G4.1 cells (section 3.3.11.).

As shown in Fig. 3.6., the addition of CHX to EL4.23 cells after 12 h PMA stimulation also led to increases in IL-3 mRNA levels and significant elevation in levels of IL-2 and IL-10 mRNAs (Lanes a,b,i,j). In contrast to the GM-CSF gene, increases in the transcriptional activities of the IL-3 and IL-10 genes and a significant elevation in the rate of IL-2 gene transcription were observed in response to the addition of CHX during the course of PMA

stimulation (Fig. 3.4.(e,f)). These observations suggest that the "superinduction" of the IL-2, IL-3 and IL-10 mRNAs by CHX may be attributable, at least in part, to stimulation in the transcriptional activities of their genes. This may involve the suppression by CHX of a labile repressor which regulates their transcription.

As shown in Fig. 3.4., the increases in IL-2 and IL-3 gene transcription which were induced by CHX, were almost completely abolished by Cs A (f,h). This is consistent with previous observations which suggest that the expression of these two genes are largely regulated by signals mediated via a Cs A-sensitive pathway (section 3.3.6.). The presence of Cs A during the course of CHX treatment did not, however, reduce the accumulation of the IL-2 and IL-3 mRNAs to the same extent as transcription of their genes (Fig. 3.6., Lanes i,j,m,n). This raises the possibility that the enhanced accumulation of their mRNAs in the presence of CHX may also be attributable to their stabilization by CHX, as well as increases in transcriptional activity.

The stimulation of IL-10 gene transcription by CHX, and the accumulation of its mRNA was also noticeably reduced by Cs A (Fig. 3.4.(f,h); Fig. 3.6., Lanes i,j,m,n). The addition of Cs A from the outset or during the course of PMA stimulation was previously found, however, not to significantly affect the rate of IL-10 gene transcription (Fig. 3.4.(b,d,e,g)). This observation raises the possibility that Cs A-sensitive signals, as well as Cs A-resistant signals, may regulate the expression of the IL-10 gene in EL4.23 cells.

In contrast to the IL-2, IL-3 and IL-10 genes, the transcriptional activity of the IL-5 gene in EL4.23 cells was almost completely abolished when CHX was added during the course of PMA stimulation (Fig. 3.4.(e,f)). This

suggests that ongoing IL-5 gene transcription in EL4.23 cells, as well as in D10.G4.1 cells (section 3.3.11.), requires continual synthesis of its specific regulatory factor(s). The accumulation of IL-5 mRNA was, however, not significantly affected by the addition of CHX (Fig. 3.6., Lanes a,b,i,j). This supports previous observations which suggest that the IL-5 mRNA is relatively stable, compared to those of other lymphokines, notwithstanding the cessation of transcription of its gene (section 3.3.8.).

To further verify the nature of the mechanisms by which CHX "superinduces" the levels of GM-CSF, IL-2, IL-3 and IL-10 mRNAs, their accumulation patterns were compared between EL4.23 cells which were stimulated with PMA for 12 h and subsequently exposed to Act D for 4 h either in the absence or presence of CHX. As shown in Fig. 3.6., the levels of GM-CSF, IL-2, IL-3 and IL-10 mRNAs were noticeably higher in the presence of Act D and CHX (Lane q), than in the presence of Act D alone (Lane o). Levels of IL-5 mRNA, in the absence of ongoing transcription, were not significantly affected by CHX. The levels of GM-CSF, IL-2, IL-3 and IL-10 mRNAs in the presence of Act D and CHX did not noticeably differ whether PMA was continually present or absent (Lanes q,r). These observations provide further evidence that CHX alone may stabilize GM-CSF mRNA and also IL-2, IL-3 and IL-10 mRNAs. "Superinduction" of the IL-2, IL-3 and IL-10 mRNAs by CHX may therefore not only involve increases in the transcriptional activities of their genes, but also a reduction in the rate of their degradation. As previously described in sections 3.3.8. and 3.3.11., the stabilization of the GM-CSF, IL-2, IL-3 and IL-10 mRNAs by Act D treatment alone may involve suppression of the expression of a labile factor(s) which mediates their degradation. The combination of Act D and CHX may be presumably more effective in enhancing mRNA stabilization by their inhibiting the expression of such a "degradative" factor at both the transcriptional and translational levels.

As shown in Fig. 3.6., treatment of EL4.23 cells with CHX alone induced low levels of GM-CSF mRNA (Lane s). This induction was not due to an increase in the rate of GM-CSF gene transcription (data not shown). Other lymphokine mRNAs were not induced by CHX in the absence of PMA stimulation. This observation suggests that CHX may stabilize the GM-CSF transcripts which are constitutively expressed in unstimulated EL4.23 cells (Fig. 3.4.(a)). The labile mechanism which degrades specific mRNAs in EL4.23 cells, as well as in D10.G4.1 cells (section 3.3.11.), appears to be constitutively active.

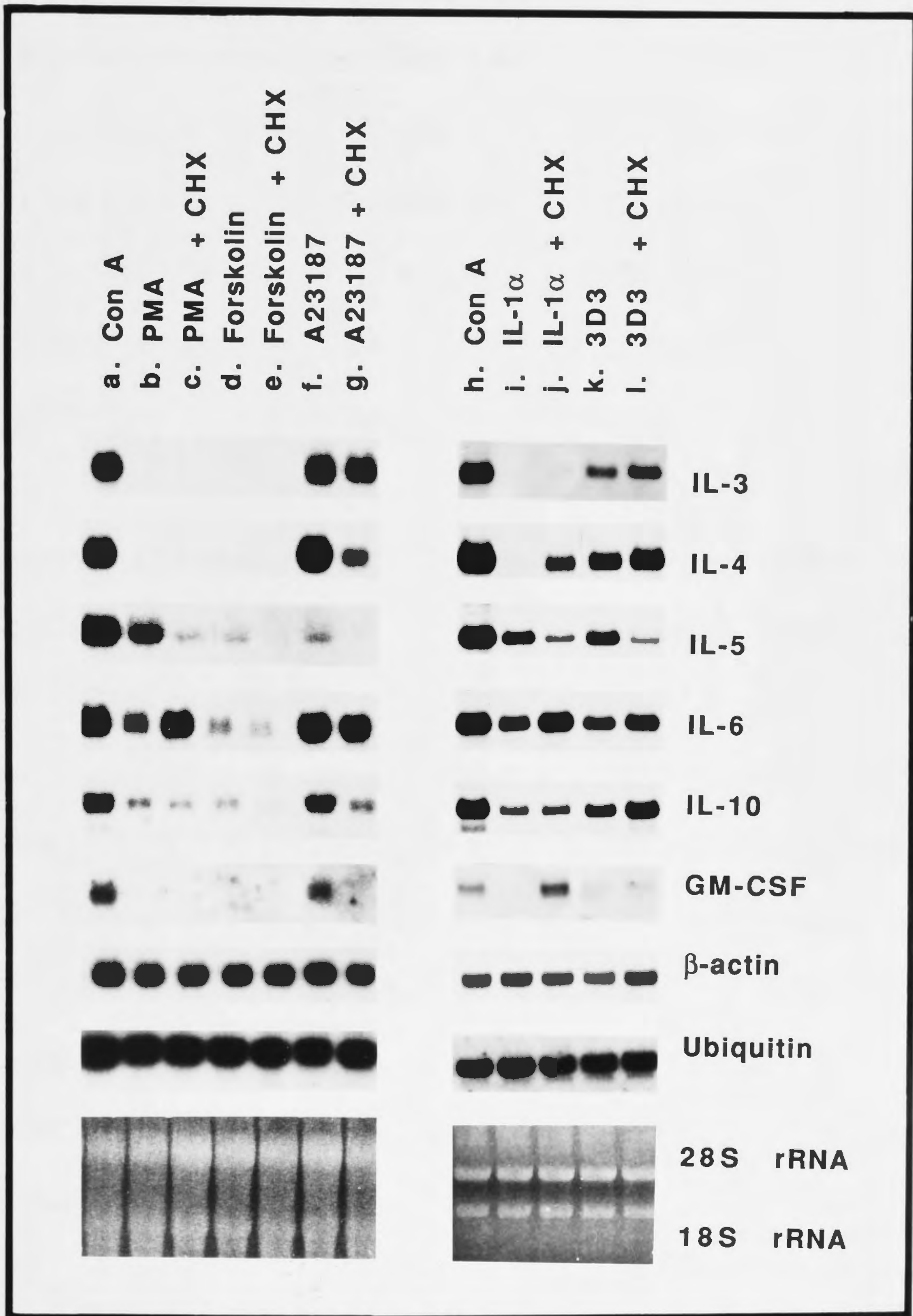
3.3.13. Selective activation of lymphokine gene transcription by different signalling pathways in D10.G4.1 cells

Studies described in Chapter 2 suggested that intracellular signal transduction pathways emanating from stimulation of the TCR/CD3 complex and the IL-1 receptor may differentially regulate the accumulation of lymphokine mRNAs in D10.G4.1 cells. The mechanisms by individual, and distinct groups of, lymphokine genes are induced in response to these specific signalling pathways were further investigated by Northern blot and nuclear run-off analyses.

As previously observed in Chapter 2, the GM-CSF, IL-3, IL-4, IL-5, IL-6 and IL-10 mRNAs were coordinately induced in D10.G4.1 cells in response to cellular stimulation by either Con A, the antigen recognition site-specific antibody 3D3 or the calcium ionophore A23187 (Fig. 3.7.). Observations described in section 3.3.1. suggest that the induction of these mRNAs in response to Con A may be attributable, at least in part, to increases in the transcriptional activity of their respective genes. As shown in Fig. 3.7., A23187 stimulation induced levels of GM-CSF, IL-3, IL-4, IL-6 and IL-10

Fig. 3.7. Induction of lymphokine mRNAs in D10.G4.1 cells by lectin, antigen recognition site-specific antibody or IL-1 receptor stimulation, PKC or adenylate cyclase activation or elevation in $[Ca^{2+}]_i$, and the effect of protein synthesis inhibition upon their induction.

Total cellular RNA was prepared from D10.G4.1 cells stimulated for 3 h at 2 x 10^6 cells/ml with either Con A (5 μg/ml), PMA (10 ng/ml), forskolin (20 μM), A23187 (1 μg/ml), IL-1α (1 ng/ml) or 3D3 (1:1000 dilution). Cells were also stimulated with these activators in the continual presence of CHX (20 μg/ml) from the outset of stimulation. mRNA accumulation was analysed by Northern blot analysis as previously described.


a. Con A
b. PMA
c. PMA + CHX
d. Forskolin
e. Forskolin + CHX
f. A23187
g. A23187 + CHX
h. Con A
i. IL-1α
j. IL-1α + CHX
k. 3D3
l. 3D3 + CHX
IL-3
IL-4
IL-5
IL-6
IL-10
GM-CSF
β-actin
Ubiquitin
28S rRNA
18S rRNA

mRNAs comparable to those optimally induced by Con A. Significant increases in the transcription rates of their genes were observed in response to A23187 (Fig. 3.8.(f)). Only a very small increase in IL-5 gene transcription and a low level of IL-5 mRNA accumulation were induced by A23187. This suggests that signals other than Ca^{2+} mobilization are required to fully potentiate IL-5 gene transcription. The relative increases in transcription of the IL-3, IL-4, IL-5, IL-6 and IL-10 genes in response to 3D3 stimulation broadly correlated with the relative increases in accumulation of their mRNAs (Fig. 3.7., Lane k; Fig. 3.8.(j)). Only a very low level of GM-CSF mRNA was detected in response to 3D3 and an alteration in the transcriptional activity of its gene was not noticeable by nuclear run-off analysis.

As previously observed in Chapter 2, the IL-5, IL-6 and IL-10 mRNAs were selectively induced by cellular stimulation with either PMA, forskolin or IL-1α (Fig. 3.7., Lanes b,d,i). Stimulation with either PMA, forskolin or IL-1α increased the transcriptional activity of the IL-5, IL-6 and IL-10 genes (Fig. 3.8.(b,d,h)). The basal level of GM-CSF gene transcription did not alter in response to stimulation by any of these agents, while the IL-3 and IL-4 genes remained transcriptionally silent. This suggests that the selective activation of IL-5, IL-6 and IL-10 gene expresesion by the PKC-, cAMP- or IL-1- dependent signalling pathways is predominantly regulated at the transcriptional level.

3.3.14. Involvement of newly synthesized and pre-existing factors in the signal-specific activation of lymphokine gene transcription in D10.G4.1 cells

To investigate whether these signalling pathways activate the expression of different lymphokine genes by inducing the synthesis of

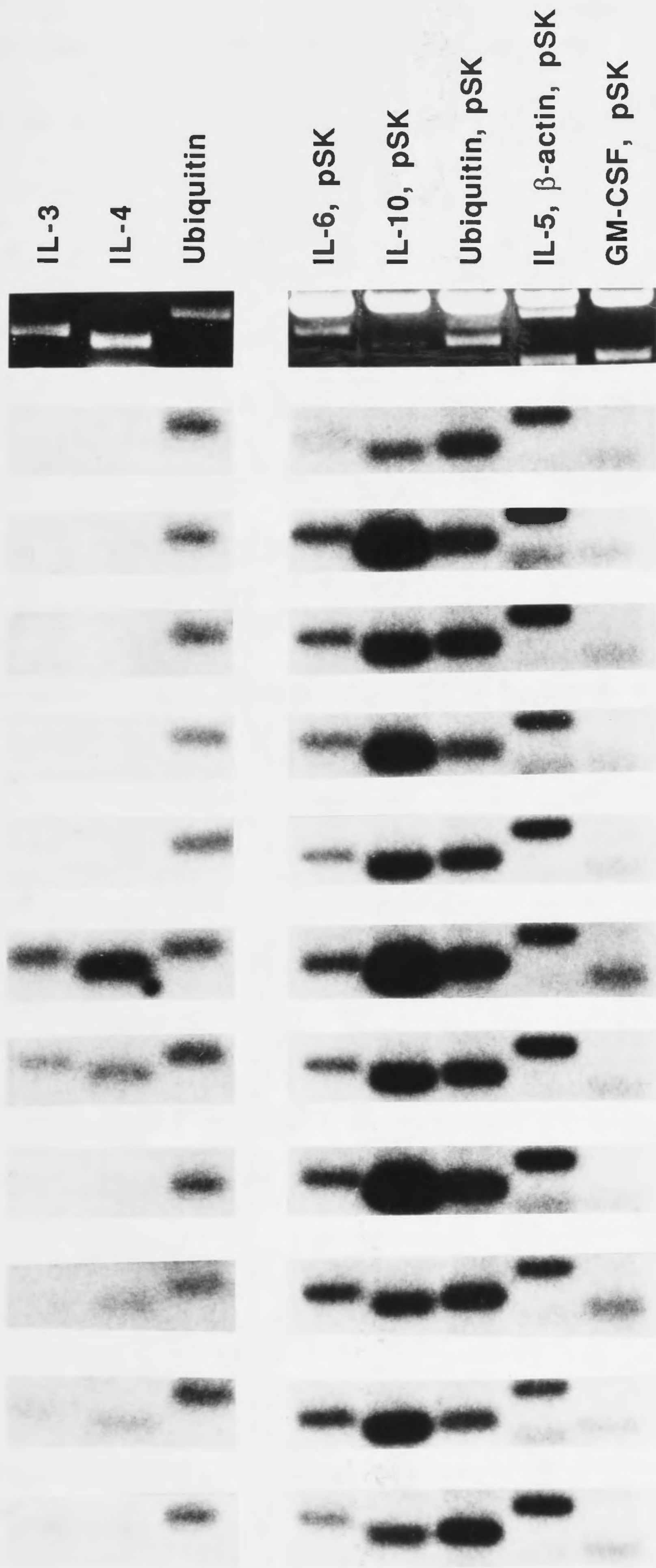
regulatory factors or by activating pre-existing factors, the effect of CHX upon their transcriptional activity and mRNA accumulation was examined. As shown in Fig. 3.7., the induction of IL-5 mRNA by either PMA, forskolin, A23187, IL-1α or 3D3 was inhibited by CHX when present from the outset of stimulation by these agents. CHX completely inhibited the activation of IL-5 gene transcription in response to stimulation by all of these agents (Fig. 3.8.). This suggests that the activation of IL-5 gene transcription by pathways which emanate from TCR/CD3 or IL-1 receptor stimulation and which involve either Ca^{2+} mobilization, PKC or adenylate cyclase activation requires the induced synthesis of an obligatory transcription factors(s).

The presence of CHX from the outset of A23187 stimulation inhibited the induction of GM-CSF mRNA and partially inhibited levels of IL-4 mRNA (Fig. 3.7., Lanes f,g). IL-3 mRNA levels were, however, not significantly affected by CHX. CHX reduced the rate of GM-CSF gene transcription to that observed in unstimulated D10.G4.1 cells (Fig. 3.8.(a,g)), and decreased the level of IL-4 gene transcription. The activation of IL-3 gene transcription in response to A23187 was not significantly affected by CHX. These observations suggest that the Ca^{2+} signal induces the synthesis of a factor(s) which enhances the transcriptional activity of the GM-CSF gene, and activates a pre-existing factor(s) which induces IL-3 gene transcription. Maximal activation of IL-4 gene transcription in response to the Ca^{2+} signal appears to require protein synthesis. The presence of CHX from the outset of 3D3 stimulation, however, led to small increases in the levels of IL-3, IL-4 and GM-CSF mRNAs without any noticeable increase in the transcriptional activity of their respective genes (Fig. 3.7., Lanes k,l; Fig. 3.8.(j,k)).

Although IL-1α did not activate GM-CSF or IL-4 gene transcription (Fig. 3.8.(h)) nor induce their mRNAs (Fig. 3.7., Lanes i), the addition of

Fig. 3.8. Differential activation of lymphokine gene transcription by multiple signalling pathways in D10.G4.1 cells

Nuclei were isolated from unstimulated D10.G4.1 cells (a) or cells stimulated for 3 h with either PMA, forskolin, A23187, IL-α or 3D3 (b,d,f,h,j). Cells were also stimulated with these activators in the continual presence of CHX from the outset of stimulation (c,e,g,i,k). Cells were treated with these agents under the conditions previously indicated in Fig. 3.7. Nascent RNA, initiated *in vivo*, were ^{32}P-labeled by elongation in isolated nuclei *in vitro*, extracted and hybridized to the indicated sequences. The IL-3, IL-4, Ubiquitin, IL-6, IL-10, Ubiquitin, β-actin, IL-5 and GM-CSF target sequences (from left to right) were, respectively, 467 bp *Hind* III/*Nco* I, 242 bp *Rsa* I/*Pst* I, 594 bp *Bgl* II/*Hind* III, 827 bp *Nla* IV/*Dra* I, 552 bp *Sac* I/*Pst* I, 594 bp *Bgl* II/*Hind* III, 2.1 kb *Xho* I, 304 bp *Sty* I and 334 bp *Hae* III fragments. These sequences were derived from the original plasmids described in sections 2.2.1.(3) and 3.2.1.(2), sub-cloned into the pSK vector, re-isolated by restriction digestion and agarose gel electrophoresis, as shown, and immobilized on filters.



a. unstimulated

b. PMA

c. PMA + CHX

d. Forskolin

e. Forskolin + CHX

f. A23187

g. A23187 + CHX

h. IL-1α

i. IL-1α + CHX

j. 3D3

k. 3D3 + CHX

CHX, at the outset of IL-1α stimulation, induced noticeable increases in the transcriptional activity of these two genes (Fig. 3.8.(i)) and significant accumulation of their mRNAs (Fig. 3.7., Lane j). Transcription of the IL-3 gene and accumulation of its mRNA, which are also not responsive to IL-1α stimulation alone, were not induced by CHX under the same conditions. These observations suggest that a labile transcriptional repressor may selectively inhibit the expression of the GM-CSF and IL-4 genes, but not the IL-3 gene, in IL-1-stimulated cells. CHX did not induce GM-CSF or IL-4 gene expression in cells stimulated with either PMA or forskolin which alone, like IL-1α, are unable to activate their expression (Fig. 3.7., Lanes b-e; Fig. 3.8.(b-e)). This suggests that the mechanism which suppresses GM-CSF and IL-4 gene transcription in IL-1-stimulated cells may be independent of the PKC- and cAMP- dependent signalling pathways.

Increases in IL-10 gene transcription activated by either PMA, forskolin, A23187, IL-1α or 3D3 were partially inhibited by CHX (Fig. 3.8.). The levels of IL-10 mRNA induced by PMA, forskolin or A23187 were partially inhibited by CHX to varying degrees, while corresponding decreases in IL-1α- or 3D3-induced mRNA levels were not observed (Fig. 3.7.). Increases in IL-6 gene transcription activated by either PMA, forskolin, A23187, IL-1α or 3D3 were not noticeably affected by CHX. Levels of IL-6 mRNA induced by PMA, IL-1α or 3D3, but not forskolin nor A23187, were noticeably enhanced by CHX to varying degrees. These observations raise the possibility that these mRNAs may be stabilized by CHX, and also by different signals to different degrees.

3.4. DISCUSSION

A number of recent studies have investigated the molecular mechanisms which regulate the expression of several individual lymphokine genes in activated T cells, in particular those of IL-2, IL-3 and GM-CSF (section 3.1.). Studies which compare the mechanisms by which different lymphokine genes are regulated have, however, been very limited. Previous studies have, furthermore, been largely conducted using transformed T cell lines. As an initial phase of a longer term project to understand how numerous, coordinately expressed lymphokine genes regulate immune responses as a "network", the studies described in this chapter sought to investigate how the expression of a variety of different lymphokine genes are regulated in both non-transformed and transformed T cells. These studies were conducted in the Th2 clone D10.G4.1 and also the transformed T cell line EL4.23. As well as the regulation of the IL-2, IL-3 and GM-CSF genes, the studies examined the regulation of the IL-4, IL-6 and IL-10 genes and, in particular, that of the IL-5 gene, the mechanisms of which were unknown.

Studies described in this chapter suggest that the accumulation of each different lymphokine mRNA species is dynamically regulated by the relative rates of its synthesis and degradation. This involves distinct and specific sets of transcriptional and/or post-transcriptional control mechanisms. The expression of certain lymphokine genes, such as those of IL-4, IL-5, IL-6 and IL-10, appear to be predominantly regulated at the transcriptional level, while the regulation of GM-CSF gene expression appears, in contrast, to largely involve modulation of its mRNA stability. Expression of certain other genes, such as those of IL-2 and IL-3, appear to be regulated at both the transcriptional level and the level of mRNA stability to different degrees.

These studies also revealed that the expression of many lymphokine genes may be dynamically regulated at either the transcriptional or post-transcriptional level by the relative activities of positive and negative regulatory factors. Expression of the IL-2 gene, for example, appears to be regulated by coordinately acting factors which activate and repress its transcription, while the regulation of GM-CSF gene expression appears to involve mechanisms which coordinately enhance stabilization and mediate degradation of its mRNA. The specific factors which regulate the expression of different lymphokine genes at either the transcriptional or post-transcriptional level were also distinguished by whether their synthesis was induced *de novo* in response to specific T cell activation signals, or whether they were pre-existing factors whose activities were modulated by specific signalling pathways. The nature and characteristics of the mechanisms which appear to predominantly regulate the expression of the variety of lymphokine genes in D10.G4.1 and EL4.23 cells are summarized in Table 3.1., and are described in further detail below.

3.4.1. Transcriptional regulation of lymphokine gene expression: Involvement of multiple positively and negatively acting mechanisms

Northern blot analysis indicated that the kinetics of mRNA accumulation are largely unique to each lymphokine species. IL-6 and IL-10 mRNAs were rapidly induced in D10.G4.1 cells soon after the outset of Con A stimulation, while maximal levels of IL-3, IL-4 and IL-5 mRNAs were not attained until after 6 h stimulation (section 3.3.1.). The accumulation of the IL-5, IL-6 and IL-10 mRNAs in D10.G4.1 cells was significantly more prolonged than the IL-3, IL-4 or GM-CSF mRNAs. While maximal levels of IL-2, IL-3, IL-5, IL-10 and GM-CSF mRNAs were attained in EL4.23 cells after 8 to 12 h PMA

Table 3.1. Summary of the nature and characteristics of mechanisms which appear to predominantly regulate the expression of different lymphokine genes in D10.G4.1 and EL4.23 cells

Regulation at either the transcriptional level or at the level of mRNA stability appears, for many lymphokine genes studied, to involve dynamic interplay between positively and negatively acting mechanisms which either activate or repress transcription, or which mediate stabilization or degradation of mRNA.

The mechanisms which appear to activate transcription of different genes in response to cellular stimulation were distinguished by their requirement for protein synthesis. The obligatory requirement of protein synthesis for transcriptional activation of genes such as IL-5 and IL-2 is denoted by **+++**. Other genes such as IL-4 and IL-10 appear to require protein synthesis for maximal transcriptional activation, and the variable degrees of this requirement are denoted by **++** or **+**. Transcriptional activation of genes such as IL-6, for example, appear to be mediated by a pre-existing factor(s) (denoted by **-**) which is presumably activated by the cellular signal. The factors which appear to activate transcription were distinguished by whether their syntheses or activities were responsive to signals mediated by a Cs A-sensitive or Cs A-resistant pathway (denoted by✓). The signalling pathways which appear to enhance the stabilization of specific mRNAs were also distinguished by their Cs A-sensitivity or resistance. A number of lymphokine mRNAs (denoted by ✓) also appeared to be specifically degraded by a labile mechanism.

Cell line: D10.G4.1
Stimulus: Con A

		IL-3	IL-4	IL-5	IL-6	IL-10	GM-CSF
Regulation of transcription							
* Activators	Requirement for protein synthesis	-	++	+++	-	+	+++
	Cs A-sensitive	✓	✓	-	-	-	✓
	Cs A-resistant	-	-	✓	✓	✓	-
* Labile Repressors		-	-	-	-	-	-
Regulation of mRNA stability							
* Signal-dependent stabilization	Cs A-sensitive	✓	-	-	-	-	✓
	Cs A-resistant	-	-	-	?	-	-
* Labile degradative mechanism		✓	-	-	✓	✓	✓

Cell line: EL4.23
Stimulus: PMA

		IL-2	IL-3	IL-5	IL-10	GM-CSF
Regulation of transcription						
* Activators	Requirement for protein synthesis	+++	?	+++	++	-
	Cs A-sensitive	✓	✓	-	-	-
	Cs A-resistant	-	-	✓	✓	-
* Labile Repressors		✓	✓	-	✓	-
Regulation of mRNA stability						
* Signal-dependent stabilization	Cs A-sensitive	?	✓	-	?	✓
	Cs A-resistant	-	-	-	-	-
* Labile degradative mechanism		✓	✓	-	✓	✓

stimulation, the accumulation of IL-5 mRNA was more prolonged than those of other lymphokines (section 3.3.2.).

While no lymphokine mRNAs were detected in unstimulated D10.G4.1 or EL4.23 cells, low levels of GM-CSF gene transcription were detected in these cells. Constitutive transcription of the GM-CSF gene has been extensively reported in a variety of cell types which express this gene. These include T cells (Bickel *et al.,* 1990; Iwai *et al.,* 1992), B cells (Akahane & Plutznik, 1992), macrophages (Thorens *et al.,* 1987), fibroblasts (Koeffler *et al.,* 1988), mast cells (Wodnar-Filipowicz & Moroni, 1990), several myeloid cell lines (Schuler & Cole, 1988) and acute myeloblastic leukemia cells (Ernst *et al.,* 1989a). A significant level of IL-10 gene transcription was observed in unstimulated D10.G4.1 cells, while a low level was also detected in unstimulated EL4.23 cells. A low level of IL-6 gene transcription was also observed in unstimulated D10.G4.1 cells. Other lymphokine genes appeared to be transcriptionally silent in both T cell types in the absence of stimuli.

Nuclear run-off analysis indicated that the patterns of accumulation of the IL-4, IL-5, IL-6 and IL-10 mRNAs in D10.G4.1 cells may be attributable, to a large degree, to changing rates in transcription of their respective genes (section 3.3.1.). The transcriptional activities of these genes appear to be differentially regulated by distinct sets of factors, the synthesis or activities of which appear to be induced by specific signals (Table 3.1.). In contrast to the IL-4, IL-6 and IL-10 genes, the IL-5 gene appears to require signals other than those mediated by Ca^{2+} mobilization to fully potentiate its transcriptional activation. Transcription of the IL-5, IL-6 and IL-10 genes, in contrast to the IL-4 gene, appears to be predominantly regulated by signals mediated via a Cs A-resistant pathway(s). Transcription of these genes may also be selectively and independently induced in response to PKC or adenylate

cyclase activation or IL-1 receptor stimulation (sections 3.3.5.;3.3.13.). The PKC-, cAMP- or IL-1- dependent pathways were previously observed in Chapter 2 to be Cs A-resistant.

In contrast to the IL-6 and IL-10 genes, the activation of IL-5 gene transcription by numerous signalling pathways, including those emanating from TCR/CD3 or IL-1 receptor stimulation, appears to require obligatory *de novo* protein synthesis. This may presumably be that of a transcription factor(s) (or subunit of a factor) (sections 3.3.9.;3.3.14.). Enhancement of IL-6 gene transcription by the same signalling pathways appears to be mediated by a pre-existing factor(s). Protein synthesis appears to be required for maximal transcriptional activation of the IL-4 and IL-10 genes. Maximal transcriptional activation of these genes may require the induction of synthesis of a regulatory factor which is distinct from, and cooperatively acts with, a pre-existing factor. Alternatively, maximal potentiation of transcription may require the enhanced expression of a pre-existing factor in response to specific activation signals. In contrast to the IL-10 gene, the syntheses and activities of the factors which regulate IL-4 gene transcription appear to be specifically regulated by a Cs A-sensitive Ca^{2+}-dependent pathway (sections 3.3.5.; 3.3.14.).

Cessation of the syntheses and/or activities of the factors which specifically regulate the transcription of the IL-4, IL-5, IL-6 and IL-10 genes was observed to rapidly abrogate the transcriptional activities of these genes. In contrast to the IL-6 and IL-10 genes, abrogation of IL-4 and IL-5 gene transcription did not lead to a significant reduction in their steady state mRNA levels (sections 3.3.5.;3.3.7.;3.3.11.). This suggests that the IL-4 and IL-5 mRNAs are relatively stable, compared to those of IL-6 and IL-10.

The responsiveness of the IL-5, IL-6 and IL-10 genes to the variety of signalling pathways which emanate from the TCR/CD3 complex or the IL-1 receptor may be mediated by either one or a combination of two possible mechanisms. Transcriptional activation of these genes may involve the cooperative action of numerous different factors, the synthesis or activity of each of which is responsive to a specific signal. The pleiotropic responsiveness of these genes may alternatively be mediated by a factor whose synthesis or activity may be induced by numerous different signals. One well-characterized example of such a factor is NF-κB, whose translocation to the nucleus may be induced in response to a wide variety of cellular signals. NF-κB, for example, appears to be involved in mediating the transcriptional activation of the IL-2 and GM-CSF genes in response to phorbol esters and the HTLV-1 *trans*-activator *tax* (Hoyos *et al.,* 1989; Shibuya *et al.,* 1989; Schreck & Baeuerle, 1990), the IL-6 gene in response to IL-1 and TNF-α (Shimuzu *et al.,* 1989; Zhang *et al.,* 1990), the TNF-α gene in response to lipopolysaccharide and IFN-γ (Collart *et al.,* 1990) and the IFN-β gene in response to double-stranded RNA and virus infection (Lenardo *et al.,* 1989; Visvanathan & Goodbourn, 1989).

Specific signalling pathways also appear to negatively modulate the expression of specific lymphokine genes at the transcriptional level. Observations described in section 3.3.14. suggest that a labile repressor may selectively inhibit transcription of the GM-CSF and IL-4 genes in D10.G4.1 cells stimulated with IL-1, but not with other activators including those which activate PKC or adenylate cyclase. Furthermore, the transcriptional inactivity of the IL-3 gene in IL-1-stimulated cells does not appear to involve such a mechanism.

The mechanisms which regulate IL-5 gene transcription in EL4.23 cells appear similar to those observed in D10.G4.1 cells. In EL4.23 cells, transcriptional activation of the IL-5 gene also appears to be regulated by a factor(s) whose synthesis is induced *de novo* in response to signals mediated via a Cs A-resistant pathway (sections 3.3.6.;3.3.10.). Furthermore, IL-5 mRNA also appears to be relatively stable in EL4.23 cells, compared to those of other lymphokines, notwithstanding cessation of its gene transcription (sections 3.3.8.;3.3.12.). IL-10 gene transcription in EL4.23 cells, as well as in D10.G4.1 cells, appears to require protein synthesis for maximal activation. A labile repressor appears, however, to be also involved in regulating IL-10 gene transcription in EL4.23 cells. Although signals mediated via a Cs A-resistant pathway appear to predominantly regulate IL-10 gene transcription in both T cell types, Cs A-sensitive signals may possibly also modulate IL-10 gene transcription in EL4.23 cells (sections 3.3.6.;3.3.12.).

Transcription of the IL-2 gene in EL4.23 cells appears to be subject to the coordinate control of positively and negatively acting regulatory factors. The positive regulatory factor(s) appears to be labile and responsive to signals mediated via a Cs A-sensitive pathway. The *trans*-activator NF-AT, which plays a critical role in activating IL-2 gene transcription in both human (Durand *et al.,* 1988) and mouse (Novak *et al.,* 1990), has recently been reported to be comprised of a newly synthesized nuclear subunit and a pre-existing cytoplasmic subunit, which is induced to translocate to the nucleus in response to Cs A-sensitive signals (Flanagan *et al.,* 1991; Clipstone & Crabtree, 1992; O'Keefe *et al.,* 1992). Studies described in section 3.3.12. suggest that the repressor which negatively modulates IL-2 gene transcription in EL4.23 cells may also be labile. A negative regulatory element has also been identified in the human IL-2 promoter (Nabel *et al.,* 1988).

Mechanisms other than transcription appear to predominantly regulate the accumulation of IL-3 and GM-CSF mRNAs in both D10.G4.1 and EL4.23 cells. The studies indicate, however, that GM-CSF gene transcription is enhanced in D10.G4.1 cells by a factor(s) which is synthesized *de novo* in response to signals mediated via a Cs A-resistant Ca^{2+}-dependent pathway. These signals also appear to activate a pre-existing factor which induces IL-3 gene transcription in D10.G4.1 cells. Transcriptional activation of the IL-3 gene in EL4.23 cells in response to Cs A-sensitive signals was very weak and appeared to be subject to the control of a labile repressor. Both positive and negative regulatory mechanisms also appear to coordinately control human IL-3 gene transcription (Mathey-Prevot *et al.*, 1990; Shoemaker *et al.*, 1990). The strength of the activity of the repressor relative to that of the positive regulator may account for, at least in part, the low levels of IL-3 mRNA which accumulate in EL4.23 cells.

3.4.2. Regulation of lymphokine mRNA stability: Specific enhancement and degradation

Results of the current study suggest that the expression of the GM-CSF gene is predominantly regulated at the level of mRNA stability in both D10.G4.1 and EL4.23 cells. Only a modest increase in GM-CSF gene transcription was detected upon stimulation of D10.G4.1 cells, while no significant change in the rate of GM-CSF gene transcription was observed upon stimulation of EL4.23 cells (sections 3.3.1.;3.3.2.). Regulation at the level of mRNA stability has been reported to be a predominant mode of control of GM-CSF gene expression in several T cell systems and other non-T cell types which express this gene (Thorens *et al.*, 1987; Bickel *et al.*, 1990; Akahane & Plutznik, 1992; Iwai *et al.*, 1992). Enhanced stabilization of IL-3 mRNA also appears to be an important mechanism in the control of its accumulation in both D10.G4.1 and EL4.23 cells. Regulation of IL-3 gene

expression at the level of mRNA stability has also been recently reported in transformed human T cells (Ryan *et al.,* 1991) and mast cells (Wodnar-Filipowicz & Moroni, 1990).

Signals mediated via a Cs A-sensitive pathway appear to coordinately enhance the stability of GM-CSF and IL-3 mRNAs in both D10.G4.1 and EL4.23 cells. Furthermore, these signals appear to stabilize IL-3 mRNA more efficiently than GM-CSF mRNA (sections 3.3.5.; 3.3.6.; 3.3.11.; 3.3.12.). Although expression of the IL-6 gene appears to be predominantly regulated at the transcriptional level in D10.G4.1 cells, the possibility that Cs A-resistant pathways may also enhance the stability of its mRNA cannot be discounted. Several recent studies indicate that certain cellular signals may either coordinately or selectively modulate the stability of different cytokine mRNAs in a given cell type. CD28-mediated signals, for example, appear to stabilize IL-2, GM-CSF, IFN-γ and TNF-α mRNAs in human peripheral blood lymphocytes (Lindsten *et al.,* 1989). Both the G-CSF and M-CSF mRNAs are coordinately stabilized in monocytes by phorbol esters. G-CSF mRNA, however, is selectively stabilized by lipopolysaccharide while M-CSF mRNA is selectively stabilized by IL-3 or GM-CSF (Ernst *et al.,* 1989b).

The stability of a given mRNA species may also be modulated by different signals to different degrees in a given cell type. Phorbol ester treatment, for example, is reported to be more effective than lectin stimulation in stabilizing GM-CSF mRNA in a murine T cell line (Iwai *et al.,* 1992). Phorbol ester also appears to stabilize IL-1β mRNA in fibroblasts to a greater degree than TNF-α stimulation (Yamato *et al.,* 1989). Ca^{2+} ionophore, however, appears more effective than phorbol ester in stabilizing IL-3 mRNA in mast cells (Wodnar-Filipowicz & Moroni, 1990). Studies described in section 3.3.14. suggest that IL-6 mRNA may be stabilized by phorbol ester to

a greater degree than Ca^{2+} ionophore in D10.G4.1 cells. The modulation of stability of different lymphokine mRNAs in D10.G4.1 cells by different signalling pathways requires more detailed investigation, including examination of their relative mRNA levels, induced by different activators, in the presence of Act D.

The stability of a number of labile mRNAs encoding inducible regulators of cellular growth and differentiation have been reported to be enhanced by cellular treatment with protein synthesis inhibitors, once their induction is under way. These include oncogenes such as *c-myc*, *c-fos* and *c-myb* (Dani *et al.,* 1984; Mitchell *et al.*, 1985; Thompson *et al.,* 1986; Brewer & Ross, 1989) and several cytokines such as IL-1β (Yamato *et al.,* 1989), IFN-β (Raj & Pitha, 1981), G-CSF and M-CSF (Ernst *et al.,* 1989a,b). Such enhanced stabilization has been generally ascribed to the suppression by protein synthesis inhibitors of a labile mechanism which mediates the degradation of these specific mRNAs. Once initial induction was under way, GM-CSF mRNA levels were able to be dramatically elevated (or "superinduced") in both D10.G4.1 and EL4.23 cells by the addition of CHX without any significant change in the rate of GM-CSF gene transcription (sections 3.3.11.;3.3.12.). Significant increases in IL-3 and IL-6 mRNA levels were also induced in D10.G4.1 cells without noticeable alterations in the transcription rates of their genes. These three mRNA species were also stabilized by CHX alone when transcription of their genes was inhibited by Act D. CHX has been reported to enhance the stabilization of GM-CSF mRNA in fibroblasts (Shaw & Kamen, 1986; Koeffler *et al.,* 1988; Akashi *et al.,* 1991) and IL-3 mRNA in mast cells (Wodnar-Filipowicz & Moroni, 1990). Observations of the current study suggest that the stability of GM-CSF mRNA in D10.G4.1 and EL4.23 cells and of IL-3 and IL-6 mRNAs in D10.G4.1 cells is dynamically regulated by a labile

mechanism which selectively mediates their degradation and by specific signal-dependent mechanisms which enhance their stabilization (Table 3.1.).

A labile mechanism also appears to mediate the specific degradation of the IL-2, IL-3 and IL-10 mRNAs in EL4.23 cells (section 3.3.12.). In contrast to the GM-CSF mRNA, the observed "superinduction" of the IL-2, IL-3 and IL-10 mRNAs by CHX appears partially attributable to an increase in the transcription rates of their genes, as well as to a reduction in the rate of their degradation. Previous reports in a different EL4 variant (Shaw *et al.,* 1987; 1988a) and other T cell systems (Kronke *et al.,* 1985; Kaempfer *et al.,* 1987) have, however, observed no stimulatory effect of CHX upon the rate of IL-2 gene transcription and suggest that "superinduction" of its mRNA may be largely attributable to a reduction in the rate of its degradation.

Observations in the current study suggest that the labile mechanism which degrades specific lymphokine mRNAs in both D10.G4.1 and EL4.23 cells may be constitutively active. CHX alone, in the absence of stimuli, appeared to stabilize the small pools of GM-CSF, IL-6 and IL-10 transcripts which are expressed in unstimulated D10.G4.1 cells and are normally undetectable because of their rapid turnover. CHX alone also stabilized the GM-CSF transcripts which are constitutively expressed at a constant rate in EL4.23 cells. Stabilization by CHX of the very low basal levels of IL-10 transcripts in unstimulated EL4.23 cells may require detection methods more sensitive than Northern blot analysis such as PCR analysis to verify this proposal.

One structural characteristic shared by many labile oncogene and cytokine mRNAs is a conserved region in their 3'-UTRs which contains several, often tandem, copies of the motif AUUUA (Caput *et al.,* 1986).

Insertion of the AU-rich region from the 3'-UTR of the GM-CSF mRNA into the 3'-UTR of the β-globin mRNA has been demonstrated to destabilize the normally stable β-globin mRNA (Shaw & Kamen, 1986). Conversely, deletion of the AU-rich region from the 3'-UTR of the c-*fos* mRNA renders it more stable than its unmodified counterpart (Wilson & Treisman, 1988). Instability conferred by these AU-rich sequences has been shown to be alleviated by cellular treatment with CHX (Shaw & Kamen, 1986; Akashi *et al.,* 1991). Recent RNA-protein binding studies have identified cellular factors which specifically bind to these AUUUA motifs (Malter, 1989; Bohjanen *et al.,* 1991;1992; Vakalopoulou *et al.,* 1991). The lability of these factors appears, as yet, unknown. A recent model proposes that these AU-rich sequences are not a target for a specific "RNase"-like molecule *per se*, but rather a binding site for a specific protein(s), the interaction of which destabilizes the association of PABP with the poly(A) tail. Such destabilization of the PABP/poly(A) complex reduces its ability to protect the poly(A) tail and mRNA body from degradation, presumably by endogenous non-specific RNases (Bernstein & Ross, 1989). The results of the current study appear largely consistent with this proposed model. Lymphokine mRNAs, such as those of GM-CSF, IL-2, IL-3, IL-6 and IL-10, which were highly labile and stabilized by CHX, appear to contain multiple, often tandem, repeats of the AUUUA motif in their 3'-UTRs. The IL-4 and IL-5 mRNAs appear to contain only a few widely dispersed single copies of this motif in their 3'-UTRs. These two mRNA species were observed to be significantly more stable than the other lymphokine mRNAs and their stabilities were not enhanced by CHX. The minimum number of AUUUA repeats, which confer instability and are required for protein binding, has as yet not been determined. Observations in this study suggest that the presence of single dispersed copies may be insufficient.

Stabilization of GM-CSF mRNA by several cellular activators, as well as CHX, has been recently reported to be mediated via these AU-rich sequences in its 3'-UTR (Akashi *et al.,* 1991). While no possible mechanism was speculated by the authors, it is possible that cellular signals may inhibit the synthesis of the labile "destabilizing" factor which binds to these sequences. Cellular signals may alternatively inactivate this factor by direct modification or by mediating its sequestration by a signal-induced inhibitory ligand. Akashi *et al.* (1991) reported, however, that TNF-α stimulation, unlike CHX, IL-1β or activators of PKC and G proteins, appears to enhance GM-CSF mRNA stabilization by a mechanism which is independent of these AU-rich sequences. Several other lines of evidence indicate that other elements, apart from the AU-rich sequences, may be involved in regulating the stability of several labile mRNAs (Rabbitts *et al.,* 1985; Jones & Cole, 1987; Kabnick & Housman, 1988; Band *et al.,* 1990; Iwai *et al.,* 1991; Ross *et al.,* 1991). These reports implicate the involvement of numerous common and unique *cis*-acting elements and *trans*-acting factors in the control of mRNA stability.

A major objective in recent years has been the identification and characterization of the *cis*-acting elements which regulate the expression of lymphokine genes at either the transcriptional and/or post-transcriptional levels, and of the regulatory factors which interact with these control regions. The following two chapters describe investigations of DNA-protein interactions which may be involved in regulating the transcription of the murine IL-5 gene.

CHAPTER 4:

REGULATION OF MURINE IL-5 GENE EXPRESSION: ROLE OF CHROMATIN STRUCTURE IN TRANSCRIPTIONAL ACTIVITY

4.1. INTRODUCTION

Previous studies, described in Chapters 2 and 3, have suggested that the expression of a number of lymphokine genes in activated murine T lymphocytes may be coordinately and/or differentially regulated by distinct sets of transcriptional mechanisms in response to specific activation signals in different T cell systems. As previously described in section 1.1.1., molecular "programs" for modulating the expression patterns of individual, or groups of, eukaryotic genes at the transcriptional level are believed to be conferred by the particular composition and spatial organisation of the multiple DNA elements utilized by, and frequently located 5' to, a given gene. These *cis*-acting elements appear to interact with specific DNA-binding proteins, the activities and/or syntheses of which may be modulated by specific signals in specific cell types.

IL-5 has been the subject of considerable interest in the laboratory in recent years, particularly given the linkage between the cell-specific nature of eosinophilia and IL-5 expression (Coffman *et al.,* 1989; Limaye *et al.,* 1989; Owen *et al.,* 1989; Sanderson, 1992). Such clinical observations implied the existence of regulatory mechanisms specific to IL-5 gene expression, which, at the commencement of the current project, had not been extensively characterized. Studies described in Chapters 2 and 3 indicated that IL-5 gene expression is predominantly regulated at the transcriptional level by a variety

of different signalling pathways. Furthermore, the activation of IL-5 gene transcription by these various pathways appears to be differentially modulated from other lymphokine genes by its dependence upon *de novo* synthesis of an obligatory *trans*-activating factor(s). These observations imply the existence of specific molecular "programs" which may be unique to regulation of IL-5 gene transcription.

The development of techniques enabling expression of exogenous DNA in both cell-free and cellular systems have been major methodological advances in the identification and characterization of transcriptional control elements. The general scheme in both systems involves assaying the effect of modification of the native cloned sequence by deletion or mutation upon transcription of an appropriate "reporter" to which these sequences are linked. Transcription of the "reporter" may be measured either (i) directly in *in vitro* systems, comprising of soluble cellular extracts which accurately initiate transcription of the exogenous DNA construct (Dignam *et al.,* 1983; Manley *et al.,* 1980, Manley, 1985) or (ii) by reference to the biological activity of the reporter gene product after the exogenous DNA construct has been introduced into, and transiently expressed in, eukaryotic cells (Banerji & Schaffner, 1983; Spandidos & Wilkie, 1985).

In preliminary investigations, two commonly used cell-free systems, originally described by Manley *et al.* (1980) and Dignam *et al.* (1983), were utilized by the investigator to identify potential elements which may regulate murine IL-5 gene transcription in response to T cell activation signals. Soluble nuclear and whole-cell extracts were prepared from unstimulated and Con A-stimulated D10.G4.1 cells, and from unstimulated and PMA-stimulated EL4.23 cells. All of these extracts were assayed for transcriptional activity

under the direction of the Adenovirus 2 major late (AdML) promoter[1], a commonly used positive control for the transcriptional competence of cell-free systems (Gorski *et al.,* 1986; Flanagan *et al.,* 1991). A series of progressive 5' deletion mutants of the murine IL-5 gene were constructed. These contained sequences from -3724, -1707, -1481, -1173, -458, -312, -172 and -88 to +162, and generated a "run-off transcript" of 162 bp when assayed *in vitro*. No significance difference in transcriptional activity between cell-free systems prepared from unstimulated or stimulated T cells was detected using any of these various constructs, and using either nuclear or whole-cell extracts prepared from either D10.G4.1 or EL4.23 cells. In preliminary experiments conducted by other members of the laboratory, 5'-flanking sequences of the murine IL-5 gene, similar to those described above but extending to +26, were linked to the CAT reporter gene and transfected into D10.G4.1 and EL4.23 cells (P. Bourke, pers. comm.). No significant difference in transcriptional activity was detected between unstimulated and stimulated T cells, in which these various constructs were transiently expressed.

The inability to detect increases in murine IL-5 gene transcription in response to T cell activation signals in either the cell-free or transient expression systems contrasts markedly with the significant activation of IL-5 gene transcription which was observed by nuclear run-off analysis described in Chapter 3. Inherent differences in the premises upon which these various methodologies are based may account for these preliminary observations. The use of exogenous DNA as templates for transcription in either cell-free or transient expression systems relies upon the ability of putative regulatory factors, together with basal transcription factors, to interact with one another and sequences of the naked DNA to accurately initiate transcription. The nuclear run-off analysis, in contrast, uses endogenous chromatin as the

1. AdML constructs were kindly provided by Dr.D.Tremethick (JCSMR)

template for transcription and measures levels of nascent RNA, initiated *in vivo* and elongated in isolated intact nuclei, in which the chromatin integrity is maintained in its native state. Such correlation between methodological premise and observation implies the critical role which chromatin structure may play in regulating transcription of the IL-5 gene.

A large body of structural and functional evidence over the past decade suggests that the transcriptional regulation of gene expression may be ultimately controlled by the packaging of DNA into nucleosomes and higher order chromatin structures, an intrinsically dynamic phenomenon specific to eukaryotes. Studies using *in vitro* chromatin assembly systems have demonstrated that nucleosome assembly over promoter sequences may render a DNA template refractory to transcription, while the formation of transcriptional preinitiation complexes on DNA templates prior to or during nucleosome assembly may alleviate promoter repression (Knezetic & Luse, 1986; Matsui, 1987; Workman & Roeder, 1987). Transcriptionally active chromatin demonstrates preferential "hypersensitivity" to non-specific nucleases such as DNase I, a phenomenon generally attributed to local disruption of nucleosomal organisation and which suggests that "open" chromatin structures may enable accessibility of the gene to the transcriptional machinery (reviewed in Eissenberg *et al.,* 1985; Yaniv & Cereghini, 1986; Elgin, 1988; Gross & Garrard, 1988; Grunstein, 1990). Furthermore, such "hypersensitive" sites have frequently been found to correspond to putative regulatory sequences. The immediate 200 bp region surrounding a hypersensitive site that was reportedly induced in the 5'-flanking regions of the IL-2 gene in response to T cell activation signals (Siebenlist *et al.,* 1986) has subsequently been found to contain multiple *cis*-acting elements which are critical for activation of IL-2 gene transcription, including those which bind NF-AT, NF-κB, AP-1 and Oct-1 (Durand *et al.,* 1988; Shaw *et al.*, 1988b;

Emmel *et al.*, 1989; Hoyos *et al.*, 1989; Serfling *et al.*, 1989; Kamps *et al.*, 1990).

Analysis of DNase I hypersensitive sites, as markers of non-nucleosomal organisation, is therefore a useful tool to initially "map" approximate locations of putative regulatory regions of genes in their native chromatin environment. Such analysis is particularly relevant in respect of the IL-5 gene, given the apparent importance of chromatin structure in its transcriptional regulation. In the current study, alterations in chromatin structure of the IL-5 gene in response to mitogenic stimulation of D10.G4.1 cells were investigated by DNase I hypersensitivity analysis.

4.2. MATERIALS AND METHODS

The source and maintenance of the murine Th2 clone D10.G4.1. has been previously described in section 2.2.1.(2). Subcloning, isolation, restriction digestion and agarose gel electrophoresis of plasmid DNA has been previously described in section 2.2.2.

4.2.1. Genomic DNA probes

IL-5 genomic sequences which were used as probes in DNase I hypersensitivity studies were derived from a 10.0 kb *Hind* III fragment containing the murine IL-5 gene (Campbell *et al.,* 1988). The 2.15 kb *Hind* III/*Eco* RI, 1.54 kb *Sac* I/*Hind* III and 0.7 kb *Stu* I/*Eco* RI fragments were isolated by the investigator from the original 10 kb genomic clone and subcloned into plasmid vectors which, upon subsequent digestion with appropriate restriction enzymes, released the inserts with suitable single recessed 3' ends for ^{32}P-end-labeling.

4.2.2. DNase I hypersensitivity analysis

(1) Isolation of nuclei and DNase I digestion

Nuclei were isolated from unstimulated and stimulated cells and digested with DNase I, and genomic DNA subsequently purified as described by Wu (1980) with modifications. Cells were harvested, washed in ice-cold PBS and lysed at 2×10^7 cells/ml in ice-cold lysis buffer (60 mM KCl; 15 mM NaCl; 15 mM Tris-Cl [pH 7.4]; 0.5 mM DTT; 3 mM $MgCl_2$; 0.1 mM ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA); 0.5 mM spermidine; 0.25 M sucrose and 0.2% Nonidet P-40). Following centrifugation at 800 x g for 5 min at 4°C, the nuclear pellet was

resuspended at 1×10^8 nuclei/ml in digestion buffer (60 mM KCl; 15 mM NaCl; 15 mM Tris-Cl [pH 7.4]; 0.5 mM DTT; 3 mM $MgCl_2$; 0.5 mM spermidine; 0.25 M sucrose; 0.05 mM $CaCl_2$). Various amounts of DNase I (RQ1, Promega) were added to the resuspended nuclei and incubated at 37°C for 3 min. The reaction was terminated by addition of EDTA and SDS to 12.5 mM and 0.5% respectively. The DNA was purified by proteinase K digestion (100 μg/ml) and repeated extraction with phenol and chloroform:isoamylalcohol (24:1). The DNA was precipitated by the addition of sodium acetate [pH 5.2] and ethanol to 0.3 M and 70% respectively and resuspended at a concentration of 1-2 mg/ml.

(2) Southern blot analysis of genomic DNA

Restriction enzyme digestion of genomic DNA was carried out according to manufacturers' directions. Agarose gel electrophoresis and Southern transfer of DNA to nylon membrane (Hybond N+, Amersham) has been previously described in sections 2.2.2.(3) and 3.2.2.(3) respectively. The nylon membrane was prehybridized for 2 to 16 h at 65°C in hybridization solution containing 5 x SSPE (0.75 M NaCl; 10 mM sodium phosphate [pH 7.0]; 1 mM EDTA); 5 x Denhardt's solution; 0.5% SDS and 500 μg/ml denatured salmon sperm DNA. Fresh hybridization solution was added prior to addition of the ^{32}P-end-labeled probe (see below) and hybridization allowed to proceed for 16 to 24 h at 65°C. Following hybridization, the filter was washed as previously described in section 2.2.3.(4).

(3) ^{32}P-end-labeling of recessed 3' ends

The recessed 3' end of the isolated DNA fragment was filled and ^{32}P-labeled according to Maniatis *et al.* (1989) with modifications. 1 μg of purified fragment was incubated in a total volume of 50 μl containing 50 mM Tris-Cl [pH 7.2]; 10 mM $MgSO_4$; 0.1 mM DTT; 50 μg/ml BSA; 40 μCi

[α-^{32}P] dATP (3000 Ci/mmole, Amersham) and 40 μM each of dGTP, dCTP and dTTP with 5 units of Klenow DNA Polymerase (Pharmacia) for 30 min at room temperature. The reaction was stopped by addition of EDTA to 20 mM and unincorporated labeled nucleotides were removed by chromatography as described previously in section 2.2.3.(5). The probe was denatured by boiling for 5 min prior to addition to the hybridization solution.

4.3. RESULTS

4.3.1. Low resolution analysis of chromatin structure of the IL-5 gene before and after mitogenic stimulation.

Nuclei were isolated from unstimulated D10.G4.1 cells and cells stimulated for 6 h with Con A (5 μg/ml), conditions which were previously found to induce maximal transcriptional activity of the IL-5 gene (Chapter 3). Nuclei were incubated with different concentrations of DNase I (0, 1 or 3 units) and the DNA was isolated and subsequently digested with *Hind* III. Southern blots containing *Hind* III-digested genomic DNA, derived from unstimulated and stimulated cells, were hybridized with a 2.15 kb *Hind* III/*Eco* RI fragment, which contains sequences 5' to the IL-5 gene and was ^{32}P-labeled at the 5' abutting *Hind* III end (Table 4.1.). As shown in Fig. 4.1.(A), the 2.15 kb *Hind* III/*Eco* RI probe hybridized to the original 10.0 kb *Hind* III genomic DNA fragment, in addition to several sub-fragments generated by partial digestion with DNase I which became more visible with increasing amounts of DNase I. The size of sub-fragments generated by partial DNase I digestion differed between DNA isolated from unstimulated and stimulated cells, and the location of putative hypersensitive sites were mapped relative to the 5' *Hind* III site by reference to the size of these bands (Table 4.1.). A putative inducible hypersensitive site was mapped to a position just 5' of the cap site by reference to a 3.7 kb sub-fragment which was detectable in stimulated T cell DNA and absent in unstimulated T cell DNA (Fig. 4.1.(A)).

To verify these initial results and identify any other hypersensitive sites, genomic DNA, isolated from DNase I-treated chromatin and digested with *Hind* III, was hybridized with a 1.54 kb *Sac* I/*Hind* III fragment whose ^{32}P-

Fig.4.1. **Analysis of DNase I hypersensitive sites in chromatin of the murine IL-5 gene.**

(A) 3 x 10^7 nuclei, isolated from unstimulated or Con A-stimulated D10.G4.1 cells, were digested with either 0, 1 or 3 units DNase I under the conditions described in section 4.2.2. Genomic DNA was purified, 30 µg of which was digested with *Hind* III, electrophoresed on 1.2% agarose gels, transferred on to nylon membrane and hybridized with the ^{32}P-end-labeled 2.15 kb *Hind* III/*Eco* RI fragment. The original 10.0 kb *Hind* III genomic DNA fragment and the sizes of sub-fragments generated by DNase I cleavage are indicated.

A

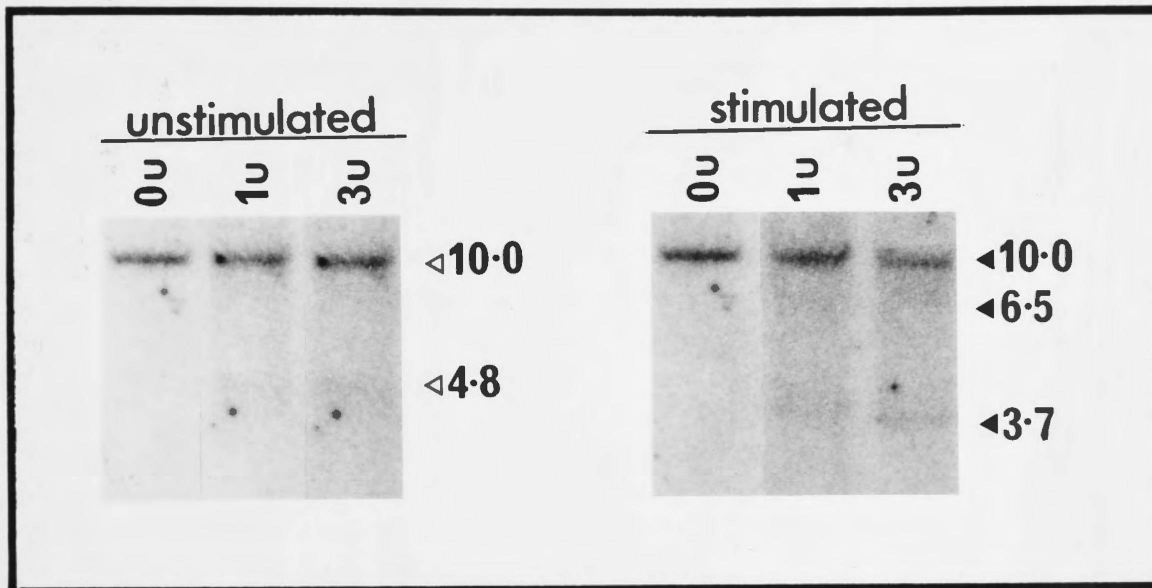
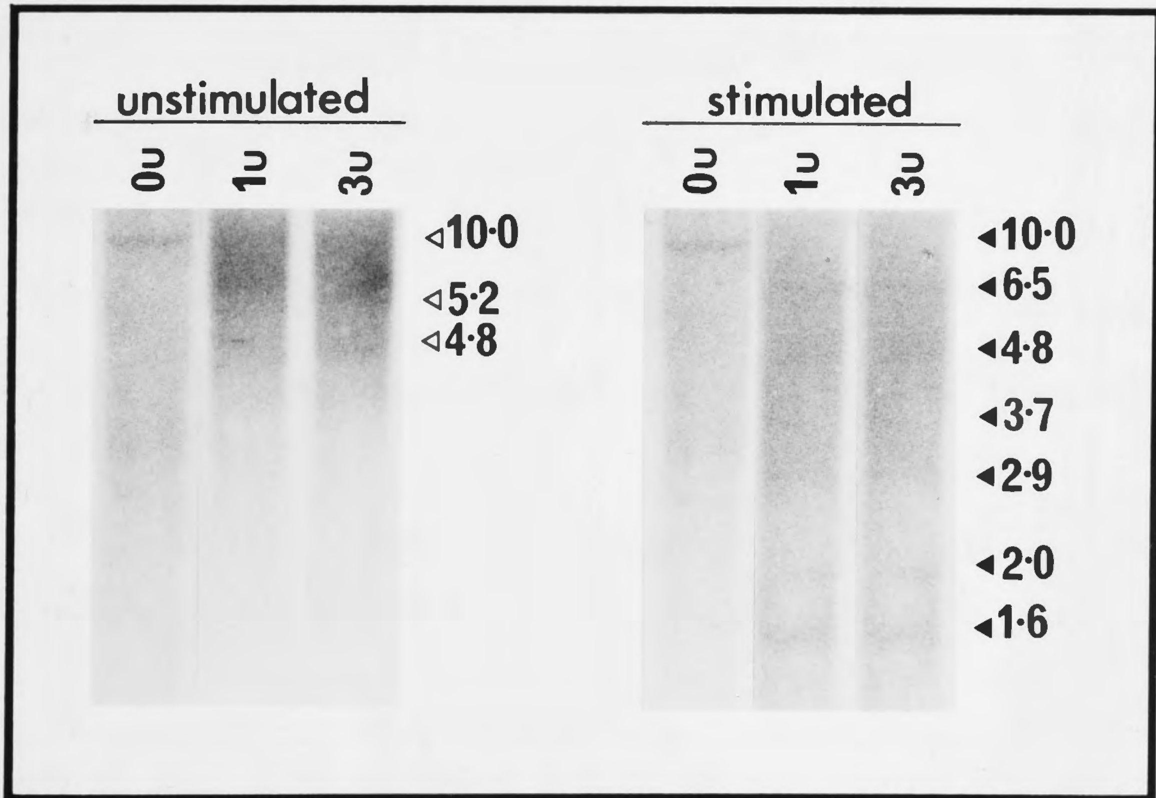


Fig.4.1. **Analysis of DNase I hypersensitive sites in chromatin of the murine IL-5 gene**

(B) Genomic DNA, purified from DNase I-treated nuclei of unstimulated or Con A-stimulated D10.G4.1 cells, were digested with *Hind* III and hybridized with the ^{32}P-end-labeled 1.54 kb *Sac* I/*Hind* III fragment. The original 10.0 kb *Hind* III genomic DNA fragment and sizes of sub-fragments generated by DNase I cleaveage are indicated.

B



labeled end abuts the 3' *Hind* III site downstream of the IL-5 gene (Table 4.1.). In addition to the original 10.0 kb *Hind* III genomic DNA fragment, a number of sub-fragments generated by DNase I digestion were detected (Fig. 4.1.(B)). The faint 5.2 kb sub-fragment detected in unstimulated T cell DNA and the 6.5 kb and 3.7 kb sub-fragments detected in stimulated T cell DNA verified the location of hypersensitive sites identified using the previous 2.15 kb *Hind* III/*Eco* RI probe (Table 4.1.). Three smaller sub-fragments of 2.9 kb, 2.0 kb and 1.6 kb were particularly distinct in stimulated T cell DNA (Fig. 4.1.(B)). The size of the 1.6 kb sub-fragment maps a putative hypersensitive site just 3' of the IL-5 gene (Table 4.1.).

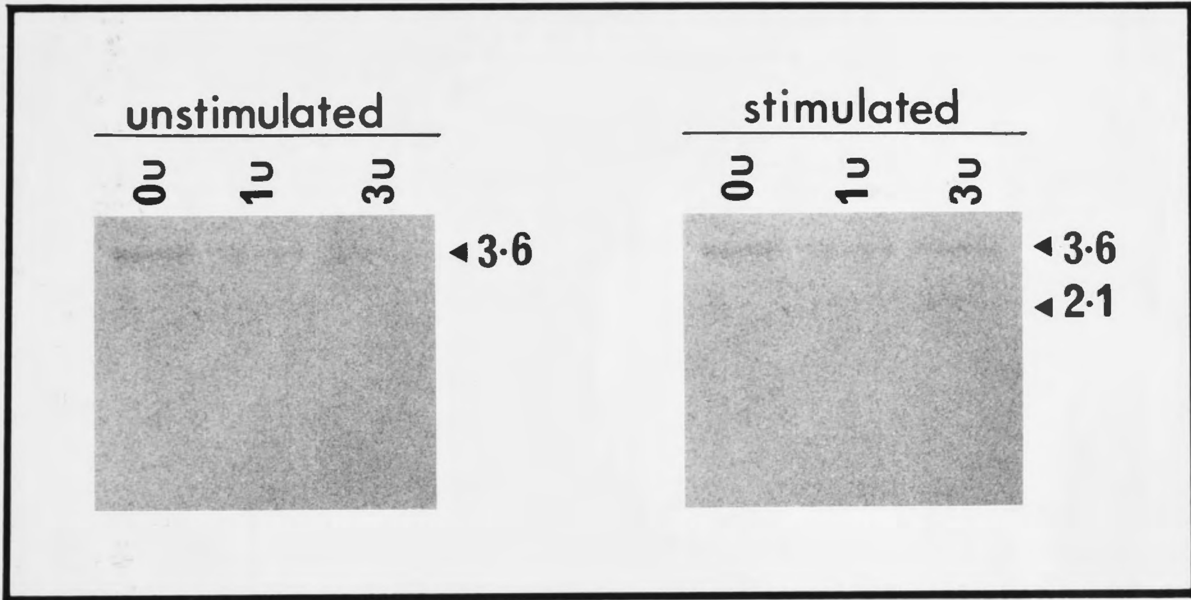
4.2.2. Higher resolution analysis of chromatin structure of the 5'- flanking region of the IL-5 gene

In view of the large body of evidence indicating that transcriptional control elements are frequently located in the 5'-flanking regions of eukaryotic genes (reviewed in Breathnach & Chambon, 1981; Yaniv, 1984; Serfling *et al.,* 1985; Maniatis *et al.,* 1987; Johnson & McKnight, 1989; Mitchell & Tijan, 1989), the hypersensitive site induced in the proximal promoter region of the IL-5 gene by mitogenic stimulation was further analysed at higher resolution. Genomic DNA, extracted from nuclei isolated from unstimulated or stimulated cells as described above, were digested with *Eco* RI. A Southern blot containing *Eco* RI-digested genomic DNA was hybridized with a 0.7 kb *Stu* I/*Eco* RI fragment which was ^{32}P-labeled at the 3' abutting *Eco* RI end (Table 4.1.). This probe hybridized to the original 3.6 kb *Eco* RI genomic DNA fragment in both unstimulated and stimulated T cell DNA and to a distinct DNase I-generated 2.1 kb sub-fragment which was present in stimulated T cell DNA but absent in unstimulated T cell DNA (Fig. 4.1.(C)). By reference to the size of this sub-fragment, the position of the inducible hypersensitive site in the 5'-flanking region proximal to the cap site was verified (Table 4.1.). The

Fig.4.1. **Analysis of DNase I hypersensitive sites in chromatin of the murine IL-5 gene**

(C) Genomic DNA, purified from DNase I-treated nuclei of unstimulated or Con A-stimulated D10.G4.1 cells, were digested with *Eco* RI and hybridized with the ^{32}P-end-labeled 0.7 kb *Stu* I/*Eco* RI fragment. The original 3.6 kb *Eco* RI genomic DNA fragment and the 2.1 kb sub-fragment generated by DNase I cleavage are indicated.

C

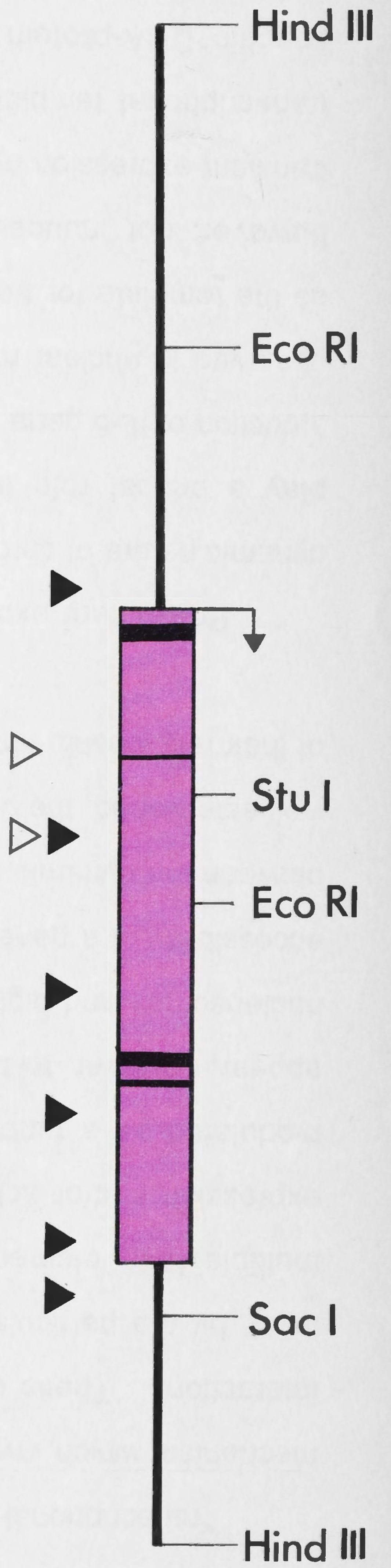


DNase I hypersensitive site in the intron 1 which was mapped by reference to the very faint bands observed in unstimulated T cell DNA by previous low resolution analysis (section 4.3.1.) was, however, not observed using this shorter probe.

<u>Table 4.1.</u> Mapping of DNase I hypersensitive sites in chromatin of the murine IL-5 locus in unstimulated or activated T cells.

The location of restriction sites within the murine IL-5 gene (pink box) and its 5'- and 3'- flanking regions are indicated. The restriction fragments used as probes in this study, (A) 2.15 kb *Hind* III/*Eco* RI, (B) 1.54 kb *Sac* I/*Hind* III and (C) 0.7 kb *Stu* I/*Eco* RI, are indicated by white boxes, and the ends which were ^{32}P-labeled are denoted by black dots. Also shown are the sub-fragments which were generated by DNase I cleavage of chromatin isolated from unstimulated (white lines) or stimulated (black lines) D10.G4.1 cells, and hybridized to the respective probes. The positions of DNase I hypersensitive sites, which were generated in chromatin of unstimulated cells (white arrows) or stimulated cells (black arrows), were localized by reference to the sizes of these sub-fragments.

1 kb



4.4. DISCUSSION

Transcriptional regulation of eukaryotic gene expression is a complex mechanism which involves multiple, specific DNA-protein and protein-protein interactions. These coordinate interactions appear to be regulated, at one level, by the particular sequences, composition and spatial organisation of multiple DNA elements which may be utilized by a given gene, and the expression and/or activities of the specific regulatory factors which may be modulated as a function of cell state or type. Such multiple interactions appear, however, to be ultimately governed by the organisation of DNA into nucleosomes and higher order chromatin structures, which may regulate the accessibility of a gene to the transcriptional machinery. While the correlation between the dynamic nature of chromatin and transcriptional activity has been well established, the underlying mechanisms which govern the intrinsic nature of their relationship remain unclear (section 1.1.2.).

Preliminary experiments described in section 4.1. suggested that the dynamic nature of chromatin structure and the nuclear micro-environment may play a critical role in regulating transcription of the murine IL-5 gene. Induction of IL-5 gene transcription in response to T cell activation signals was observed in nuclear run-off assays, where endogenous chromatin was used as the template for transcription (Chapter 3). Transcriptional activation was, however, not induced in cell-free (*in vitro* transcription) systems nor in transient expression systems where exogenous naked DNA was used as the transcriptional template. Higher order structure and topology, apart from specific DNA-protein and protein-protein interactions, therefore appear necessary to activate IL-5 gene transcription. This proposal has been supported by recent findings which demonstrate inducibility of the IL-5 gene promoter in response to T cell activation signals under conditions where the

exogenous DNA is stably integrated into the chromosomes of eukaryotic host cells, as opposed to being transiently expressed (P. Bourke, unpublished results).

Studies described in this chapter indicate that the chromatin structure of the murine IL-5 gene undergoes significant alterations in response to T cell activation signals. Increased hypersensitivity to DNase I correlated strongly with increased transcriptional activity of the IL-5 gene. Such hypersensitivity is generally believed to be attributable to decondensation in chromatin structure, by the local disruptions in nucleosomal organisation. Such dynamic alterations in chromatin structure are believed to allow accessibility of the gene to *trans*-activating factors, a proposal supported by the frequent association of hypersensitive sites with protein-binding regulatory regions of genes (reviewed in Eissenberg *et al.,* 1985; Yaniv & Cereghini, 1986; Elgin, 1988; Gross & Garrard, 1988; Grunstein, 1990).

Mitogenic stimulation of D10.G4.1 cells induced several novel DNase I hypersensitive sites in the non-coding regions of the IL-5 gene, including one site in the large second intron and three sites in the 3'-UTR, two being proximal to the polyadenylation signal (Table 4.1.). One inducible hypersensitive site was identified just upstream of the cap site. A large body of recent evidence indicates that *cis*-acting regulatory elements of several lymphokine genes, including the IL-2 (Durand *et al.,* 1988; Brunvand *et al.,* 1988; Emmel *et al.,* 1989; Serfling *et al.,* 1989), IL-3 (Mathey-Prevot *et al.,* 1990; Shoemaker *et al.,* 1990), IL-4 (Abe *et al.,* 1992) and GM-CSF (Miyatake *et al.,* 1988a,b, 1991; Nimer *et al.,* 1988, 1989a, 1990) genes, which mediate responsiveness to T cell activation signals, are generally located within a 300 bp region 5' of their respective cap sites. Given their proximity to the cap site of the IL-5 gene, the sequences which surround the

observed inducible hypersensitive site may therefore be of significant interest as they may contain putative regulatory elements.

The reported proximal 5'-flanking sequences of the IL-5 gene demonstrate overall poor homology to those of other lymphokine genes with the notable exception of a short region extending just 5' of the TATA boxes of the murine and human genes encoding IL-5, IL-4 and GM-CSF (Campbell *et al.,* 1988). This highly conserved region in the GM-CSF gene has been demonstrated to bind specific nuclear proteins (Nimer *et al.,* 1988; Miyatake *et al.,* 1991) and mediate inducibility of human GM-CSF promoter activity in T cell lines (Nimer *et al.,* 1988, 1989a, 1990), fibroblasts (Nimer *et al.,* 1989b) and endothelial cells (Kaushansky, 1989). The regulatory role of these conserved sequences in GM-CSF gene transcription, the similar positioning of these sequences relative to the TATA boxes and the fact that lymphokine genes demonstrate very limited homology in their 5'-flanking regions, give this conserved region additional significance as a potential transcriptional control element of the murine IL-5 gene which may bind specific regulatory factors. Investigations of the interactions between these DNA sequences and specific nuclear proteins are described in the following chapter.

CHAPTER 5:

REGULATION OF MURINE IL-5 GENE EXPRESSION: IDENTIFICATION AND POTENTIAL INVOLVEMENT OF MULTIPLE, SPECIFIC DNA-PROTEIN INTERACTIONS

5.1. INTRODUCTION

As previously discussed, the underlying mechanisms by which many sequence-specific DNA-binding proteins may regulate the spatial, temporal or inducible expression patterns of many eukaryotic genes at the transcriptional level appear to be inextricably associated with their being intracellular "downstream" targets for signals emanating from events external to the genomic micro-environment. In this regard, the identification and characterization of these DNA-binding proteins is a major approach in elucidating the complex linkage between the transduction of cellular signals and consequent modulation of the genetic program.

The development of techniques enabling the detection of DNA-protein interactions *in vitro* using crude cellular extracts have been major methodological advances in the identification and characterization of DNA-binding regulatory factors and has, in recent years, led to the subsequent purification of several factors and cloning of their respective genes (Kadonga *et al.,* 1987; Singh *et al.,* 1988; Staudt *et al.,* 1988; Meyer *et al.,* 1991). Early DNA-protein binding assays included the nitrocellulose filter binding assay, which involves the separation of stable DNA-protein complexes by filtration through nitrocellulose (Riggs *et al.,* 1970) and south-western blotting, which involves hybridization of DNA to proteins separated by electrophoresis and immobilized on to nitrocellulose membrane (Bowen *et al.,* 1980;

Miskimins *et al.,* 1985). One of the most sensitive and popular methods is the gel shift (retardation) assay which is based on the differential electrophoretic mobilities of DNA-protein complexes versus free unbound DNA (Hendrickson, 1985; Garner & Revzin, 1986; Crothers, 1987; Revzin, 1989). This assay allows determination of the abundance and relative affinity or specificity of DNA-binding proteins by assessing qualitative and quantitative changes in DNA-protein interactions when complex formations are challenged by unlabeled competitor DNA containing sequences identical or non-homologous to the labeled recognition element. Other strategies to identify specific DNA sequences involved in protein binding include protection and interference assays. Protection or "footprinting" assays determine the influence of protein binding on the subsequent reactivity of DNA molecules to non-specific cleavage by chemical agents such as methidiumpropyl-EDTA-Iron(II) (MPE) (Van Dyke *et al.,* 1988; Landolfi *et al.,* 1989), or nucleases such as Exonuclease III (Wu, 1985; Cordingley & Hager, 1988) or DNase I (Galas & Schmitz, 1978; Jones *et al.,* 1985). Interference assays, conversely, determine the influence of DNA modification, for example, by methylation, upon the subsequent binding of proteins (Hendrickson & Schleif, 1985).

As previously discussed in Chapter 4, studies using cell-free or transient expression systems were unable to identify sequences involved in the transcriptional regulation of the IL-5 gene in murine T cells. DNase I hypersensitivity studies of endogenous chromatin, however, identified a region of non-nucleosomal organisation just 5' of the cap site of the IL-5 gene in activated, but not unstimulated, T cells, which may represent a site of inducible sequence-specific protein-DNA interactions. The sequences surrounding this inducible hypersensitive site in the IL-5 gene are highly homologous to corresponding regions in the GM-CSF and IL-4 genes (Table 5.1.), which otherwise demonstrate little significant homology in their

5'-flanking regions. This highly conserved region in the GM-CSF gene has been demonstrated to bind specific nuclear proteins (Nimer *et al.,* 1988; Miyatake *et al.,* 1991) and mediate inducibility of human GM-CSF promoter activity in several GM-CSF-expressing cells including T cells, fibroblasts and endothelial cells in response to stimulation by mitogens, the HTLV-1 *tax* protein and IL-1 (Kaushansky, 1989; Nimer *et al.,* 1988; 1989a,b; 1990).

In addition to the regulatory role of these highly conserved sequences in GM-CSF gene transcription, several other features give these conserved sequences considerable significance as a potential transcriptional control element of IL-5 gene expression which may bind specific regulatory factors. These include the similar positioning of these sequences relative to the TATA boxes of the IL-5, GM-CSF and IL-4 genes (Table 5.1.) and the fact that these lymphokine genes otherwise demonstrate very limited homology in their 5'-flanking regions. Several recent lines of evidence suggest a strong correlation between regulatory functionality and conservation of non-coding sequences. Conserved NF-κB binding sites, which are located proximally to the TATA boxes of the IL-2 (Hoyos *et al.,* 1989; Shibuya *et al.,* 1989), IL-6 (Shimuzu *et al.,* 1990; Zhang *et al.,* 1990) and GM-CSF (Schreck & Baeuerle, 1990), and AP-1 sites sites located upstream of the IL-2 (Emmel *et al.,* 1989) and IL-3 (Mathey-Prevot *et al.,* 1990; Shoemaker *et al.,* 1990) genes appear, for example, to play important regulatory roles in mediating the transcriptional activation of these genes in response to cellular signals. It seemed pertinent, therefore, to identify and characterize the possible interactions of specific nuclear proteins with these DNA sequences in the murine IL-5 gene, and investigate the possible modulation of such interactions by specific T cell activation signals.

Table 5.1. Comparison of 5'-flanking sequences of the murine and human genes encoding IL-5, GM-CSF and IL-4.

Positions of upstream sequences, relative to the cap sites of the respective genes, are indicated. The "TATA" boxes and conserved CATT(A/T) motifs are underlined. Two sub-regions in the murine IL-5 gene, termed mIL-5A (-61 to -41) and mIL-5B (-76 to -62), are indicated. Oligonucleotide probes containing sequences of these two sub-regions were used in gel shift analysis. Oligonucleotide probes containing sequences of the murine GM-CSF and IL-4 genes which exhibit high homology to the mIL-5A region (-60 to -40 and -70 to -50 respectively) were also used in gel shift analysis.

```
                 ┌──── mIL-5B ────┐┌──────── mIL-5A ────────┐
mIL-5     TCCTCTATCTGATTGTTAGCAATTATTCATTTCCTCAGAGAGAGAATAAA
          ^               ^                   ^
          -76             -61                 -41

hIL-5     TTCTCTATCTGATTGTTAGAAATTATTCATTTCCTCAAAGACAGACAATAAA
                                              ^
                                              -42

mGM-CSF   CCCTGGAGTTCTGTGGTCACCATTAATCATTTCCTCTAACTGTGTATATAA
                          ^                   ^
                          -60                 -40

hGM-CSF   CCCTGGCATTTTGTGGTCACCATTAATCATTTCCTCTGTGTATTTAA
                                              ^
                                              -33

mIL-4     TGTAA-TAAAATTTTCCAATGTAAACTCATTTTCCCTT-20bp-TATATATA
                          ^                   ^
                          -70                 -50

hIL-4     TGTAACGAAAA-TTTCCAATGTAAACTCATTTTCCCTC-20bp-TATATATA
                                              ^
                                              -50
```

5.2. MATERIALS AND METHODS

The sources and preparation of reagents used to treat tissue culture cells in this study have been previously in section 2.2.1.(1). Isolation and Northern blot analysis of total cellular RNA has been previously described in section 2.2.3.

5.2.1. Cell lines

The source and maintenance of the conalbumin-specific Th2 clone D10.G4.1 (Kaye *et al.,* 1983) has been previously described in section 2.2.1.(2). HDK-1 is a keyhole limpets hemocyanin-specific Th1 clone derived from Balb/c mice (Cherwinski *et al.,* 1987) and was obtained from DNAX. HDK-1 cells were stimulated at 5 x 10^5 cells/ml every 10 days with a 10-fold excess of irradiated Balb/c spleen cells and keyhole limpets hemocyanin (150 μg/ml) (Sigma), and maintained in DMEM medium (GIBCO), supplemented with 10% fetal calf serum (CSL), 1 mM sodium pyruvate, 2 mM glutamine, 0.05 mM β-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin and recombinant IL-2 (50 units/ml). The source of IL-2 has been previously described in section 2.2.1.(2).

5.2.2. Preparation of cell extracts

Crude nuclear and cytoplasmic extracts were prepared from unstimulated or stimulated cells as described by Miskimins *et al.* (1985) with modifications. Cells were harvested, washed in ice-cold PBS and lysed at 4 x 10^7 cells/ml in ice-cold lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH [pH 8.0]; 50 mM NaCl; 0.5 M sucrose; 0.1 mM EDTA; 0.5% Triton X-100; 1 mM DTT and 5 mM $MgCl_2$).

Following centrifugation at 800 x g for 5 min at 4°C, the cytoplasmic supernatant was retained on ice and the nuclear pellet was resuspended in lysis buffer at 8 x 10^7 nuclei/ml. Spermidine and NaCl were added to final concentrations of 5 mM and 0.42 M respectively. The suspension was incubated on ice for 30 min with gentle stirring and then centrifuged at 150,000 x g for 15 min at 4°C. The nuclear and cytoplasmic supernatants were dialysed against 500 volumes of dialysis buffer (10 mM HEPES-KOH [pH 8.0]; 50 mM NaCl; 50% glycerol (vol/vol); 1 mM DTT and 1 mM $MgCl_2$) for 12 h at 4°C. The dialysates were centrifuged at 12,000 x g for 10 min at 4°C and aliquots of the supernatants frozen in liquid nitrogen until use. The protein concentrations of cellular extracts were determined using the BioRad Bradford Protein Assay Kit.

5.2.3. Oligonucleotide probes

(1) Oligonucleotide synthesis

Oligonucleotides were synthesized by Dr. P. Milburn and Mr. C. McCrae (Biomolecular Resource Facility) on an Applied Biosystems Model 380A DNA Synthesizer using the phosphoramidite method and purified by HPLC. Pairs of synthetic oligonucleotides containing complementary sequences were annealed by boiling equimolar concentrations of each strand for 10 min and allowing the mixture to slowly cool in the water bath to room temperature.

(2) Synthesis of ^{32}P-end-labeled DNA probes

Four pmoles of annealed oligonucleotides were incubated in a 50 μl reaction containing 50 mM Tris-Cl [pH 7.5]; 10 mM $MgCl_2$; 5 mM DTT, 50 μg/ml BSA; 150 μCi [γ-^{32}P] ATP with 10 units of T4 polynucleotide kinase (Pharmacia) for 30 min at 37°C. The reaction was terminated by

addition of EDTA to 20 mM and unincorporated nucleotides were removed by chromatography as described previously in section 2.2.3.(5). For *in vitro* DNase I footprinting reactions, one oligonucleotide strand was labeled at the 5' terminus by T4 polynucleotide kinase and annealed to an equimolar concentration of unlabeled oligonucleotide strands containing complementary sequences.

5.2.4. Gel shift analysis

Gel shift analysis was performed essentially according to Singh *et al.* (1986) with modifications. 10 fmole of ^{32}P-end-labeled oligonucleotides were incubated in a 20 μl reaction containing 10 mM Tris-Cl [pH 7.9]; 1 mM EDTA; 1 mM DTT; 10% glycerol; 100 mM NaCl; 5 mM $MgCl_2$; 1 μg poly(dI.dC).poly(dI.dC) (Pharmacia) with 8 μg of crude cellular extracts for 20 min at room temperature. In competition experiments, a 10 to 200 molar excess of unlabeled oligonucleotides, relative to the probe, was added to the binding reaction. Following incubation, bromophenol blue and xylene cyanol were added to 0.05% and resulting complexes were resolved on low ionic strength 6% polyacrylamide gels (acrylamide:bisacrylamide weight ratio 30:1) containing 6.7 mM Tris-Cl [pH 7.9]; 3.3 mM sodium acetate and 1 mM EDTA by electrophoresis at 11 Vcm^{-1} at room temperature. The gel was subsequently dried and exposed to X-ray film (Kodak XAR) at -70°C.

5.2.5. *In vitro* DNase I footprinting

In vitro DNase I footprinting analysis was performed essentially according to Henninghausen & Lubon (1987) with modifications. 10 fmole of ^{32}P-end-labeled DNA was incubated in a 50 μl reaction containing 10 mM Tris-Cl [pH 7.9]; 1 mM EDTA; 1 mM DTT; 10% glycerol; 100 mM NaCl;

5 mM $MgCl_2$; 1 µg poly(dI.dC).poly(dI.dC) (Pharmacia) with 0 to 100 µg of crude cellular extracts for 20 min at room temperature. As a control, end-labeled DNA was incubated with BSA instead of cellular extracts under the same conditions. $CaCl_2$ was added to 1 mM and DNase I (RQ1, Promega) was added to 1 to 3 units/ml. After incubation for 1 min at 37°C, the reaction was terminated by addition of Tris-Cl [pH 8.0], NaCl, EDTA, SDS and yeast tRNA to final concentrations of 100 mM, 100 mM, 10 mM, 0.5% and 100 µg/ml respectively. The mixture was incubated with proteinase K (100 µg/ml) for 20 min at 50°C, followed by phenol and chloroform:isoamylalcohol (24:1) extractions and ethanol precipitation. The reaction products were resuspended in 90% formamide containing 0.05% bromophenol blue and xylene cyanol and resolved by electrophoresis on denaturing 8 M urea/12% polyacrylamide gels containing 0.089 M Tris-base, 0.089 M boric acid and 2 mM EDTA. The gel was subsequently dried and exposed to X-ray film at -70°C.

5.3. RESULTS

5.3.1. DNase I footprinting analysis of protein interactions with 5'- flanking sequences of the murine IL-5 gene

DNase I footprinting analysis was initially performed to identify any qualitative differences in the interactions between nuclear proteins extracted from unstimulated or stimulated T cells and 5'-flanking regions of the murine IL-5 gene. Of particular interest was the region immediately 5' of the TATA box which is highly conserved in the GM-CSF, IL-4 and human IL-5 genes (Table 5.1.) and includes sequences homologous to those reported to be involved in inducing GM-CSF gene transcription (Kaushansky, 1989; Nimer *et al.*, 1988; 1989a,b; 1990). A unlabeled synthetic oligonucleotide containing sequences from -107 to -32 of the murine IL-5 gene was annealed to a ^{32}P-end-labeled oligonucleotide containing complementary (non-coding) sequences. This duplex was incubated with crude nuclear extracts prepared from unstimulated D10.G4.1 cells or cells which were stimulated with Con A for 6 h. These stimulatory conditions were previously found to induce maximal transcriptional activity of the IL-5 gene (Chapter 3) and thus may involve temporally elevated levels of potential DNA-binding *trans*-activating factors. Although numerous reports have demonstrated DNase I protection using enriched or purified sequence-specific DNA-binding proteins, this analysis has been successfully extended to using crude nuclear extracts (Ohlsson & Edlund, 1986; Nimer *et al.*, 1988). The free and protein-bound DNA was subjected to limiting DNase I digestion and purified DNA resolved by denaturing gel electrophoresis.

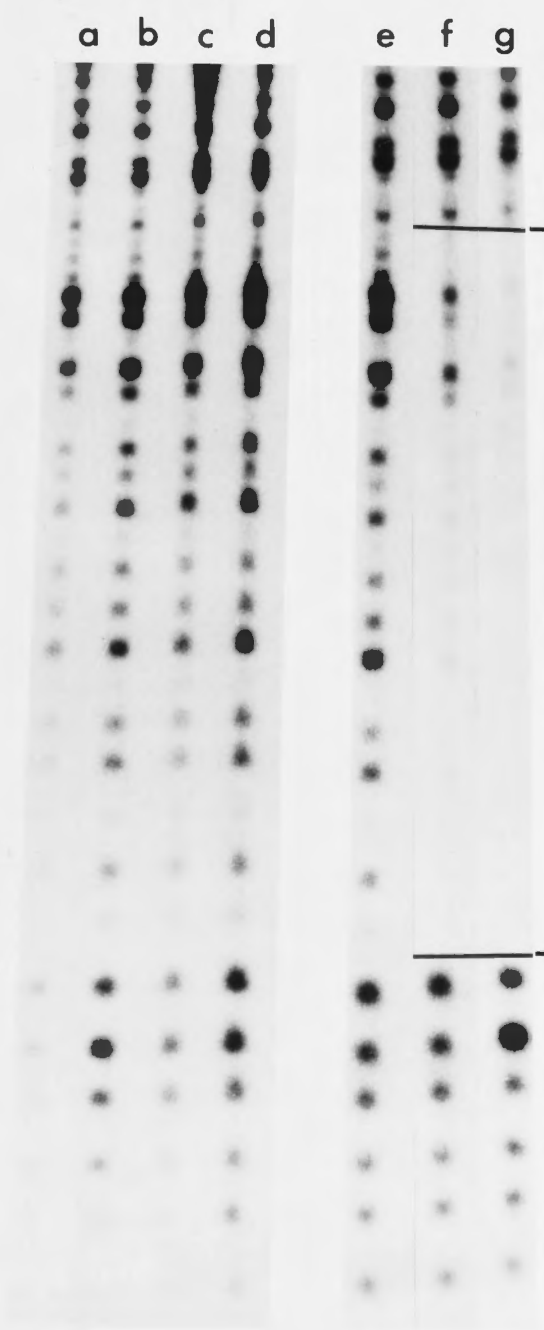
As shown in Fig. 5.1.(A), incubation of the DNA with crude nuclear extracts prepared from unstimulated D10.G4.1 cells resulted, upon DNase I

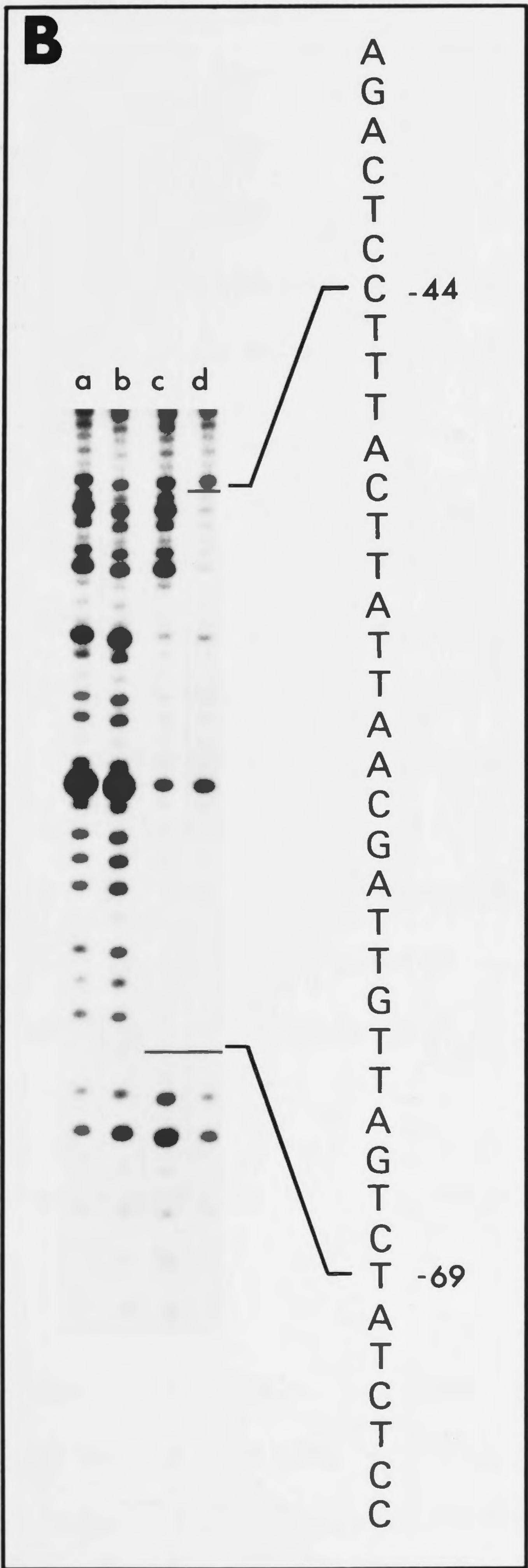
Fig. 5.1. DNase I footprinting analysis of protein interactions with sequences upstream of the murine IL-5 gene.

The non-coding strand (A) or coding strand (B) of DNA sequences from -107 to -32 were ^{32}P-end-labeled. 10 fmole of DNA was incubated with 30 μg or 100 μg of either BSA or crude nuclear extracts prepared from unstimulated or Con A-stimulated D10.G4.1 cells, as indicated below, under the conditions described in section 5.2.5. Unbound and protein-bound DNA was subjected to digestion with DNase I at 1 or 3 units/ml as indicated, and the DNA was subsequently purified and resolved by denaturing gel electrophoresis. The positions of sequences protected from DNase I digestion are indicated.

		Protein		DNase I
(A)	(a)	BSA	30 μg	1 unit/ml
	(b)	BSA	30 μg	3 units/ml
	(c)	unstimulated	30 μg	1 unit/ml
	(d)	unstimulated	30 μg	3 units/ml
	(e)	BSA	30 μg	3 units/ml
	(f)	stimulated	30 μg	3 units/ml
	(g)	stimulated	100 μg	3 units/ml
(B)	(a)	BSA	30 μg	3 units/ml
	(b)	unstimulated	30 μg	3 units/ml
	(c)	stimulated	30 μg	3 units/ml
	(d)	stimulated	100 μg	3 units/ml

A




B
a b c d
A
G
A
C
T
C
C -44
T
T
T
A
C
T
T
A
T
T
A
A
C
G
A
T
T
G
T
T
A
G
T
C
T -69
A
T
C
T
C
C

digestion and resolution by gel electrophoresis, in a pattern of bands not dissimilar to the pattern resulting from incubation of the probe with non-specific proteins such as BSA (Lanes a-d). Incubation of the probe with crude nuclear extracts prepared from Con A-stimulated D10.G4.1 cells, however, resulted in a considerably different pattern (Lanes f,g). The presence and position of an observable "gap" in the resultant ladder of bands suggests that sequences between -68 and -47 are protected from DNase I digestion by the binding of a specific nuclear protein(s) which is present only in stimulated, but not unstimulated, D10.G4.1 cells.

Footprinting analysis was repeated using a probe composed of the ^{32}P-end-labeled coding strand annealed to the unlabeled non-coding strand. As shown in Fig. 5.1.(B), sequences between -69 and -44 were protected from DNase I digestion when incubated with nuclear extracts prepared from Con A-stimulated D10.G4.1 cells (Lanes c,d), but not from unstimulated cells nor with BSA (Lanes a,b). No other protein interactions were observed with sequences extending further upstream to -107 using nuclear extracts prepared from either unstimulated or stimulated D10.G4.1 cells.

5.3.2. Gel shift analysis of protein interactions with two discrete elements in the proximal 5'- flanking region

To further investigate the interactions between sequences upstream of the murine IL-5 gene and the putative inducible nuclear protein(s) identified by DNase I footprinting analysis, gel shift assays were performed. A synthetic oligonucleotide containing sequences from -76 to -41, which include those protected from DNase I digestion, was annealed to a second oligonucleotide containing complementary sequences. The resulting duplex, termed mIL-5(-76/-41) was ^{32}P-end-labeled, incubated with either BSA or crude

nuclear extracts prepared from unstimulated or Con A-stimulated D10.G4.1 cells and unbound and protein-bound DNA resolved by native gel electrophoresis. As shown in Fig. 5.2., no DNA probe was bound when incubated with either BSA or nuclear extracts prepared from unstimulated D10.G4.1 cells (Lanes 2,3). Incubation of the probe with nuclear extracts prepared from Con A-stimulated D10.G4.1 cells resulted, however, in the formation of several DNA-protein complexes of different electrophoretic mobilities (Lane 1).

To resolve the DNA-protein complexes associated with sequences located between -76 and -41, gel shift assays were conducted using two shorter oligonucleotide probes, termed mIL-5A(-61/-41) and mIL-5B(-76/-62), which respectively contain sequences of adjacent sub-regions tentatively referred to here as mIL-5A (-61 to -41) and mIL-5B (-76 to -62). Sequences of mIL-5A are highly homologous to those of corresponding regions in the GM-CSF and IL-4 genes (Table 5.1.). Sequences of the adjacent mIL-5B sub-region are highly conserved between the murine and human IL-5 genes, but do not demonstrate significant homology to corresponding regions in other lymphokine genes.

Distinct complexes of different electrophoretic mobilities were observed to form independently to oligonucleotides mIL-5A(-61/-41) and mIL-5B(-76/-62) using crude nuclear extracts prepared from Con A-stimulated D10.G4.1 cells (Fig. 5.2., Lanes 4,7). The proteins associated with these complexes, respectively termed NF-IL-5A and NF-IL-5B, appeared to be present (or demonstrate active DNA-binding ability) only in the nuclei of activated D10.G4.1 cells. No protein interactions with either of these two oligonucleotides were detected using cytoplasmic extracts prepared from

Fig. 5.2. **Gel shift analysis of interactions between sequences 5' of the murine IL-5 gene and nuclear proteins extracted from unstimulated or stimulated D10.G4.1 cells.**

^{32}P-labeled DNA containing sequences of the murine IL-5 gene from -76 to -41 (mIL-5(-76/-41)) (Lanes 1-3), from -61 to -41 (mIL-5A(-61/-41)) (Lanes 4-6) or from -76 to -62 (mIL-5B(-76/-62)) (Lanes 7-9) were incubated with either BSA (Lanes 3,6,9) or nuclear extracts prepared from unstimulated (Lanes 2,5,8) or Con A-stimulated (Lanes 1,4,7) D10.G4.1 cells under the conditions described in section 5.2.4. Unbound and protein-bound DNA was resolved by native gel electrophoresis.

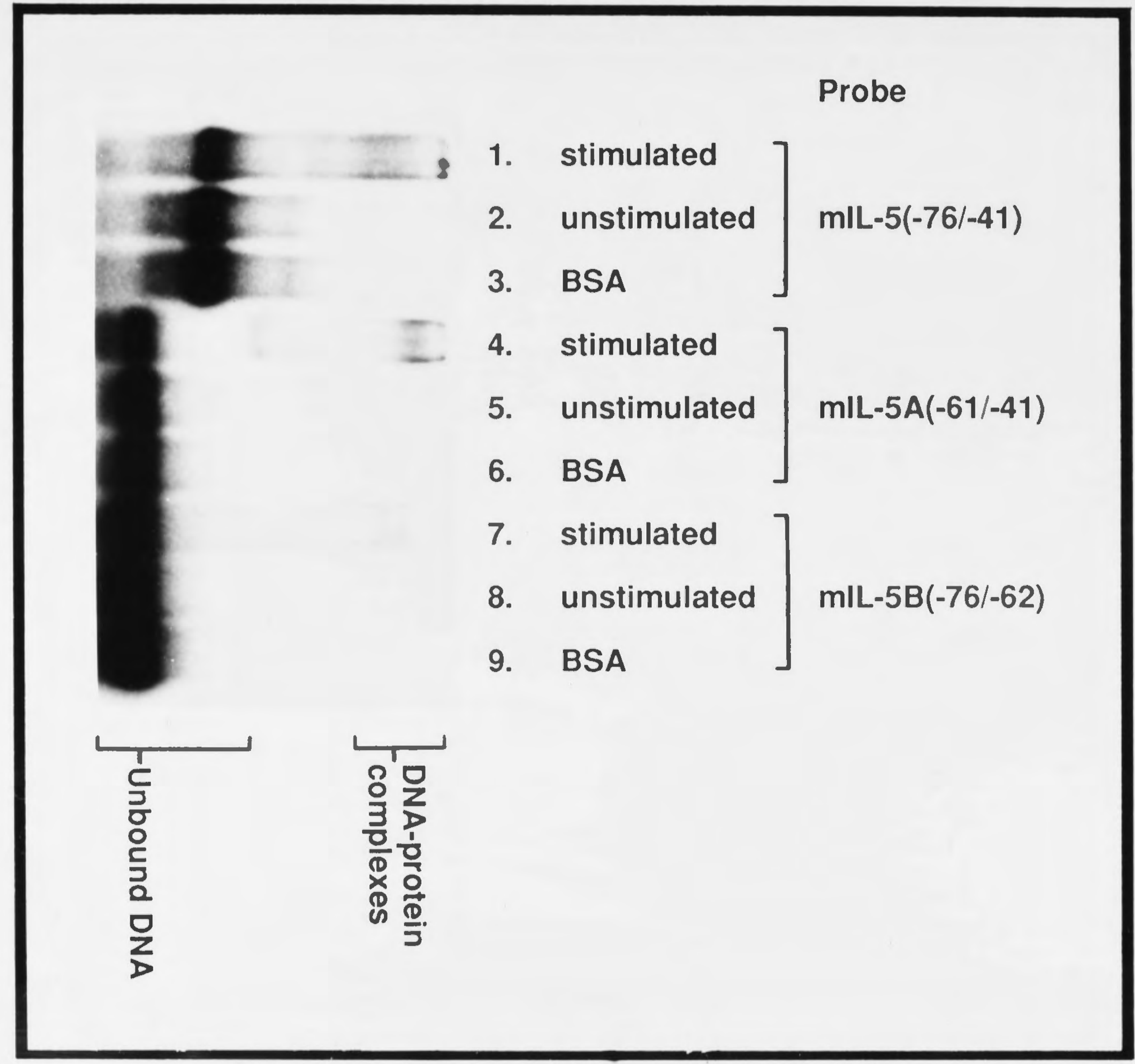
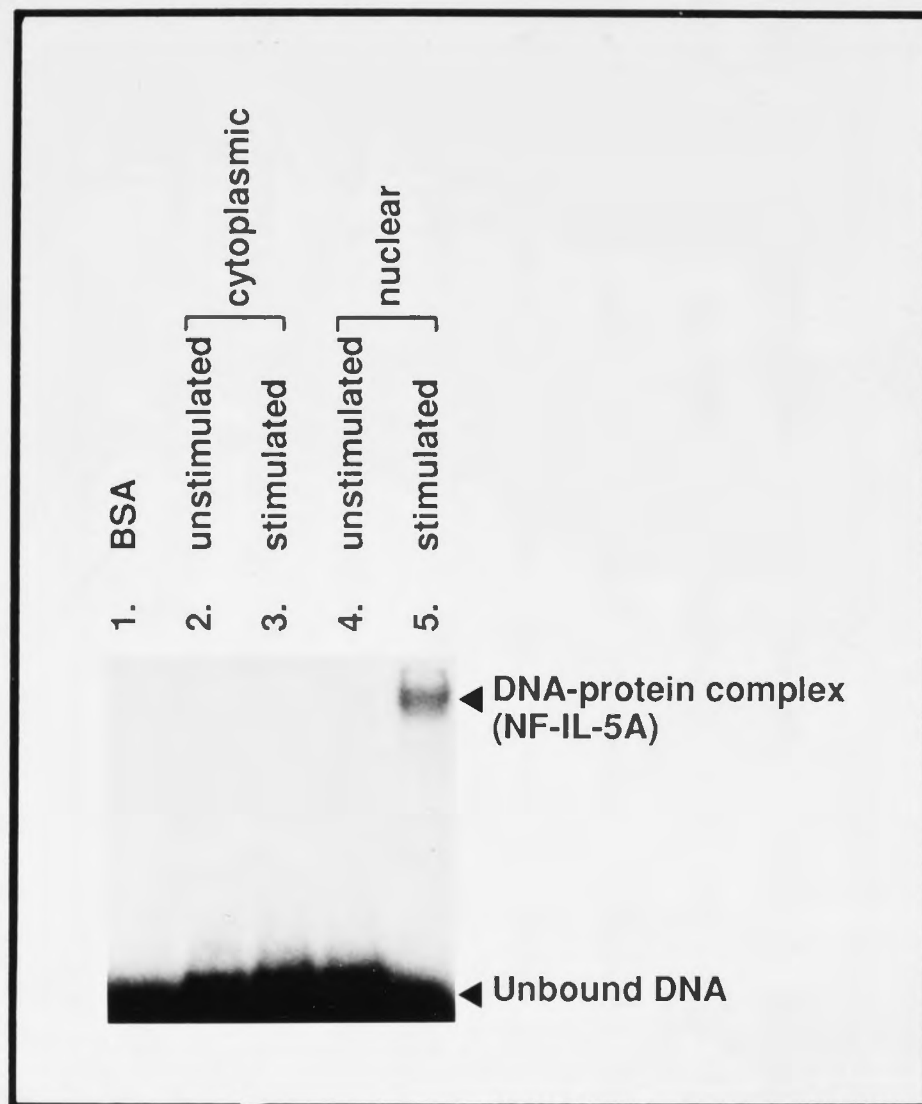

Probe
1. stimulated
2. unstimulated
3. BSA
mIL-5(-76/-41)
4. stimulated
5. unstimulated
6. BSA
mIL-5A(-61/-41)
7. stimulated
8. unstimulated
9. BSA
mIL-5B(-76/-62)
Unbound DNA
DNA-protein complexes

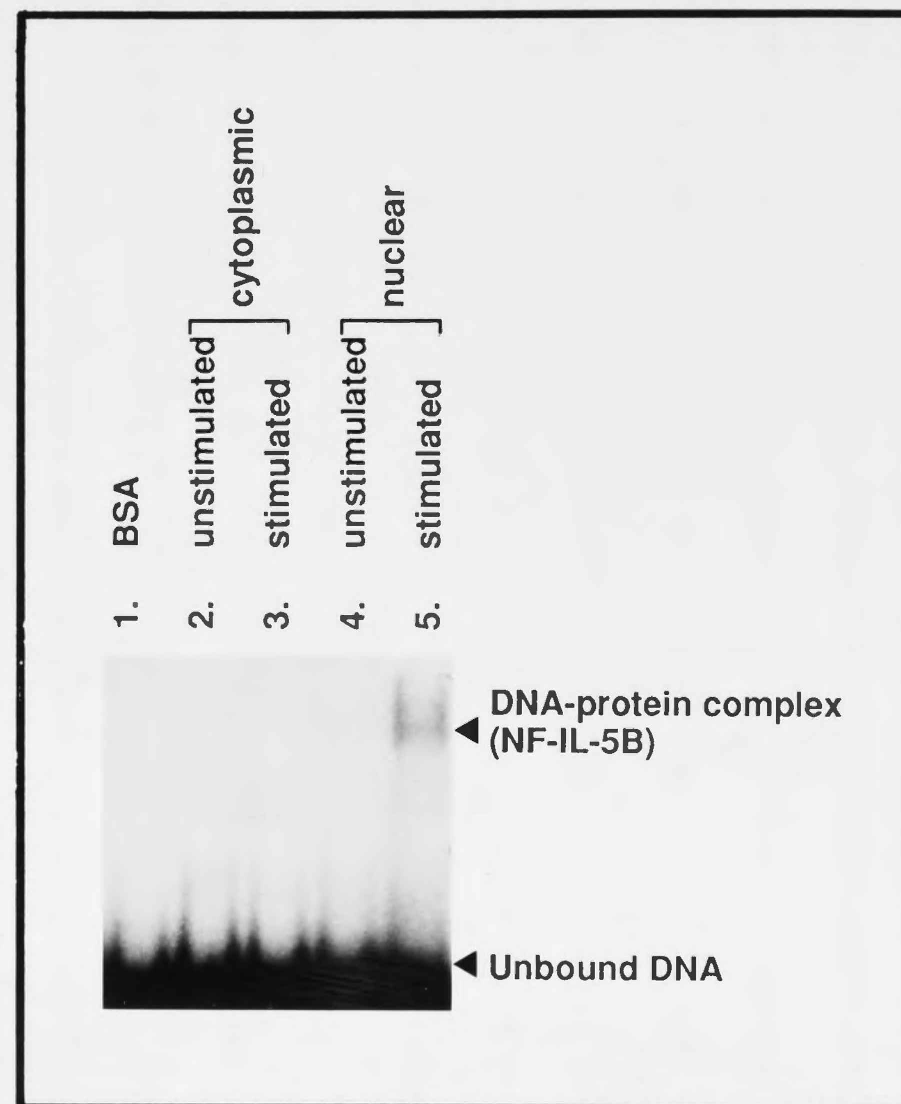
Fig. 5.3. **Inducible nuclear protein interactions with mIL-5A and mIL-5B, two discrete DNA elements located 5' of the murine IL-5 gene.**

^{32}P-labeled oligonucleotide probes containing sequences (a) from -61 to -41 (mIL-5A(-61/-41)) or (b) from -76 to -62 (mIL-5B(-76/-62)) were incubated with either BSA (Lane 1) or cytoplasmic or nuclear extracts prepared from unstimulated (Lanes 2,4) or Con A-stimulated (Lanes 3,5) D10.G4.1 cells. DNA-protein complexes formed with mIL-5A(-61/-41) and mIL-5B(-76/-62)) are respectively termed NF-IL-5A and NF-IL-5B.

(a) Probe: mIL-5A(-61/-41)



(b) Probe: mIL-5B(-76/-62)



unstimulated or stimulated D10.G4.1 cells or unstimulated nuclear extracts (Fig. 5.3.(a)(b)).

To investigate the sequence-specificity of protein interactions with the mIL-5A and mIL-5B elements, competition assays were performed. The addition to the binding reaction of increasing amounts of unlabeled competitor DNA, containing the same sequences as the probe, increasingly inhibited protein interactions with either mIL-5A(-61/-41) (Fig. 5.4.(a), Lanes 3-5) or mIL-5B(-76/-62) (Fig. 5.4.(b), Lanes 3-5). The addition to the binding reaction of the same increasing amounts of unlabeled competitor DNA, containing sequences non-homologous to those of either probe was, in contrast, significantly less effective in perturbing the formation of the NF-IL-5A or NF-IL-5B complexes (Fig. 5.4.(a)(b), Lanes 6-8). These non-homologous or "non-specific" competitor sequences themselves were observed not to specifically bind proteins (data not shown).

The addition of increasing amounts of unlabeled mIL-5B(-76/-62) to the binding reaction did not significantly perturb formation of NF-IL-5A (Fig. 5.4.(a), Lanes 10-12), when compared with competition assays using the same increasing amounts of sequences non-homologous to either mIL-5B(-76/-62) or mIL-5A(-61/-41) (Fig. 5.4.(b), Lanes 6-8). Conversely, the addition of increasing amounts of unlabeled mIL-5A(-61/-41) did not significantly perturb formation of NF-IL-5B (Fig. 5.4.(b), Lanes 10-12), when compared with competition assays using non-homologous sequences (Fig. 5.4.(b), Lanes 6-8). These competition assays together suggest that two discrete adjacent elements, described here as mIL-5A (-61 to -41) and mIL-5B (-76 to -62), specifically and independently form distinct complexes, respectively termed NF-IL-5A and NF-IL-5B, with putatively different nuclear

Fig. 5.4. **Specificity of inducible nuclear protein interactions with the mIL-5A and mIL-5B elements.**

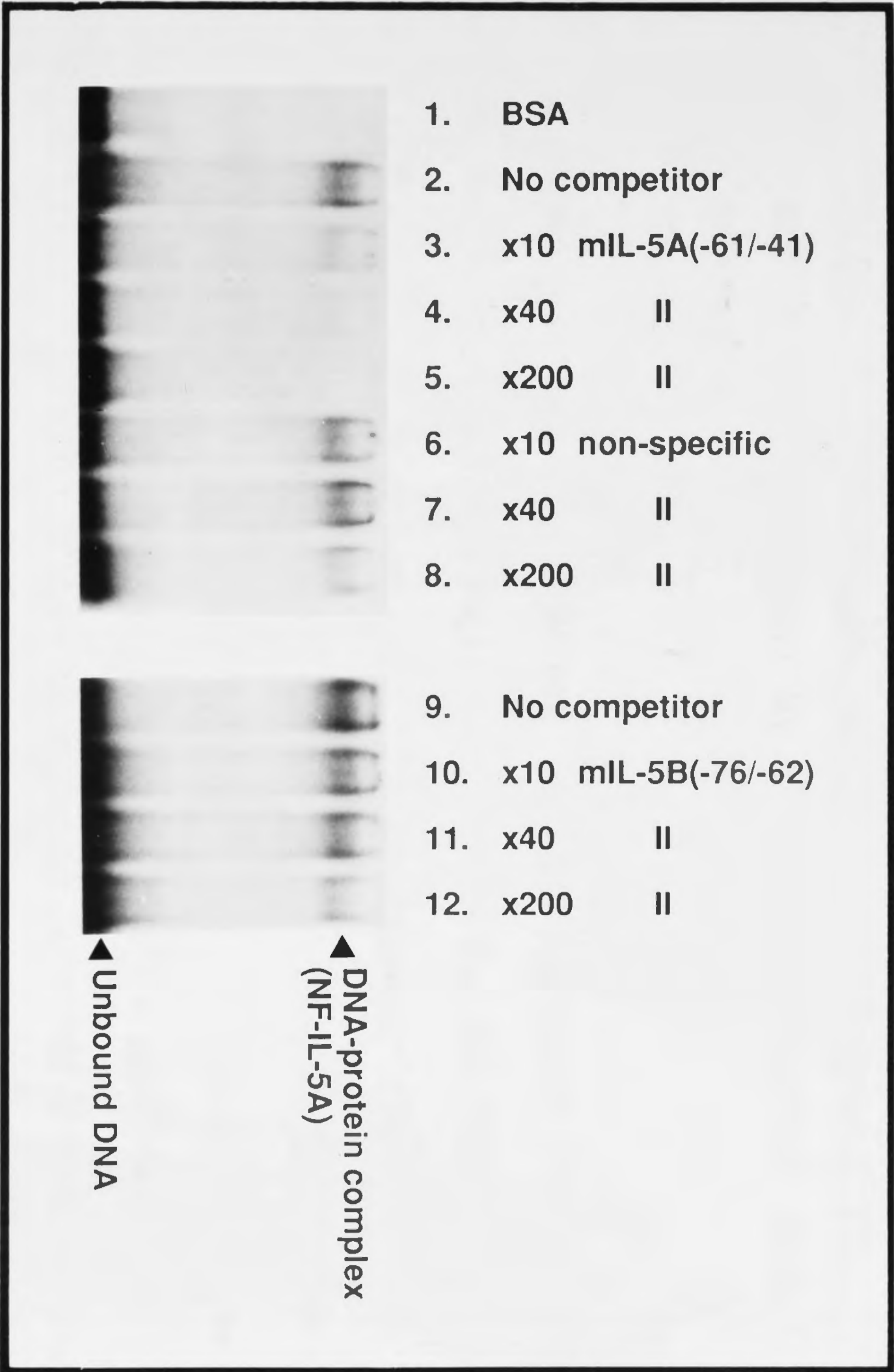
^{32}P-labeled mIL-5A(-61/-41) (a) or mIL-5B(-76/-62) (b) were incubated with either BSA (Lane 1) or Con A-stimulated D10.G4.1 nuclear extracts in the absence of competitor DNA (Lane 2) or presence of the indicated molar excess, relative to the probe, of unlabeled competitor DNA containing sequences identical to those of the respective probes (Lane 3-5) or sequences non-homologous ("non-specific") to those of either probe (Lanes 6-8). These "non-specific" competitors were of the same lengths as the probe used and their sequences are indicated below. Protein interactions with mIL-5A(-61/-41) (NF-IL-5A) were also challenged by addition to the binding reaction of the indicated molar excess of unlabeled mIL-5B(-76/-62) (a, Lanes 9-12), while protein interactions with mIL-5B(-76/-62) (NF-IL-5B) were challenged with the same increasing molar excesses of unlabeled mIL-5A(-61/-41) (b, Lanes 9-12).

Non-specific competitor against mIL-5A(-61/-41):

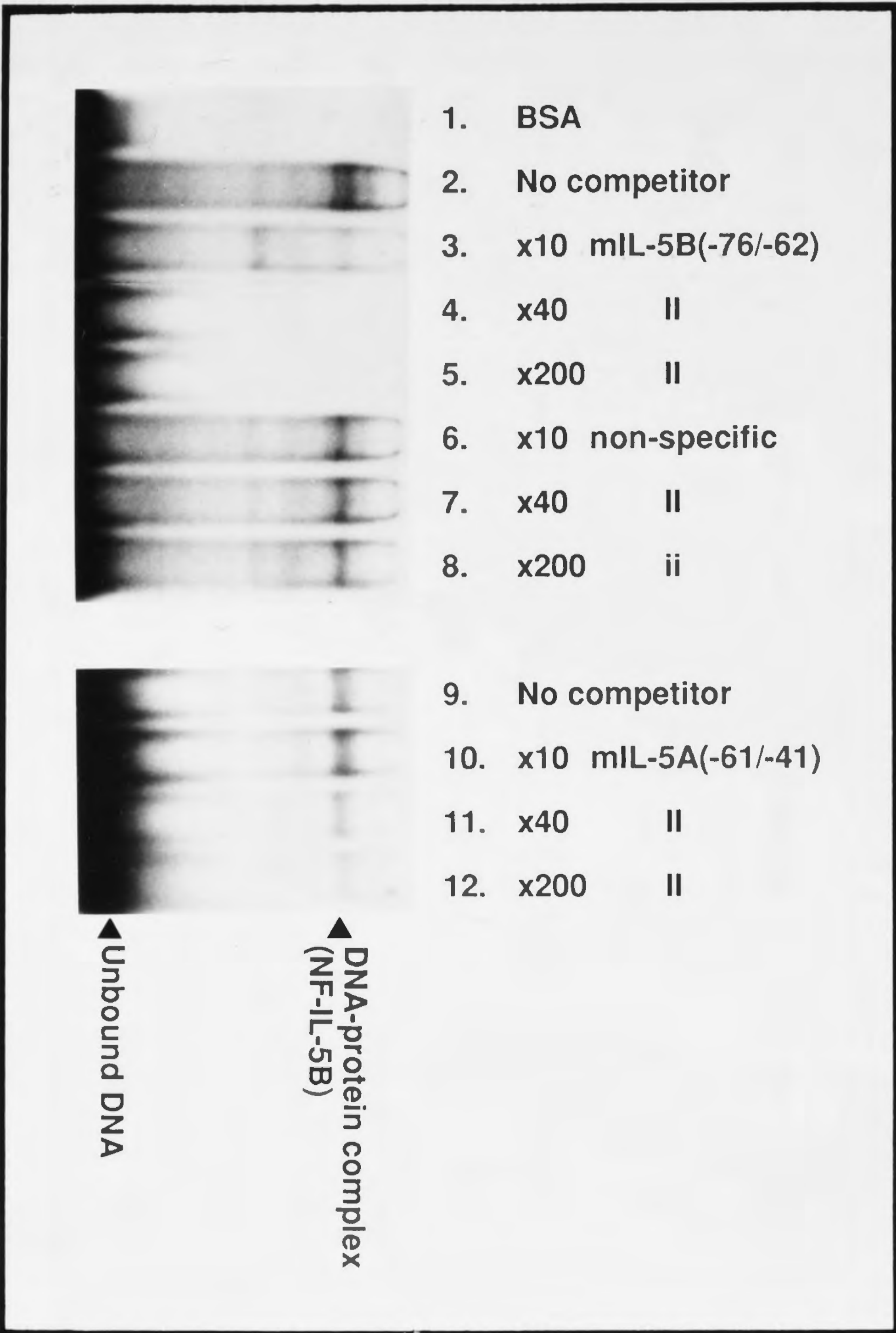
5'-CCGATGGCCGACTGCACTTCT-3'

Non-specific competitor against mIL-B(-76/-62):

5'-CTTCTCGCTCAGCCA-3'



(a) Probe: mIL-5A(-61/-41)
1. BSA
2. No competitor
3. x10 mIL-5A(-61/-41)
4. x40 II
5. x200 II
6. x10 non-specific
7. x40 II
8. x200 II
9. No competitor
10. x10 mIL-5B(-76/-62)
11. x40 II
12. x200 II
Unbound DNA
DNA-protein complex (NF-IL-5A)



(b) Probe: mIL-5B(-76/-62)
1. BSA
2. No competitor
3. x10 mIL-5B(-76/-62)
4. x40 II
5. x200 II
6. x10 non-specific
7. x40 II
8. x200 ii
9. No competitor
10. x10 mIL-5A(-61/-41)
11. x40 II
12. x200 II
Unbound DNA
DNA-protein complex (NF-IL-5B)

proteins which are present (or active) in stimulated, but not unstimulated, D10.G4.1 cells.

5.3.3. Protein interactions with the conserved TCATTT-containing element in the IL-5, GM-CSF and IL-4 genes

As shown in Table 5.1., sequences of the mIL-5A region (-61 to -41) are highly homologous to analogous regions present in the GM-CSF (-60 to -40) and IL-4 (-70 to -50) genes and include a 100% conserved TCATTT motif. To determine whether nuclear proteins associated with the NF-IL-5A complex also interact with the highly conserved sequences in other lymphokine promoters, gel shift assays were performed using Con A-stimulated D10.G4.1 nuclear extracts to compare protein interactions with mIL-5A(-61/-41) and homologous sequences in the murine GM-CSF (mGM-CSF(-60/-40)) and IL-4 (mIL-4(-70/-50)) genes. As shown in Fig. 5.5.A., DNA-protein interactions of similar electrophoretic mobility and affinity to NF-IL-5A were observed to form with mGM-CSF(-60/-40), while only weak interactions were observed to mIL-4(-70/-50).

Competition assays were subsequently performed to further assess the affinity of nuclear proteins for these lymphokine elements. As shown in Fig. 5.5.B., NF-IL-5A formation was increasingly inhibited by increasing amounts of unlabeled mIL-5A(-61/-41) (Lanes 2,3) and inhibited to a slightly lesser degree by the same increasing amounts of unlabeled mGM-CSF(-60/-40) (Lanes 4,5). Unlabeled mIL-4(-70/-50), in comparison, only weakly inhibited protein interactions with mIL-5A(-61/-41) (Lanes 6,7), though not as weakly as sequences non-homologous to any of these elements (Lanes 8,9).

Fig. 5.5. **Comparison of DNA-protein complex formations associated with mIL-5A and homologous elements in the murine GM-CSF and IL-4 genes.**

A. The indicated oligonucleotide probes containing sequences from -61 to -41 of the murine IL-5 gene (mIL-5A(-61/-41)) (Lane 1), from -60 to -40 of the murine GM-CSF gene (mGM-CSF(-60/-40)) (Lane 2) or from -70 to -50 of the murine IL-4 gene (mIL-4(-70/-50)) (Lane 3) were incubated with nuclear extracts from Con A-stimulated D10.G4.1 cells.

	5' 3'
mIL-5A(-61/-41)	TTAGCAATTATTCATTTCCTC
mGM-CSF(-60/-40)	GTCACCATTAATCATTTCCTC
mIL-4(-70/-50)	CCAATGTAAACTCATTTTCCC

A

1. mIL-5A(-61/-41)
2. mGM-CSF(-60/-40)
3. mIL-4(-70/-50)

◀ DNA-protein complexes

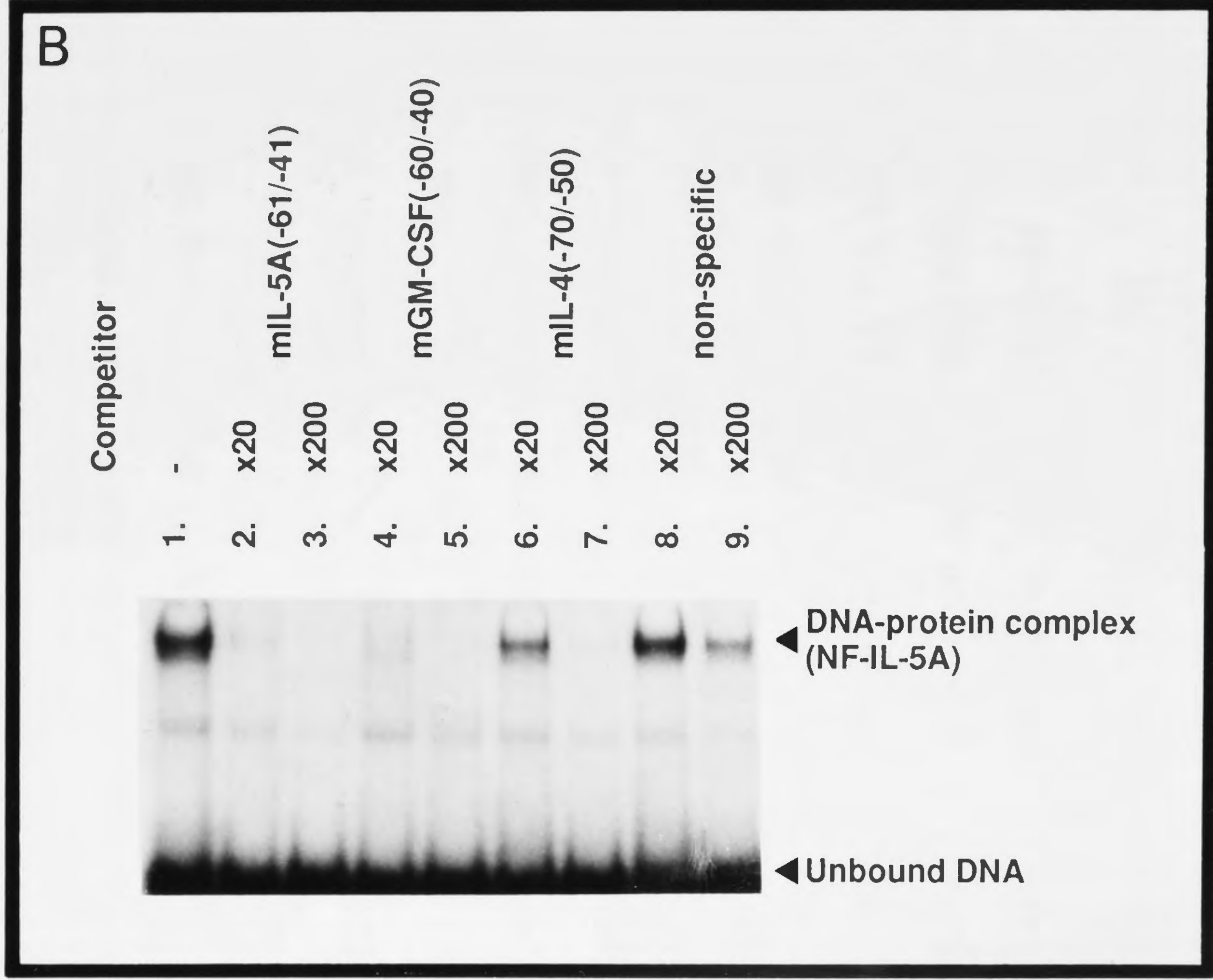
◀ Unbound DNA

Fig. 5.5. Comparison of DNA-protein complex formations associated with mIL-5A and homologous elements in the murine GM-CSF and IL-4 genes.

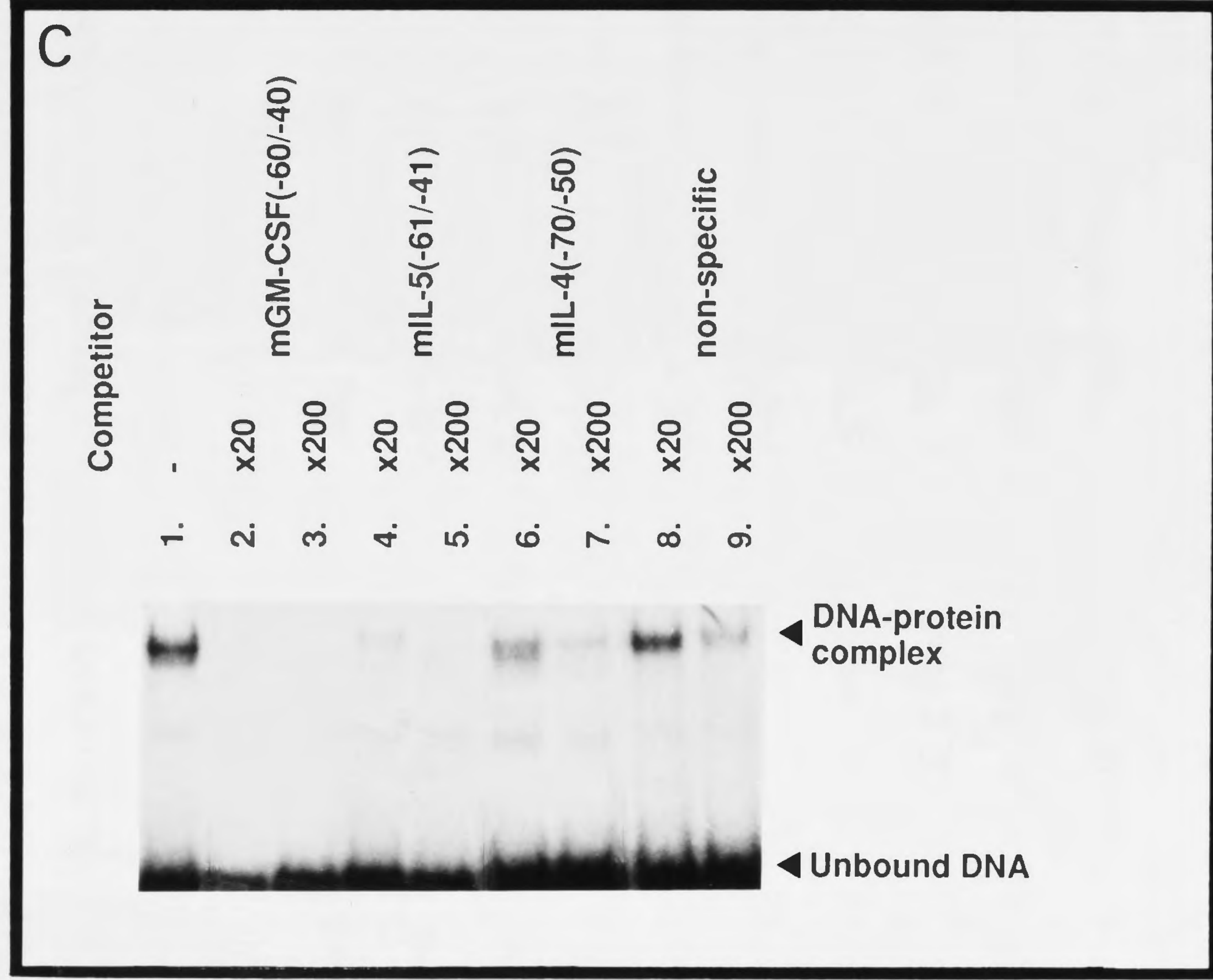
B. ^{32}P-labeled mIL-5A(-61/-41) was incubated with Con A-stimulated D10.G4.1 nuclear extracts either in the absence of competitor DNA (Lane 1) or presence of the indicated molar excess, relative to the probe, of unlabeled mIL-5A(-61/-41) (Lanes 2,3), mGM-CSF(-60/-40) (Lanes 4,5), mIL-4(-70/-50) (lanes 6,7) or sequences non-homologous to any of these elements ("non-specific") (Lanes 8,9). These "non-specific" competitor sequences have been previously described in Fig. 5.4.

C. ^{32}P-labeled mGM-CSF(-60/-40) was incubated with Con A-stimulated D10.G4.1 nuclear extracts either in the absence of competitor DNA (Lane 1) or presence of the indicated molar excess of unlabeled mGM-CSF(-60/-40) (Lanes 2,3), mIL-5A(-61/-41) (Lanes 4,5), mIL-4(-70/-50) (Lanes 6,7) or sequences non-homologous to any of these elements (non-specific) (Lanes 8,9). The "non-specific" competitor was the same as that used against mIL-5A(-61/-41).

Probe: mIL-5A(-61/-41)



Probe: mGM-CSF(-60/-40)



In a converse experiment, protein interactions with mGM-CSF(-60/-40) were found to be increasingly inhibited by increasing amounts of unlabeled sequences identical to the probe (Fig. 5.5.C., Lanes 2,3), to a slightly lesser degree by mIL-5A(-61/-41) (Lanes 4,5) and only weakly by mIL-4(-70/-50) (Lanes 6,7). These results indicate that nuclear proteins associated with the NF-IL-5A complex bind with highest affinity to mIL-5A(-61/-41), with slightly reduced affinity to mGM-CSF(-60/-40) and with significantly weaker affinity to the less conserved analogous element in the murine IL-4 gene. Furthermore, these results suggest that similar and closely related, but possibly not identical, proteins may recognise the conserved element in the IL-5 and GM-CSF genes.

The significantly weak affinity of nuclear proteins for mIL-4(-70/-50) suggests that other sequences apart from, and located 5' to, the 100% conserved TCATTT motif, may be critical for binding of inducible nuclear proteins. Such sequences are highly conserved between the IL-5 and GM-CSF genes, and are less conserved in the IL-4 gene (Table 5.1., Fig. 5.5.A.). A report published during the course of this study indicated, however, that mutations of the TCATTT motif eliminated inducible human GM-CSF promoter activity in T cells (Nimer *et al.,* 1990). To determine whether the TCATTT motif was critical for recognition by the inducible proteins, competition assays were performed using murine IL-5 and GM-CSF sequences which contained the same base-substitutions introduced into the TCATTT motif as those reported by Nimer *et al.* (1990) (Fig. 5.6.). These TCATTT mutants were respectively termed mIL-5(-61/-41)(mut) and mGM-CSF(-60/-40)(mut). Both of these mutant recognition elements failed to bind nuclear proteins (Fig. 5.6.A., Lane 1; Fig. 5.6.B., Lane 1). NF-IL-5A complex formation with the wild-type element (mIL-5(-61/-41)(wt)) was weakly perturbed by increasing amounts of mutated sequences (mIL-5(-61/-41)(mut))

Fig. 5.6. **Effect upon protein-binding of substitution mutations in the conserved TCATTT motif in the murine IL-5 and GM-CSF genes.**

A. Oligonucleotide probes containing wild type sequences of the murine IL-5 gene from -61 to -41 (mIL-5(-61/-41)(wt)) (Lane 8) or harboring substitution mutations in the conserved TCATTT motif (mIL-5(-61/-41)(mut)) (indicated below) (Lane 1) were incubated with Con A-stimulated D10.G4.1 nuclear extracts. Protein interactions with the wild type sequences (mIL-5(-61/-41)(wt)) were challenged by addition to the binding reaction of the indicated molar excess of unlabeled oligonucleotides containing the mutated binding site (Lanes 4,5), the wild type binding site (Lanes 6,7) or sequences non-homologous to either the wild-type or mutated sequences (Lanes 2,3). The "non-specific" competitor was the same as that previously described in Fig. 5.4.

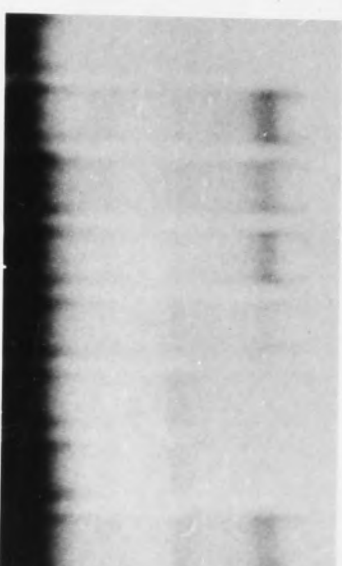
B. Oligonucleotide probes containing wild type sequences of the murine GM-CSF gene from -60 to -40 (mGM-CSF(-60/-40)(wt)) (Lane 8) or harboring the indicated substitution mutations in the conserved TCATTT motif (mGM-CSF(-60/-40)(mut)) (Lane 1) were incubated with Con A-stimulated D10.G4.1 nuclear extracts. Protein interactions with the wild type sequences (mGM-CSF(-60/-40)(wt)) were challenged by the indicated molar excess of unlabeled oligonucleotides containing the mutated binding site (Lanes 4,5), the wild type binding site (Lanes 6,7) or sequences non-homologous to either the wild type or mutated sequences (Lanes 2,3).

```
                    5'                   3'
mIL-5(-61/-41)(wt)  TTAGCAATTATTCATTTCCTC
mIL-5(-61/-41)(mut) ------------CG-AA-----
mGM-CSF(-60/-40)(wt) GTCACCATTAATCATTTCCTC
mGM-CSF(-60/-40)(mut)------------CG-AA-----
```

A

	Probe	Competitor	
1.	mIL-5(-61/-41)(mut)	-	
2.	mIL-5(-61/-41)(wt)	x20	non-specific
3.	"	x200	non-specific
4.	"	x20	mIL-5(-61/-41)(mut)
5.	"	x200	mIL-5(-61/-41)(mut)
6.	"	x20	mIL-5(-61/-41)(wt)
7.	"	x200	mIL-5(-61/-41)(wt)
8.	"	-	

B



	Probe	Competitor	
1.	mGM-CSF(-60/-40)(mut)	-	
2.	mGM-CSF(-60/-40)(wt)	x20	non-specific
3.	"	x200	non-specific
4.	"	x20	mGM-CSF(-60/-40)(mut)
5.	"	x200	mGM-CSF(-60/-40)(mut)
6.	"	x20	mGM-CSF(-60/-40)(wt)
7.	"	x200	mGM-CSF(-60/-40)(wt)
8.	"		

(Fig. 5.6.A., Lanes 4,5), though inhibition was stronger than that observed in analogous competition assays using sequences non-homologous to either the wild-type or mutant elements (Lanes 2,3). Similarly, complex formation with wild-type GM-CSF sequences (mGM-CSF(-60/-40)(wt)) was only weakly inhibited by increasing amounts of mutated GM-CSF sequences (mGM-CSF(-60/-40)(mut)) (Fig. 5.6.B., Lanes 4,5). These results together suggest that the intact TCATTT motif and several adjacent bases 5' to this motif are both required for recognition by inducible nuclear DNA-binding factors..

5.3.4. Signal modulation and cell-specificity of protein interactions with the conserved TCATTT-containing element

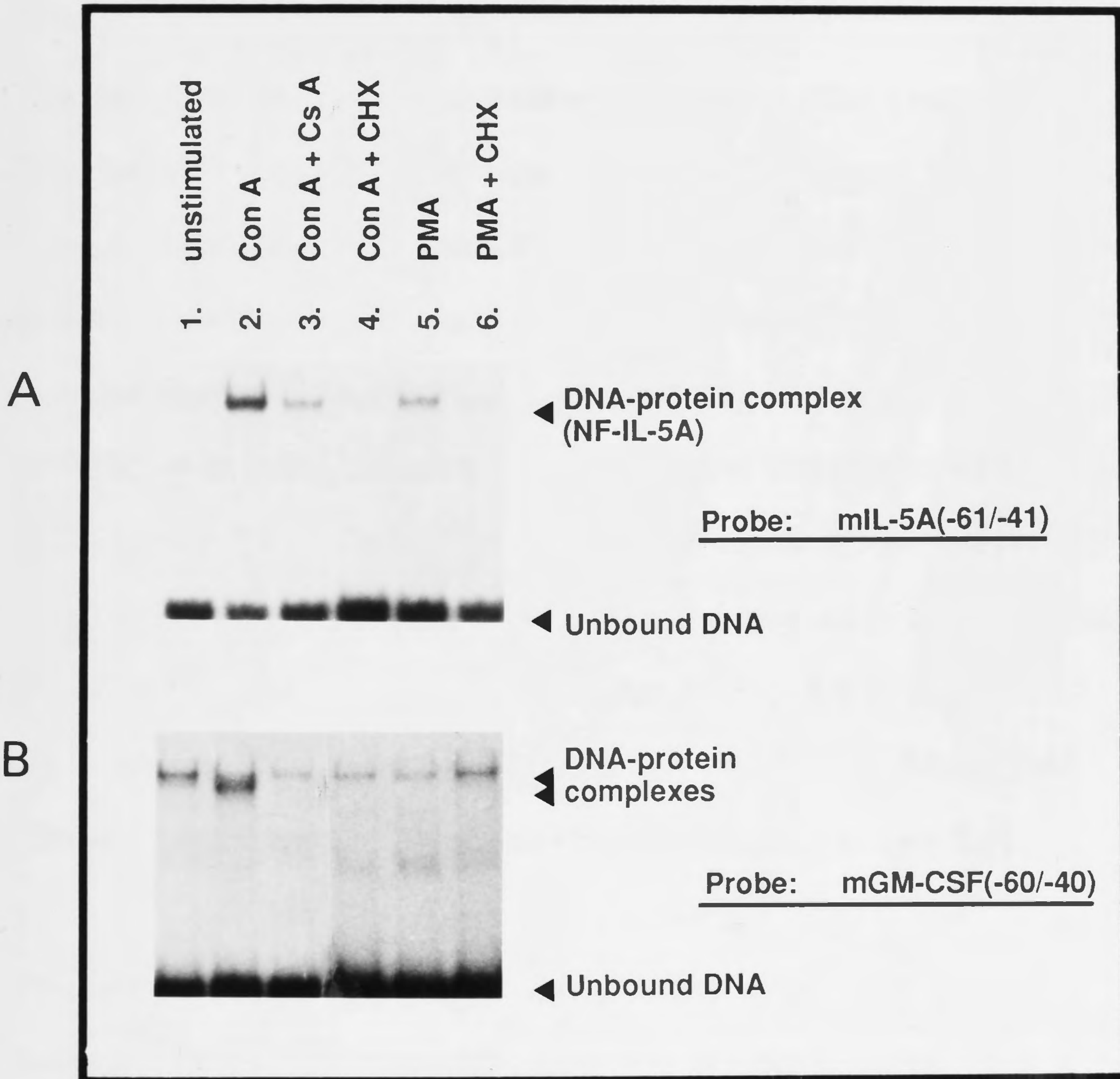
Studies described in Chapter 3 indicated that the GM-CSF gene is constitutively transcribed in unstimulated D10.G4.1 cells while the IL-5 gene is transcriptionally silent. While the transcriptional activities of both genes were coordinately elevated in response to Con A stimulation, activation of GM-CSF gene transcription, but not IL-5 gene transcription, appeared to be regulated by a Cs A-sensitive mechanism. Furthermore, IL-5 gene transcription was independently activated in response to PMA stimulation and appeared dependent upon an obligatory factor(s) synthesized *de novo* in response to Con A or PMA stimulation. Enhancement of GM-CSF gene transcription in response to Con A stimulation also appeared to be dependent upon a newly synthesized factor(s). These studies suggested that transcription of the IL-5 and GM-CSF genes was differentially regulated in D10.G4.1 cells. It seemed pertinent, therefore, to investigate whether protein interactions with the conserved TCATTT-containing element in these two genes could be differentially modulated by these different signalling pathways.

As previously shown in Fig. 5.5.A., complexes of similar electrophoretic mobilities and affinities were observed to form with mIL-5A(-61/-41) and mGM-CSF(-60/-40) using crude nuclear extracts prepared from Con A-stimulated D10.G4.1 cells. As shown in Fig. 5.7., no protein interactions were detected to mIL-5A(-61/-41) using unstimulated nuclear extracts. A complex was observed to form with mGM-CSF(-60/-40) using unstimulated nuclear extracts, the electrophoretic mobility of which was slower than the complex formed using stimulated nuclear extracts (Lanes 1,2). This suggests that the TCATTT-containing element in the GM-CSF gene, but not the IL-5 gene, forms a complex with a constitutive factor(s), which may be distinct from the inducible factor(s) which recognises this element in both genes. No protein interactions were detected to mIL-5A(-61/-41) using extracts prepared from D10.G4.1 cells which were stimulated with Con A in the presence, from the outset, of CHX (Fig. 5.7.A., Lane 4). Incubation of these extracts with mGM-CSF(-60/-40) resulted in a binding pattern similar to that observed using unstimulated extracts (Fig. 5.7.B., Lane 4). This suggests that similar, related nuclear proteins which recognises the TCATTT-containing element in the IL-5 and GM-CSF genes may be synthesized *de novo* in response to Con A stimulation.

As shown in Fig. 5.7.A., complexes of similar electrophoretic mobilities were formed with mIL-5A(-61/-41) using extracts prepared from D10.G4.1 cells which were stimulated with either Con A or PMA (Lanes 2,5). This suggests that activation pathways mediated by these two agents may induce the synthesis of at least similar, if not the same, factor(s) which binds specifically to this IL-5 element. Incubation of mGM-CSF(-60/-40) with extracts prepared from PMA-stimulated D10.G4.1 extracts, however, resulted in a binding pattern more similar to that observed using extracts prepared from unstimulated rather than Con A-stimulated cells (Fig. 5.7.B., Lanes 1,2,5).

<u>Fig. 5.7.</u> **Signal-induced modulation of protein interactions with the conserved TCATTT-containing elements in the murine IL-5 and GM-CSF genes.**

^{32}P-labeled mIL-5A(-61/-41) (A) or mGM-CSF(-60/-40) (B) were incubated with nuclear extracts prepared from unstimulated D10.G4.1 cells (Lane 1), D10.G4.1 cells stimulated for 6 h with Con A in the absence or presence of either Cs A or CHX (Lanes 2,3,4), or D10.G4.1 cells stimulated for 6 h with PMA in the absence or presence of CHX (Lanes 5,6).


1. unstimulated
2. Con A
3. Con A + Cs A
4. Con A + CHX
5. PMA
6. PMA + CHX
A
DNA-protein complex
(NF-IL-5A)
Probe: mIL-5A(-61/-41)
Unbound DNA
B
DNA-protein
complexes
Probe: mGM-CSF(-60/-40)
Unbound DNA

NF-IL-5A complex formation was observed using extracts prepared from D10.G4.1 cells which were stimulated with Con A in the presence, from the outset, of Cs A (Fig. 5.7.A., Lanes 2,3). Incubation of mGM-CSF(-60/-40) with these extracts, however, resulted in a binding pattern more similar to that observed using unstimulated rather than Con A-stimulated extracts (Fig. 5.7.B., Lanes 1,2,3).

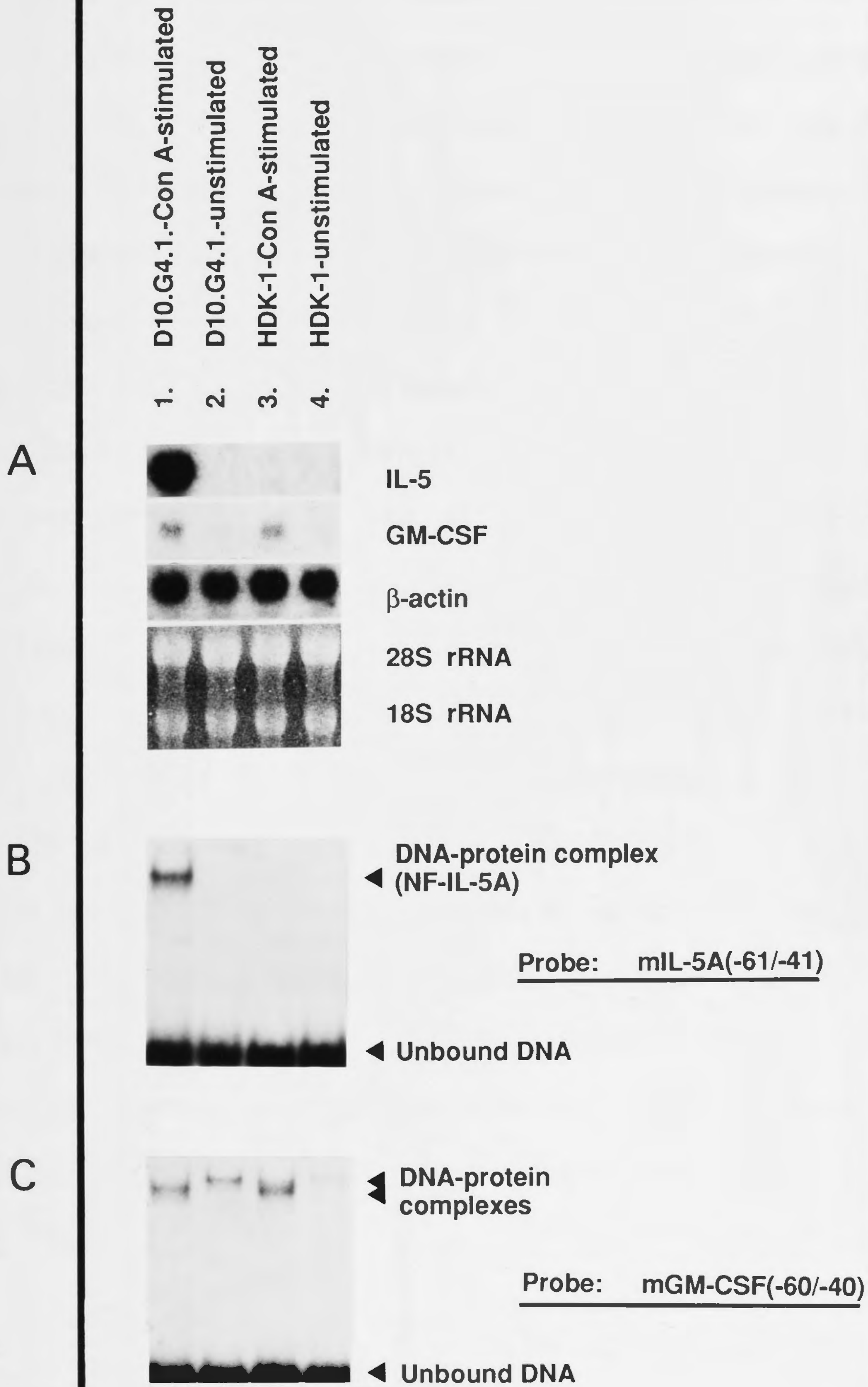
The inducible DNA-protein interactions were also investigated in the Th1 helper clone HDK-1, which was found, by Northern blot analysis, to express the GM-CSF but not the IL-5 gene in response to Con A stimulation (Fig. 5.8.A.). As observed in assays using unstimulated D10.G4.1 nuclear extracts, a complex of slow electrophoretic mobility was observed to form with mGM-CSF(-60/-40) using extracts prepared from unstimulated HDK-1 cells (Fig. 5.8.C., Lanes 2,4). Complexes of higher electrophoretic mobility were observed to associate with mGM-CSF(-60/-40) using extracts prepared from D10.G4.1 or HDK-1 cells stimulated with Con A (Fig. 5.8.C., Lanes 1,3). The NF-IL-5A complex was not observed to form with mIL-5A(-61/-41) using unstimulated or stimulated HDK-1 extracts (Fig. 5.8.B., Lanes 3,4).

These observations together suggest that signalling pathways which differentially modulate the expression patterns of the IL-5 and GM-CSF genes in murine Th clones may also differentially modulate the nature of nuclear protein interactions with the conserved TCATTT-containing element present in the 5'-flanking regions of these two genes. The strong correlation between signal-dependent and cell-specific modulation of gene expression patterns and DNA-protein interactions implicates the potential role which these DNA-binding proteins and their recognition sequences may play in the transduction of intracellular activation signals to the transcriptional machinery of these genes.

Fig. 5.8. Cell-specificity of gene expression patterns and protein interactions with the conserved TCATTT-containing elements in the murine IL-5 and GM-CSF genes.

A. 20 μg of total cellular RNA, prepared from unstimulated D10.G4.1 (Lane 2) or HDK-1 (Lane 4) cells, or cells stimulated for 6 h with Con A (Lanes 1,3), were used to prepare Northern blots which were hybridized with the ^{32}P-labeled murine IL-5 and GM-CSF cDNA probes. Northern blots were also hybridized with the ^{32}P-labeled β-actin cDNA probe as a control for constitutive mRNA accumulation. Equalization of RNA content was confirmed by visualization of 28S and 18S ribosomal RNAs (rRNA) on ethidium bromide-stained RNA gels.

B,C. ^{32}P-labeled mIL-5A(-61/-41) (B) or mGM-CSF(-60/-40) (C) were incubated with nuclear extracts prepared from unstimulated D10.G4.1 (Lane 2) or HDK-1 (Lane 4) cells, or cells stimulated for 6 h with Con A (Lanes 1,3). Unbound and protein-bound DNA was resolved by native gel electrophoresis.



1. D10.G4.1.-Con A-stimulated
2. D10.G4.1.-unstimulated
3. HDK-1-Con A-stimulated
4. HDK-1-unstimulated
A
IL-5
GM-CSF
β-actin
28S rRNA
18S rRNA
B
DNA-protein complex (NF-IL-5A)
Probe: mIL-5A(-61/-41)
Unbound DNA
C
DNA-protein complexes
Probe: mGM-CSF(-60/-40)
Unbound DNA

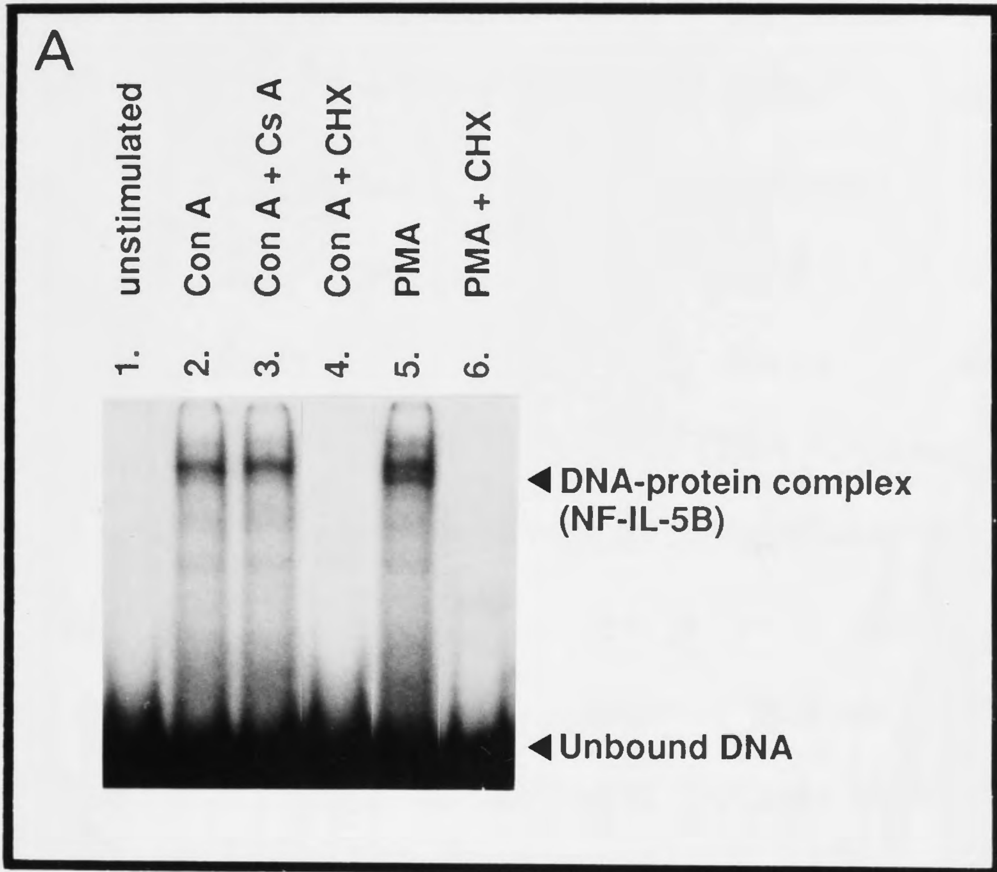
5.3.5. Protein interactions with the putatively unique mIL-5B element

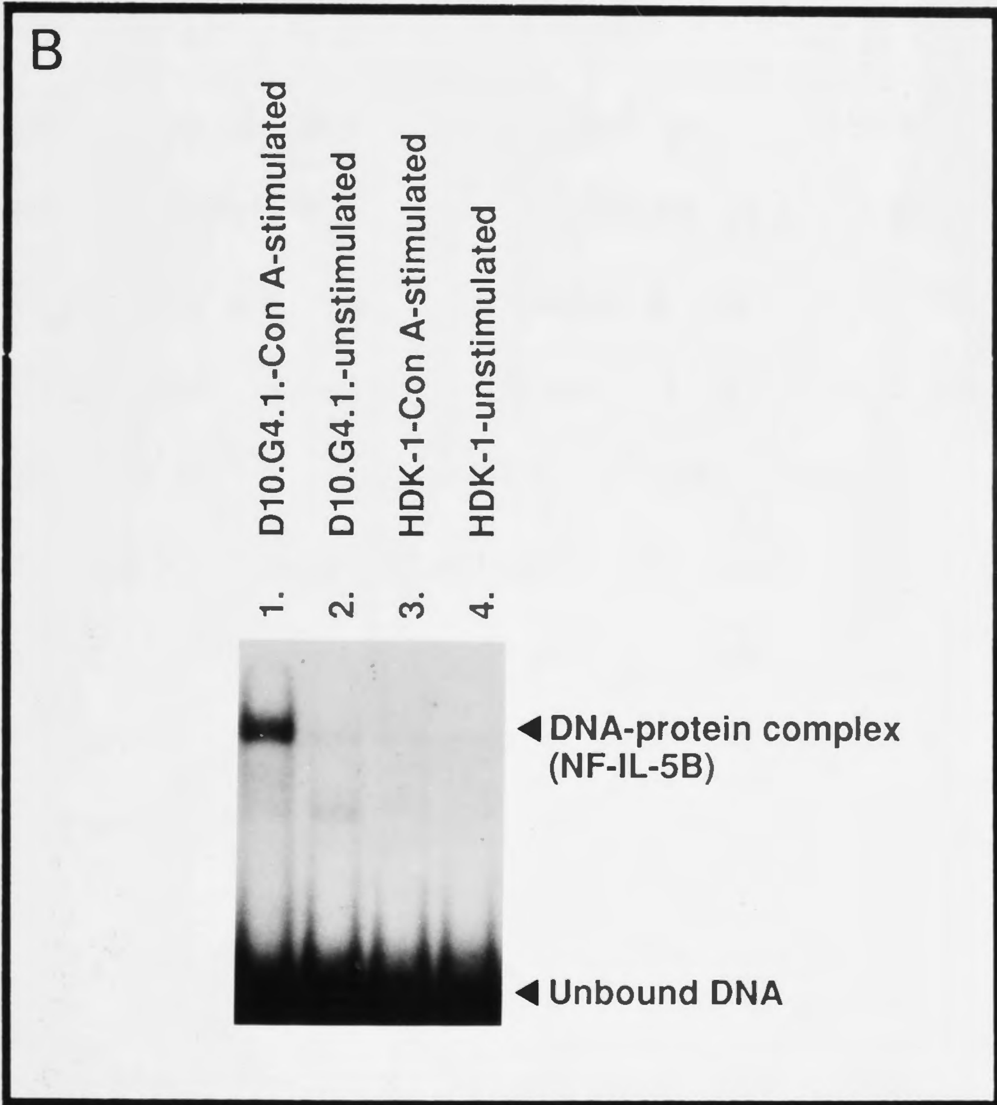
Modulation of protein interactions with the adjacent mIL-5B element (-76 to -62) by different signalling pathways was also investigated. As shown in Fig. 5.9.A., no protein interactions were detected to mIL-5B(-76/-62) using unstimulated D10.G4.1 extracts (Lane 1), while complexes of similar electrophoretic mobility were observed using extracts prepared from D10.G4.1 cells which were stimulated with PMA or Con A either in absence or presence of Cs A (Lanes 2,3,5). These inducible DNA-protein interactions were, however, not observed using extracts prepared from D10.G4.1 cells which were stimulated with either Con A or PMA in the presence of CHX (Lanes 4,6). This suggests that the nuclear protein(s) associated with the NF-IL-5B complex is synthesized *de novo* in response to signalling pathways activated by Con A or PMA stimulation. The NF-IL-5B complex was not detected using extracts prepared from unstimulated or Con A-stimulated HDK-1 cells which do not express the IL-5 gene (Fig. 5.9.B.. Lanes 3,4). The correlation between the patterns of transcriptional activity of the IL-5 gene and the modulation of nuclear protein interactions with the mIL-5B region suggests that the DNA-binding protein(s) which specifically bind to mIL-5B may, together with those which interact with the TCATTT-containing mIL-5A element, play a role in coordinating the induction of IL-5 gene transcription in D10.G4.1 cells.

__Fig. 5.9.__ **Gel shift analysis of signal-modulation and cell-specificity of protein interactions with the mIL-5B element.**

A. ^{32}P-labeled mIL-5B(-76/-62) was incubated with nuclear extracts prepared from unstimulated D10.G4.1 cells (Lane 1), D10.G4.1 cells stimulated for 6 h with Con A in the absence or presence of Cs A or CHX (Lanes 2,3,4) or D10.G4.1 cells stimulated for 6 h with PMA in the absence or presence of CHX (Lanes 5,6).

B. ^{32}P-labeled mIL-5B(-76/-62) was incubated with nuclear extracts prepared from unstimulated D10.G4.1 (Lane 2) or HDK-1 (Lane 4) cells, or cells stimulated for 6 h with Con A (Lanes 1,3).


A
1. unstimulated
2. Con A
3. Con A + Cs A
4. Con A + CHX
5. PMA
6. PMA + CHX
DNA-protein complex
(NF-IL-5B)
Unbound DNA


B
1. D10.G4.1.-Con A-stimulated
2. D10.G4.1.-unstimulated
3. HDK-1-Con A-stimulated
4. HDK-1-unstimulated
DNA-protein complex
(NF-IL-5B)
Unbound DNA

5.4. DISCUSSION

In recent years, a number of DNA elements have been identified in the 5'-flanking regions of several lymphokine genes which appear to mediate their transcriptional activation in response to T cell activation signals. These DNA sequences are generally located proximally to the TATA boxes of these genes and appear to specifically interact with numerous constitutive or inducible DNA-binding factors. While the overall DNA homology between 5'-flanking regions of different lymphokine genes is relatively poor, conserved sequences have frequently been identified as putative *cis*-acting elements which specifically bind *trans*-activating regulatory factors. The ubiquitous factors NF-κB, AP-1 and Oct-1, for example, appear to bind recognition elements which are conserved in several lymphokine genes, and co-operatively interact with one another and other factors to mediate the transcriptional activation of these respective genes (Emmel *et al.,* 1989; Hoyos *et al.,* 1989; Serfling *et al.,* 1989; Shibuya *et al.,* 1989; Kamps *et al.,* 1990; Mathey-Prevot *et al.,* 1990; Schreck & Baeuerle, 1990; Sugimoto *et al.,* 1990). Regulation of lymphokine gene transcription also appears to involve more specific mechanisms. NF-AT, which plays a critical role in mediating induction of IL-2 gene transcription, for example, appears to be a T cell-specific factor (Durand *et al.,* 1988; Shaw *et al.,* 1988b), while transcriptional activation of the IL-4 gene appears to be mediated by an enhancer element which may be unique to this particular gene (Abe *et al.,* 1992).

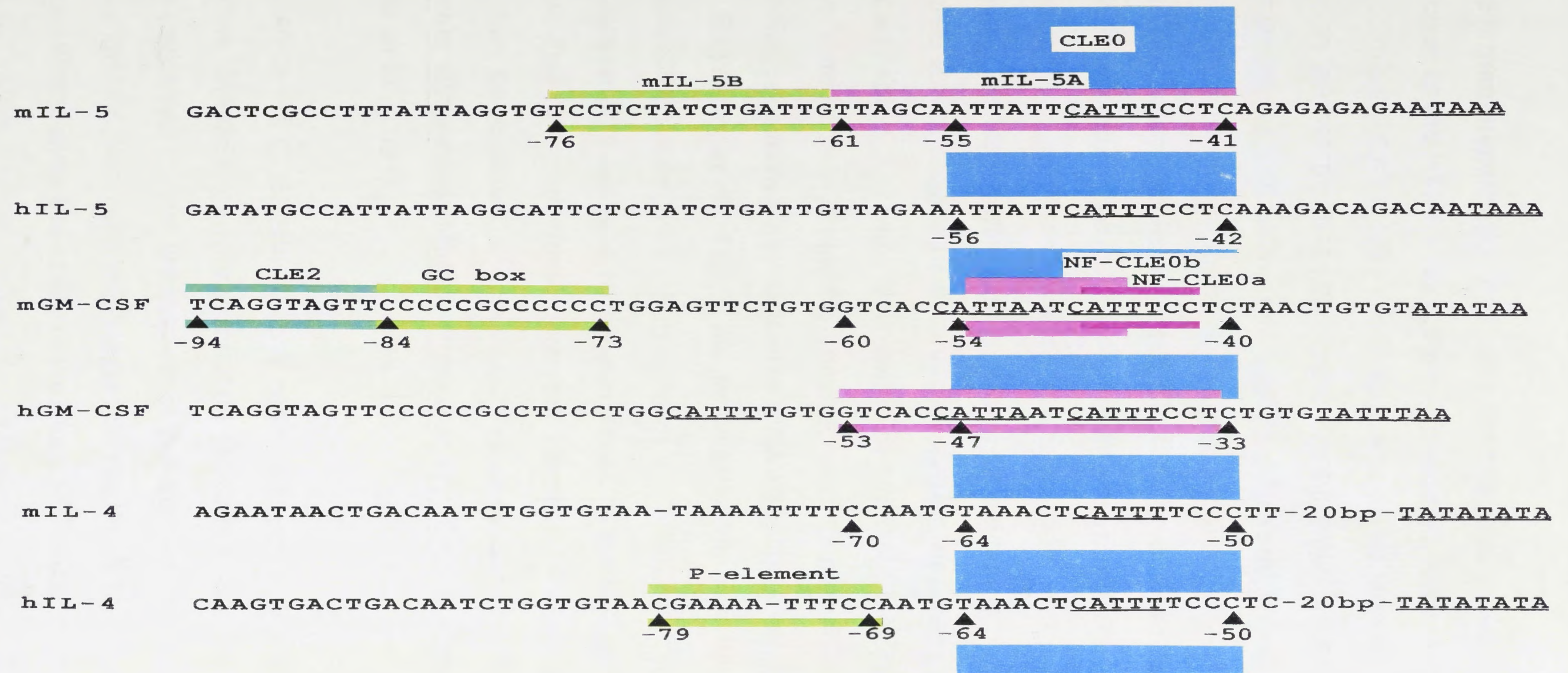
Results of the current study demonstrate that cell-specific inducible nuclear proteins specifically interact with two distinct, adjacent DNA elements located just 5' of the TATA box of the murine IL-5 gene. One element, referred to here as mIL-5A (-61 to -41), is highly conserved at similar positions in

human and murine genes encoding IL-5, GM-CSF and IL-4 (Table 5.2.) and has recently been termed conserved lymphokine element 0 (CLE0) (Miyatake *et al.,* 1991). The other element, referred to here as mIL-5B (-76 to -62), is highly conserved between the murine and human IL-5 genes, but demonstrates little significant homology to corresponding regions in other lymphokine genes. Distinct nuclear proteins in Con A- or PMA- stimulated D10.G4.1 cells, which express the IL-5 gene (Chapter 2), specifically and independently bound to the mIL-5A (or IL-5 CLE0) and mIL-5B elements, No protein interactions with either of these two elements were detected using extracts prepared from unstimulated D10.G4.1 cells in which the IL-5 gene is transcriptionally inactive (Chapter 3). The synthesis of nuclear proteins which specifically recognised mIL-5A (IL-5 CLE0) and mIL-5B appeared to be induced *de novo* in D10.G4.1. cells in response to Con A or PMA stimulation, and their syntheses and/or activities appeared to be regulated by Cs A-resistant signalling pathways. Furthermore, nuclear protein interactions with either of these two elements were not observed using extracts prepared from unstimulated or stimulated HDK-1 cells which do not express the IL-5 gene. The strong correlation between signal-dependent and cell-specific modulation of the transcriptional activity of the IL-5 gene and of specific nuclear protein interactions with these two elements implicates the potential role which these DNA-protein interactions may play in the transduction of activation signals to the transcriptional machinery of the IL-5 gene.

The regulatory role of the CLE0 element in mediating induction of human GM-CSF gene transcription has been extensively studied in a variety of cell systems. Unlike the IL-5 gene, the analogous region in the human GM-CSF gene contains three repeats of the CATT(A/T) motif (Table 5.2.). Sequences up to -53, which include the two downstream motifs, have been demonstrated to be sufficient to mediate cell-specific induction of human

Table 5.2. **Location of putative *cis*-acting regulatory elements in the proximal 5'-flanking regions of the murine and human genes encoding IL-5, GM-CSF and IL-4.**

Positions of upstream sequences, relative to the cap sites of the respective genes, are indicated. The "TATA" boxes and conserved CATT(A/T) motifs are underlined. Two protein-binding sites in the murine IL-5 gene, termed mIL-5A (-61 to -41) and mIL-5B (-76 to -62), were identified in this study to bind inducible nuclear factors and are indicated respectively by pink and green boxes. Also indicated by green boxes are the CLE2/GC box elements (-94 to -73) and P-element (-79 to -69) which respectively are reported to be critical for mediating transcriptional activation of the murine GM-CSF (Miyatake *et al.*, 1988a,b) and human IL-4 (Abe *et al.*, 1992) genes in response to T cell activation signals. Sequences which are reported to mediate inducibility of human GM-CSF promoter activity and to be protected from DNase I digestion by the binding of nuclear proteins in activated T cells (Nimer *et al.*, 1988, 1989a, 1990) are also indicated. The region defined by Miyatake *et al.* (1991) in these lymphokine genes as the CLE0 element is denoted by the blue box. Sequences within the CLE0 element of the murine GM-CSF gene, which are reported to be required for the formation of the NF-CLE0a and NF-CLE0b complexes (Miyatake *et al.*, 1991) are indicated by pink boxes.



CLE0
mIL-5B
mIL-5A
mIL-5 GACTCGCCTTTATTAGGTGTCCTCTATCTGATTGTTAGCAATTATTCATTTCCTCAGAGAGAGAATAAA
-76 -61 -55 -41
hIL-5 GATATGCCATTATTAGGCATTCTCTATCTGATTGTTAGAAATTATTCATTTCCTCAAAGACAGACAATAAA
-56 -42
CLE2
GC box
NF-CLE0b
NF-CLE0a
mGM-CSF TCAGGTAGTTCCCCCGCCCCCCTGGAGTTCTGTGGTCACCATTAATCATTTCCTCTAACTGTGTATATAA
-94 -84 -73 -60 -54 -40
hGM-CSF TCAGGTAGTTCCCCCGCCTCCCTGGCATTTTGTGGTCACCATTAATCATTTCCTCTGTGTATTTAA
-53 -47 -33
mIL-4 AGAATAACTGACAATCTGGTGTAA-TAAAATTTTCCAATGTAAACTCATTTTCCCTT-20bp-TATATATA
-70 -64 -50
P-element
hIL-4 CAAGTGACTGACAATCTGGTGTAACGAAAA-TTTCCAATGTAAACTCATTTTCCCTC-20bp-TATATATA
-79 -69 -64 -50

GM-CSF gene transcription in T cells or fibroblasts in response to stimulation by mitogen or the HTLV-1 *tax* protein (Nimer*et al.*, 1988; 1989a,b; 1990). The third upstream CATT(A/T) motif appears to be additionally required for activation of human GM-CSF gene transcription in endothelial cells in response to IL-1 (Kaushansky, 1989). Several other reports indicate, however, that deletion or mutation of other sequences, located upstream of the CLE0 element, abolishes inducibility of the murine GM-CSF promoter in response to mitogenic or *tax* stimulation (Miyatake *et al.,* 1988a,b). Comparison of regulatory mechanisms between species is somewhat ambiguous as these upstream regulatory elements in the murine GM-CSF gene (-95 to -73), termed conserved lymphokine element 2 (CLE2) and the GC box (Table 5.2.), were delineated by transient expression studies in a human T cell line. These elements appear to specifically interact with a number of constitutive and inducible DNA-binding proteins (Shannon *et al.,* 1988; Sugimoto *et al.,* 1990), the latter of which may be related to NF-κB (Schreck & Baeuerle, 1990; Sugimoto *et al.,* 1990). Although the CLE2 and GC box elements are also highly conserved in the IL-3 gene, recent studies suggest that other additional upstream elements, including conserved AP-1 sites, may be required in activating IL-3 gene transcription in response to mitogenic stimulation (Mathey-Prevot *et al.,* 1990; Shoemaker *et al.,* 1990; Nishida *et al.,* 1991).

While the CLE2 and GC box elements appear critical for the activation of murine GM-CSF gene transcription (Miyatake *et al.,* 1988a,b), a recent report published during the course of the current study demonstrated that substitution mutations of every single base in the 3' half of the CLE0 element (-49 to -39) abolishes the ability of the murine GM-CSF promoter to respond to T cell activation signals despite the presence of intact CLE2 and GC box elements (Miyatake *et al.,* 1991). Mutations in the 5' half of the CLE0 element

(-52 to -50) appeared, however, to upregulate both basal and inducible promoter activity. The CLE0 element was observed to form an inducible DNA-protein complex NF-CLE0, which consisted of two complexes of slightly different electrophoretic mobilities. The recognition sequences of the higher mobility complex, NF-CLE0a, (-47 to -42) appear to be required for induction of promoter activity, while the recognition sequences of the lower mobility complex, NF-CLE0b (-53 to -46) includes sequences which suppress promoter activity (Table 5.2.). These results imply that NF-CLE0b may serve as a repressor, while NF-CLE0a may function as a positive regulator.

An earlier report demonstrated that sequences between -57 and -24 of the human GM-CSF promoter are protected from DNase I digestion by the binding of specific nuclear proteins in unstimulated T cells, while a shorter region (-52 to -34) is protected in activated T cells (Nimer *et al.,* 1988; Table 5.2.). This may reflect displacement of a constitutive repressor in response to cellular activation. Signal-induced modulation of nuclear protein interactions with the murine GM-CSF CLE0 element was also observed by gel shift analysis in the current study. A complex of slower mobility was observed using extracts prepared from unstimulated D10.G4.1 cells under conditions in which the GM-CSF gene is constitutively transcribed at a low basal level, while a complex of higher mobility was observed using extracts prepared from stimulated cells in which transcriptional activity of the GM-CSF gene is enhanced (section 5.3.4.). Furthermore, the slower mobility complex was not observed to form with the murine IL-5 CLE0 element (mIL-5A) in extracts prepared from HDK-1 cells, or unstimulated or CHX-treated D10.G4.1 cells in which the IL-5 gene is transcriptionally inactive. These observations bear interesting correlation to recent findings of Miyatake *et al.* (1991) which demonstrate only weak affinity of the putative repressor complex NF-CLE0b to the IL-5 CLE0 element despite its high homology to the GM-CSF CLE0

element (Table 5.2.). These observations together suggest that interactions of a putative repressor with the CLE0 element in the GM-CSF gene may be involved in regulating its constitutive transcription at a low basal rate in unstimulated T cells. This repressor may be displaced in response to specific T cell activation signals by inducible positive regulators.

As expected from the homology data (Table 5.2.), the affinity of the CLE0 elements in the GM-CSF and IL-5 genes for the inducible NF-CLE0 complex was higher than that of the analogous IL-4 CLE0 element (section 5.3.3.). A recent study has identified a unique 11 bp motif termed the P-element (-79 to -69) upstream of, and adjacent to, the CLE0 element in the human IL-4 gene, which confers responsiveness to T cell activation signals (Abe *et al.,* 1992; Table 5.2.). Data, available at the time of thesis preparation, from stable transfection studies using the murine thymoma cell line EL4.23 in this laboratory, indicate that the CLE0 element alone is insufficient to mediate inducibility of the IL-5 promoter (P. Bourke, unpublished results). Substitution mutations in the conserved TCATTT motif, which were found in the current study to abolish formation of inducible DNA-protein complexes (section 5.3.3.), however, diminished responsiveness to T cell activation signals conferred by other sequences further upstream . Detailed stable transfection studies in the D10.G4.1 cell system are, however, essential to establish the regulatory role of the CLE0 element and delineate other elements which may regulate IL-5 gene transcription. One likely candidate is the adjacent mIL-5B region (-76 to -62) which was found in this study to form complexes with inducible, cell-specific nuclear proteins (section 5.3.5.). Another feature which gives the mIL-5B region additional significance as a potential regulatory element is its position relative to the CLE0 element, given that elements in the IL-4 and GM-CSF genes which confer responsiveness to

T cell activation signals have been identified in similar locations (Miyatake *et al.,* 1988a,b; 1991; Abe *et al.,* 1992; Table 5.2.).

Although the CLE0 element alone appears insufficient to confer responsiveness of the GM-CSF, IL-5 and IL-4 promoters to T cell activation signals, the conserved spatial organisation of the CLE0 elements relative to the TATA boxes of these genes and its apparently critical requirement for induction of their promoters suggests that inducible factors which specifically interact with the CLE0 element in these genes may function to co-operatively link the basal transcriptional machinery at the "core promoter" with other multiple regulatory factors which bind to other, possibly gene-specific, elements further upstream. This hypothesized model may be analysed by investigating the effect, upon promoter inducibility, of alterations in the relative spatial organisation of the TATA boxes, CLE0 elements and other *cis*-acting regulatory sites.

CHAPTER 6:

DISCUSSION

Alterations in patterns of cellular growth and differentiation may essentially be regarded as manifestations of the dynamic interactions between the genetic program and information external to the genomic micro-environment, the generation and/or transmission of which may largely involve inter-cellular communication and cell-to-cell interactions. In this regard, the role of the T lymphocyte in "inducible haemopoiesis" may essentially be envisaged as that of a "signal transducer" which converts specific signals, largely communicated by antigen-presenting cells, into pleiotropic mediators which interact with, and thereby modulate the genetic programs of, numerous haemopoietic target cells. In effecting this critical response to immunological stress, the genetic program of the T lymphocyte is itself dynamically modulated, the most significant manifestation of which being the expression of a battery of lymphokine genes.

In Chapter 1, several broad concepts were described and espoused as general underlying themes for the studies conducted in this thesis. One included the notion of the intrinsically dynamic and "integrated" nature of mechanisms which regulate gene expression in eukaryotic systems. Another involved the increasing conceptualization of lymphokines as coordinating "inducible" haemopoiesis as a "network". In the light of such concepts, this thesis, as an initial phase of a longer term project, sought to investigate and compare the mechanisms which regulate the expression of the IL-5 gene and a variety of other different lymphokine genes in murine T lymphocytes. This broader comparative perspective was somewhat of a departure from the

approach adopted by the majority of previous studies which have tended to focus upon investigating mechanisms which regulate a specific individual lymphokine gene in isolation. Furthermore, while a number of previous studies have been conducted using transformed T cell lines, the mechanisms which regulate the expression of a variety of different lymphokine genes were investigated in this project in both non-transformed and transformed T cells.

The studies described in this thesis essentially investigated the mechanisms which regulate the synthesis of steady state translatable lymphokine mRNAs. Although earlier studies largely investigated the regulation of synthesis of a particular lymphokine in terms of its bioactivity, such biological analysis would be more complex and less specific if applied to a broader comparative investigation of a number of different lymphokines. While it cannot be discounted that the synthesis of a biologically active lymphokine gene product may be influenced by mechanisms including translation, glycosylation and secretion, various studies have demonstrated strong correlation between the steady state levels of mRNA and bioactivity of a number of lymphokines induced in response to stimulation by several of the cellular activators in the T cell systems studied in this project (Cherwinski *et al.,* 1987; Lichtman *et al.,* 1987; Shaw *et al.,* 1988a; Bickel *et al.,* 1990; Zubiaga *et al.,* 1990).

The findings of the various studies described in the bulk of this thesis essentially suggest that the expression of the IL-5 gene and a number of other lymphokine genes are differentially regulated in T lymphocytes by specific signalling pathways emanating from distinct cell surface events, which are either dependent or independent of TCR/CD3 stimulation. Such differential control appears to be largely mediated at either the transcriptional level and/or at the level of mRNA stability. Studies described in the remainder of the thesis describe the identification and characterization of DNA-protein interactions

which may be potentially involved in regulating IL-5 gene expression at the transcriptional level. The following sections seek to further discuss these various regulatory mechanisms in the context of their coordination and integration, and the significance of such coordination and integration *in vivo.*

6.1. Differential regulation of lymphokine gene expression by signalling pathways emanating from distinct cell surface events

The inducibility of expression of a variety of lymphokine genes in response to TCR/CD3 stimulation has been well-established by a number of studies conducted in murine T cell clones (Cherwinski *et al.,* 1987; Bohjanen *et al.,* 1990; Gajewski *et al.,* 1990). Studies described in Chapter 2 suggest that the expression of the IL-3, IL-4 and GM-CSF genes in the murine Th2 clone D10.G4.1 is exclusively regulated by TCR/CD3-dependent mechanisms. The expression of the IL-5, IL-6 and IL-10 genes appears, however, to be additionally and selectively regulated by cell surface mediated-mechanisms independent of direct TCR/CD3 stimulation. These include signalling pathways emanating from the CD45 or CD2 cell surface molecules or the IL-1 receptor.

The exclusive control of IL-4 gene expression in D10.G4.1 cells by TCR/CD3-dependent mechanisms may be functionally associated with the ability of IL-4 to act as an autocrine factor which regulates the clonal expansion of Th2 cells following antigenic stimulation (Kupper *et al.,* 1987; Lichtman *et al.,* 1987). The gene encoding IL-2, which is exclusively expressed in, and is believed to act as an autocrine factor for, Th1 cells, also appears to be exclusively controlled by TCR/CD3-dependent mechanisms (Herold *et al.,* 1986; Lichtman *et al.,* 1988). Furthermore, IL-4 also appears to selectively induce the expression of the IL-5 (Bohjanen *et al.,* 1990), IL-6 and IL-10 genes (H. Naora, unpublished observations) in D10.G4.1 cells. The prolonged nature

of the kinetics of IL-5, IL-6 and IL-10 gene expression, compared to those of the IL-3, IL-4 and GM-CSF genes (Chapter 3), may be partially attributable to their "additional" regulation via the autocrine IL-4 pathway. IL-3 and GM-CSF are not known to be autocrine factors for T cells and the restriction of their gene regulation to TCR/CD3-dependent mechanisms may be linked to their coordinate expression by both Th1 and Th2 populations. The flexibility and multiplicity of signalling mechanisms which regulate the expression of the IL-5, IL-6 and IL-10 genes, in contrast, may be functionally associated with their expression being restricted to the Th2 subset.

The ability to "mimick" TCR/CD3-mediated responses by simultaneously activating PKC and inducing elevation in $[Ca^{2+}]_i$ has been well-established (Truneh *et al.,* 1985; Weiss *et al.,* 1986; Kumagai *et al.,* 1987). Studies described in Chapter 2 indicate, however, that these two intracellular second messanger pathways which emanate from the TCR/CD3 complex may differentially regulate the expression of lymphokine genes in D10.G4.1 cells. Signals mediated by a Cs A-sensitive Ca^{2+}-dependent pathway appear obligatory for the induction of IL-3, IL-4 and GM-CSF gene expression. Such a pathway may involve the Cs A-sensitive Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Takahashi *et al.,* 1989; Liu *et al.,* 1991; Clipstone & Crabtree, 1992; Fruman *et al.,* 1992). In contrast to the IL-3 and IL-4 genes, the GM-CSF gene appears to additionally require PKC-mediated signals for maximal induction. PKC-mediated signals may, however, selectively and independently of Ca^{2+} mobilization, induce the expression of the IL-5, IL-6 and IL-10 genes. A basis for the differential control of these genes may lie in the ability of TCR/CD3 stimulation to induce significantly lower $[Ca^{2+}]_i$ in Th2 cells than in Th1 cells, as recently reported by Gajewski *et al.* (1990). Furthermore, Ca^{2+}-dependent and PKC-mediated signals appear to synergize for optimal induction of the IL-5 and IL-6 genes, but not the IL-10 gene. PKC-mediated

signals appear obligatory for the inducibility of the IL-5 gene which, in comparison to the other five lymphokine genes studied, was very weak in response to the Ca^{2+} signal alone. A recent study suggests that the inhibition of human IL-5 gene expression by dexamethasone may involve its interference with a PKC-dependent pathway (Rolfe *et al.,* 1992).

Although the nature of the IL-1 signal transduction pathway and its role as an additional, TCR/CD3-independent signal in T cell activation have been extensively studied (Mizel, 1982;1990; Abraham *et al.,* 1987; Rosoff *et al.,* 1988; Shirakawa *et al.,* 1988), the modulation of lymphokine gene expression by the IL-1 signal has been little studied to date. Studies described in Chapter 2 suggest that IL-1 may selectively, and independently of TCR/CD3 stimulation, induce the expression of the IL-5, IL-6 and IL-10 genes. The selective inducibility of these Th2-specific lymphokine genes by the IL-1 signal may be inherently and functionally associated with the fact that Th2, but not Th1, cells express IL-1 receptors on their surface (Lichtman *et al.,* 1988).

The studies described in Chapter 2 suggest that the IL-1 receptor is coupled to a variety of signal transduction pathways which appear to differentially regulate the expression of the IL-5, IL-6 and IL-10 genes. While a number of studies suggest that the IL-1 receptor is coupled to the PKA pathway (Shirakawa *et al.,* 1988; Chedid *et al.,* 1989; Chedid & Mizel, 1990), a recent study in the D10.A sub-line demonstrates that IL-1 stimulation induces both PKC activation and elevation in $[cAMP]_i$ (Munoz *et al.,* 1990). In the parental clone, both the PKC- and PKA- dependent pathways emanating from the IL-1 receptor appear to regulate IL-6 gene expression, while the PKC-dependent pathway appears to predominantly regulate the expression of the IL-5 gene. Other pathways, possibly independent of PKC and PKA activation, appear to regulate IL-10 gene expression in response to IL-1 stimulation. The

involvement of kinases other than PKC or PKA in IL-1 signal transduction has been suggested by O'Neill *et al.* (1990). The PKA involvement in IL-1 signal transduction in D10.G4.1 cells observed in this study may explain the weaker antagonistic effect of PKA pathway activators upon Th2 proliferation, compared to Th1 proliferation as reported by Gajewski *et al.* (1990).

A number of recent lines of evidence have implicated the role of CD45 in T cell activation as essentially that of an integral intermediary which couples the TCR/CD3 complex and also the CD2 molecule to the PIP_2 signal transduction pathway (Mustelin *et al.,* 1989;1992; Koretzy *et al.,* 1990;1991; Danielian *et al.,* 1991). Studies described in Chapter 2 indicate that CD45 may, independently of direct stimulation of either the TCR/CD3 complex or CD2, selectively induce the expression of the IL-5, IL-6 and IL-10 genes in D10.G4.1 cells. These observations suggests that the CD45 molecule may initiate and/or regulate signal transduction pathways that are distinct from those activated by TCR/CD3 or CD2 stimulation. The physiological significance of this CD45-mediated mechanism and the possibility that different CD45 isoforms may regulate different signalling pathways in T cells remains to be elucidated. Furthermore, the ability of CD2 to also selectively induce IL-5, IL-6 and IL-10 gene expression, independently of direct TCR/CD3 stimulation, raises the possibility that it may regulate a pathway which may be independent of PIP_2 hydrolysis. Of potential significance to the gene-specificity of CD2 modulation may be the recently reported ability of IL-6 to act as an accessory signal in CD2-mediated, but not TCR/CD3-mediated, T cell proliferation (Lorre *et al.,* 1990). The role which CD2 plays in T cell activation and the nature of its functional inter-relationship with the TCR/CD3 complex remains to be clarified. It will be of interest in future studies to investigate if and how CD45 and CD2 modulate TCR/CD3-induced lymphokine gene expression in Th1 cells, and whether they

may regulate signalling pathways, independently of direct TCR/CD3 stimulation, in this subset.

Another interesting basis for future studies is the possibility that CD28 stimulation may differentially modulate the expression of a variety of lymphokine genes. This accessory molecule has gained increasing attention over recent years and is believed to initiate and/or regulate a signal transduction pathway that is distinct from those emanating from the TCR/CD3 complex (June *et al.,* 1987;1989). CD28-mediated signals have been shown to synergize with those mediated by CD2 in T cell proliferation (Pierres *et al.,* 1988; Van Lier *et al.,* 1988). Furthermore, CD28-mediated signals appear to augment TCR/CD3-induced expression of the IL-2, TNF-α, LT, IFN-γ and GM-CSF genes, but not that of the IL-4 gene, in normal human T lymphocytes (Thompson *et al.,* 1989). Although human T cell populations appear not to be distinguished by Th1/Th2 phenotypes, it is of significant interest to note that the genes whose expression were reportedly enhanced by CD28-mediated signals are expressed by the Th1 subset in the murine system. Furthermore, CD28 surface expression does not appear to be restricted to the Th1 subset (June *et al.,* 1990b; Galvin *et al.,* 1992). The basis of possible differential control may, however, not lie in the heterogeneity of Th1/Th2 phenotypes *per se*. CD28-mediated signals appear to augment TCR/CD3-induced expression of the IL-2, TNF-α, LT, IFN-γ and GM-CSF genes largely via a mechanism involving stabilization of their mRNAs (Lindsten *et al.,* 1989). Observations described in Chapter 3, and elaborated upon in further detail below, suggest that signal-dependent mechanisms which enhance mRNA stabilization are not involved in regulating IL-4 and IL-5 gene expression.

Another interesting corollary which emerges from the comparative studies described in this thesis is the physiological implication of the ability of

phorbol esters to activate pathways in transformed T cell lines such as EL4.23, which appear analogous to those activated in T cell clones by TCR/CD3 or lectin stimulation. PMA stimulation appears to activate both Cs A-resistant and Cs A-sensitive pathways in EL4.23 cells. This may involve the activation of "cross-talk" between the PKC- and Ca^{2+}- dependent pathways, and suggests that signalling pathways in transformed cell lines may be less discrete. "Promiscuous" utilization of signal transduction mechanisms may be a feature of cellular transformation.

6.2. Differential regulation of lymphokine gene expression by multiple transcriptional and post-transcriptional mechanisms

The comparative studies described in Chapter 3 essentially suggest that the accumulation of each different lymphokine mRNA species is dynamically and differentially regulated by the relative rates of its synthesis and degradation. This appears to involve distinct and specific sets of transcriptional and post-transcriptional control mechanisms. Transcriptional and/or post-transcriptional control of many of the lymphokine genes studied appears to involve dynamic interplay between positively and negatively acting factors. These specific factors were distinguished by whether their synthesis was induced *de novo* in response to specific T cell activation signals, or whether they were pre-existing factors whose activities were presumably modulated by specific signalling pathways.

The expression of the IL-4, IL-5, IL-6 and IL-10 genes appears to be predominantly regulated at the transcriptional level in D10.G4.1 cells. Whether this is functionally significant or merely coincidental to their Th2-specificity remains to be elucidated. Transcription of the IL-5, IL-6 and IL-10 genes may be independently activated by largely Cs A-resistant signalling pathways

emanating from the TCR/CD3 complex and the IL-1 receptor. In EL4.23 cells, the expression of the IL-5 and IL-10 genes also appear to be predominantly regulated at the transcriptional level by Cs A-resistant signalling pathways. IL-5 gene transcription appears to require signals other than those mediated by Ca^{2+} mobilization for maximal activation in D10.G4.1 cells, and requires obligatory *de novo* protein synthesis in both T cell systems, presumably that of a transcription factor(s). Enhancement of IL-6 gene transcription in D10.G4.1 cells appears, in contrast, to be mediated by pre-existing factors. Protein synthesis of putative regulatory factors appears to be required for maximal activation of IL-10 gene transcription in both D10.G4.1 and EL4.23 cells. A labile repressor may also be involved in regulating IL-10 gene transcription in EL4.23 cells. Transcription of the IL-2 gene in EL4.23 cells also appears to be subject to the coordinate control of labile positively and negatively acting factors, the labile positive regulator being responsive to Cs A-sensitive signals. Both negatively acting and labile Cs A-sensitive positively acting mechanisms have been reported to coordinately control human IL-2 gene transcription (Durand *et al.,* 1988; Nabel *et al.,* 1988; Shaw *et al.,* 1988b; Emmel *et al.,* 1989; Williams *et al.,* 1991).

In contrast to the IL-5, IL-6 and IL-10 genes, transcription of the IL-4 gene appears to be exclusively regulated in D10.G4.1 cells by signals mediated by a Cs A-sensitive, Ca^{2+}-dependent pathway. Its maximal activation, like that of the IL-10 gene, appears to require protein synthesis. This may involve the induction of synthesis of a regulatory factor which is distinct from, and cooperatively acts with, a pre-existing factor. Alternatively maximal transcriptional activation may require the enhanced expression of a pre-existing positive regulator. Specific signals may also negatively modulate the expression of specific lymphokine genes at the transcriptional level. A labile repressor appears to selectively inhibit GM-CSF and IL-4 gene transcription in

IL-1-stimulated D10.G4.1 cells. The transcriptional inactivity of the IL-3 gene in IL-1-stimulated cells does not appear, however, to involve a similar mechanism.

Transcription of the IL-3 and GM-CSF genes in D10.G4.1 cells, like that of the IL-4 gene, also appears to be exclusively regulated by Cs A-sensitive, Ca^{2+}-dependent signals. Enhancement of GM-CSF gene transcription appears, however, to be mediated by a newly synthesized factor(s), while activation of IL-3 gene transcription largely involves a pre-existing factor(s). IL-3 gene transcription is similarly regulated in EL4.23 cells by a Cs A-sensitive pathway, and also appears to involve a labile repressor. Both positively and negatively acting mechanisms are reported to coordinately regulate IL-3 gene transcription in transformed human T cell lines (Mathey-Prevot *et al.,* 1990; Shoemaker *et al.,* 1990). The expression of the IL-3 gene in both D10.G4.1 and EL4.23 cells, and of the GM-CSF gene in D10.G4.1 cells also appear to be regulated at the level of mRNA stability. This mode of control appears to be the predominant mechanism in the regulation of GM-CSF gene expression in EL4.23 cells.

In both T cell systems studied, the IL-3 and GM-CSF mRNAs appear to be stabilized by Cs A-sensitive signals, IL-3 mRNA being more efficiently stabilized than GM-CSF mRNA. The dynamic regulation of the stability of these two species appears, however, not only to involve their enhanced stabilization by signal-dependent mechanisms, but also their specific degradation by a labile mechanism. IL-2, IL-6 and IL-10 mRNAs also appear to be selectively degraded by a labile mechanism. Furthermore, this labile mechanism appears to be constitutively active and may be involved in regulating the rapid turnover of the GM-CSF, IL-6 and IL-10 transcripts which are expressed in unstimulated T cells. Constitutive transcription of the GM-CSF gene has been extensively reported in a variety of cell types which express this gene (Thorens *et al.,* 1987;

Koeffler *et al.,* 1988; Schuler & Cole, 1988; Ernst *et al.,* 1989a; Bickel *et al.,* 1990; Wodnar-Filipowicz & Moroni, 1990; Akahane & Plutznik, 1992; Iwai *et al.,* 1992). Constitutive transcription of the IL-6 and IL-10 genes in other T and non-T cell types which express these genes has not been reported, and the significance of the gene-specificity of this phenomenon remains to be elucidated.

Regulation of mRNA stability is generally believed to be a predominantly cytoplasmic phenomenon (Nevins, 1983, Hentze, 1991), although signal-dependent modulation of the stability of certain intranuclear RNA precursors has been reported (Akahane & Plutznik, 1992). The studies described in Chapter 3 support a growing body of evidence which implicates conserved AUUUA motifs in the 3'-UTRs of many unstable, inducible eukaryotic mRNAs as targets for a labile cytoplasmic mechanism which mediates their specific degradation (Caput *et al.,* 1986; Shaw & Kamen, 1986; Wilson & Treisman, 1988; Shaw *et al.,* 1988a; Brewer & Ross, 1989; Akashi *et al.,* 1991). Such a mechanism has been proposed not to involve a specific "RNase"-like molecule *per se*, but rather destabilization of the PABP/poly(A) complex by the interaction of a specific protein(s) with these AU-rich sequences (Bernstein & Ross, 1989; Malter, 1989; Bohjanen *et al.,* 1991). Although the minimum number of AUUUA motifs required for the mediation of mRNA instability has yet to be determined, the studies described in Chapter 3 suggest that single, dispersed copies of this motif may be insufficient. GM-CSF, IL-2, IL-3, IL-6 and IL-10 mRNAs, which are highly labile and stabilized by CHX, contain multiple, often tandem, repeats of the AUUUA motif in their 3'-UTRs. In contrast, the IL-4 and IL-5 mRNAs, which are significantly more stable than the other lymphokine mRNAs studied and whose stabilities are not affected by CHX, contain only a few, widely dispersed single copies of this motif in their 3'-UTRs.

At least two models may be proposed for the dynamic and specific regulation of lymphokine mRNA stability. Firstly, cellular signals may stabilize specific mRNAs by inhibiting the synthesis of the labile "destabilizing" factor, or inactivating this factor either by direct modification or by mediating its sequestration by a signal-induced inhibitory ligand. A recent study, which demonstrates the ability of a variety of cellular signals, as well as CHX, to stabilize GM-CSF mRNA via the AU-rich sequences in its 3'-UTR (Akashi *et al.,* 1991), may arguably support this model. Alternatively, the regulation of mRNA stability may involve the coordinate actions of two operationally independent mechanisms, namely a signal-dependent mechanism which enhances stabilization of specific mRNAs and a distinct labile mechanism which mediates their specific degradation. This model implicates the involvement of specific elements other than the AU-rich sequences in the regulation of mRNA stability. A number of elements, apart from the AU-rich sequences, appear to be involved in regulating the stability of several labile mRNAs (Rabbitts *et al.,* 1985; Jones & Cole, 1987; Kabnick & Housman, 1988; Band *et al.,* 1990; Iwai *et al.,* 1991; Ross *et al.,* 1991).

The molecular basis for the plasticity and specificity of eukaryotic gene expression essentially lies in the particular sequence, unique composition and spatial organisation of regulatory elements constituting the complex set utilized by a given gene. Utilization of common elements by different genes potentiates a basis for coordinate control. The recognition element of NF-κB, for example, is highly conserved in, and is involved in regulating the transcription of, numerous inducible eukaryotic genes (reviewed in Lenardo & Baltimore, 1989), while AUUUA repeats, conserved in the 3'-UTRs of a variety of inducible eukaryotic mRNAs, appear to mediate their instability as previously described. Essentially similar paradigms involving multiple specific, nucleotide-protein and

protein-protein interactions have been proposed for the mechanisms of transcriptional control and the regulation of mRNA stability.

The DNA-protein and protein-protein interactions which regulate eukaryotic gene expression at the transcriptional level are inherently more complex as they are inextricably associated with two dynamic phenomena intrinsic to all eukaryotic systems. The first feature concerns the packaging of DNA into nucleosomes and higher order chromatin structures, which are generally believed to regulate the accessibility of a gene to the transcriptional machinery (reviewed in Reeves, 1984; Eissenberg *et al.,* 1985; Yaniv & Cereghini, 1986; Gross & Garrard, 1988; Grunstein, 1990). As described in Chapter 4, the ability to activate IL-5 gene transcription in nuclear run-off assays using endogenous chromatin as the template for transcription *vis-a-vis* the inability to activate IL-5 gene transcription in either cell-free or transient expression systems using exogenous "naked" DNA as the template, suggests that IL-5 gene transcription is dynamically regulated in T lymphocytes by higher order chromatin structures. For this reason, the analysis of DNase I hypersensitive sites, as "markers" of non-nucleosomal organisation, was a particularly relevant means of initially identifying approximate locations of putative protein-binding regulatory regions of the IL-5 gene in its native chromatin environment. The significance of these regions is discussed in further detail below.

The second "intrinsically eukaryotic" phenomenon pertaining to transcriptional regulation concerns "inter-compartmental communication". The factors which activate lymphokine gene transcription may essentially be regarded as integral "downstream" nuclear targets in a progression of signalling events emanating from the cell surface, and their identification and characterization is an important approach in understanding the transduction of

specific T cell activation signals to the nucleus. The responsiveness of the IL-5, IL-6 and IL-10 genes to the variety of signalling pathways which emanate from the TCR/CD3 complex, IL-1 receptor and the CD45 and CD2 molecules, may be mediated by either one or a combination of two possible mechanisms. Under a "divergent" model, transcriptional activation of these genes may involve the cooperative action of numerous different factors, the synthesis or activity of each of which is responsive to a specific signal. Under a more "convergent" model, the pleiotropic responsiveness of these genes may be mediated by a more limited number of factors, one or several of which may be responsive to a broad range of signals. One example of a particularly "promiscuous" inducible factor is NF-κB, which is activated in response to a variety of different signals in a range of cell types (reviewed in Lenardo & Baltimore, 1989). A basis for its pleiotropic responsiveness may lie in the ability of different kinases, such as PKC and PKA, to phosphorylate, and thereby induce the dissociation of, its inhibitory ligand, IκB (Baeuerle & Baltimore, 1988a,b; Shirakawa & Mizel, 1989; Ghosh & Baltimore, 1990).

The synthesis or activity of certain other transcription factors may, however, be regulated by highly specific signal-dependent mechanisms. The activity of NF-AT, for example, which plays a critical regulatory role in IL-2 gene transcription (Durand *et al.,* 1988; Shaw *et al.,* 1988b) and has recently been demonstrated to enhance transcription of the human IL-3 and GM-CSF genes (Cockerill *et al.,* 1993), appears to require the two second messangers generated by TCR/CD3-mediated PIP_2 hydrolysis (Hivroz-Burgaud *et al.,* 1991). These intracellular pathways appear to independently regulate two critical components of NF-AT: (i) a pre-existing T cell-specific cytoplasmic subunit which is induced to translocate to the nucleus by signals delivered by a Cs A-sensitive Ca^{2+}-dependent pathway, involving the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Flanagan *et al.,* 1991; Clipstone &

Crabtree, 1992; O'Keefe *et al.,* 1992), and (ii) a nuclear subunit which is synthesized *de novo* in response to Cs A-insensitive signals. Recent studies, which demonstrate that this nuclear subunit may comprise of AP-1, suggest that its synthesis may be regulated by PKC activation (Jain *et al.,* 1992; Cockerill *et al.,* 1993).

The assembly of heterologous subunits, the synthesis and/or activity of each of which may be independently regulated by different signals, to form multimeric transcription factors such as NF-AT, potentiates a versatile mechanism by which different signalling pathways may effectively cross-talk and coordinately modulate the expression of a gene via a specific individual element. Both pleiotropic and specific responsiveness of an inducible gene may be potentially mediated by numerous unique combinations of a relatively limited number of "interchangeable" components, each of which may be responsive to a particular signal. The pleiotropic responsiveness of the IL-5, IL-6 and IL-10 genes may involve such an alternative "convergent" paradigm.

The utilization of different, but closely related, factors which interact with a common recognition element may potentiate another versatile transcriptional control mechanism. Recent evidence of relationships between several components of NF-κB (Blank *et al.,* 1992), Ets-related proteins (Thompson *et al.,* 1992) or octamer factors, which themselves are members of a larger family of homeodomain proteins (Wright *et al.,* 1989; Kemler & Schaffner, 1990), suggest that such "regulatory factor families" may be an integral feature of eukaryotic gene regulation. Studies described in Chapter 5 suggest that the factors which recognise the highly conserved CLE0 element in the proximal 5'-flanking regions of the IL-5, GM-CSF and IL-4 genes may be closely related, but differentially modulated, members of a regulatory factor family. The strong correlation observed between the differential cell-specificity and signal-

dependent modulation of transcription of the IL-5 and GM-CSF genes, and of the specific inducible nuclear protein interactions with the CLE0 elements in these respective genes, implicates the potential involvement of these DNA-protein interactions in the transduction of specific activation signals to the transcriptional machinery of these two genes.

The CLE0 element alone appears insufficient to confer responsiveness of the IL-5 (P. Bourke, pers. comm.), GM-CSF (Miyatake *et al.,* 1988a,b; 1991) or IL-4 (Abe *et al.,* 1992) promoters to T cell activation signals. Mutations in the CLE0 elements of the IL-5 and GM-CSF genes were, however, found in the studies described in Chapter 5 to abolish their interactions with inducible nuclear proteins, and also appear to diminish the inducibility of the IL-5 (P. Bourke, pers. comm.) and GM-CSF (Miyatake *et al.,* 1991) promoters conferred by sequences further upstream. Given the critical requirement of the CLE0 elements for the activity of the IL-5 and GM-CSF promoters, and also possibly the IL-4 promoter, and the conserved spatial organisation of the CLE0 elements relative to the TATA boxes of these genes, the inducible factors which specifically interact with the CLE0 elements in these genes may be essential in cooperatively linking the core promoter machinery with regulatory factors which interact with possibly gene-specific enhancer elements further upstream. One likely candidate is the mIL-5B element, which is located immediately upstream of the CLE0 element and appears unique to the IL-5 gene. The strong correlation observed between the patterns of IL-5 gene transcription and the modulation of inducible nuclear protein interactions with the mIL-5B element, suggests that this element may cooperatively act with the CLE0 element in regulating IL-5 gene transcription. Although the functional significance of the mIL-5B element and the nature of its inter-relationship with the CLE0 element require investigation in transcriptional assays, another feature which gives the mIL-5B element additional significance as a potential regulatory element is its

position relative to the CLE0 element, given that enhancer elements in the GM-CSF and IL-4 genes have been identified in similar locations (Miyatake *et al.,* 1988a,b;1991; Abe *et al.,* 1992).

6.3. Concluding remarks

In the light of recent suggestions that Th1/Th2 phenotypes may, at least in the murine system, represent mature stages of helper T cells (Swain *et al.,* 1988; Mosmann & Coffman, 1989a,b), the differential utilization of multiple signal transduction mechanisms observed in the Th2 clone D10.G4.1 may be reflective of the development of heterogenous mature T cell populations. Furthermore, such differential utilization of signal transduction mechanisms in the regulation of expression of different lymphokine genes may be functionally significant in the provision by heterogenous T cell populations of an appropriately "balanced" network of mediators to coordinate immune responses.

A current perplexing question is one pertaining to the cell-specificity of mechanisms which regulate lymphokine gene expression in T lymphocytes. Similar mechanisms of control appear to regulate the expression of several lymphokine genes in other non-T cell types which express these genes. IL-3 gene expression, for example, also appears to be regulated by a Ca^{2+}-dependent pathway at the level of mRNA stability in mast cells (Wodnar-Filipowicz & Moroni, 1990). Signal-dependent stabilization mechanisms also appear to play a predominant role in regulating the accumulation of GM-CSF mRNA in macrophages, fibroblasts, mast cells and B cells (Thorens *et al.,* 1987; Koeffler *et al.,* 1988; Wodnar-Filipowicz & Moroni, 1990; Akahane & Plutznik, 1992). Furthermore, the labile mechanism which mediates degradation of specific lymphokine mRNAs in T cells, appears to mediate the

degradation of several other unstable mRNAs in a variety of other cell types as described earlier. NF-κB, which appears to be involved in the transcriptional regulation of the IL-2 and GM-CSF genes in T cells (Hoyos *et al.,* 1989; Shibuya *et al.,* 1989; Schreck & Baeuerle, 1990), also appears to be involved in regulating the transcription of the IL-6 (Shimuzu *et al.,* 1990; Zhang *et al.,* 1990) and TNF-α (Collart *et al.,* 1990) genes (and also a number of other cellular genes) in non-T cell types. Furthermore, the CLE0 element has been reported to be involved in regulating GM-CSF gene transcription in fibroblasts and endothelial cells, as well as in T cells (Nimer *et al.,* 1988;1989a,b;1990; Kaushansky, 1989). The cytoplasmic subunit of NF-AT appears to be the only well-established T cell-specific regulatory factor reported to date (Flanagan *et al.,* 1991; Clipstone & Crabtree, 1992; O'Keefe *et al.,* 1992).

Given that a number of receptors expressed on the surfaces of a variety of non-haemopoietic cells appear, like the TCR/CD3 complex, to be coupled to the PIP_2 signal transduction pathway (Berridge, 1987; Nishizuka, 1986;1988), the identification and characterization of T cell-specific regulatory factors "downstream" of the early signal transduction events, and the investigation of their roles in regulating the expression of a variety of lymphokine genes is central to understanding the molecular basis of T cell activation. The isolation and use of signal transduction mutant T cells will be of enormous value in future studies. "Piecing" together these "missing components" of the downstream signal transduction events will in the longer term expand our understanding of the molecular basis of development of many haemopoietic disorders and dysfunctional immune responses, given that many such disorders appear to be attributable to an aberrantly "balanced" network of lymphokines.

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