Molecular Mechanisms of Interleukin-2 Gene Inducibility: Developmental Control and Combinatorial Action of Transcription Factors

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This work is dedicated to my parents Chen Keji and Chen Weiyang.

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Abstract

Interleukin-2 is one of the lymphokines secreted by T helper type 1 cells upon activation mediated by T-cell receptor (TCR) and accessory molecules. The ability to express IL-2 is correlated with T-lineage commitment and is regulated during T cell development and differentiation. Understanding the molecular mechanism of how IL-2 gene inducibility is controlled at each transition and each differentiation process of T-cell development is to understand one aspect of T-cell development. In the present study, we first attempted to elucidate the molecular basis for the developmental changes of IL-2 gene inducibility. We showed that IL-2 gene inducibility is acquired early in immature $CD4^-CD8^-TCR^-$ thymocytes prior to TCR gene rearrangement. Similar to mature T cells, a complete set of transcription factors can be induced at this early stage to activate IL-2 gene expression. The progression of these cells to cortical $CD4^+CD8^+TCR^{lo}$ cells is accompanied by the loss of IL-2 gene inducibility. We demonstrated that DNA binding activities of two transcription factors AP-1 and NF-AT are reduced in cells at this stage. Further, the loss of factor binding, especially AP-1, is attributable to the reduced ability to activate expression of three potential components of AP-1 and NF-AT, including c-Fos, FosB, and Fra-2. We next examined the interaction of transcription factors and the IL-2 promoter *in vivo* by using the EL4 T cell line and two non-T cell lines. We showed an all-or-none phenomenon regarding the factor-DNA interaction, i. e., in activated T cells, the IL-2 promoter is occupied by sequence-specific transcription factors when all the transcription factors are available; in resting T cells or non-T cells, no specific protein-DNA interaction is observed when only a subset of factors are present in the nuclei. Purposefully reducing a particular set of factor binding activities in stimulated T cells using pharmacological agents cyclosporin A or forskolin also abolished all interactions. The results suggest that a combinatorial and coordinated protein-DNA interaction is required for IL-2 gene activation.

The thymocyte experiments clearly illustrated that multiple transcription factors are regulated during intrathymic T-cell development, and this regulation in turn controls the inducibility of the lineage-specific IL-2 gene. The *in vivo* study of protein-DNA interaction stressed the combinatorial action of transcription factors to stably occupy the IL-2 promoter and to initiate its transcription, and provided a molecular mechanism for changes in IL-2 gene inducibility in T cells undergoing integration of multiple environmental signals.

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INTRODUCTION

Tissue specification during development is characterized by the expression of a battery of tissue specific genes. The control of tissue-specific gene expression can be achieved by selectively turning on or off tissue specific genes in response to developmental cues. Selective gene activation is considered the dominant mechanism in higher organisms during cellular differentiation based on the thought that it is more efficient to turn on the appropriate cell type-specific genes in limited cells than to repress them in all other cells. Unlike many prokaryotic genes, many eukaryotic genes are not expressed, or are expressed *in vivo* at relatively low levels, unless enhanced by the sequence-specific *trans*-acting factors which interact with *cis*-elements in the enhancer region and directly or indirectly with the basic transcriptional machinery to stimulate the initiation or completion of pre-initiation complex assembly (Ptashne, 1988; Ptashne & Gann, 1990; Johnson & McKnight 1989; Mitchell & Tjian 1989; Hahn 1993). As one of the pre-requisites, cells capable of expressing a particular gene would possess necessary *trans*-activators, which include both ubiquitous *trans*-acting factors and tissue-specific activators. Accompanying this gene activation process is the repression of inappropriate genes. Instead of being economic, progenitor or precursor cells are adapted for the evolutionary flexibility with pluri-potentialities. Therefore, negative regulation of gene expression can be equally important as positive regulation in development. For instance, activation of the β-globin locus control region as represented by the presence of erythrocyte-specific DNase I sites is seen in both lymphomyeloid stem cells and myeloid stem cells preceding their commitment to the erythroid lineage. Selective repression of the locus control region is then seen whenever multipotential stem cells differentiate to cells other than erythroid lineage (Jiménez et al., 1992). In accord with this result is the finding that the transcription factor GATA-1 which is responsible for erythroid development and expression of erythroid-specific genes, is actively transcribed in cycling erythroid-myeloid precursors. Differentiation to the erythroid lineage is accompanied by increased GATA-1 expression; differentiation to the granulocyte-macrophage pathway

abruptly shuts off the expression (Sposi et al., 1992). This result suggests a two-step control for the expression of transcription factor expression: cell cycle-dependent initiation and lineage-dependent maintenance or repression. The development of T lymphocytes in mouse or human may apply the same mechanism. In the development of T helper type 1 cells (Th1), for example, a hematopoietic stem cell proliferates and differentiates into lymphoid lineage and non-lymphoid lineage; lymphoid cells then decide to become T or B lymphoid lineage; T lineage cells still have to decide to become CD4 T helper cells or CD8 T killer cells; CD4 T helper cells finally make their determination to become Th1 or Th2 cells (Fig. 1). During this whole process, each differentiated cell turns on or maintains the ability to activate some genes and switches off the potentiality to turn on some other genes which are characteristic of the other lineage(s) derived from the same precursor cell. Thus, understanding tissue or cell-specific gene regulation is to understand cell fate control. My thesis work is focused on the regulation of a Th1 cell-specific gene-interleukin-2 (IL-2) during T cell receptor αβ lineage T cell development. As described above, clarification of the mechanisms determining the regulation of IL-2 gene expression would shed light on the developmental control of the Th1 cell differentiation process.

Interleukin-2 is not merely a simple marker for type 1 T helper cells; it is one of the most important lymphokines secreted by activated Th1 cells. As in the discovery of most growth factors, IL-2 was first found by its T-cell mitogenic activity in T-cell cultures in the presence of plant lectins, or specific antigens, and in conditioned medium from allogeneic mixed leukocyte cultures. Subsequent studies further demonstrated that the way that antigens or plant lectins stimulate T cells to proliferate is that they induce IL-2 and IL-2 receptor expression in T cells. It was the interaction of the IL-2 with the IL-2R that drives T cells into the cell cycle (Leonard et al., 1982; and Smith et al., 1983). This finding not only negated the notion that T lymphocytes are terminally differentiated hematopoietic cells unable to proliferate, but it also provided new insights for the

regulation of immune responses. IL-2 is an essential regulator for helping to mount an immune response. It exerts its effect through interacting with the IL-2 receptor expressed on a variety of cells. The activities associated with IL-2 include not only proliferation of T cells, but also induction of high affinity IL-2 receptor expression on T cells and B cells (Depper et al., 1985; Smith & Cantrell, 1985), activation of cytotoxicity by CTL, growth and differentiation of activated B cells into antibody-secreting cells (Watson & Mochizuki, 1980), and growth of natural killer cells and monocytes (O'Garra et al., 1988). In this study we have not focused on its function, but use its expression as a molecular indicator of T-cell development. Most of my work is based on the recent progress on the biochemical studies of IL-2 gene transcription regulation. It is necessary to give a general but brief overview of these results.

Recent development in IL-2 gene regulation

The cloning of both human and mouse IL-2 genes and resolving of the IL-2 crystal structure have been accomplished (Taniguchi et al. 1983; Fujita et al. 1983; Fuse et al., 1986; Holbrook et al., 1984a; Macky, 1992; Zurawski et al, 1993). The interleukin-2 gene is a single-copy gene with four exons separated by one short and two long introns, which is similar to the genomic organization of other cytokines, including IL-4 and GM-CSF. The coding regions of the mouse and human IL-2 genes are 62% identical at the amino acid level, which may explain why both the human and mouse IL-2 are functional across species. Sequence analysis and gene transfer experiments confirmed that the 5' flanking region immediately upstream of the start site shares a high degree of homology (86% identity up to -580bp) between human and mouse and contains enhancer activity (Fujita et al., 1986; Fuse et al, 1984; Holbrook et al., 1984b; Novak et al., 1990; Durand et al., 1988; Williams et al., 1988). These data suggest the functional and regulatory conservation of the IL-2 gene.

Further detailed analysis of the *cis*-acting elements in the proximal IL-2 enhancer region and the definition of their cognate *trans*-acting factors have been the focus of study for the past six years. The attempt to connect the proximal T-cell activation signalling apparatus and the distal triggering of transcription factor activity also provides a system to understand signal transduction events in T cells (Crabtree, 1989).

Regulatory elements of the IL-2 gene. In addition to its cell-specificity and activation-dependence, IL-2 also has a transient expression pattern, with IL-2 mRNA appearing at 40 min, peaking at about 6 to 8 hours, and disappearing 24 hours after stimulation under certain conditions. Compared to some constitutively expressed house-keeping genes, activated T cells make only a moderate levels of IL-2 mRNA per cell (200 to 800 molecules/cell) (McGuire & Rothenberg. 1987; McGuire et al. 1988). Although four DNase I hypersensitive sites (upstream of the promoter, -300, -100, and first intron) have been identified in the human IL-2 gene locus in stimulated T cells (Siebenlist et al., 1986), gene transfer studies indicate that the 321 bp sequence immediately upstream of the start site is sufficient to confer all the known regulatory characteristics of the endogenous IL-2 gene (Durand et al., 1988), which is also true for the highly homologous murine IL-2 gene (Novak et al., 1990). Detailed analysis of the *cis*-acting elements in the 321 bp regulatory sequence and of the putative *trans*-acting factors which could interact with this region not only revealed the identity of many of the factors but also provided valuable information on how signals were transmitted intracellularly. As diagrammed in figure 2, the transcription factors which may contact the IL-2 300 bp promoter/enhancer region and regulate IL-2 gene activation can be divided into three classes. They are: 1) T-cell specific inducible factors (NF-AT, CD28RE); 2) general inducible factors (NF-κB/Rel, AP-1, TGGGC factor); and 3) general constitutively expressed factors (Octamers, Sp1-like). I will give a general overview of these factors in relation to IL-2 gene activation.

NF-AT. The nuclear factor of activated T cells (NF-AT) was the name given to the factor which binds to the -290/-260 and -145/-120 regions of the IL-2 enhancer. The distal binding site was first named NF-IL2E or purine-box binding site and was identified by *in vitro* DNase I footprinting studies (Durand et al., 1988; Shaw et al., 1988; Serfling et al., 1989). A distal sequence, containing AAAGAGGAAA, can also be found in the promoters of multiple genes, including the human IL-4, the human γIFN, and the murine GM-CSF, in the HIV-LTR, and in enhancer regions of SV40 and lymphotropic papovavirus (Fiegel et al., 1992; Pettersson, M. & W. Schaffner, 1987). The inducible binding activity to NF-AT sites is reported to be T-cell specific and is completely inhibited by the immunosuppressants cyclosporin A and FK-506 (Flanagan et al., 1991; Banerji et al. 1991). The factor which binds to this site is a multi-protein complex which contains AP-1 and an immunosuppressant-sensitive cytoplasmic component. The former is regulated by the activation of protein kinase C and requires protein synthesis, while the latter is present in the cytoplasm and is transported to nucleus by Ca^{2+} mobilization (Flanagan et al., 1991; Jain et al., 1992). Since cyclosporin A and FK-506 block the Ca^{2+} branch of signal transduction pathway by inhibiting the calcium/calmodulin-dependent phosphatase calcineurin, it is known that the nuclear localization of the cytoplasmic component is a downstream consequence of calcineurin activation (Liu et al. 1991; Clipstone et al., 1992). A number of proteins have been reported to able to bind the NF-AT site. An ubiquitously expressed 60kD protein was cloned which binds to the NF-AT site in the HIV LTR (Li et al., 1992). A recent report indicated that a 110-140 kDa phosphoprotein might be the actual cytoplasmic NF-AT component (McCaffrey et al., 1993). However, since Ets family proteins bind the same site as NF-AT (Wang et al., 1992), and Ets family genes are expressed predominantly in thymus (Bhat, N. K. 1987 and 1989), it has been suspected that an Ets gene product is the cytoplasmic component of NF-AT. In particular, Elf, an Ets family member, was shown to be able to bind the NF-AT site in the IL-2 regulatory region (Thompson et al., 1992). Although Ets-1 was

also shown to be able to cooperate with AP-1 for activation of transcription (Wasylyk et al., 1990; Wu et al., 1994), the phosphoprotein identified by McCaffrey et al. is now generally considered to be the most likely candidate for the actual cytoplasmic component of NF-AT. The nuclear component of NF-AT is known to be AP-1; however, different combinations of AP-1 family members were reported for NF-AT binding depending on the cell types tested. This will be discussed further below (Boise et al., 1993; Jain et al., 1992).

CD28RE. CD28 is a T-cell surface marker (Hansen et al. 1980). It is a 44 kD homodimeric glycoprotein and belongs to the Ig supergene family. Its ligand B7 (also for CTLA4), a 44-to 54--kD glycoprotein, is found on dendritic cells, on monocytes, and on activated B cells (Clark et al. 1986, Linsley et al. 1991). Signalling to T cells via anti-CD28 antibodies synergizes strongly with TCR signals for IL-2 production (Thompson et al. 1989, June et al. 1989) and is insensitive to a cyclosporin A block (June, et al. 1987). Upon stimulation of human Jurkat T cells in the presence of anti-CD28 antibody, a factor is induced which binds to the "CD28 response element" (CD28RE) (-164/-154) immediately upsteam of the proximal AP-1 site (Fraser et al., 1991 and see figure 2). Closely related *cis*-elements are also found in the 5' flanking regions of several other cytokine genes, including GM-CSF, IL-3, G-CSF and IFN-γ , which are also subject to CD28 signal modulation (Fraser et al., 1992). The identity of the protein bound to this site has not been determined. Although a number of proteins with a molecular weight from 30 to 44 kD have been implicated in the binding, it is not clear whether any of them could be a general inducible factor like members of the AP-1 family (Fraser et al., 1992). There is some evidence indicating that this binding activity can be induced in the absence of anti-CD28 antibody (Civil et al., 1992). Our experiments with thymocyte extracts also showed inducible binding for this site without utilizing the anti-CD28 antibody (see chapter 4). Further characterization of both the proximal signalling events and factor binding to the CD28RE sequence need to be done. In addition, it has also been found that

signals transduced by CD28 can regulate IL-2 gene activation post-transcriptionally by increasing stability of the message, indicating that the CD28 signal could regulate the IL-2 gene at more than one level (Lindsten et al., 1989; Umlauf et al., 1993).

NF-κB/Rel. NF-κB was initially identified as a B-cell specific *trans*-acting factor which regulated Ig κ light chain expression (Sen and Baltimore, 1986). It was later found that this transcription factors can be induced in multiple types of cells by a number of stimuli (see Rev. by Grilli et al., 1993). The *cis*-acting element is found in a number of genes other than the Ig κ light chain, including IL-2, IL-2Rα, MHC, and genes encoding other cytokines (Grilli et al., 1993). Protein purification and cloning work have resolved a family of NF-κB proteins whose DNA binding domains are related to the *rel* oncogene and the Drosophila morphogen, dorsal (Ghosh et al., 1990; Kieran et al., 1990). The NF-κB/Rel family contains p50/p105 (KBF-1), p50B/p97 (p49), p52 (NFKB2), v-rel, c-rel, RelA (p65), RelB, and the dorsal. These proteins remain in the precursor form, or else form heteromers with a negative regulator protein called IκB and remain in the cytoplasm (Baeuerle and Baltimore, 1988; Baeuerle et al., 1988). The activation of NF-κB in association with IκB is regulated through modification of the IκB molecules. Once IκB is phosphorylated by protein kinases, it releases NF-κB/Rel proteins, which then translocate to the nucleus and bind DNA as homo or heterodimers by dimerizing through the rel-like domain (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). A number of proteins with IκB function have been purified or cloned, including IκB α (37 kD, MAD-3, pp40), IκB β (43 kD), IκB γ (70 kD, C-terminal portion of p105), bcl 3 (46-56 kD), Δbcl 3 (37 kD), and cactus.

NF-κB is also shown to be important for IL-2 gene activation. A non-canonical κB site is found in the IL-2 promoter (-206/-195), which can form complexes with NF-κB (p65/p50) and KBF (p50/p50) in activated T cells. Mutation of this site results in a significant reduction in transcription (Hoyos et al., 1989). Inhibition of protein

synthesis does not affect NF-κB activation, but it does affect IL-2 gene expression, suggesting that NF-κB is necessary but not sufficient for IL-2 gene activation.

NF-κB/Rel regulation in B cell development seems to indicate that not only does the synthesis of the specific NF-κB/Rel members change, but the IκB molecules also vary with B cell maturation, and the combination of both determines the nuclear components of the NF-κB/Rel family. Such variations of the nuclear NF-κB/Rel may influence the transcription of developmentally and functionally regulated genes including the Ig κ gene. How are these activators regulated in the context of T-cell development? How do different dimerizations affect IL-2 transcription activity? How do signals activating or inhibiting NF-κB/Rel activation influence IL-2 gene activation? Those are some questions I attempted to answer in my work.

AP-1. There are two putative AP-1 sites in the 300 base pair enhancer sequence. *In vitro* DNase I footprinting did not reveal any occupancy of the distal AP-1 site (-185/-179) in human leukemia T cell line Jurkat (Brunvand et al., 1988). But IL-1 and PHA stimulated murine T lymphoma LBRM cells showed inducible binding activity to this site (Muegge et al., 1989). The proximal site (-151/-145) is footprinted by DNase I footprinting analyses, and is gel shifted with nuclear extracts from activated T cells (Serfling et al., 1989). AP-1 proteins also form the nuclear component of the two NF-AT binding complexes and compose OAP^{40} , which cooperates with octamer proteins to form a protein-DNA complex at the octamer site (Ullman et al., 1991; Ullman et al, 1992). The multiple engagements of AP-1 in the limited enhancer region indicate that it may play a very important role in IL-2 gene regulation (see fig. 2). Furthermore, the AP-1 factor involves either a Jun-Jun dimer or a Jun-Fos dimer. These jun and fos family members are each regulated by multiple signals and at multiple levels. Different combinations of AP-1 and different signal transduction events may well result in differences in IL-2 gene activation. By utilizing specific AP-1 antibodies in gel shift analysis or by transfecting particular jun or fos cDNA to T cells, several reports from a

number of laboratories suggested that different combinations of AP-1 can participate in the AP-1 or NF-AT complexes (Hentsch et al., 1992; Jain et al., 1992). However, because different T cell lines or T cell clones were tested and different stimuli were used in each experiment, there has not been a consensus in the results on this issue. In my work, I focused on two questions concerning AP-1 regulation in T cells: (1) how AP-1 binding activity is controlled in developing T cells, and (2) how signals which synergize or antagonize with the TCR transduced signals affect AP-1 activation. One aspect of AP-1 regulation which has not yet been under study in T cells is the status of kinases which potentially mediate the DNA binding or *trans*-acting activity of AP-1 in resting or activated T cells. Understanding this will provide more evidence as to which members of the AP-1 family are actually the players in IL-2 activation.

OAP^{40}/Oct. The octamer motif ATGCAAAT was first reported as a conserved sequence motif in the promoters of Ig genes and was later found in enhancers of other genes such as the histone genes, small nuclear RNA genes and viral genes (Fletcher et al., 1987; Gerster & Roeder, 1988). The *trans*-acting activity of Oct-1 is induced by phosphorylation (Segil et al., 1991). Its association with the viral protein VP16 can activate viral promoters (Gerster & Roeder, 1988). The *cis*-acting element at -90/-60 (NFIL-2A) contains noncanonical AP-1 (OAP^{40}) and octamer binding sites. It was discovered that both c-Jun and JunD are associated with this region in conjunction with Oct-1 protein in activated human Jurkat cells, so either or both may provide OAP^{40} function (Ullman et al., 1991; Ullman et al, 1992). Although Oct-1, Oct-2A, and Oct-2B can interact with the Oct site in gel shift studies, it is likely that Oct-1 alone can mediate binding and activation, since in human Jurkat cells, human peripheral T cells and IL-2 producing murine thymocytes, only Oct-1 is found to form complexes with the element (Granelli-Piperno & Nolan, 1991; Ullman et al., 1991; and see chapter 4). In EL4 cells Oct-1, Oct-2A and Oct-2B can all interact with this site (Hentsch et al., 1992; see chapter 2 & 3). Cotransfection of the NFIL-2A containing enhancer construct with either Oct-1,

Oct-2A, c-Jun, JunD, and c-Fos, but not JunB, can increase enhancer activity (Bielinska et al., 1990; Hentsch et al., 1992). Both deletion and mutation analyses also indicate that this region plays a positive role for IL-2 gene activation (Durand et al., 1988; William et al., 1988; Bielinska et al., 1990). Furthermore, this element is the target of the inhibitory effects of CsA, TGFβ, and retinoic acid (RA) (Ullman et al., 1992; Barbletz et al., 1993; Felli et al., 1991). In those situations, the *in vitro* DNA binding activity of octamer proteins is not affected by the addition of TGFβ and RA, and the binding activities of both octamer proteins and OAP^{40} are unaffected by CsA, but the transactivation activity of the concatamerized octamer site is significantly reduced. These results suggest that the inhibition is directed at the level of the transactivation activity, which is dependent on Ca^{2+} influx (Ullman et al., 1992).

Novel cis-elements and their corresponding factors. In analyzing the factor binding activities in thymocytes, we identified a novel site upstream of the distal NF-AT site. The *in vivo* footprinting analysis confirmed the presence of the distal CACCC site along with the discovery of a proximal CACCC site, a TGGGC site, and a ATGG site which were not reported in previous *in vitro* DNAase I footprinting studies. The identity of the factors bound to these elements is not known. Gel mobility shift assays indicated that the factor bound to the TGGGC site is induction dependent and protein kinase A sensitive. The binding cannot be competed individually with oligos containing NF-κB, AP-2, AP-3, or Sp-1 sequences. Two GC rich elements (CACCC) in the IL-2 promoter region might belong to one of the two elements present in a very large number of eukaryotic promoters and appear to be generally important for promoter function (Mitchell et al., 1989). Although Sp-1 has been suggested to be the factor bound to the site, studies have shown that there are a number of zinc-finger proteins which can interact with this element (Kingsley & Winoto, 1992). Interestingly, a T-cell specific zinc-finger factor called Ikaros binds to a GC-rich sequence (TGGGGGT) in the CD3-δ enhancer, and it is exclusively expressed in the fetal liver and the thymus (Georgopoulos et al.,

1992 & 1990). Therefore, Ikaros may be one of the proteins interacting with CACCC sites in IL-2 promoter, and may also contribute to the T-cell specificity of the IL-2 gene.

The different transcription factors have been described individually. In general, these factors do not act individually, since mutation of any particular site or decreasing availability of one or two factors will decrease the transcriptional efficiency. The activation-dependent nature of IL-2 gene expression indicates that in T cells all the necessary factors required for IL-2 activation are present. It is necessary to clarify the mechanism of how these factors function coordinately to initiate transcription in response to activation signals .

Acquisition of IL-2 gene inducibility during T cell development

Maturation of T cells occurs in the thymus. T precursor cells from fetal liver and bone marrow migrate to the thymus, where they become immunocompetent mature T cells and emigrate to the secondary lymphoid organs (spleen and lymph nodes, etc). T cells are categorized according to the type of TCR expressed, i.e., TCR$\gamma\delta$ or TCR$\alpha\beta$ T cells. The latter accounts for the majority of T cells. Certain T cells bearing specific TCR $\gamma\delta$ chains are produced early in ontogeny and home to certain locations in the body. For example, Vγ3Vδ1 and Vγ4Vδ1 cells are only produced in late fetal life and home to the skin and reproductive tract respectively. Other $\gamma\delta$ cells as well as $\alpha\beta$ cells are generated both late in fetal and adult thymus and home to the spleen (Vγ4 and $\alpha\beta$), the intestine (Vγ5) or to lymph nodes ($\alpha\beta$). During intrathymic development, the $\alpha\beta$ lineage T cells can be further defined by the expression of a number of other surface markers, like CD4 and CD8. Immature early thymocytes in the subcapsular region of the thymus do not express CD4, CD8, and TCR ($CD4^-CD8^-TCR^-$ or triple negative cells). As they differentiate, they progress to rearrange and express their TCR genes and become $CD4^+CD8^+TCR^{lo}$ thymocytes (double positive cells) localized to the cortex of the organ, in which stage they are also referred to as cortical cells. The $CD4^+CD8^+TCR^{lo}$ cells are

subject to a selection process, in which cells bearing self antigen-reactive TCR undergo apoptosis (negative selection) and cells bearing TCR against foreign antigens remain alive and mature into either $CD4^+TCR^{hi}$ or $CD8^+TCR^{hi}$ cells (single positive or mature cells) localized in the medulla and ready to migrate to the periphery (positive selection). In general, T cells in the thymus are educated to recognize and to respond to foreign antigens. In addition to obtaining antigen recognition specificity by expressing TCR, T cells also acquire helper ($CD4^+$) or cytotoxic ($CD8^+$) functions during the intrathymic maturation process. These effector functions enable $CD4^+$ T cells to produce lymphokines to facilitate the functions of other immune cells, and enable $CD8^+$ T cells to punch holes in pathogen-infected cells and release cytotoxic molecules which lyse target cells and induce programmed cell death (Reviewed by Rothenberg, 1992; Weissman, 1994). A large body of evidence showed that IL-2 gene expression in mature $CD4^+$ T helper cells depends on activation signals delivered by engagement of TCR with antigen in the context with appropriate MHC on antigen-presenting cells and engagement of accessory molecules like CD28. Resting CD4+ T cells do not make IL-2. However, IL-2 gene inducibility and possibly other effector functions in mature T cells are not acquired during the final stage of T cell maturation post-positive selection, but early in T cell development.

For TCR $\alpha\beta$ lineage, pre-T cells, early immature $CD4^-CD8^-TCR^-$ thymocytes and thymocytes from severe combined immune deficient mice (SCID), whose thymocytes arrest at the immature triple negative stage (see fig. 2, "ImmT"), are already competent to be induced to make IL-2 (McGuire & Rothenberg, 1987; Rothenberg et al., 1993; Rothenberg & Diamond, 1994). The expression of IL-2 is induced when these immature cells are stimulated with TCR mimicking pharmacological agents (TPA/A23187) and interleukin-1 (IL-1). Therefore, in the adult thymus, prior to T-cell receptor gene rearrangement and therefore independent of positive selection, immature thymocytes already possess the competence to make IL-2. This finding indicates that early immature

T cells already have established internal signalling pathways and all the necessary IL-2 gene transcriptional apparatus. Interestingly, this competence is lost or repressed when immature thymocytes start rearranging their TCR β chain gene. In addition, the IL-2 receptor α chain gene, like IL-2, cannot be activated in the late triple negative cells and throughout the entire $CD4^+CD8^+TCR^+$ stage (Rothenberg et al. 1993). Positively selected mature single positive thymocytes behave similarly to peripheral spleen lymphocytes. They regain the competence to make IL-2 and IL-2 receptor. Unlike their immature precusors, however, mature thymocytes do not require IL-1 as a co-stimulus, indicating developmental changes with respect to signalling requirement after positive selection (Rothenberg et al., 1990; Rothenberg & Diamond, 1994).

The earliest cells found to be competent to make IL-2 are triple negative immature thymocytes. Unfractionated bone marrow cells which contain T-precursor cells and other lineages of hematopoietic cells cannot be induced to make IL-2 (Rothenberg et al., 1990). However, it is possible that precursor T cells in the bone marrow, lymphoid progenitor cells, and hematopoietic stem cells are already in the process of approaching the activatable state. The ability to make IL-2 may be gradually acquired in an accumulative developmental process. The initial point could be at the generation of hematopoietic stem cells (Fig. 1). With each step of differentiation closer to mature $CD4^+$ T lineage, cells are one step closer to becoming IL-2 inducible cells. On the other hand, cells that diverge away from $CD4^+$ T lineage would either maintain or lose the partially gained IL-2 gene inducibility. For IL-2 inducible cells, they are still in the process to refining this inducibility. This is shown by the different activation requirements in immature and mature T cells.

What is the biochemical basis which makes immature triple negative thymocytes and mature $CD4^+$ T cells able to make IL-2? What makes mature $CD4^+$ T cells independent of IL-1 co-stimulation? As I mentioned in the overview, a number of transcription factors have been implicated in regulating IL-2 gene activation. However,

most of the work was done in T cell lines, and it is not clear whether non-transformed primary T cells also use the same set of transcription factors and whether these factors are similarly regulated. To address these questions, we first tested a thymoma cell line (EL4) which has similar responsiveness to activation signals as mature T cells. These EL4 cells, like immature T cells also respond to IL-1 co-stimulation by expressing higher levels of IL-2. In the first chapter, we defined a set of transcription factor DNA-binding activities in EL4 cells which are correlated with IL-2 gene activation. We also identified two positive regulators, AP-1 and NF-κB, which are sensitive to IL-1 regulation. The DNA-binding activities of both AP-1 and NF-κB are activation dependent and are further increased in the presence of IL-1 during the activation. In chapter 2 we analyzed the DNA-binding activities of these transcription factors in immature, cortical, and mature thymocytes under both stimulated and unstimulated conditions. The results show that the ability to mobilize the same set of transcription factors is correlated with IL-2 gene inducibility in developing thymocytes. The inducible DNA-binding activities of two factors including NF-κB are developmental stage dependent. Higher DNA-binding activity of NF-κB is correlated with more mature cells. Interestingly, the low inducible binding ability of NF-κB in immature cells can be alleviated by addition of IL-1 to the cell culture, which may explain their IL-1 dependent IL-2 gene activation. Therefore, we concluded that the acquisition of IL-2 gene inducibility during T-cell development is by acquiring both signalling pathways and activitable transcription factors.

Acquisition and control of IL-2 gene inducibility

The IL-2 gene inducibility is restricted to Th1 cells. The so-called inducibility means that under normal conditions T cells do not make IL-2, whereas upon stimulation through TCR and accessory molecule engagement, they are induced to produce IL-2. Non-T cells, especially non-hematopoetic cells, do not have this inducibility, even if they receive activation signals that mimic the TCR signal. This inducibility or pre-activation

state can be interpreted molecularly at several levels during development as diagramed in figure 1. Like most tissue specific genes, the IL-2 gene most likely is neither expressed nor activatable in early embryogenesis. The generation of hematopoietic stem cells marks the first access toward IL-2 gene activation (marked 1 in Fig. 1), which may be represented by the alteration of chromatin structure (Siebenlist et al., 1986). Those non-hematopoietic cells would retain their IL-2 gene in the same state carried from the early embryo. The non-hematopoietic cells have the first level of negative control of IL-2 gene. Differentiation of hematopoietic stem cells towards T lineage cells is accompanied by gaining more requisites for IL-2 gene activation, while differentiation towards other hematopoietic cells is accompanied by maintaining or losing the partially gained necessities. The differentiation towards either myeloid or lymphoid lineage has the second level of control, while differentiation to T or B cells marks the third level of control. The fourth level control is intrathymic T-cell development when IL-2 gene inducibility first becomes on, then off, then on again. The final control is at the terminal differentiation of CD4 vs. CD8 and Th1 vs. Th2 cells. Unlike the IL-2 non-inducible cells diverged from other points, a less stringent control is also applied to $CD8^+$ T cells, Th2 cells, and possibly B cells. Bone marrow cells and cortical $CD4^+CD8^+TCR^{lo}$ cells cannot be induced to express IL-2 gene under conditions when immature triple negative and mature $CD4^+$ cells can be stimulated to do so (Rothenberg et al., 1990). On the other hand, mature $CD8^+$ T cells, whose function is to recognize antigen in association with class I MHC molecules and to activate a set of killer-cell specific genes, can be induced to express IL-2 gene under certain conditions *in vitro* (McGuire & Rothenberg, 1987; McGuire et al., 1988). This result implies that the IL-2 gene is less stringently repressed in $CD8^+$ T cells. Similar results are also obtained in B cells and Th2 helper cells. Different from levels 1 and 2, controls from level 3 deal with cells whose precursors are able to activate IL-2 gene. My work is mainly focused on the intrathymic control of IL-2

gene inducibility, but the mechanisms used in this case may also apply to other levels of control.

The newly generated naive $CD4^+$ T helper cells are able to produce IL-2 along with some other lymphokines upon activation. However, depending on the antigen encountered and the genetic background of the animals, $CD4^+$ cells can further differentiate to different effector T cells as defined by the sets of lymphokines they produce. In addition to common lymphokines they secrete, T helper cells can be either type 1 cells (Th1) which secrete IL-2, IFNγ, TNFα and TNFβ; or type 2 cells (Th2) which produce IL-4, IL-5, IL-6, IL-10, and IL-13 (Mosmann et al., 1986; Heinzel et al., 1989; Paul & Seder, 1994). However, similar to $CD8^+$ T cells, Th2 cells under certain conditions can also make IL-2 (McGuire & Rothenberg, 1987; McGuire et al., 1988). EB-virus transformed B cells which are the last cells diverged from T lineage cells are also reported to be able to make IL-2 under certain conditions (Amigorena et al., 1992; Bonerot et al., 1992).

In the first section of the introduction, I discussed the first acquisition of IL-2 gene inducibility early during T-cell development. In chapter 2, we show that upon activation immature T cells already possess a complete set of transcription factors necessary for IL-2 gene activation. It is possible that the develomental process prior to T lineage commitment is a process where cells are gaining these transcription factors or gaining the inducibility of these transcription factors. The temporary loss of the IL-2 gene inducibility in T cells at cortical stage of development may be due to temporary loss of the ability to utilize certain transcription factors. To understand how at each stage of development IL-2 gene can be regulated I will discuss several mechanisms which are generally used to control tissue-specific gene expression and discuss their application to IL-2 gene regulation.

Methylation. Methylation of CpG dinucleotides in the genome has a potential role in regulating gene expression. A correlation between gene methylation and expression is

well-established. Hypomethylation of promoters is correlated in general with gene expression, and methylation is associated with the silencing of genes (Cedar, 1988; Brandeis et al., 1993). Gene methylation is considered responsible for maternal imprinting (Enver et al., 1988) and allelic X-chromosome inactivation (Riggs & Pfeifer, 1992). Methylated genes can be stably propagated *in vivo*, in transgenes, or transfected into cells grown in culture. Treatment of cells with 5-azacytidine can activate normally inactive genes in the cells and make cells differentiate (Jones et al., 1990). Methylation was also shown to be essential for development, since mice homozygous for a mutant DNA methytransferase gene have stunted development and die at midgestation (Li et al., 1992). Molecular mechanisms for transcription inhibition by DNA methylation can be categorized as follows:

1. Methylated DNA can directly interfere with *trans*-acting factor binding. Activation of genes, therefore, depends on DNA demethylation. For example, methylation of the muscle-specific α-actin promoter results in transcriptional repression in both non-muscle and muscle cells (Paroush et al., 1990). Similarly, B cells cannot transcribe a methylated Ig κ chain gene even if it is already rearranged, that is, with its promoter and enhancer brought close together for optimal expression (Lichtenstein et al., 1994). Comparison of the human phosphoglycerate kinase gene promoters on inactive and active X chromosomes showed that the active unmethylated PGK promoter is heavily occupied by factors, whereas the inactive methylated promoter is free of footprints. Furthermore, the inactived PGK can be reactivated by treatment with 5-azacytidine. These results suggest that methylation of DNA prevents active regulators from binding to the promoter (Pfeifer et al., 1990).

2. Methylated CpGs can be bound by methyl CpG-binding proteins (MeCP1 and MeCP2). MeCP1 binds *in vitro* to sites containing multiple CpGs, while MeCP2 can bind to a single CpG (Meehan et al., 1989; Lewis et al., 1992). The binding of *trans*-activators can be blocked directly or indirectly by MeCPs (Boyes & Bird, 1992). It has

been shown that transcription inhibition by methylation depends on both CpG density and promoter strength. Transcription of CpG-poor, weak promoters can be inhibited by methylation; while CpG poor, strong promoter will be less susceptable to methylation (Boyes & Bird, 1992). Tissue-specific genes are mostly CpG-poor and are methylated in non-expressing tissues (Bird, 1986). It is proposed that genes with a limited amount of methylated CpG in a weak promoter have a weak binding to MeCP, and that activation of genes during development depends upon the appearance of factors which are capable of displacing the weakly bound MeCP. Adding a strong enhancer, however, can restore transcription to sparsely methylated, but not to heavily methylated promoters (Boyes & Bird, 1992). In reference to the recent discovery, a function of a strong enhancer is to direct demethylation in addition to its transcription enhancing activity (Lichtenstein et al. 1994). This is not only shown with transfected genes, but also with endogenous genes in development. For example, the expression of the methylated Ig κ chain gene is enhancer-dependent in pre-B cells, but upon differentiation to mature B cells, the gene loses its methylation, and expression is no longer enhancer-dependent (Kelley et al., 1988). These data suggest a competition between the activity of transcription factor and that of MeCPs.

3. Methylation may change the chromatin structure. Unlike the PGK gene mentioned above, the promoter of the tyrosine aminotransferase (a CpG-deficient promoter), is occupied by sequence-specific transcription factors in expressing cells but not in non-expressing cells, even through the transcription factors are ubiquitous (Becker et al., 1987). Demethylating the gene in non-expressing cells by 5-azacytidine does not lead to factor binding nor gene activation (Weih et al., 1991). It has also been shown that methylated DNA is more resistant to nucleases even in cells deficient of MeCPs (Levine et al., 1991). These results suggest that something other than or in addition to methylation represses the gene expression. The nuclease-hypersensitivity experiments suggest that the chromatin structure changed upon methylation. Other evidence comes from studies of Ig gene V(D)J rearrangement. Introducing a densely methylated

minichromosome bearing the recombination signal sequences into a murine pre-B cell line showed a cell-replication dependent inhibition of V(D)J recombination, suggesting that replication induces methylated DNA into a heterochromatic structure (Hsieh & Lieber, 1992).

Alteration of chromatin structure. DNA in the nucleus is packaged into octamer nucleosomes composed of (H2A, H2B, H3, and H4)$_2$ with two superhelical turns of 164 bp of DNA wrapped around the octamer and histone H1 bound to the outside of nucleosomes. The polynucleosome filament is further compacted into a 30 nm fiber by coiling to form a solenoidal structure with about 6 nucleosomes per turn. Higher order structures which account for the various forms of condensed and decondensed chromatin have been proposed. Transcriptionally active and inactive chromatin may each be characterized by different degrees of condensation. Endonucleases like DNase I preferentially cleave genes which are transcriptionally active. It has been suggested that active and potentially active genes reside within a chromatin configuration that is different from that of inactive genes. Active genes contain 'open' regions which are physically accessible to cleavage by nucleases. The appearance of a DNase I hypersensitive site corresponds to the disruption of one or two specifically phased nucleosomes (Gross & Garrard, 1988; Carr & Richard-Foy, 1990). The presence of ordered or phased nucleosome structure in chromatin correlates with tissue-specific gene repression and has been investigated using periodic chromatin cleavage patterns generated by various nucleases or chemical cleavage reagents. The phased nucleosome structure has been found in the enhancer region of the β-globin gene, and the loss of nucleosome phasing is correlated with the capacity for β-globin transcription in murine erythroleukemia cells (Benezra et al., 1986). In yeast depletion of histone proteins results in the activation of many genes which are normally silent. In this specific case, the expression of newly induced genes in this system is *trans*-acting factor independent. Thus, the 'closed' chromatin structure can function as a general repressor of transcription

(Han & Grunstein, 1988). In another yeast example, a proposed global multiprotein transcriptional activator composed of SWI1, SWI2/SNF2, SWI3, SNF5, and SNF6 regulates a large number of genes. Mutations in any of the activator components leads to repression of target genes. Interestingly, revertants which express target genes include the alteration of histone H3, inactivation of an HMG1-like protein, and deletion of H2A and H2B (Peterson & Herskowitz, 1992; Sternberg et al, 1987; Kruger & Herskowitz, 1991; Happel et al., 1991; Winston & Carlson, 1992). These results suggest that the multiprotein activator could interact with nucleosomes or with chromosome-binding proteins to antagonize the repressive effects of chromatin and facilitate the binding of other transcription factors.

Chromatin structure affects both transcription initiation and elongation (Lorch et al., 1987). I will focus on the former issue in the present discussion. A number of tissue-specific genes examined by *in vivo* footprinting studies showed that promoters in non-expressing cells are free of sequence-specific *trans*-acting factors and that promoter occupancy occurs only in expressing cells. Among them are genes encoding liver tyrosine aminotransferase (TAT) (Becker et al., 1987), muscle creatine kinase (Mueller & Wold, 1989), MHC class II (Kara & Glimcher, 1991), MHC class I (Dey et al., 1992), transthyretin (Mirkovitch & Darnell. 1991), chicken β-globin (Jackson et al., 1989). Although most studies do not directly address nucleosome assembly or disassembly in both non-expressing or expressing cells, it has been implied that at least in non-expressing cells DNA is packaged into nucleosome and H1-containing chromatin. As was mentioned earlier, a large body of evidence has indicated that both nucleosomal cores and histone H1 can act as global repressors of transcription and that gene activation is achieved by sequence-specific transcription factors which function both to counteract the chromatin-mediated repression (antirepression) and to facilitate the inherent transcripiton process (Adams & Workman, 1993; Croston & Kadonaga 1993). Two

models have been proposed regarding the role of chromatin structure in transcriptional repression (Felsenfeld, 1992).

The first model suggests that there is a dynamic equilibrium involving competition between the transcription factors and histone octamers for sites in non-dividing cells. In two cases, glucocorticoid receptor (GR) has been indicated to compete for DNA binding and induces, in a cell-cycle independent manner, remodeling of the chromatin structure, which allows binding of other transcription factors. For instance, the mouse mammary tumor virus (MMTV) promoter has a phased array of six nucleosomes in the nucleus. One of the positioned nucleosomes excludes a group of transcription factors including the glucocorticoid receptor (GR) and nuclear factor 1 (NF1) from the promoter before glucocorticoid treatment. Hormone induction of the promoter does not change the concentration and DNA binding affinity of NF1, but results in hypersensitivity around the nucleosome to nucleolytic agents and hormone-dependent loading of a transcription preinitiation complex including NF1 following the chromatim structural transition (Archer et al., 1992; Pina et al., 1990). Although this finding is from a viral promoter, similar results are also obtained from the promoter of a cellular gene, a liver-specific tyrosine aminotransferase (TAT) gene. A DNase I-hypersensitive site which results from the displacement of two specifically phased nucleosomes is seen at position -2500 of the promoter following hormone induction. Further analysis revealed that this region contains a GRE and a liver-specific transcription factor HNF5 binding site that are partially overlapping. GR is able to bind its site *in vitro*. However, glucocorticoid treatment only results in HNF5 binding to the site *in vivo*. A natural internal control has shown that a hormone-independent DNase I-hypersensitive site further upstream is constitutitively bound by HNF5 (Rigaud et al., 1991). These findings clearly indicate that HNF5 binding to the proximal site depends on a hormone-induced alteration of chromatin structure. Another well-illustrated example is the control of the yeast acid phosphatase gene PHO5. The promoter of the PHO5 gene contains two pairs of sites for

trans-activating proteins PHO2 and PHO4. One of the PHO4 sites is located in the linker DNA between two phased nucleosomes, which also overlaps the DNase I hypersensitive site. The other three sites are buried in the two phased nucleosomes. During induction of the gene in the absence of phosphate, displacement of the adajcent four nucleosomes neighboring the linker DNA containing both DNase I hypersensitive site and PHO4 site is observed, which results in exposing the second PHO4 site and two PHO2 sites (Almer et al., 1986). Replacing the DNA wrapped around nucleosome covering two sites with either a satellite DNA which has a high affinity for histones or a derivative of a pBR322 DNA segment alters the promoter, making it either more resistant or more susceptible to induction, respectively (Straka & Hörz, 1991). This suggests that resistence to nucleosome displacement by satellite DNA does not relieve of the repression. The nucleosome displacement is shown to be initiated by PHO4 binding to its site at the linker sequence, since deleting this PHO4 site inhibits the transcription initiation of the PHO5 gene (Fascher et al., 1993).

The second model proposes that there is a 'pre-emptive' competition for binding sites. As shown in a number of *in vitro* chromatin assembly experiments, depending on the order of factor addition, adding pol II transcription factor first will exclude the histone octamer binding and vice versa (Workman & Roeder, 1987). In the presence of transcription factors, the assembly of a transcriptionally active complex can be less dependent on the order of TFIID and histone additions (Workman et al., 1990; 1991). Extending the *in vitro* data would predict that chromatin changes would only occur during DNA replication when the site is exposed at the replication fork before chromatin assembly. Acquisition or repression of a tissue-specific gene expression, or establishment of a pre-activation state in proper cells, occurs most likely in dividing cells and is irreversible once the cell is terminally differentiated. As mentioned earlier, by *in vitro* DNase I footprinting method, *trans*-acting factors present in TAT-non-expressing cell nuclei bind to the TAT promoter region equally well as in TAT-expressing liver cell

nuclei. Demethylating the gene in non-expressing cells also failed to activate it, indicating that the chromatin structure is in a permanently repressed configuration inaccessible to activators once cells acquire the non-liver cell fate (Becker et al., 1987; Weih et al., 1991)

However, there are some experimental data which seem difficult to reconcile with the pre-emptive model. In heterokaryons from cell fusion experiments, tissue-specific genes can either be turned on in the nuclei of cells which normally do not express them or turned off in nuclei which expressed them previously (Blau, 1989). In contrast to the differentiation process when cells are in cycle, the conversion of gene expression in heterokaryons is independent of DNA replication. Although it is thought that the degree of activation is contingent upon the history of the nuclei exposed to a series of developmental cues to progress from undifferentiated zygotes to specific tissue, this process is not understood molecularly. *In vitro* biochemical studies demonstrate that a promoter template with phased nucleosomes assembled in the absence of H1 can be activated with a sequence-specific DNA-binding activator, Gal4-VP16, but it cannot be activated when H1 is also packaged into the chromatin template except during the process of template replication (Kamakaka et al., 1993). In heterokaryons as long as tissue-specific genes which are normally silent in one partner nucleus are not heavily packaged with H1, they may be activated by factors diffused from the other nucleus. The ability to reactivate tissue-specific genes in heterokaryons may depend upon the concentration and modification of histone proteins.

Methylation and chromatin structure may both be involved in IL-2 gene regulation early in development, during the generation of hematopoietic stem cells and in early hematopoiesis which corresponds to stage 1 as shown in figure 1. General inspection of the gene reveals that like other tissue-specific genes, the IL-2 gene promoter/enhancer region is poor in CpG. Seventeen CpG pairs are found in 2800 bp 5' regulatory sequence with the frequence of 1/165, equivalent to the human γ-globin gene

(1/126) of which it has been shown that methylation alone is sufficient to inhibit the transcription (Boyes & Bird, 1992). Unfortunately, little is known about the methylation status of the IL-2 gene especially in relation to its inducibility. However, there are two examples of gene regulation in thymocytes by developmentally regulated demethylation. The demethylation of a gene encoding a T-cell specific CD3 ζ chain is tightly correlated with its expression in thymocytes on day 18 of gestation (Hsu et al, 1992), while the demethylation of the CD8 gene is also coupled with the onset of its expression during the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$ stage (Carbone et al., 1988). Similarly, methylated Ig κ gene in pre-B cells becomes demodified once the cells progress to the B cell stage. This developmental stage-specific demodification is independent of Ig gene rearrangement and transcription (Kelley et al., 1991; Lichtenstein et al., 1994). It is possible that at stage 1 there is a demethylation process accompaning the generation of hematopoietic stem cells.

The chromatin structure for the IL-2 locus, on the other hand, is better understood but our knowledge is far from satisfactory. There are at least three different configurations of IL-2 chromatin structure according to DNase I hypersensitivity studies (Siebenlist et al., 1986). Non-hematopoietic cells do not have any nuclease-sensitive sites, while resting T cells and some hematopoietic cell lines have three tissue-specific sites (upstream of the promoter, -300bp, and first intron). Activated IL-2 producing T cells have a fourth nuclease-hypersensitive site around the promoter region (-100). The interpretation for the lack of nuclease-sensitive sites in non-hematopoietic cells would be that the tightly packaged chromatin is resistant to direct nucleosome and histone H1 disruption or displacement by *trans*-acting factors. The relatively 'open' chromatin structure in resting T cells and T-cell related hematopoietic cells distinguishes them from the former cells. These two distinct chromatin configurations could be acquired by the pre-emptive mechanism during the tissue differerentiation process. It can be hypothesized that in response to specific developmental cues IL-2 gene-specific

demethylation may occur and may be followed by alteration of the chromatin conformation during the progression to become hematopoietic stem cells only. The chromatin structure of IL-2 locus in non-hematopoietic cells would remain in a locked configuration which is constitutively inherited in all cell derivatives. It would be predicted that introducing *trans*-activators from activated T cells to their nuclei by forming heterokaryons with T cell nuclei would not be able to activate the gene. For these non-hematopoietic cells both methylation and repressed chromatin structure are likely to be the major mechanisms used to repress the IL-2 gene. Other repression controls may also exist but probably play a minor role.

What does the presence of constitutive nuclease-sensitive sites in hematopoietic cells mean? It could indicate a relatively 'open' structure which may make the proximal promoter region more accessible to basal transcription factors than it would be with a closed configuration. Biochemically, this can be achieved by changing the positioning or phase of the nucleosomes which flank these hypersensitive sites. In doing so, two short sequences that are separated by the equivalent of one superhelical turn of DNA could be brought into close lateral proximity by assembling them into the same, rather than into neighboring nucleosomes. Alternatively, changes in the position of nucleosomes could be responsible for defining the rotational orientation of the major and minor grooves relative to the histone octamer and could place a sequence within an accessible linker region, thus, poising the promoter for *trans*-acting factor assembly. The hypersensitive site in the -300 bp region may, in particular, be essential for repositioning the sites for CACCC and NF-AT binding, which may serve to create the inducible hypersensitive site further downstream (to be discussed more in later sections). Regions of DNase hypersensitiy may have sites for specific *trans*-acting factor binding, which may exert an anti-repressive effect (Croston & Kadonaga, 1993). Overall, the specific protein-DNA interactions at these sites may serve as a pre-requisite for IL-2 gene expression. And this step may possibly occur prior to the differentiation to T lineage cells. However, these

induction-independent hypersensitive sites are necessary but not sufficient to activate IL-2. IL-2 expression is correlated with the stimulation-dependent nuclease-hypersensitive site. The appearance of this site is likely to be cell-cycle independent, since IL-2 mRNA can be detected 30 min after stimulation, while it takes 18-24 h to complete a cycle. In contrast to the other three sites, disruption of nucleosomal cores and histone H1 at the promoter site is likely to be a dynamic quasi-equilibium competition between stimulation-dependent *trans*-acting factors and histone octamers. The hypersensitive sites examined in limited types of cells suggest that the inability to express IL-2 gene in non-Th1 hematopoietic cells (stages 2 through 5 in Fig. 1) is unlikely due to a repressed chromatin structure, but instead repression is more likely due to a repressor or a lack of activators. In support to this hypothesis, $CD8^+$ T cells and some malignantly transformed B cell lines can make IL-2 under certain conditions, while under the same conditions non-lymphoid cells are not able to make IL-2 (Rothenberg et al., 1990; McGuire & Rothenberg, 1987; McGuire et al., 1988; Amigorena et al., 1992; Bonnerot et al., 1992).

Repressors and negative regulators. By interacting with the *cis*-elements in a promoter region a repressor could directly inhibit the general transcription machinery. A negative regulator-mediated gene repression can occur at multiple levels in the transcription initiation process. For example, it may interact directly with *trans*-activators to block their DNA binding domain or the activation domain.

The Drosophila homeodomain gene *even-skipped* (*eve*) is an example of a repressor interfering with the pre-initiation complex assembly. Upon binding to the hemoedomain-binding sites *eve* can inhibit activator-independent transcription, suggesting that *eve* can directly interact with the general transcription machinery (Johnson & Krasnow, 1992). Direct binding of repressors to the promoter region is also illustrated in β-interferon gene regulation. Upon induction the repressor bindings are either dissociated or displaced by an activator protein (Zinn & Maniatis, 1986). It is

likely that the repressor binding activity is inactivated or is competed by the activator following viral infection. In another example, studies using a fusion cell system showed that in hepatoma-fibroblast hybrids liver-specific genes like tyrosine aminotransferase and albumin are repressed. This inhibitory effect is directed by a tissue-specific control element found in the albumin gene, and may implicate a possible repressor interaction (Petit et al., 1986).

A more efficient way to control gene expression has recently been described. A single gene can generate either a positive or a negative regulator depending on various post-transcriptional modifications, in most cases by alternative RNA splicing. In these cases, full length proteins act as activators, while truncated ones which have usually lost the activation domain act as repressors. Some known genes which are subject to this form of modification are $erbA_2$ (Koenig et al., 1989; Rentoumis et al., 1990), a proto-oncogene encoding the α subtype of the nuclear thyroid hormone receptor; mTFE3, a gene encoding an Ig enhancer binding protein (Roman et al., 1991); fosB (Dobrzanski et al., 1991; Yen et al., 1991; Nakabeppu & Nathans, 1991); and CREM, a gene encoding a cAMP-response element modulator which is an antagonist of cAMP-induced transcription (Foulkes et al., 1991). Another way to generate activator and repressor from the same gene is by alternative translation initiation. For example, by doing so, the mRNA that encodes a liver-enriched transcription factor, called LAP, can also encode a transcription inhibitor LIP, due to initiation downstream of an N-terminal activation domain by translation initiation 3' to LAP (Descombes & Schibler, 1991). In all of these cases, the DNA binding and dimerization domains remain intact and inhibition of gene activation is achieved by either competing directly with activator DNA binding, by formation of inactive heterodimers with activator proteins, or by competition for accessory transcription factors.

One other interesting aspect of this type of regulation as well as other regulations in general is that the ratio of activator vs. repressor can be modulated in a temporal and

spatial fashion. Thus, developmental cues also influence the post-transcriptional and translational modification steps, which in turn regulate gene expression. An extension to these data is the discovery of chromosomal relocalization of a heat-shock transcription factor (HSF) during heat shock, which can act as both an activator and a repressor. Heat shock induces puffs in Drosophila polytene chromosome, which include heat-shock genes that are actively transcribed. HSF is known to be an active regulator for heat shock genes. Normally HSF forms an oligomer with homogenous chromosomal distribution, upon heat-shock induction the inactive oligomers form active multimers and relocalize to discrete sites in the chromosome. Interestingly, these discrete sites include not only the heat-shock puff sites but also some developmental loci that are normally repressed during heat shock. Genes within this depressed loci include the ets-related protein and a steroid receptor DNA-binding protein. Thus, HSF can act both as an activator to induce heat-shock genes and as a repressor to inactivate normal cellular transcription. The assumption from this experiment argues the original thought that repression during heat shock was controlled by some general stress-induced defect of the transcriptional machinery instead of a specific transcription factor (Westwood et al., 1991).

Negative regulation of the IL-2 gene by a repressor or a negative regulator may be implied at two levels. One is by regulating initiation and rate of the RNA synthesis, and the other is by regulating the transiency of IL-2 expression. A zinc-finger protein induced in stimulated T cells binds the promoter region at -101/-110, and this can negatively regulate activity of the -548 bp IL-2 promoter (Williams et al., 1991). Although the IL-4 secreting Th2 cell clone (D10) cannot normally express IL-2, treatment with cycloheximide 3 hr after stimulation induces IL-2 expression and suggests that the synthesis of an IL-2 gene repressor is required to inhibit IL-2 expression and the repressor is inhibited by the cycloheximide treatment (Muñoz et al., 1989). Both experiments might address the effect of the same repressor which controls the transient fashion of expression once the gene is activated. Mapping the regulatory element by

comparing the activities of promoters containing different lengths of 5' promoter sequences revealed that pIL2(-1219) has two fold lower activity than pIL2(-578), while pIL2(-753), pIL2(-1219) with deletion of -1002 to -579, and pIL2(-578) express equally well (Novak et al., 1990). These results suggest that a sequence from -1000 to -750 has a negative effect on transcription. This region is not very well conserved between mouse and human yet it contains stretches of poly d(CA), which may be significant since poly d(CA) stretches are also found in other murine cytokine genes as well (Novak et al., 1990). On the other hand, this fragment is not conserved, so that even if it has an inadvertent negative regulatory effect, it may not be used for human IL-2 gene regulation.

Using Xenopus oocytes as an assay system, Mouzaki et al. reported the presence of a repressor activity in resting spleen T cell extracts. This is shown by injecting the IL-2 gene and spleen cell extracts into Xenopus oocytes. Since whole-cell extracts were used, much of what is assayed may be cytoplasmic factors. The injected IL-2 gene has a basal level of expression which can be repressed after injecting extracts from resting spleen cells. In contrast to the extract from resting spleen T cells, extracts from brain, liver, and kidney cells do not have this repressor activity. Surprisingly, injecting extracts from stimulated spleen cells does not augment the transcription unless it is coinjected with unstimulated extracts, indicating that there is an anti-repression activity in the stimulated extract (Mouzaki et al., 1991). As $CD4^+$ T cells differentiate further to become memory T cells they lose the repressor activity (Mouzaki et al., 1993). The repressor activity is exerted at (-292/-264) which overlaps the distal NF-AT site (Mouzaki et al., 1991). Could this repressor factor be the cytoplasmic NF-AT component (NF-ATc)? Since whole cell extract is used, the inactivated phosphorylated NF-ATc may interact with its cognate site in association with AP-1 from the oocyte and inhibit IL-2 gene transcription. It has been shown that phosphorylated NF-ATc in the presence of AP-1 can bind to DNA (Flanagan et al., 1991; Clipstone et al., 1992). The anti-repressive effect of activated extracts can be attributed to either the active form of NF-ATc in

activated extracts which competes with the inactive form of NF-ATc, or to the activation of bound NF-ATc through dephosphorylation with phosphatase from activated extracts (Schreiber & Crabtree, 1992). Evidence supporting the role of NF-ATc as a repressor include results from injecting extracts of the brain, liver, and kidney cells which do not have this repressor activity, and the fact that NF-ATc was reported to be a T-cell specific factor (Shaw et al., 1988). If NF-ATc is the repressor in this experiment, does this kind of repression necessarily occur in resting T cells? Probably not. NF-ATc in resting T cells is located in the cytoplasm, and only upon receiving the activation signals does it become dephosphorylated and is able to mobilize to the nucleus. It is not clear how this repressor activity as measured by the injection assay disappears as $CD4^+$ T cells differentiate further to become memory T cells (Mouzaki et al., 1993). Unfortunately, the memory T cells generated in this experiment were not checked for their ability to make IL-2. They could be still in activated state and are making IL-2, or they are anergized and unable to make IL-2. It would also be interesting to see whether stimulated memory T cells are similar to stimulated spleen T cells and have anti-repression activity. This comparison would allow us to determine whether activation requirements change when T cells differentiate.

Regulation of positive regulator availability. Control of tissue-specific genes by a gradient distribution of transcription factors is best shown by several Drosophila morphogens. The mRNA for the anterior determinant bicoid is initially concentrated in the anterior pole of the egg. After fertilization, the bicoid protein subsequently diffuses and is selectively degradated to form an anterior to posterior concentration gradient that extends over the anterior two thirds of the embryo. The bicoid protein is a sequence-specific DNA-binding protein, and regulates a number of gap genes, including a gene called hunchback. In contrast to the anterior-posterior gradient distribution of the bicoid gene product, the hunchback protein is distributed homogeneously in the anterior half of the embryo and has a sharp posterior boundary. Varying the concentration of either

bicoid protein or the number of bicoid binding sites in the hunchback promoter can move the hunchback boundary forward or backward (Johnston & Nüsslein-Volhard, 1992). Concentrations of bicoid protein below a certain limit result in no activation of the hunchback gene. This activator dosage dependent transcription is called a threshold phenomenon. Another example of the developmental control of transcription factors is the differential regulation of GATA-1 and Sp-1 in primitive and definitive lineages of chicken erythrocytes. Higher levels of GATA-1 and Sp-1 in primitive erythrocytes are correlated with the expression of a primitive lineage-specific globin gene (Minie et al., 1992).

Decreasing protein synthesis or increasing protein degradation is one way to physically reduce the number of activator molecules, while specific modification or dimerization can be used to mask the nuclear localization signal or DNA-binding domain and affect the protein's function. For example, the gradient of dorsal protein, another Drosophila morphogen which determines the dorsoventral axis, is controlled by nuclear localization. Different zygotic genes are activated in response to a spectrum of concentrations of nuclear localized dorsal protein (Johnston & Nüsslein-Volhard, 1992). The nuclear localization of the dorsal protein in the ventral and lateral side of the embryo is triggered by the IL-1 receptor-like membrane protein Toll in response to the active form of spätzle protein. It is proposed that the gradient distribution of the dorsal protein along the ventral-dorsal axis is generated by the localized proteolytic processing, which generates processed, active spätzle protein at the ventral side of the embryo (Morisato & Anderson, 1994).

Direct blocking of an activation domain by a negative regulator has been demonstrated for c-myc gene regulation. The cell-cycle control gene c-myc is developmentally regulated in B cells. It is on in early B cells but off in terminally differentiated plasma cells. Its expression during development is inversely correlated with the expression of a negative regulator called myc-PRF, which binds to the c-myc

promoter and shuts off transcription (Kakkis et al., 1987). Biochemical studies showed that the site for myc-PRF is adjacent to the site for myc-CF1, an activator for the c-myc gene. Both factors can bind to their sites simultaneously and are physically associated. This implies that in plasma cells high levels of myc-PRF allow formation of a multi-unit complex containing myc-PRF, myc-CF1, and myc DNA, which masks myc-CF1 (Kakkis et al., 1989; Riggs et al., 1991). Masking the activation domain can also be achieved by post-translational modification of activation domains.

There are many examples of variation in the availability of *trans*-activators by dimerization with an inhibitory protein. Negative regulators which function by complexing with activator proteins include IκB, IP-1, hsp90, and Id. IκB dimerizes with NF-κB, but upon activation by TPA, IL-1, or TNFα, IκB becomes phosphorylated and releases NF-κB which subsequently binds DNA (Baeuerle & Baltimore, 1988). IP-1 behaves similarly to IκB but instead it associates with AP-1 and prevents its DNA binding. Phosphorylation of IP-1, mediated by cAMP-dependent protein kinase A, releases AP-1 in both the nucleus and the cytoplasm (Auwerx & Sassone-Corsi, 1991). A heat shock protein encoded by the hsp90 gene associates with the glucocorticoid receptor, and blocks its transcriptional activation function. Binding of steroids to the receptor causes dissociation of hsp90 and releases the receptor, which is then able to bind DNA (Picard et al., 1988). Myoblasts express MyoD family proteins which cannot activate muscle-specific genes at that developmental stage. Id, an HLH protein, associates with MyoD in the myoblasts and the resulting Id-MyoD heterodimers fail to bind DNA. During myocyte differentiation, down-regulation of the Id protein permits dimerization of MyoD family member proteins which can now bind DNA (Benezra et al., 1990).

How does a reduction in the nuclear concentration of positive regulators result in no or significant transcription? What is the mechanism of this threshold phenomenon? *In vitro* biochemical studies using a 5xVAG4-VP16-site containing the adenovirus E4 minimal promoter template either naked or assembled with nucleosomes and H1 proteins

showed that with increasing concentrations of GAL4-VP16 proteins, the naked template has a gradual linear increase in transcription over the basal level of activation seen in the absence of GAL4-VP16. On the other hand, the chromatin template shows a quantum non-linear increase in transcription from no expression at zero or low levels of GAL4-VP16 to high expression at higher levels of GAL4-VP16. The reproduced threshold of activation with this template indicates that it is the repressive effect of chromatin structure which accounts for lack of expression with an insufficient amount of activator. Higher amounts of activator are required for both anti-repression and *trans*-activation of genes (Laybourn & Kadonaga, 1992).

As briefly mentioned earlier, the differentiation stages 2 and 3 in figure 1 might be points where lymphoid and T cells become more competent to express IL-2 gene by acquiring the ability to activate T-cell specific transcription factors and the ability to express high levels of both T-cell specific and certain general transcription factors upon activation. Non-lymphoid or non-T cells at each of these points do not undergo this acquisition process. For instance, a pre-mast cell line (32D) (differentiated from stage 2) has been shown to have decreased NF-AT and AP-1 binding activities upon stimulation (chapter 4). A similar mechanism could also apply to B cells developed from stage 3. However, different from pre-mast 32D cells, B cells are one or more steps closer to T cells. This may indicate that they have more characteristics of T cells in terms of IL-2 gene inducibility. In fact, it has been shown that B cells from transgenic mice with (NF-AT site)$_3$-SV40 large T antigen clearly express T antigen upon induction *in vitro*. But their kinetically slow expression of IL-2 gene may suggest that their ability to mobilize NF-AT or some other transcription activators is less ready than T cells and they require longer time to accumulate high enough concentration of certain factors (Verweij et al., 1990). Also as mentioned earlier, that EB-virus transformed B cells can make IL-2 under certain conditions (Amigorena et al., 1992; Bonnerot et al., 1992). Most of the transcription factors described above are not T-cell specific or IL-2 gene specific.

Teleologically, in addition to requiring the ability to express T-cell specific transcription activator NF-AT and being able to activate it, IL-2 gene inducibility could also be regulated through increasing the nuclear concentration or availability of those T-cell non-specific factors. This may be particularly true for T cells at stages 4 and 5. For example, in chapter one we showed that increasing the DNA-binding activities of AP-1 and NF-κB by co-stimulation with IL-1 superinduced the level of IL-2 gene activation. Similarly, the requirement of IL-1 co-stimulation in immature T cells is another example showing that NF-κB, a non-T cell specific transcription activator can be regulated developmentally. In IL-2 producing cells, specifically reducing the nuclear concentration of certain factors or affecting the *trans*-activation activity of certain factors has been shown to decrease the IL-2 inducibility. For example, inhibition of a Ca^{2+}-calmodulin-dependent phosphatase by treatment with an immunosuppressant cyclosporin A specifically blocks the nuclear translocation of NF-ATc and results in no IL-2 gene activation (Liu et al., 1991; Clipstone et al., 1992).

We have discussed the first acquisition of IL-2 gene inducibility in immature triple negative T cells. Point 4 in figure 1 indicates the transitions from immature triple negative T cells to cortical double positive TCR^{lo}, and to single positive TCR^{hi} T cells. We also know the fact that the transition from $CD4^-CD8^-TCR^-$ immature cells to $CD4^+CD8^+TCR^{lo}$ cortical thymocytes, and then positive selection to single positive TCR^{hi} mature cells are accompanied by changes in the regulation of the IL-2 gene from inducible, to non-inducible, and then to inducible again. Could the inability of $CD4^+CD8^+TCR^{lo}$ thymocytes to make IL-2 be attributed to a repressed chromatin structure? Most likely not. Remodeling of chromatin structure can be dependent or not be dependent on DNA-replication. However, those dependent on DNA-replication are usually more stably inherited, while those independent seem more reversible. The transition from IL-2 inducible $CD4^-CD8^-TCR^-$ immature thymocytes to IL-2 non-inducible $CD4^+CD8^+TCR^{lo}$ cells does require several rounds of proliferation, which may

give cells an opportunity to remodel the chromatin structure and to render the promoter-enhancer region inaccessible to *trans*-acting factors. However, the positive selection process which transforms the $CD4^+CD8^+TCR^{lo}$ back to IL-2 inducible $CD4^+TCR^{hi}$ or $CD8^+TCR^{hi}$ cells does not involve cell proliferation. Thus in the absence of proliferation there is little chance for the chromatin structure to transform back to an activatable state. Furthermore, since a pattern of hypersensitive sites associated with the activatable configuration is also stably inherited in other hematopoietic non-T cells which cannot be induced to express IL-2 (Siebenlist, et al., 1986), it is not inconsistent that these sites remain in $CD4^+CD8^+TCR^{lo}$ cells. Although circumstantial evidence seems to favor that there is no change of chromatin structure during intrathymic development, it would still be necessary to demonstrate this either directly by performing the DNase I mapping or indirectly by showing its IL-2 expression under certain circumstances.

The cortical stage in T-cell development is coupled with many changes. The IL-2 gene is not the only gene whose inducibility is repressed. For example, the IL-2 receptor α gene is constitutively expressed in early triple negative immature cells, non-constitutive, but inducible in late stage triple negative immature cells, and completely non-inducible in $CD4^+CD8^+TCR^{lo}$ cells. It is reversed to be non-constitutive, but inducible again in post-positively selected cells (Boyer & Rothenberg, 1988; Rothenberg et al., 1993). In addition to these inducible genes, a number of constitutively expressed genes like the IL-2 receptor γ gene and the apoptosis-protective bcl-2 gene are also temporarily repressed during this transition (Cao et al., 1993; Moore et al., 1994). Instead a number of genes associated with programmed cell death, including Fas, are upregulated or become inducible in cortical thymocytes (Andjeli´c et al, 1993; Owens et al., 1991); as if these cells switch to a semi-suicidal mode ready to undergo apoptosis at any moment. Both the ectopic expression of bcl-2 in bcl-2 transgenic cortical thymocytes and the *in vivo* administration of soluble Fas protein, which blocks the Fas ligand can reduce thymocyte apoptosis (Sentman et al., 1991; Strasser et al., 1991; Cheng et al., 1994).

Cortical cells may not interpret signals transduced through Fas in addition to signals generated by the high avidity engagement of epithelial cells as an activation signal as mature T cells do but as a death signal (Hogguist et al., 1994; Ashton-Rickardt et al., 1994). Transcription factors used to induce genes like IL-2 may not be activated in cortical cells because of the nature of the signal, or the factors may be activated but then are used instead to activate genes for programmed cell death. In chapter 2 and 3, we will specifically examine the availability of the transcription activators in cortical cells by comparing DNA-binding activities in immature, cortical, and mature thymocytes and we will analyze at which level the factors with altered binding activity are regulated.

Once mature $CD4^+$ T cells acquire this competence, the regulation of IL-2 gene continues during the final differentiation to Th1 and Th2 cells (Fig. 1, stage 5). Naive $CD4^+$ cells produce IL-2 upon stimulation. Memory $CD4^+$ cells, on the other hand, produce either IL-2, IFNγ, TNFα and TNFβ, or IL-4, IL-5, IL-6, IL-10, and IL-13 upon induction. Memory cells making the former set of cytokines are designated Th1 cells, while memory cells making the latter are Th2 cells. Activation of a Th1 response favors the development of cellular immunity by enhancing the microbicidal activity of macrophages; while activation of a Th2 response facilitates humoral immunity by enhancing B cell antibody production. By varying culture conditions, Th1 and Th2 cells may develop directly from a naive T cell which produces IL-2 predominantly (Röcken et al., 1992). Alternatively, Th1 and Th2 cells may arise from a IFNγ and IL-4 producing Th0 cell, which is the cell in the developmental stage between naive and memory T cells. A common progenitor for Th1 and Th2 cells is best demonstrated in transgenic mice expressing the IL-4 promoter driving herpes simplex virus thymidine kinase. Activation of the cytokine promoter in IL-4 expressing cells induces the synthesis of thymidine kinase and renders the cell sensitive to killing by ganciclovir. Culture of naive $CD4^+$ T cells from transgenic mice in conditions which allow naive T cells to differentiate to either IFNγ-producing Th1 or IL-4 producing Th2 cells does not give rise to IL-4

producing Th2 cells nor IFNγ producing Th1 cells when ganciclovir is included in the beginning of the culture. Native CD4+ T cells from negative control littermates produce both types of cells under same conditions (Kamogawa et al., 1993). The progression to Th1 or Th2 is shown to be more instructive. Differentiation from either naive T cells or Th0 cells to Th1 or Th2 cels can be induced by cytokines like IL-2, IL-4, IL-12 and IFNγ. Both *in vitro* and *in vivo* studies have demonstrated that IL-2+IL-4 directs T cells to produce a Th2-like response, which is inhibited by IFNγ; whereas IL-2+IL-12 drives a Th1-like response, which can be inhibited in the presence of IL-4 (Seder et al., 1992). The preferential induction of Th1 development by macrophages is also correlated with their secretion of IL-12 cytokines (Hsieh et al., 1993). It appears that these cytokines may cause irreversible differentiation to Th2 or Th1 end states, not just a strongly biased short-term response. Therefore, cytokine-cytokine receptor mediated signals are able to determine Th1 and Th2 differentiation. It is then necessary to address what nuclear targets are controlled by these differentiation signals. A number of experiments discussed below indicate that the repression of either IL-2 or IL-4 gene inducibility is reversible under certain conditions. Therefore, DNA methylation or repressed chromatin structure is less likely to be involved in Th1 and Th2 differentiation, but sequence-specific transcriptional activators or repressors are more likely to be the nuclear target of these differentiation signals.

The proximal promoter region of IL-4 gene contains four NF-AT-like sites. Recent studies have shown that the factor(s) bound to one of the NF-AT-like site in the IL-4 gene promoter did not contain an AP-1 factor (Rooney et al., 1994), in other words, AP-1 may not be involved in IL-4 gene activation. Cotransfection of a Th2 clone with the eukaryotic initiation factor 4E (eIF-4E) was able to induce IL-2 gene expression and augment NF-AT binding activity. Since AP-1 is the component of NF-AT which requires *de novo* protein synthesis, the above results may imply that the overexpression of eIF-4E induced the synthesis of proteins including AP-1 and other IL-2 regulatory factors

which are deficient in Th2 cells (Barve et al., 1994). Not surprisingly, there are also Th2-specific IL-4 gene regulators which are similarly controlled in Th1 cells. For instance, when using either the entire IL-4 5' regulatory sequence or a multimerized site from the IL-4 promoter, the reporter gene was only induced in IL-4 producing Th2 clones but not in IL-2 producing Th1 clones (Bruhn et al., 1993; Lederer et al., 1994). Therefore, it appears that the divergence of cytokine gene expression in Th1 and Th2 cells may be limited at least in part by the amount of gene-specific positive regulatory factors. This was also supported by the finding that hybrids produced by the fusion of BW5147 (a Th1-like thymoma cell line) and a Th2 clone express both IL-2 and IL-4 (Hagiwara et al., 1988). Is AP-1 a likely nuclear target that are involved in Th1 and Th2 determination? Could AP-1 also be differentially regulated by IL-2+IL-4 and IL-2+IL-12? One study using a human myeloid cell line, HL-60, demonstrated that the commitment to either macrophage or granulocyte lineage pathways is correlated with the level of AP-1 induction (Mollinedo et al., 1993). Cross-linking the γ chain which is now known to be shared by both IL-2 receptor and IL-4 receptor has been shown to be able to activate c-myc, c-fos and c-jun (Arso et al., 1993). The exact nature of the combined signal from IL-2R and IL-4R or from IL-2R and IL-12R remains unknown. However, experiments based on the *in vitro* assay system can be designed to determine what controls the differentiation pathways of naive T cells.

In summary, although four general mechanisms of gene regulation are mentioned here, they do not work independently. Each event can be in sequential order or act simultaneously. For example, demethylation of DNA can lead to an alteration of chromatin structure which in turn becomes accessible to transcription activators. However, this open or partially open chromatin structure together with general or basic transcription factors may be necessary but may not be sufficient to ensure gene activation. Inhibition of repressor activity or activation of transcription activators may be required to sufficiently induce gene expression, particularly the expression of those tissue-specific

genes. As for the IL-2 gene, at differentiation stage 1 demethylation and alteration of chromatin structure may be involved to initiate a pre-activatable IL-2 locus. At points 2 and 3, an active process of activating both T-cell specific and non-T cell specific transcription activators which are implicated for IL-2 gene regulation could occur. At points 4 and 5, fine tuning of the DNA binding activity or *trans*-activation activity induced by differentiation signals determines whether to maintain or lose IL-2 gene inducibility.

Study of IL-2 gene regulation by *in vivo* footprinting techniques

Work from enhancer mapping, *cis*-element footprinting, gel shift binding assays, and finally the purification and cloning of specific factors yielded significant information which laid the foundation for us to understand how the IL-2 gene can be regulated. A number of transcription factors examined apparently do not require an activation signal in order to bind DNA, such as the octamer protein and factors which bind the CACCC sites. Others, however, definitely need activation signals. PKC activation induces the synthesis of AP-1, the nuclear translocation of NF-κB, and the binding of the lesser known TGGGC factor; while Ca^{2+} mobilization causes dephosphorylation of NF-ATc which is then able to translocate to the nucleus. Almost all of these factors, except NF-ATc, are not T-cell specific. We still do not understand how the cell-type specificity of the IL-2 gene is achieved given the fact that many of the positive regulatory factors mentioned above are not specific to IL-2-producing cells. Different gene repression mechanisms, like DNA modification, repressed chromatin structure, repressor binding and lack of activator, etc. have been discussed above. Previous studies from this lab and others have shown that a lack of certain factors correlates with lack of gene expression, but it is not clear exactly how this is accomplished, e.g., how other factor DNA-binding activities are affected by the lack of certain factors.

The *in vivo* footprinting technique has been used to study the regulation of a number of genes and has proven to be a useful approach to address the DNA binding or similar kinds of questions which cannot be solved by other *in vitro* biochemical analyses. These studies revealed several patterns of protein-DNA interaction with respect to genes and cells examined. Some constitutively expressed tissue specific genes, like the Ig heavy chain gene (Ephrussi et al., 1985), the liver-specific TAT gene (Becker et al., 1987), the MHC class I gene (Dey et al., 1992), the class II gene (Kara & Glimcher, 1991), and the muscle specific MCK gene (Mueller & Wold, 1989), etc, have their regulatory regions occupied with *trans*-acting factors only in gene-expressing cells. In cells which do not express the gene, the regulatory region is free of specific protein-DNA interactions. Neither repressor nor ubiquitous positive regulatory proteins are bound to DNA in these cells. The unoccupied state remains in the TAT gene which is not blocked by methylation (Weih et al., 1991). These results strongly suggest that the chromatin structures may be differentially constructed which enable some genes to be expressed constitutively in one but not the other configuration.

Studies of β-interferon, a relatively ubiquitously expressed and induction-dependent gene showed that two sites in the promoter region are occupied by repressors in uninduced cells, but upon viral infection, the repression-associated binding is displaced with activator binding (Zinn et al., 1986). In this case, it is the repressor binding that results in non-expression. The regulation of the metallothionein-1 gene is another example in which the basal level of expression is controlled by the interaction of a set of transcription factors with the enhancer sequence. Upon metal induction, new protein-DNA complexes are assembled and transcription activity increases, suggesting that increased transcription is correlated with new factor binding (Mueller et al., 1988). One exception that is different from all the cases given above is the regulation of two immediate early genes c-fos and c-jun, which encode components of AP-1. In the non-expressing state, the serum-response element on c-fos gene promoter is occupied even

before serum-induction, and serum induction does not change the binding (Herrera et al., 1989). Similarly, multiple sites in the c-jun promoter are occupied before induction by UV-irradiation and remain unchanged after irradiation (Rozek & G. P. Pfeifer, 1993). The situation with c-jun and c-fos may indicate a committed state.

The examples given are of either tissue-specific genes or induction-dependent genes, both of which are not like the IL-2 gene. As a rule, gene expression is accompanied by the presence of a set of sequence-specific protein-DNA interactions at the regulatory region. No gene expression, on the other hand, can have various footprinting patterns, which may be correlated with the mechanisms used to repress the gene. What can be said about the IL-2 gene? We can be sure that in activated, IL-2 producing T cells, the IL-2 gene promoter region is occupied by *trans*-acting factors. We could guess that in the IL-2 gene non-expressing state, there would be either no sequence-specific factors bound to the DNA as seen for tissue-specific genes in non-expressing cells; or a repressor bound to the DNA as seen in the β-interferon gene; or a constitutively expressed factor bound to DNA as in c-fos and c-jun genes. The last type might be expected in resting T cells. In any case, resolving this question is essential for us to understand the mechanism of how the IL-2 gene is repressed in non-T cells, and in resting T cells, and how it is activated. In chapter 4 and 5 , we report the results of *in vivo* footprinting studies for the IL-2 gene. This work was begun in close collaboration with Paul Garrity and Barbara Wold.

Modulating IL-2 gene expression by accessory signals

The T-cell activation signal transduction pathway can be simplified as follows: activation signals delivered by T cell receptor and accessory receptors first induce tyrosine phosphorylation on CD3 γ and ζ chains, two components of TCR, by $p56^{lck}$ or $p59^{fyn}$. The SH2 domain of ZAP70, another tyrosine kinase, is then able to associate with the ζ chain (Chan et al., 1992; Irving et al., 1993). Tyrosine kinases recruited by the

phosphorylated ζ chain, like ZAP70, are able to activate phospholipase Cγ, which in turn catalyzes the cleavage of PIP_2 into IP_3 and DAG. IP_3 will induce Ca^{2+} mobilization and DAG will activate protein kinase C (Reviewed by Weiss and Imboden, 1987; Weiss and Littman, 1993). Both initial PTK activation and down-stream PKC activation can independently lead to Ras activation and trigger the Raf-1, Mek, and MAK signalling pathways (Downward et al., 1992; Crews and Erikson, 1993). Activation of primary mature T cells requires both a TCR signal and a costimulatory signal. Induction through TCR alone will render T cells unable to make IL-2 (anergized state), even if the accessory signal is added later (Schwartz et al. 1989). The current view regards the CD28 transduced signal as the accessory signal, although the precise biochemical events initiated by CD28 are not clear at the moment. The basic two branch-signal pathways triggered by TCR alone in T leukemic or tumor lines can together activate some transcription factors, and can independently activate different factors as well. For example, NF-AT activation requires PKC for AP-1 and Ca^{2+} for the cytoplasmic component; NF-κB and c-fos, on the other hand, can apparently be activated by PKC alone.

Cell-cell interaction involves multiple types of receptor-ligand engagement. T cells express multiple molecules on their surface, and recognition of antigen presented by APC is not only limited to the TCR-Ag/MHC ligation. Surface receptors like CD28, CD45, and some adhesion molecules also participate in recognition and activation by interacting with their ligands. Thus, the significantly increased avidity of the cell-cell interaction will greatly facilitate the transduction of activation signals. Signals delivered by other accessory molecules on T cells and by hormone receptors may synergize with or antagonize the activation signal in regulating IL-2 gene transcription. One such example is the interaction of the IL-1 receptor with interleukin-1 which is secreted from antigen-presenting cells. We are interested in the mechanisms of how cells integrate the TCR activation signals with signals from interleukin-1. The activation of certain serine-

threonine kinases by IL-1 may synergize with PKC. Ultimately all these different signals will be interpreted at the level of *trans*-acting factor activation and in turn gene activation. For instance, as described above, anti-CD28 costimulation induces activation of a novel transcription factor and synergizes with the TCR signal in IL-2 gene transcription (Fraser et al., 1991).

As discussed earlier in the introduction, the other significance of studying the IL-1 effect is its developmental relevance. Immature $CD4^-CD8^-TCR^-$ thymocytes can be induced to express IL-2, but in addition to signals that mimic TCR activation they also require IL-1. Mature T cells do not need an IL-1-mediated accessory signal to be induced to make IL-2. Although in chapter 1, we investigated the mechanism of the IL-1 synergistic effect using the EL4 thymoma cell line, the results may apply to immature cells as well.

In addition to the synergistic effect of certain accessory signals, there are signals delivered through other molecules or by pharmacological agents which antagonize the TCR signal and down-regulate the IL-2 gene. For example, the immunosuppressant cyclosporin A can selectively suppress IL-2 gene activation. As reviewed earlier in the introduction, cyclosporin A blocks the Ca^{2+} branch of the activation signal by inhibiting a calcium-calmodulin dependent phosphatase activity, which in turn blocks the translocation of NF-ATc. Reduction of activator availability was one of the gene repression mechanisms I discussed before. But a remaining question is how this affects further down-stream events, such as the DNA binding of other cyclosporin A-insensitive factors. Could they still form protein-DNA complexes without NF-AT and simply slow down the transcription? Could some other factors fill the sites, or is there no binding at all? These are the questions we study in chapter 4. Similarly, we also examine the effect of another signal mediated through the cAMP-dependent protein kinase A pathway in chapter 5. It has long been observed that elevating cAMP could antagonize the TCR activation signals and reduce IL-2 transcription. Although it is not known exactly how

the activation of protein kinase A inhibits the TcR activation signal, it has been shown that the type I PKA regulatory subunit becomes associated with the TCR complex upon T cell activation, suggesting that part of the block may be at an early step of signal transduction (Skålhegg et al. 1994). We have also shown, however, that signalling downstream of the TCR is also affected, as cAMP even inhibits activation through TPA+A23187. We analyzed whether the activity of any of the nuclear factors would be interfered with by this signal and examined the *in vivo* protein-DNA interaction in the IL-2 promoter region in activated T cells in the presence of the cAMP-elevating agent, forskolin. Understanding how reducing the availability of certain, but not all, *trans*-acting factors could result in gene inactivation will certainly enlighten us as to how, in general, gene repression can be achieved, especially regarding different types of cells or tissues.

T cell lines vs. primary T cells

Because of the difficulties in isolating and transfecting mature helper T cells, we selected EL4 cells for some of the work because they are easy to populate and to transfect. The murine thymoma cell line EL4 and the human T leukemic cell line Jurkat, are two commonly used model T helper cell lines for studying lymphokine gene regulation and signal transduction. As regarding to IL-2 gene expression, EL4 cells do respond similarly to real T cells, although not identically. Accessory signals which up or down-regulate the IL-2 gene in immature or mature T cells also have same effect in EL4 cells. There are, however, several characteristics which separate EL4 cells from normal mature IL-2 producing T cells. First, EL4 cells can be induced to express the IL-4 gene as well, which is a type-2 helper T cell specific gene (Novak & Rothenberg, 1990). Second, activation of protein kinase C by TPA in the absence of calcium ionophore is sufficient to induce IL-2 expression. Third, EL4 cells are cycling cells, but they are not refractory to stimulation signals. These 'abnormalities' may be attributed to their tumor

phenotype or to the fact that they represent a specific stage of T cell development. Therefore, these cells may not truthfully reflect the state of mature T helper cells *in vivo*, nor a particular stage of T-cell development. Nevertheless, the accuracy in IL-2 gene expression provides a convenient and direct system to study the transcriptional regulation of T helper specific genes. Naturally, caution and more extensive studies are necessary when generalizing the results to normal cells.

A recapitulation of the contents of each chapter is as follows. Chapter 1 describes the synergism of interleukin-1 with the T-cell receptor signalling pathways for IL-2 gene induction, and further reveals that IL-1 costimulation enhances the DNA binding of transcription factors NF-κB and AP-1. This enhancement is not coupled with cAMP elevation. Chapter 2 studies the molecular basis for changes in IL-2 gene inducibility during thymocyte development and shows that under certain stimulation conditions, both early immature and mature thymocytes are equally able to mobilize a set of transcription factors in order to express the IL-2 gene. The inability to activate the IL-2 gene in cortical thymocytes is correlated with their defective DNA-binding activity of NF-AT and AP-1 upon stimulation. Chapter 3 further shows that in cortical thymocytes the induction of c-fos, fosB, and fra-2 genes is reduced at the mRNA level and implies that the defective DNA-binding activity of NF-AT and AP-1 may be due to the reduction of Fos proteins and the subsequent failure of Jun-Fos dimer formation. In chapter 4, utilizing *in vivo* footprinting analysis, we demonstrated that the contact of *trans*-acting factors and the IL-2 promoter is an all-or-none pattern. The interaction is only correlated with the presence of both induction-dependent and induction independent factors in the stimulated T cells. A lack of protein-DNA complexes at the IL-2 promoter is correlated with the presence of only induction-independent factors in resting T cells and in non-T cells. Failure to activate factors including NF-AT upon stimulation in the presence of cyclosporin A also results in a lack of factor-DNA complex assembly in the promoter

region. These results indicate a combinatorial and coordinated interaction of transcription factors with the IL-2 gene. In chapter 5, a similar approach, which combines the *in vitro* and *in vivo* DNA binding assays, is taken to examine the IL-2 gene inhibition mechanism by elevating the intracellular cAMP. We demonstrate that forskolin costimulation specifically blocks the DNA binding activity of certain factors including NF-κB, but not NF-AT. This blocking also results in no occupancy in the IL-2 promoter. The results strongly indicate that the stable protein-DNA complex assembly requires the combinatorial coengagement of all the distinct factors.

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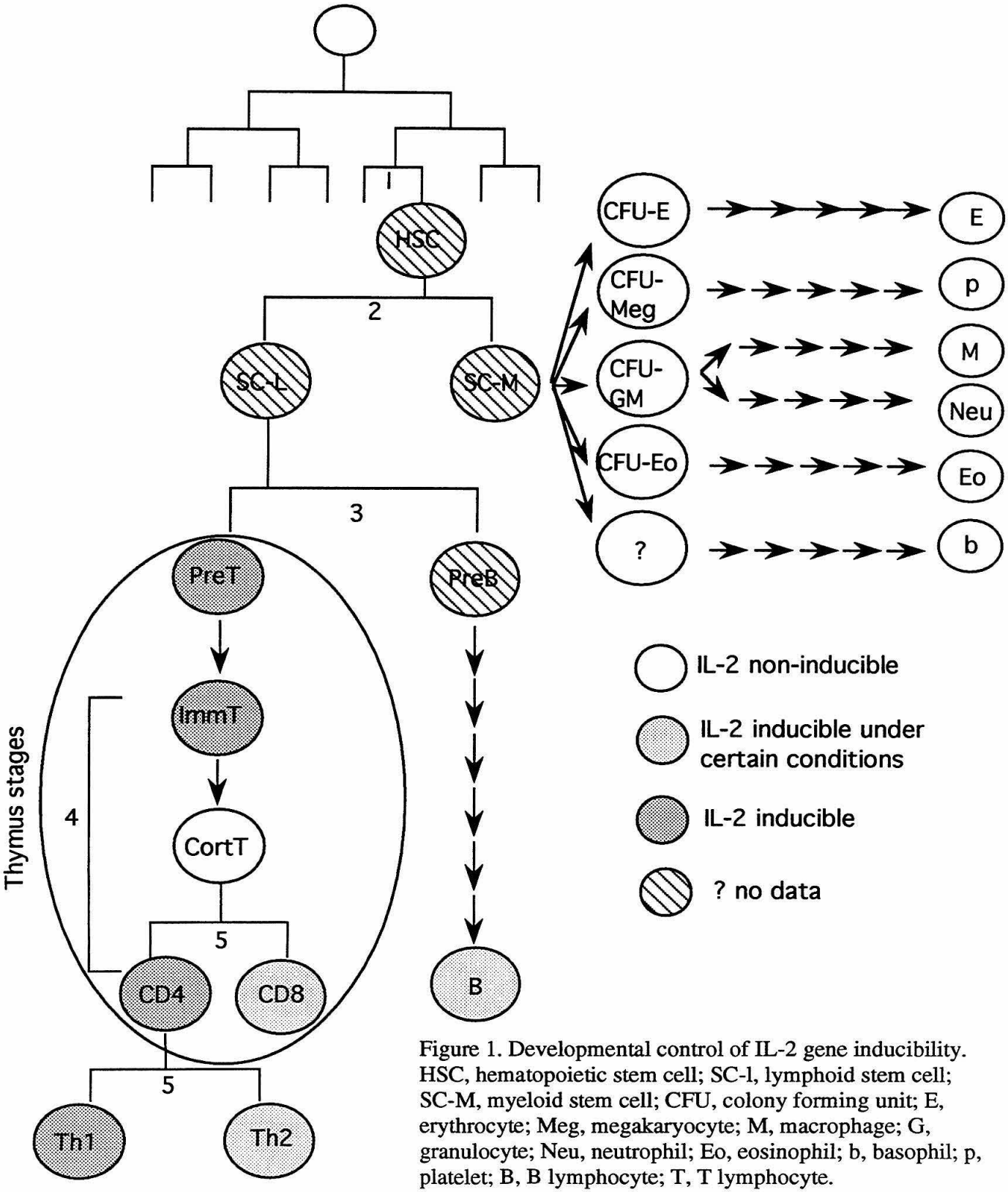

Figure 1. Developmental control of IL-2 gene inducibility. HSC, hematopoietic stem cell; SC-l, lymphoid stem cell; SC-M, myeloid stem cell; CFU, colony forming unit; E, erythrocyte; Meg, megakaryocyte; M, macrophage; G, granulocyte; Neu, neutrophil; Eo, eosinophil; b, basophil; p, platelet; B, B lymphocyte; T, T lymphocyte.

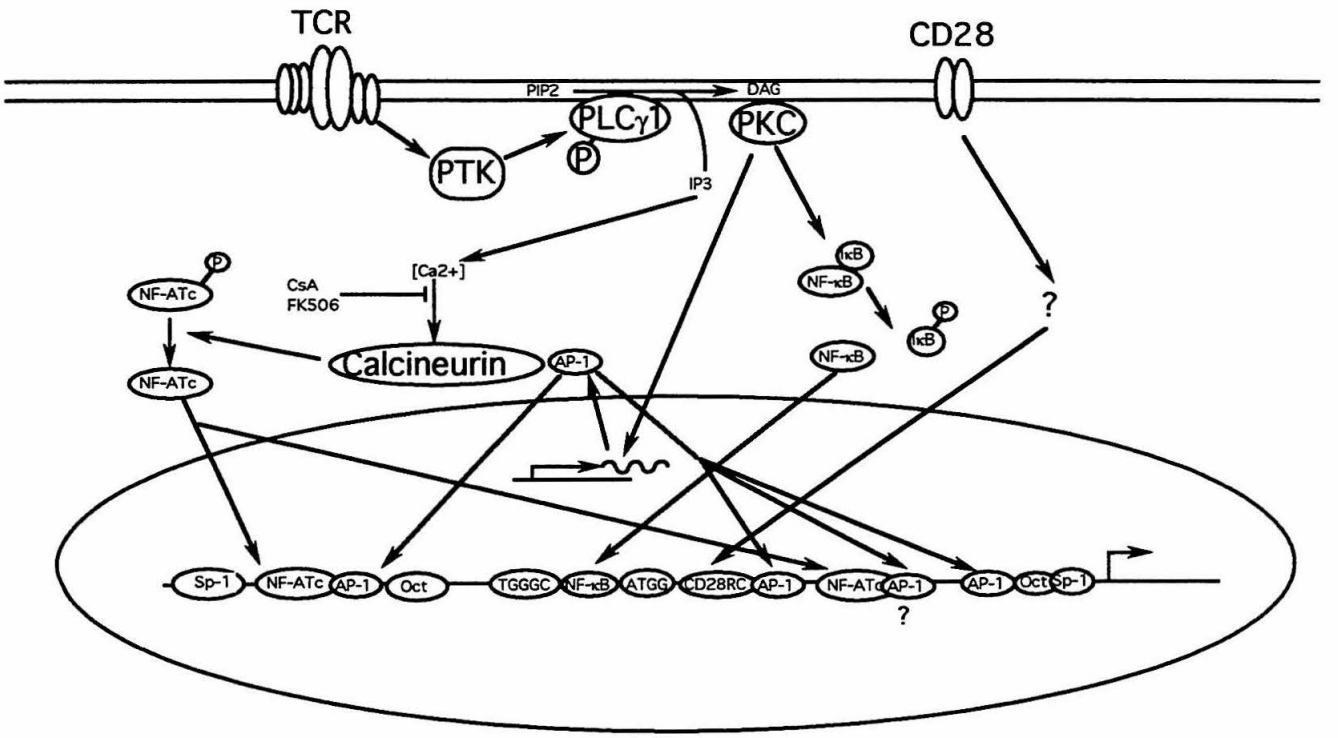

Figure 2. Diagram of signalling pathways and interaction of trans-acting factors with the IL-2 promoter.

Chapter 1

Interleukin-1 Synergy with Phosphoinositide Pathway Agonists for Induction of Interleukin-2 Gene Expression: Molecular Basis of Costimulation

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Interleukin-1 Synergy with Phosphoinositide Pathway Agonists for Induction of Interleukin-2 Gene Expression: Molecular Basis of Costimulation

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The macrophage-derived cytokine interleukin-1 (IL-1) can provide a second signal with antigen to elicit production of interleukin-2 (IL-2) by helper T cells. The pathway(s) involved remains controversial, with protein kinase C and cyclic AMP (cAMP) invoked as possible second messengers. In the murine thymoma EL4.E1, IL-1 could synergize with the phosphoinositide pathway, because the cells made higher levels of IL-2 in the presence of IL-1 than could be induced by phorbol ester plus calcium ionophore alone. IL-1 is unlikely to act through a sustained increase in cAMP in these cells because it did not raise cAMP levels detectably and because IL-1 and forskolin had opposite effects on IL-2 gene expression. Inducible expression of a transfected reporter gene linked to a cloned fragment of the murine IL-2 gene promoter was initially increased by IL-1 costimulation, implying that IL-1 can increase the rate of transcription of IL-2. The minimal promoter elements required for IL-1 responsiveness were located within 321 bp of the IL-2 RNA cap site, and further upstream sequences to −2800 did not modify this response. IL-1 costimulation resulted in enhanced activity of both an inducible NF-κB-like factor and one of two distinct AP-1-like factors that bind to IL-2 regulatory sequences. Neither was induced, however, by IL-1 alone. Another AP-1-like factor and NFAT-1, while inducible in other cell types, were expressed constitutively in the EL4.E1 cells and were unaffected by IL-1. These results are discussed in terms of the combinatorial logic of IL-2 gene expression.

Interleukin-1 (IL-1) is a potent mediator of cellular function that is produced primarily by activated macrophages (19), and a role for IL-1 in T-cell activation has been demonstrated in a number of experimental systems (7, 8, 25). In vitro, IL-1 synergizes with mitogenic lectins or anti-T-cell receptor (TcR) antibodies to induce expression of interleukin 2 (IL-2) and IL-2 receptor α-chain genes (13, 18, 44). In these roles, IL-1 can be replaced by tumor-promoting phorbol esters (e.g., 12-*O*-tetradecanoylphorbol 13-acetate [TPA]) (11). As IL-1 was known to provide a necessary, antigen-nonspecific signal for T-cell activation (9), this apparent equivalence with TPA initially suggested that its action might result from activation of protein kinase C (28). However, IL-1 does not promote phosphoinositide hydrolysis or cause protein kinase C translocation to an active membrane-bound form (1). It therefore seems unlikely that its role is to augment the low levels of diacylglycerol produced by TcR-mediated phosphoinositide breakdown. More recently, it has been demonstrated that IL-1 treatment of the human T-cell leukemia line Jurkat causes increased production of two protein kinase C cofactors, phosphatidylserine and diacylglycerol, the latter from hydrolysis of membrane phosphatidylcholine (23, 34). However, because Jurkat lacks conventional IL-1 receptors (IL-1Rs) (34), the significance of these results for other T cells is unknown.

The studies that have reported a costimulatory role for IL-1 have usually demonstrated this effect on cells suboptimally stimulated with mitogenic lectins (13, 15, 18, 44). Several laboratories have obtained results that call into doubt the notion that IL-1 is necessary at all for the high-level production of IL-2 when the TcR signal is optimized. We have shown that when fresh splenic T cells produce IL-2 in response to TPA and the calcium ionophore A23187, their response is both independent of and insensitive to added IL-1 (35, 36). Lichtman et al. (21) have obtained similar results with a panel of cloned antigen-dependent T_H1 cell lines stimulated with an anti-CD3 monoclonal antibody. Two other groups have shown that long-term T_H1 (IL-2-producing) lines may lack IL-1Rs altogether (14, 20). Thus, these systems do not provide access to any unique pathway through which IL-1 may act.

Recent data suggest, however, that a requirement for IL-1 may reflect the developmental status of a T cell. These differences are most apparent in induction of IL-2 expression, a response subject to complex regulation (16, 27, 32). Mature TcR^+ thymocytes resemble peripheral T cells in their insensitivity to IL-1 when they are stimulated to produce IL-2 in response to TPA plus A23187. These chemical proxies for mediators of the phosphatidylinositol bisphosphate hydrolysis pathway may be able to induce IL-2 expression in all mature T cells, for they can also elicit high-level responses in most fresh peripheral $CD8^+$ cells (24), cells which generally do not express IL-2 mRNA in response to either antigen or mitogenic lectins. In contrast, however, immature TcR^- double-negative thymocytes do not make IL-2 in response to TPA and A23187 at all unless IL-1 is present (35, 36). Thus, these immature thymocytes differ from their more mature descendents in two important aspects of their IL-2 inductive signal requirements. First, in these cells, phosphoinositide pathway agonists are insufficient to activate IL-1 expression. Second, they possess a pathway allowing IL-1 effects to synergize with phosphoinositide pathway mediators.

Here we report that the cloned murine thymoma EL4.E1 possesses characteristics of both immature and mature IL-2 producers. Like mature T cells, it can respond to phorbol

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ester and ionophore alone. In addition, it possesses a trait normally associated with immature thymocytes, namely, responsiveness to IL-1 as a costimulus with TPA plus A23187. We have used these cells to explore the mechanism by which the synergy between IL-1 and TPA plus A23187 occurs.

MATERIALS AND METHODS

Reagents. TPA and A23187 were from Sigma or Calbiochem. They were dissolved in dimethyl sulfoxide to final concentrations of 10 μg/ml and 0.37 mg/ml, respectively, and stored in small samples at −20°C. Recombinant human and mouse IL-1α (IL-1) was purchased from Genzyme. The specific activity, as reported by the supplier, was 10^8 U/mg.

Cells. EL4.E1, a mouse IL-2-producing thymoma cell line (originally donated by V. Paetkau, University of Alberta), and the human T-cell leukemic line Jurkat (generously provided by G. Crabtree, Stanford University) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics.

Plasmids. The series of pIL2-CAT plasmids containing varying lengths of the mouse IL-2 gene 5′ DNA linked to the bacterial gene for chloramphenicol acetyltransferase (CAT) has been described in detail (30). Briefly, cloned fragments of the IL-2 gene 5′-flanking region, all terminating 3′ at +45 (in the 5′ untranslated region), were ligated to the CAT gene from pTK-CAT in the vector pSP65 (Promega). They are designated pIL2(−X), where X is the 5′-terminal nucleotide, relative to the IL-2 transcriptional start site, that is present in the construct. All plasmids were purified in CsCl-ethidium bromide density gradients before use.

Transfections and CAT assays. Transfection of logarithmically growing cells was by DEAE-dextran facilitation as described elsewhere (30). In short, cells were washed in serum-free Dulbecco modified Eagle medium with 10 mM *N*-2-hydroxylethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0) (DME/H) and then suspended at 10^7/ml in a transfection cocktail containing 250 μg of DEAE-dextran (molecular weight, 2×10^6) per ml, 0.1 mM chloroquine, and 10 μg of supercoiled plasmid DNA per ml, all in DME/H. After incubation for 30 to 60 min at 37°C in 7% CO_2, cells were pelleted, washed, and plated into 4 to 12 identical cultures. Approximately 20 h after plating, cells were stimulated with TPA and A23187 at final concentrations of 10 and 37 ng/ml, respectively. These dosages were previously determined to be optimal for both IL-2 mRNA expression and secretion over 18 to 20 h in the population overall. Some of the cells also received IL-1 to the indicated concentrations. Human recombinant IL-1α was found to be as effective as the mouse version on EL4.E1 cells and was used in most experiments.

Cells were harvested after various times of stimulation, and extracts from equivalent numbers of cells were assayed for CAT activity in a 5-h assay as described previously (29).

RNA analysis. Cytoplasmic RNA was extracted by the method of Favaloro et al. (12). The RNA from equivalent cell numbers was electrophoresed in denaturing 1% agarose-formaldehyde gels, stained with acridine orange to visualize the rRNA, and then blotted onto nylon membranes (Nytran; Schleicher & Schuell). The RNA was fixed to the membrane by baking at 80°C for 60 min. Hybridization probes were generated by random priming of cDNAs for mouse IL-2 (46) and mouse skeletal α-actin (S. Sharp and N. Davidson, unpublished data) as described elsewhere (30). Hybridizations were carried out for 20 h at 42°C in 5× SSPE–50% formamide–0.2% sodium dodecyl sulfate (SDS)–5× Denhardt solution–10% dextran sulfate. Filters were washed three times at room temperature for 1 min each in 2× SSC(SSC is 0.15 M NaCl plus 0.015 M sodium citrate–0.2% SDS–0.05% sodium pyrophosphate, followed by two 30-min washes at 68°C in 0.2× SSC–0.1% SDS–0.05% sodium pyrophosphate. Filters were exposed to film at −70°C with an intensifying screen, and autoradiograms in the linear response range were densitometrically scanned. The amount of IL-2 mRNA was normalized with respect to the actin signal.

cAMP determination. EL4.E1 cells were resuspended in medium at a concentration of 1.2×10^6/ml and were stimulated at 37°C as indicated in Table 2. Cell extracts were assayed for cyclic AMP (cAMP) by using a competition binding assay (RPA.509; Amersham) as described elsewhere (T. J. Novak and E. V. Rothenberg, Proc. Natl. Acad. Sci. USA, in press). Extracts were acetylated before assay to increase sensitivity.

Nuclear extracts. Nuclear proteins were extracted according to published procedures (6, 8; G. Crabtree, personal communication) from cells that had been stimulated for 3 to 4 h under the conditions described for Fig. 4 and Table 3. Proteins extracted by either method gave similar results in gel mobility shift assays, and the data presented in Table 4 were obtained by using both types of extraction protocols. Extracts were stored at −80°C until use.

Gel mobility shift assay. Mobility shift assays were carried out essentially as described elsewhere (3), with minor modifications. Synthetic double-stranded oligonucleotides were end labeled by filling in with either ^{35}S- or ^{32}P-labeled dTTP and dATP. For binding assays, 2.5 to 5.0 μg of nuclear protein was incubated for 15 min at 25°C with ~0.1 ng of probe in the presence of a nonspecific competitor, usually 0.5 μg of poly(dI · dC) for NF-κB, 0.25 to 0.5 μg of poly(dI · dC) for AP-1_D and AP-1_P, or 2.5 to 10 μg of poly(dA · dT) for NFAT-1.

Complexes were separated from free probe by electrophoresis through 8% acrylamide gels which were cast and run in low-ionic-strength buffer. Gels were dried and exposed to film at −70°C with intensifying screens. For experiments with ^{35}S-labeled probes, gels were fixed in Amplify (Amersham) and complexes were detected by fluorography.

For competition binding studies, 2.5 to 5.0 μg of nuclear extract was preincubated for 15 min at 25°C with the indicated molar excess of competitor DNA (either a synthetic oligonucleotide or a cloned genomic fragment) before addition of labeled probe.

The synthetic oligonucleotides contained the binding sites for NFAT-1, NF-κB, AP-1_P and AP-1_D as found upstream of the mouse IL-2 gene (30) and were of the following sequences (coding strands on top):

AP-1 consensus (Stratagene)	5′-CTAGTGATGAGTCAGCCGGATC-3′ 5′-GATCCGGCTGACTCATCAGTAG-3′
AP-1_P	5′-AATTCCAGAGAGTCATCAG-3′ 5′-CTGATGACTCTCTGG-3′
AP-1_D	5′-AAATCCATTCAGTCAGTG-3′ 5′-CACTGTCTGAATGG-3′
NF-κB	5′-AAGAGGGATTTCACCT-3′ 5′-ATTTAGGTGAAATCCCTCTT-3′
NFAT-1	5′-AAGAGGAAAATTTGTTTCATACAGAAGGCG-3′ 5′-AATTCGCCTTCTGTATGAAACAAATTTTCCTCTT-3′

TABLE 1. Effects of different stimuli on IL-2 production by EL4.E1 cells

Stimulation[a]	IL-2 titer (U/ml)[b]	
	Expt 1	Expt 2
None	<4	<4
TPA	256	386
IL-1	<4	<4
TPA + A23187	307	448
TPA + A23187 + IL-1	1,280	614
TPA + A23187 + forskolin	106	204

[a] Cells were incubated for 20 h in the presence or absence of TPA (35 nM), A23187 (140 nM), IL-1 (20 U/ml), and forskolin (10 μM). Confirmation of the inhibitory effects of forskolin at the RNA level is presented by Novak and Rothenberg (in press).

[b] Titer in supernatants, determined by colorimetric assay on CTLL-2 cells as previously described (36). The presence of IL-1 in the assay supernatants has no effect on the indicator cells (36), and the addition of forskolin did not interfere with the quantitative detection of IL-2 in a control experiment (after adjustment to 10 μM forskolin, a sample with 450 U of IL-2 per ml gave a titer of 530 U of IL-2 per ml in this assay; E. V. Rothenberg, unpublished data).

RESULTS

IL-1 is a costimulus for IL-2 expression by phorbol ester- and ionophore-treated EL4.E1 cells. The EL4.E1 thymoma is a convenient murine model for studies of IL-2 gene expression, but it differs from normal T_H1 cells in several ways. First, it is responsive to TPA alone. A Ca^{2+} costimulus elicits higher levels of IL-2 but is not required. Second, unlike normal mature T cells (35), it shows synergy between IL-1 and TPA + A23187. These features are illustrated in Table 1, which presents typical yields of IL-2 from EL4.E1 cells after 20 h of stimulation under different conditions. The addition of calcium ionophore generally increased the levels of IL-2 relative to those elicited by TPA alone, to a maximum level that could not be surpassed by increasing the doses of TPA + A23187 (data not shown). The further addition of IL-1, however, resulted in even higher levels of IL-2 secretion. Nevertheless, IL-1 alone did not stimulate any detectable production of IL-2. Thus, the effect of IL-1 in these cells was to potentiate the effects of the TPA and A23187 stimuli on IL-2 synthesis. Such a potentiation could operate at pretranscriptional, posttranscriptional, or post-translational levels. To dissect the basis for these differences in cumulative IL-2 synthesis over 20 h, we examined the time course of IL-2 RNA accumulation.

The accumulation of IL-2 transcripts in EL4.E1 cells stimulated with TPA + A23187 alone was rapid and bimodal (Fig. 1A, lanes 2, 6, and 10). IL-2 mRNA levels peaked around 5 h and declined slowly during the next 13 h. Costimulation of EL4.E1 cells with 2.5 or 20 U of IL-1 per ml resulted in a two- or sixfold increase, respectively, in IL-2 mRNA at 5 h (Fig. 1A, lanes 1 to 4). This effect was primarily observed at early times of stimulation. At 12 h the enhancement due to IL-1 was only 1.5-fold (Fig. 1A, lanes 6 to 9),

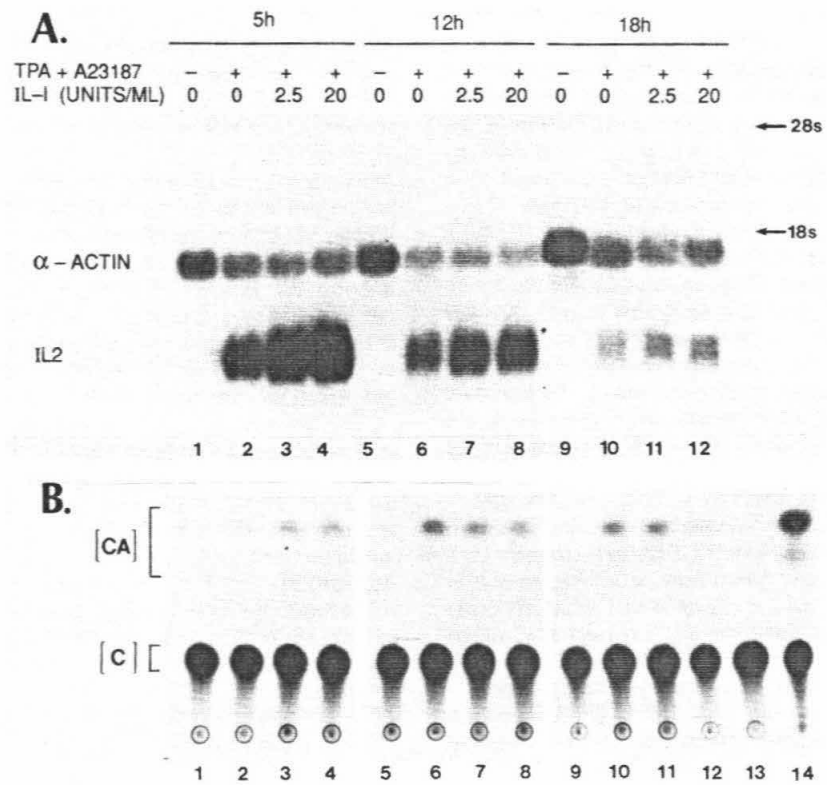

FIG. 1. IL-1 costimulation of IL-2 induction: effects on the IL-2 promoter. (A) Gel blot of cytoplasmic RNA extracted from unstimulated or stimulated EL4.E1 cells. Cells were stimulated as indicated for 5 to 18 h, and RNA from 10^6 cells was extracted, electrophoresed on a denaturing 1% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran), and hybridized to cDNA probes for IL-2 and skeletal α-actin. (B) CAT assay of extracts from EL4.E1 cells transfected with pIL2(−2800). Cells were stimulated as for panel A. Each lane represents the extract from transfected cells stimulated under conditions identical to those of the sample used for the corresponding lane of the RNA gel blot. Extracts were assayed at 37°C for 5 h. Lanes 13 and 14 represent negative and positive assay controls, respectively. Positive control contains commercial CAT enzyme. [C], Unacetylated chloramphenicol; [CA], monoacetylated chloramphenicol.

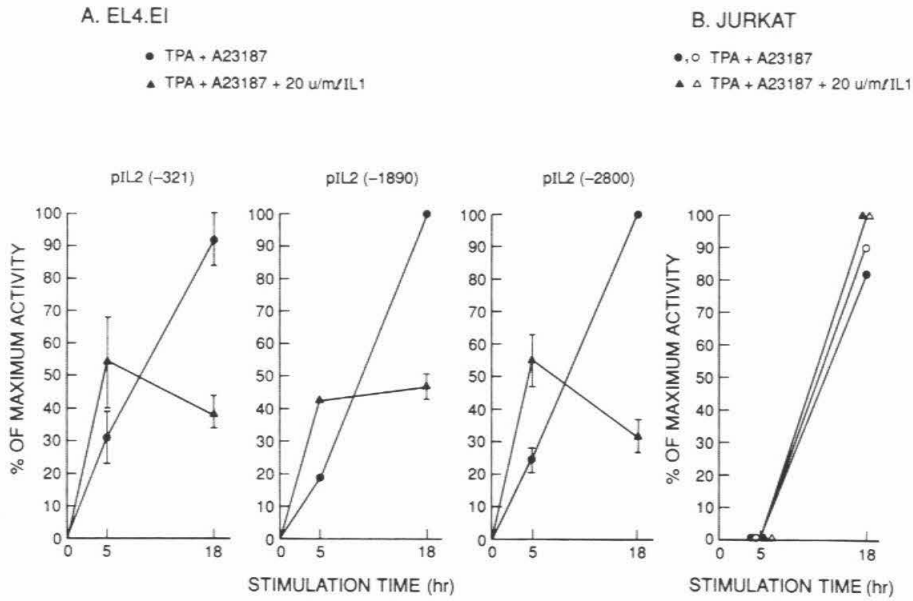

FIG. 2. Normalized time courses of IL-2–CAT expression in the presence or absence of recombinant IL-1α. (A) Time courses for EL4.E1 cells transfected with pIL2(−321), pIL2(−1890), and pIL2(−2800). Calculations for each plasmid were done separately. Each experiment consisted of six datum points: 5 h, 12 h (data not shown), and 18 h stimulated ± 20 U of human recombinant IL-1α per ml. CAT activity per 10^6 cells was normalized with respect to the highest value in that experiment (in all but one case this was the 18-h sample without IL-1). Results are the means ± standard errors of the mean of two to five separate experiments except for the pIL2(−1890) 5-h values, which were determined in one time course. (B) Normalized CAT assay curve for transfected Jurkat cells. Experimental conditions and calculations were exactly as for panel A. Open symbols, pIL2(−321); closed symbols, pIL2(−2800). Data are from one experiment.

and by 18 h IL-1 ceased to augment the level of steady-state IL-2 mRNA (Fig. 1A, lanes 9 to 12). The early increase and later loss of IL-2 RNA makes it likely that IL-1 costimulation increases the transcription rate but not RNA stability or translational efficiency.

No IL-2 mRNA was detected when EL4.E1 cells were stimulated for 5 h with 20 U of IL-1 per ml in the absence of TPA, alone or in combination with 37 ng of A23187 per ml (not shown). This inability to respond to IL-1 plus ionophore is in contrast to results reported by others for several IL-1-independent cell lines (43, 47), possibly because of the lower concentration of ionophore used here (37 ng/ml = 70 nM, compared with 250 to 500 nM). The failure of IL-1 to substitute for TPA suggests that it cannot duplicate the effects of TPA on protein kinase C.

Costimulatory effects of IL-1 are mediated through the IL-2 gene promoter. To determine whether the IL-2 gene promoter was responsible for mediating this positive effect, we used a series of pIL2-CAT plasmids that contains various lengths of IL-2 5′-flanking DNA, including the transcriptional start site, driving expression of CAT (30). In this way we could assay transcriptional initiation without concern for regulatory variations in mRNA stability (22) since, unlike authentic IL-2 mRNA, the CAT 3′ untranslated region does not contain the AUUUA motifs that target lymphokine transcripts for rapid degradation (33, 38). Note that the CAT assay measures the translation product of the induced RNA, not the RNA directly.

Transfection experiments using IL2-CAT plasmids suggest that IL-1 can increase the rate of transcription from the IL-2 gene promoter, even in cells optimally stimulated with TPA and ionophore. Several pIL2-CAT plasmids were transfected into EL4.E1 cells along with pRSV-CAT as a positive control. CAT activity was measured after 5, 12, and 18 h of stimulation with TPA and A23187 in the presence and absence of IL-1 (Fig. 1B and 2). A CAT assay of cells transfected with pIL2(−2800) is shown in Fig. 1B. Expression of the transfected IL-2–CAT gene required an appropriate inductive signal because unstimulated cells contained no enzyme activity (Fig. 1B, lanes 1, 5, and 9). Thus, even when introduced as a naked DNA template, the IL-2 gene promoter was not promiscuously transcribed in these cells. EL4.E1 transfectants stimulated with TPA plus A23187 contained easily detectable CAT activity at 5 h (Fig. 1B, lane 2), which would be expected if the peak in endogenous IL-2 mRNA seen at this time resulted from transcriptional activation of the IL-2 gene promoter. CAT activity in these cells continued to rise over the next 13 h, reaching a maximum at 18 h, the latest time examined. This increase in CAT activity occurred even as the steady-state level of endogenous IL-2 mRNA dropped (Fig. 1A), possibly indicating greater stability of the IL-2–CAT mRNA.

The addition of IL-1 to the stimulation regimen had two dramatic effects. First, cells stimulated in the presence of IL-1 contained up to 2.5 times as much CAT activity per cell at 5 h as did those stimulated with TPA and A23187 alone (Fig. 1B, lanes 1 to 4, and Fig. 2A). This effect was IL-1 dose dependent because the enhancement was greater with 20 than with 2.5 U/ml.

Second, the increase was transient, like the effect on endogenous IL-2 mRNA. Not only did IL-1 completely cease to enhance the steady-state level of IL-2 mRNA at 18 h (Fig. 1A, lanes 10 to 12), it also ceased to increase the amount of CAT activity per cell. In fact, CAT activity induced in the presence of IL-1 was now at least 60 to 70% lower than in cells stimulated without IL-1 (Fig. 2A). The mechanism of this late decrease is not fully understood but may be nonspecific. At 18 h, the positive control plasmid, pRSV-CAT, was also expressed at levels 31% lower in cells costimulated with IL-1 than in cells stimulated without IL-1

TABLE 2. Failure of IL-1 to increase cAMP in stimulated EL4.E1 cells

Stimulation conditions[a]	Time (min)	cAMP concn (fmol/10^6 cells)[b]	
		Expt 1	Expt 2
No stimulation		350	320
TPA + A23187	15	300	320
	30	250	310
	60	271	380
TPA + A23187 + 20 U of IL-1/ml	15	246	300
	30	308	340
	60	258	460
TPA + A23187 + 10^{-7} M forskolin	15	ND	460
	30	ND	470
	60	ND	750
TPA + A23187 + 10^{-5} M forskolin	15	ND	>4,000
	30	ND	>4,000
	60	ND	>4,000

[a] EL4.E1 cells were stimulated with TPA (10 ng/ml) + A23187 (37 ng/ml) at a density of 1.2×10^6/ml.

[b] Measured by using a commercial assay kit as described in Materials and Methods. All values are from assays with acetylated standards and samples. The high values for samples with 10^{-5} M forskolin were corroborated by reassay with nonacetylated standards and samples. ND, Not done.

TABLE 3. Distinct effects of IL-1 and forskolin on expression of pRSV-CAT and pIL2(−321) in EL4.E1 cells

Construct	Stimulation time (h)	CAT activity (% of control)[a]	
		+IL-1 (20 U/ml)	+10 μM forskolin[b]
pRSV-CAT	18	68 ± 0.5	427 ± 99
pIL2(−321)	5	169 ± 22	28 ± 4
	18	43 ± 6	51 ± 17

[a] Cells were stimulated for the indicated lengths of time with TPA (10 ng/ml) + A23187 (37 ng/ml) in the presence or absence of IL-1 or forskolin. CAT activity per 10^6 cell equivalents was determined, and the value for the drug-treated samples was normalized with respect to that of the drug-free control. Results are presented as means ± standard errors of the mean or as means ± ranges (in the cases with two experiments) of two to four experiments each.

[b] Results are presented in more detail elsewhere (Novak and Rothenberg, in press).

(see below and Table 3). By contrast, the early enhancement of CAT expression from the IL-2 gene promoter was promoter specific. Expression of pRSV-CAT in stimulated EL4.E1 cells was not affected by the presence of IL-1 when examined after 3 or 5 h of stimulation (unpublished data). This result suggests that at later times, IL-1 caused a general decrease in CAT expression, possibly via reduced translational efficiency, which was distinct from its promoter-specific transcriptional enhancing effects seen at 5 h.

Possible mediators contributing to the effects of IL-1 on EL4.E1 cells. (i) IL-1R. The effects of IL-1 on the IL-2 gene promoter require the expression of IL-1R, as demonstrated by the results from identical transfection experiments using IL-1R$^-$ Jurkat cells instead of EL4.E1 cells. A time course of IL-2–CAT expression in Jurkat cells is shown in Fig. 2B. These cells showed much slower kinetics of expression of pIL2-CAT plasmids than did EL4.E1 cells. No activity was detected at 5 h even in the presence of 20 U of IL-1 per ml. Cells had begun to express by 12 h, and CAT activity was still rising by 18 h. However, no significant effect, early or late, could be attributed to IL-1. These results provide evidence that surface expression of a canonical IL-1R is a prerequisite for the IL-1 effects that we have described. It is thus unlikely that the effects seen in EL4.E1 cells are due to the type of phosphatidylcholine hydrolysis reported to occur in Jurkat cells (34).

(ii) cAMP. It has been shown that IL-1 can utilize cAMP as an intracellular second messenger in a number of non-T-cell lines (42). Our results argue against a simple cAMP elevation mechanism as the pathway for IL-1 costimulation in EL4.E1 cells. First, IL-1 costimulation had minimal effects on cAMP levels in these cells (Table 2). Second, the effects of 20 U of IL-1 per ml and 10 μM forskolin were clearly different (Table 3). Expression of pRSV-CAT was enhanced at 18 h with forskolin but inhibited with the concentration of IL-1 used here. Whereas expression of pIL2(−321) was depressed at 18 h by both IL-1 and forskolin, the 5-h results clearly demonstrated the dissimilarity of these two stimuli. Forskolin was even more suppressive at 5 h than at 18 h, whereas IL-1 was stimulatory at this time point (Table 3; see also Fig. 1B, lanes 1 to 4, and Fig. 2A). We have also shown that elevation of cAMP by a variety of other agents can depress IL-2 gene expression in a dose-dependent fashion (Novak and Rothenberg, in press), supporting the conclusion that elevation of cAMP levels per se has a net negative effect on IL-2 gene promoter function. While these results do not rule out cAMP-responsive signaling molecules as participants in the IL-1 enhancement pathway, they do show that the stimulatory effect of IL-1 is unlikely to be a result of any sustained increase in cAMP concentration.

IL-2 gene promoter sequences upstream of −321 are not required for IL-1 costimulation. Our previous work on the upstream region of the mouse IL-2 gene indicated the presence of several positive and negative regulatory elements between −321 and −2800, any of which might be involved in IL-1-mediated effects. However, the data presented in Fig. 2A make it unlikely that far-upstream sequences are required.

When normalized for maximal levels of CAT activity, the kinetics of IL-1 costimulation for pIL2(−321), pIL2(−1890), and pIL2(−2800) were virtually superimposable (Fig. 2A). Thus, the degrees of both the 5-h enhancement and the 18-h suppression were unaffected by IL-2 5′-flanking sequences extending upstream of −321. It should be noted, however, that the absolute amount of CAT activity per cell at each time point was higher with pIL2(−1890) and pIL2(−2800) than with pIL2(−321), in accord with our previously reported results (30).

Further truncation of the IL-2 gene promoter region led to severe loss of inducibility, such that any IL-1-dependent enhancement was difficult to detect. We evaluated the responses of two plasmids with deletions in the region from −321 to −261. One, pIL2(−232), failed to give significant expression with or without IL-1 at any time point. Expression of the other, pIL2(−1890; ΔNFAT-1), which bears a deletion from −261 to −321 (30), was also undetectable at 5 h of stimulation, when IL-1 would be expected to exert a positive effect (P. M. White, T. J. Novak, and E. V. Rothenberg, unpublished results; 30). Thus, retention of sequences between −261 and −321 appeared to be essential for expression and IL-1 costimulation.

Effect of IL-1 costimulation on sequence-specific DNA-binding factors. The sequences from −50 to −300 in the human IL-2 gene include binding sites for several DNA-binding proteins of different specificities which have been implicated in regulation (4, 10). One factor, binding se-

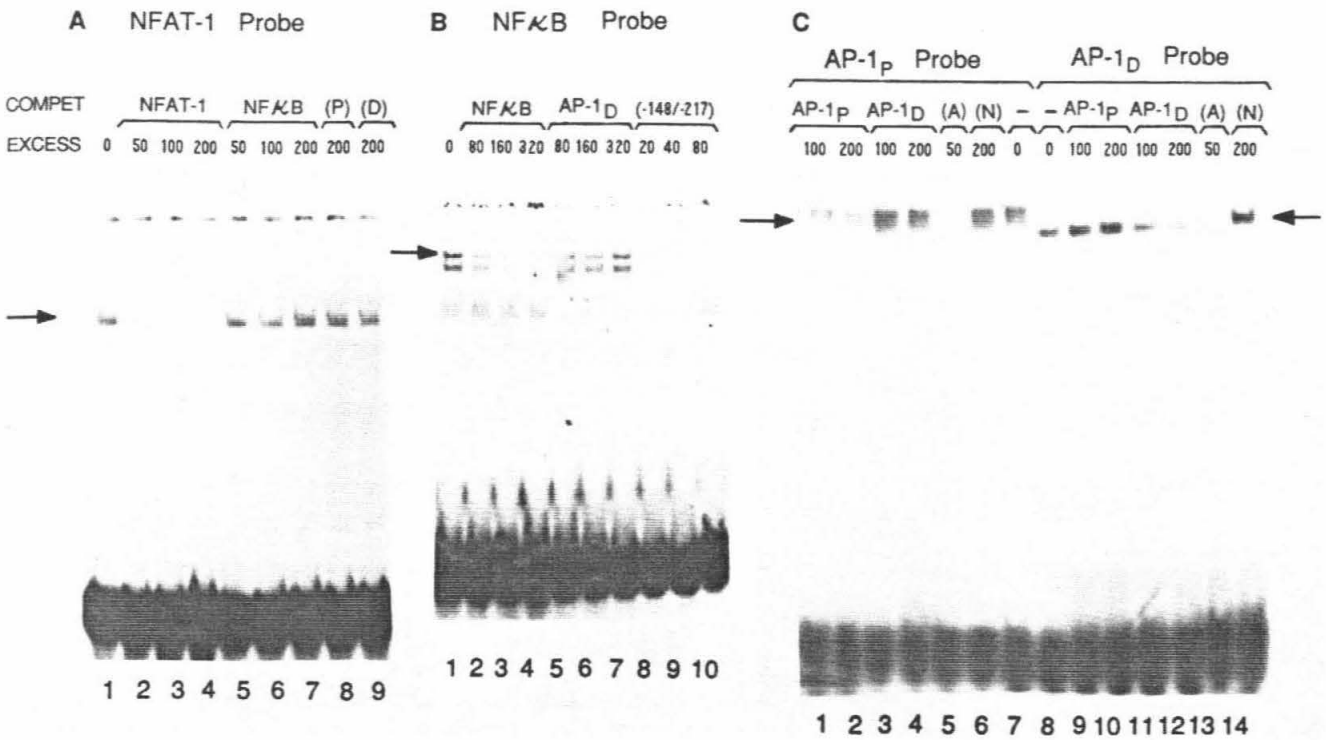

FIG. 3. Specificities of complexes with binding activity for IL-2 regulatory sequences. (A) Homologous and heterologous competition for the complex binding the NFAT-1 oligonucleotide. Competition gel shift assays were carried out as described in Materials and Methods with a labeled NFAT-1 oligonucleotide. Unlabeled competitor oligonucleotides were added as indicated, to 50- to 200-fold molar excesses. The arrow indicates the specific NFAT-1 complex. (P) and (D) represent the AP-1_P and AP-1_D oligonucleotides, respectively. Lane 1, No specific competitor. Each lane contained 2.5 μg of nuclear extract from cells stimulated with TPA + A23187 + IL-1 with 2.3 μg of poly(dA · dT) as nonspecific competitor. (B) As panel A, using labeled NF-κB oligonucleotide with the indicated competitors at the molar excesses shown. Lane 1, No specific competitor. (−148/−217) is a restriction fragment from the natural IL-2 gene promoter that contains the NF-κB site. Its greater potency as a competitor in part reflects a general effect of length (F. J. Calzone and E. H. Davidson, personal communication). Each lane contained 5 μg of extract from TPA + A23187-stimulated cells and 0.5 μg of poly(dI · dC). (C) Cross-competition of complexes binding the AP-1_P and AP-1_D oligonucleotides. Lanes: 1 to 7, AP-1_P probe with the indicated competitors (lane 7, no specific competitor); 8 to 14, AP-1_D probe with the indicated competitors (lane 8, no specific competitor). (A) denotes a commercial consensus AP-1 22-mer (Stratagene), used at 50-fold molar excess; (N) denotes the NF-κB oligonucleotide used at 200-fold molar excess. Arrows indicate the mobilities of the main AP-1_P complex (left) and the AP-1_D complex (right). Each lane contained 5 μg of extract as in panel A, with 0.125 μg of poly(dI · dC). Both extracts used in this figure were made by the method of Dignam et al. (6).

quences from −66 to −90, is the constitutive Oct-1 protein, which also appears to bind sequences between −240 and −250 (10). Factors whose binding activity is known to be dependent on cellular activation bind at −264 to −284 (NFAT-1) and −196 to −205 (NF-κB or a close relative) (5, 17, 39). In addition, there are two potential sites for the binding of the canonical TPA-response factor AP-1 (2), although neither contains a perfect consensus sequence. One, from −186 to −195 (AP-1_D), has been proposed as a critical site for IL-1 effects in another cell line (26). The other, from −145 to −153 (AP-1_P), has been shown to bind a purified AP-1 factor (37).

To dissect the mechanism of IL-1 costimulation of EL4.E1 cells, we focused on the four sites for potentially inducible factors: NFAT-1, NF-κB, AP-1_D, and AP-1_P. Synthetic double-stranded oligonucleotides containing these sites as found in the murine IL-2 gene were used in gel retardation assays to detect DNA-binding proteins in nuclear extracts from EL4.E1 cells after different types of stimulation. Typical results, representative of four to seven independent analyses, are presented in Fig. 3 and 4. A summary is provided in Table 4.

Four distinct binding specificities. Confirmation of the distinct specificities of these factors is presented in Fig. 3. The binding of NFAT-1 was highly sensitive to competition with its homologous target sequence and impervious to competition with the other oligonucleotides used (Fig. 3A). Figure 3B similarly confirms the specificity of the NF-κB complexes and their ability to be competed for by a longer IL-2 DNA fragment (−148 to −217) that contains this site We routinely observed a doublet of complexes binding the NF-κB site, both of which appear specific by competition analysis. Only the upper band was strongly induction dependent, however, and in the text that follows we will use "NF-κB" to refer only to this slower-migrating species.

The factors that bind to the two AP-1 sites are not identical, although their target sites are of related sequence This conclusion was confirmed by the cross-competition analysis shown in Fig. 3C. First, the two complexes have different mobilities (compare lanes 7 and 8). Second, it is clear that they had different binding preferences. Both were legitimately capable of binding a commercial consensus AP-1 oligonucleotide (Fig. 3C, lanes 5 and 13), and neither was detectably competed for by the NF-κB site oligonucleotide (lanes 6 and 14). However, while the AP-1_P oligonucleotide competed for the complex binding its homologous sequence (lanes 1 and 2), it did not compete for the AP- complex (lanes 9 and 10). Correspondingly, the AP-

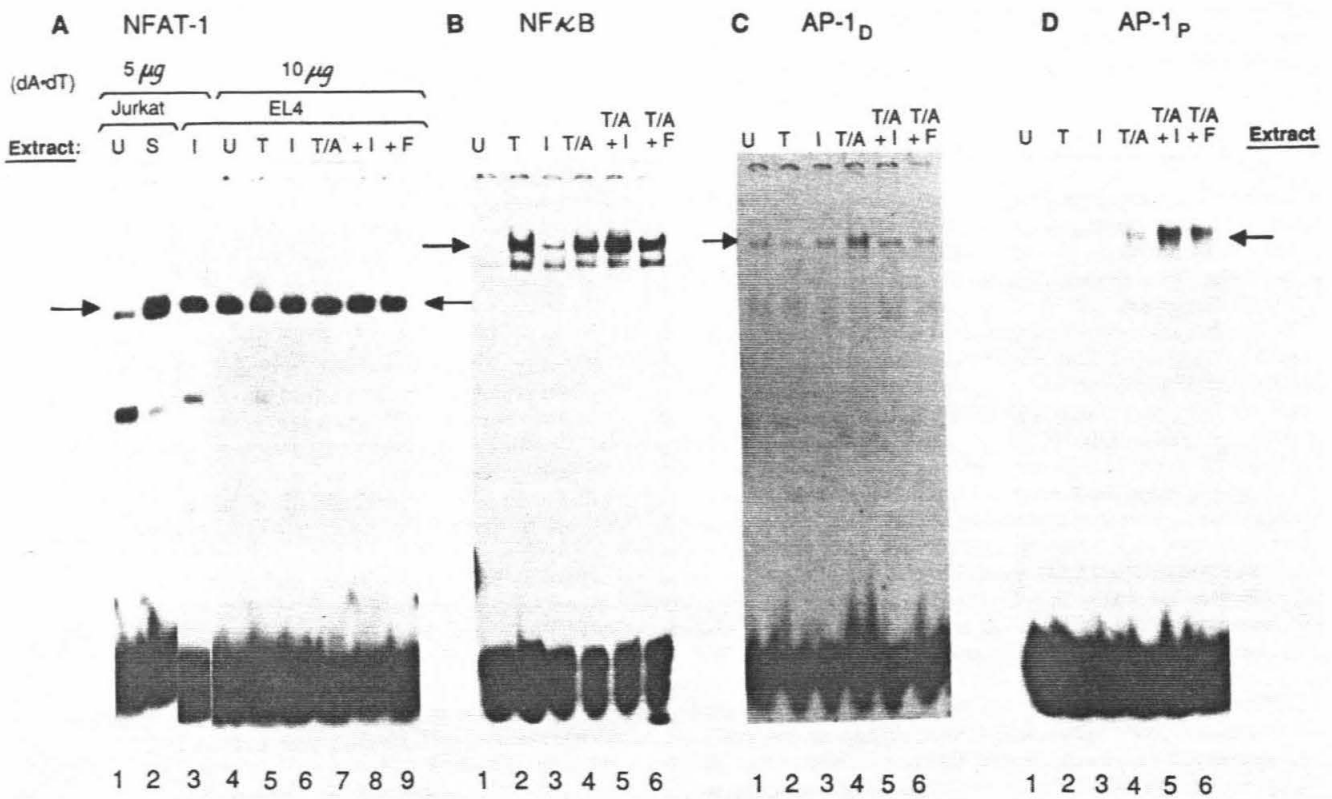

FIG. 4. Differential activation of IL-2 DNA-binding factors under different conditions of stimulation. In each panel, the indicated probes were used for gel shift analysis, each lane containing 5 μg of extract from EL4.E1 cells that were stimulated for 3.5 h under the indicated conditions: U, unstimulated; T, TPA alone; I, IL-1 alone; T/A, TPA plus A23187; T/A+I, TPA plus A23187 plus IL-1; T/A+F, TPA plus A23187 plus 10 μM forskolin. In panel A only, NFAT-1 complexes from EL4.E1 cells (lanes 3 to 9) are compared with complexes formed by nuclear extracts from Jurkat cells, either unstimulated (U) or stimulated with TPA plus A23187 (S). (A) NFAT-1 factor. The arrow indicates the NFAT-1 complex, which is inducible in Jurkat cells (lanes 1 and 2) but constitutive in EL4.E1 cells (lanes 4 to 9). Lane 3 contains a duplicate of the extract used in lane 6 with a lower amount of nonspecific competitor, to demonstrate that the lower band seen in the Jurkat sample (lane 1) is nonspecific. (B) NF-κB factors. The arrow indicates the upper complex, which is more strictly dependent on induction than the lower complex and which is the subject of discussion in the text. Each lane contained 0.5 μg of poly(dI-dC). (C) AP-1_D factor. The increased band intensity seen in lane 4 was highly irreproducible (see Table 4), in contrast to the differences seen in panel B. In panels C and D, 0.25 μg of poly(dI · dC) was used as nonspecific competitor. Arrows indicate the specific complexes. (D) AP-1_P factor, analysis as for panel C.

oligonucleotide showed homologous competition (lanes 11 and 12) but did not compete for the AP-1_P complex (lanes 3 and 4). The quantitative reciprocity of these effects, as measured at 100- and 200-fold molar excess, rules out the trivial explanation that the two factors had identical specificities but were present at different concentrations in the nuclear extract. These results show that the two AP-1 factors are distinct both in size and in specificity. As discussed below, they are also distinct in regulation.

Constitutive factors. Two of the factors detected by these assays appeared to be expressed constitutively in the EL4.E1 thymoma cells. One, unexpectedly, was NFAT-1. A complex of the expected mobility (indicated by an arrow in Fig. 4A) was highly inducible in Jurkat cells (Fig. 4A, lanes

TABLE 4. Summary of effects of different stimulation conditions on specific IL-2 DNA-binding proteins

DNA-binding factor	Stimulation conditions[a]						No. of expts[b]
	U	T	I	T/A	T/A+I	T/A+F	
NFAT-1	+/±	+	+	+	+	+	2(D) + 3(C)
NF-κB[c]	−	++/+	+/±	++/+	+++	++/+	2(D) + 2(C)
AP-1_D[c]	+	+	+	+	+	+	2(D) + 2(C)
AP-1_P[c]	±/−	+	−	++	+++	+++	2(D) + 2(C)

[a] Terminology as in the legend to Fig. 4.

[b] Number of independent experiments (separate stimulation and extract preparation), counting separately cases in which extracts were generated by the method of Dignam et al. (6) (D) or Crabtree (4) (C). Similar relative results were obtained with both kinds of extracts although the absolute concentrations of different factors were somewhat different for the two protocols.

[c] One set of data was obtained from an experiment with extracts prepared by the method of Dignam et al. (6) which compared only the U, T, T/A, and T/A+1 conditions of culture.

1 and 2). However, the corresponding complex was present at indistinguishable levels in nuclear extracts from our subline of EL4.E1 cells, whether or not they were stimulated and regardless of the stimulation conditions used (Fig. 4A, lanes 4 to 9). This complex appeared unlikely to represent a spurious DNA-binding activity, since it was specific by the competition assays in Fig. 3 but quantitatively unaffected by increasing the concentration of nonspecific competitor (Fig. 4A, lanes 3 and 6). Thus, although the NFAT-1 site appeared essential for expression, on the basis of our deletion studies, the presence of its binding factor was not sufficient to support IL-2 expression.

The other factor present at unchanged levels was the complex of well-defined mobility binding the AP-1_D site (Fig. 4C). This factor exhibited slight fluctuations in concentration in different samples, but no reproducible increase was observed upon stimulation under any conditions (Table 4). In fact, in some experiments there was an indication that its concentration diminished in stimulated cells. The possibility that it might exert a negative regulatory effect is under further study.

Inducible factors. Only two of the candidate inducible factors showed increased DNA binding in response to induction. These were NF-κB (Fig. 4B) and the factor binding the AP-1_P site (Fig. 4D). In agreement with the ability of EL4.E1 cells to make IL-2 in response to TPA alone (Table 1), both were activated in cells stimulated with TPA alone (Fig. 4B and D, lanes 2). The further addition of A23187 up regulated both binding activities in most experiments, in accord with its effect on IL-2 production (Table 1), although the extents of up regulation did not match precisely (Fig. 4B and D; compare lanes 2 and 4). The binding activities of both factors, however, were reproducibly enhanced further by addition of IL-1, which consistently yielded the highest levels observed of both complexes (Fig. 4B and D, lanes 5). This result was not due to additive activation of these factors by IL-1 and by TPA + A23187, for in most experiments IL-1 alone induced little or no binding activity of either type (compare lanes 3 in Fig. 4B and D). Thus, while IL-1 was only marginally and irreproducibly capable of activating either binding factor alone, it was a potent enhancer of the stimulating capacity of A23187 and TPA.

Summary. The results of multiple analyses of these four factors are collated in Table 4. Overall, these data agree plausibly with the IL-2 titers reported in Table 1, if it is assumed that all four factors are required for optimal promoter activity. The surprising, constitutive expression of NFAT-1 in this subline of cells, and possibly that of the AP-1_D binding factor as well, may simply provide the biochemical explanation for the unique ability of EL4.E1 cells to express IL-2 in response to TPA alone. Nevertheless, this set of factors may not be sufficient to account for all regulatory modulation of IL-2 gene expression in EL4.E1 cells. As shown previously (Novak and Rothenberg, in press; Tables 1 and 3), 10^{-5} M forskolin inhibits IL-2 induction by two- to threefold under these conditions. It is therefore surprising that forskolin, when added to TPA and A23187, induced effects on these DNA-binding proteins that were very similar to those of IL-1 (Fig. 4A, lane 9; Fig. 4B to D, lanes 6). While in several experiments the addition of forskolin appeared to decrease NF-κB expression selectively (data not shown), this effect was often modest (e.g., Fig. 4B). It is thus possible that the ratio of AP-1_P to NF-κB affects expression. However, it seems more likely that the activity of the four putative positive regulators can be influenced by other regulatory proteins not examined here. Preliminary results suggest that the net inhibitory effects of elevated cAMP depend on the presence of additional discrete sequences in the IL-2 regulatory region (P. M. White and E. V. Rothenberg, unpublished results).

DISCUSSION

IL-1 has a long history as a costimulator of T-cell responses (9, 13, 25, 44). In recent years, it has become clear that this cytokine carries out different essential functions, depending on the cell type being stimulated. Most effects of IL-1 on mature T cells, however, can be substituted for by the addition of phorbol esters, presumably via stimulation of protein kinase C. Biochemical evidence does not support the conclusion that IL-1 acts directly via protein kinase C (1), but in most cases activation of this kinase can obscure the effects of IL-1 itself on T cells. In this study we took advantage of the unusual properties of EL4.E1 cells, which respond to IL-1 as a costimulus even with optimal levels of TPA, to investigate the molecular mediators by which IL-1 can cooperate with the phosphoinositide pathway. Our results show that IL-1 costimulates IL-2 gene expression by enhancing the activity of the IL-2 gene promoter, correlated with augmented levels of an NF-κB-like factor and one of two distinct AP-1-like factors that bind to IL-2 regulatory sequences.

Recent work by Shirakawa and colleagues has shown that IL-1 can utilize cAMP as an intracellular second messenger for the induction of κ immunoglobulin light-chain synthesis in the mouse pre-B-cell line 70Z/3 (42). In these cells, IL-1-mediated induction coincides with the activation of a protein that can bind to the NF-κB consensus site in the 5′-flanking region of the κ-chain genes (40). NF-κB is not the only transcription factor implicated in IL-1 effects, however. IL-1 can also induce transcription of the c-*jun* proto-oncogene, which encodes a component of AP-1, in the mouse IL-1-dependent T-cell line LBRM-331A5 (26). In that cell line, expression of a linked reporter gene from the human IL-2 promoter required an intact AP-1 site at −185 relative to the transcriptional start site. However, the deletions tested which removed this site also removed the NF-κB site centered at −200. Therefore, the resulting loss of promoter function (26) cannot be attributed unequivocally to loss of this distal AP-1 site. In fact, deletion analysis of the human IL-2 gene promoter has not demonstrated an important role for this AP-1 site in vivo (8). Footprint analysis of the mouse IL-2 promoter has led to the same conclusion (37).

In several respects, our results are in agreement with these previous reports. Thus, our data support the interpretation that one mediator of the IL-1 effect may be NF-κB or a related factor (31, 40). The addition of IL-1 as a costimulus invariably augmented NF-κB binding activity over that induced by the cocktail of TPA and A23187, and in some experiments (e.g., Fig. 4) we could even detect enhanced NF-κB binding after stimulation with IL-1 alone. As expected if IL-1 activated NF-κB, it also enhanced the induction of IL-2 receptor α-chain transcripts in the EL4.E1 cells (T. J. Novak and E. V. Rothenberg, unpublished results). Even the failure of IL-1 to affect IL-2 gene promoter activity in Jurkat cells is also consistent with its working through NF-κB, since in these cells IL-1 does not induce appearance of this DNA-binding protein (31). The resulting implication that NF-κB may be rate limiting for IL-2 induction is in accord with published evidence that mutation of the NF-κB site depresses IL-2 gene promoter function, relative to wild type, in optimally stimulated cells (17).

In contrast to other cell types (41, 42), however, EL4.E1 cells appear to be able to respond to IL-1 as a costimulus without using cAMP as the principal mediator. As we report here and in more detail elsewhere (Novak and Rothenberg, in press), IL-2 gene promoter activity is significantly inhibited by treatments that cause a sustained elevation of intracellular cAMP. This inhibition is especially sharp at the early times when IL-1 is most potent in its stimulatory activity. Thus, the cooperative effects of IL-1 with phosphoinositide pathway stimulation and those of cAMP appear quite distinct.

It seems paradoxical, then, that both IL-1 and cAMP may enhance activation of NF-κB. Indeed, both also hyperactivate the binding factor of the proximal AP-1 site. However, while the induced DNA-binding activities that we have studied probably participate in the mechanism regulating IL-2 gene promoter activity, they may not be the only components, or the only decisive components, of a combinatorial mechanism. The resolution of this paradox is most likely to come, therefore, from more extensive monitoring of the ensemble of regulatory proteins that control IL-2 gene expression, rather than a focus on any single one. Our results with only four such factors provide compelling evidence for the combinatorial nature of IL-2 gene regulation. The NFAT-1 factor, the regulation of which may be sufficient to account for the response to TcR ligands in Jurkat cells (4, 10) is not rate limiting in EL4.E1 cells. The NFAT-1-binding activity is present at maximal levels even in cells which cannot secrete IL-2 and cannot express IL-2 promoter constructs. Yet the cooperation of this factor appears to be required for efficient IL-2 induction, as shown by the minimal activity, with or without IL-1, of constructs from which its binding site has been deleted. In this context, the shared abilities of forskolin and IL-1 to stimulate the $AP\text{-}1_p$ factor and NF-κB imply less that these agents mediate the same signal than that the set of factors we monitor may be incomplete.

How can the costimulatory effects of IL-1 on $AP\text{-}1_p$ and NF-κB activation be explained? There are two general possibilities. First, IL-1/IL-1R triggering may directly induce activation of these two factors, and possibly others uniquely responsive to IL-1, mobilizing pools that are not accessible to the calcium ionophore/phorbol ester stimulation pathway. The effects of IL-1 would then be additive with those of the phosphoinositide pathway agonists, at the level of the DNA-protein contacts in chromatin. Another possibility is that the ability of A23187/TPA to activate these DNA-binding proteins is itself modulated by an intracellular gating function that is subject to IL-1 regulation. In this case, IL-1 need not induce any DNA-binding protein activities by itself in order to be a potent regulator of the efficiency with which the major activating pathway mobilizes gene expression. While too few factors have yet been examined to confirm or rule out the first mechanism, several of our findings lend plausibility to the second. First, IL-1 alone is strikingly poor at effecting any increase or decrease in the binding activity of any of the factors studied here. In EL4.E1 cells, it is far more potent as a costimulus than as a stimulus (see, e.g., Table 4). Second, the factors that IL-1 coactivates thus far appear to be only those that are activated in any case by A23187 and TPA. Third, our survey of IL-1 effects on the expression of IL-2 promoter constructs with widely differing numbers of positive and negative modulatory sites (30) indicates that all expressible constructs are affected in parallel. Thus, IL-1 may act in these cells by enhancing the potency of A23187 and TPA as inducing agents, its signals converging with the phosphoinositide pathway before the activation of the specific DNA-binding proteins.

The mechanism of IL-1 costimulation may help to illuminate key stages in the development of T-cell functional competence. We have previously reported that immature thymocytes can activate the IL-2 gene, but only subject to an absolute requirement for IL-1 costimulation. The locus of the IL-1 effect may thus identify a stage-specific block to the exercise of function in immature cells which is later overcome by the complex physiology of positive selection in the thymus. Work is now under way to explore this possibility.

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Chapter 2

Molecular Basis for Developmental Changes in Interleukin-2 Gene Inducibility

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Molecular Basis for Developmental Changes in Interleukin-2 Gene Inducibility

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At least three stages in the intrathymic development of pre-T cells are demarcated by differences in the competence to express the interleukin-2 (IL-2) gene as an acute response to stimulation. IL-2 inducibility appears to be acquired relatively early, prior to T-cell receptor (TcR) gene rearrangement. It is then abrogated during the stage when cells are subject to positive and negative selection, i.e., the fate determination processes that select cells for maturation or death. IL-2 inducibility finally reappears in mature classes of thymocytes that have undergone positive selection. To provide a basis for a molecular explanation of these developmental transitions, we have examined the representation in different thymocyte subsets of a set of DNA-binding proteins implicated in IL-2 gene regulation. As the DNA-binding activities of many factors are elicited only by inductive stimuli, the cells were cultured in the presence or absence of the calcium ionophore A23187 and phorbol ester. Our results separate these factors into four regulatory classes: (i) constitutive factors, such as Oct-1 and probably Sp1, that are expressed in thymocytes at all stages; (ii) inducible factors, such as NF-κB and complexes binding to the region of a CD28 response element, that can be activated in all thymocytes, including those cells ($CD4^+$ $CD8^+$ TcR^{low}) that can undergo selection; (iii) inducible factors, such as NF-AT and AP-1, that can be activated in mature ($CD4^+$ $CD8^-$ TcR^{high}) and immature ($CD4^-$ $CD8^-$ TcR^-) thymocytes alike but not in the transitional stages when the cells ($CD4^+$ $CD8^+$ TcR^{low}) are subject to selection; and (iv) a factor containing CREB, which can be activated in thymocytes of all developmental stages by culture but does not require specific induction. These results verify that inducible transcription factors are targets of intrathymic developmental change. They also identify NF-AT and AP-1 as factors that are particularly sensitive to the mechanism altering thymocyte responses during the stages when thymocytes may undergo positive and negative selection.

T-cell precursors undergo a cascade of developmental changes in the thymic microenvironment which ultimately endow them with the recognition specificities, signaling mediators, and capacity for functional responses of mature T lymphocytes (reviewed in references 10, 36, and 49). Recognition specificity is the result of the rearrangement of T-cell receptor (TcR) genes and the cell surface expression of their products. The TcR complex becomes effective in triggering appropriate signaling pathways through the expression of the coreceptor molecules, CD4 and/or CD8, at the cell surface, together with a network of cytoplasmic protein kinases (1, 8). The acquisition of functional response capability, however, is more complex. Mature T-cell function is exercised only in response to antigen stimulation, through the abrupt and transient transcriptional activation of specific response genes such as the gene encoding interleukin-2 (IL-2). The competence to express IL-2 upon stimulation is largely, if not exclusively, a property of T lymphocytes. Accordingly, one of the changes induced in precursor cells by passage through the thymus is acquisition of IL-2 inducibility (39, 40).

Analysis of the competence to express IL-2 at various stages of intrathymic development has revealed a series of transitions distinct from those recognized on the basis of cell surface phenotype and TcR expression (reviewed in reference 36). In particular, immature thymocytes before TcR expression exhibit a form of IL-2 inducibility that seems to be extinguished in most of their descendants, while a different form of IL-2 inducibility is found in the minority of cells that go on to full maturation (9, 17, 24, 38, 39). Neither the early inducibility nor the extinction of inducibility is well understood in relation to the generation of mature IL-2 producers. However, these findings suggest provocative links between responsiveness to activation signals and thymocyte fate determination, as we have discussed elsewhere (36, 39). The lymphostromal interactions responsible for the changes in IL-2 inducibility may or may not be the same as those that trigger quite different effects on TcR expression. However, an advantage of using IL-2 inducibility to monitor developmental processes is that this property is relatively well understood at a molecular level. Thus, it is possible to define the impact of each transition, and gain a clue as to its mechanism, by analysis of the IL-2 gene-regulatory proteins that it affects.

Therefore, we have taken advantage of an extensive literature characterizing the minimal enhancer sequence of the IL-2 gene and many of the DNA-binding proteins that interact with it (reviewed in reference 47). Both inducible and constitutively expressed *trans*-acting factors are involved in initiating IL-2 gene transcription, including NF-κB or TcF-1 (18, 32, 42), AP-1 (20, 27), Oct-1 and other octamer-binding proteins (21), and two uncloned factors termed NF-AT (41) or Pu-box factor III (5, 33) and CD28RC or the CD28 response element (CD28RE)-binding factor (11, 48). We have used electrophoretic mobility shifts to score for the DNA-binding activities of these and other IL-2 DNA-binding factors in thymocyte nuclear extracts after in vitro stimulation. For those factors which do not require activation to reside in nuclei and bind DNA, these assays allow us to score the presence of factor proteins. For those which require specific signaling events to display DNA-binding

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activity, these assays further allow us to score cells for the existence of the signaling pathway that controls the factor modification. Our results demonstrate that inducible IL-2-regulatory factors are expressed noncoordinately in T-cell development. We further show that a full panoply of IL-2-regulatory proteins is present in immature thymocytes. Finally, our results show that the transitional $CD4^+$ $CD8^+$ TcR^{low} cortical thymocytes that are the main targets of selection are highly activatable even though functionally incompetent, responding to stimulation with intense induction of a specific but incomplete set of IL-2 DNA-binding activities.

MATERIALS AND METHODS

Reagents. Tetradecanoyl phorbol acetate (TPA) and the calcium ionophore A23187 (both from Sigma) were dissolved in dimethyl sulfoxide to the stock concentrations of 10 μg/ml and 0.7 mM, respectively. Recombinant human IL-1α was purchased from Genzyme. Antisera against Fos and Jun family proteins were kindly provided by Rodrigo Bravo. These reagents react specifically with all known members of the Fos and Jun families, respectively (23). Antiserum 244, against the W39 peptide of CREB, was a generous gift from M. R. Montminy (13). It is highly specific for recognition of CREB (26). In addition to the published documentation of the specificity of these antisera, we carried out controls for specificity under the conditions of our assays. Supershifted bands were not produced when 1 μl of a control antibody, RL172.4 (anti-CD4 ascites fluid), was added to the reactions. Furthermore, anti-CREB, anti-Fos, and anti-Jun did not affect the mobility of complexes binding the NF-κB oligonucleotide. We therefore consider the supershifted complexes to be specific.

Cells. Freshly isolated thymocytes from 4- to 6-week-old C57BL/6 mice were fractionated by complement-dependent cytolysis or panning. To isolate immature TcR^- $CD4^-$ $CD8^-$ cells, we used complement-dependent lysis with anti-CD4 (RL172.4), anti-CD8 (3.155), and anti-CD5 (CG-15) (39), or we used cells taken directly from the thymus of immunodeficient C.B-17 *scid/scid* (SCID) mutant mice, which are naturally arrested at this stage (>98% TcR^- $CD4^-$ $CD8^-$) (2). Cortical $CD4^+$ $CD8^+$ TcR^{low} thymocytes were either enriched by adherence to peanut agglutinin-coated plates or isolated by complement-dependent lysis with anti-$H\text{-}2K^b$ (28-13-33), taking advantage of their anomalously low class I major histocompatibility complex expression (4). By either method, the fraction contained over 92% $CD4^+$ $CD8^+$ cells. Mature thymocytes were either enriched by nonadherence to peanut agglutinin-coated plates (at 4°C) or isolated by complement-dependent lysis (at 37°C) with anti-CD8 antibody. By the latter method, over 70% $CD4^+$ $CD8^-$ and less than 30% $CD4^-$ $CD8^-$ cells were obtained; the fraction contained no cortical-type $CD4^+$ $CD8^+$ thymocytes. Flow cytometric analysis was used routinely to confirm the phenotypes of the fractions. Freshly isolated SCID thymocytes were put into culture directly, without prefractionation. All cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum (HyClone, Logan, Utah), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics, with or without TPA (17 nM, 10 ng/ml) plus A23187 (70 to 120 nM). In some experiments, the stimulated cells were also supplemented with IL-1 (20 or 50 U/ml).

IL-2 bioassay. Supernatants were collected from 20-h cultures with or without stimulation under the indicated conditions. The IL-2 bioassay was performed as described previously (39).

Nuclear extracts. Nuclear extracts were made from cells cultured for 2 to 4 h according to the procedure of Stein et al. (45). Briefly, 1×10^7 to 5×10^7 thymocytes were washed twice with ice-cold phosphate-buffered saline and resuspended in 100 μl of lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM Tris, 60 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and then the nuclei were pelleted at $1,200 \times g$ for 5 min at 4°C. The nuclei were washed with lysis buffer without Nonidet P-40, then resuspended in nuclear resuspension buffer (250 mM Tris [pH 7.8], 60 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and disrupted with three cycles of freezing and thawing. After clarification by centrifugation at $7,000 \times g$ for 15 min, the supernatant was collected, and the nuclear extract was stored at −80°C until use. Protein concentrations in the extracts were determined by the Bradford assay (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Calif.).

Oligonucleotides. Most double-stranded oligonucleotides were synthesized at the Microchemical Facility in our Division of Biology. Unless specified, the sequences are found in the 5'-flanking region of the mouse IL-2 gene (28) at distances from the cap site as shown below:

Spl consensus (Stratagene)	5' GATCGATCGGGGCGGGGCGATC 3' 3' CTAGCTAGCCCCGCCCCGCTAG 5'
NFI/CTF (4) (Stratagene)	5' ATTTTGGCTTGAAGCCAATATG 3' 3' TAAAACCGAACTTCGGTTATAC 5'
AP-2 consensus (Stratagene)	5' GATCGAACTGACCGCCCCGCGGCCCGT 3' 3' CTAGCTTGACTGGCGGGGCGCCGGGCA 5'
AP-1 (CREB) (−161 to −143)	5' AATTCCAGAGAGTCATCAG 3' 3' GGTCTCTCAGTAGTC 5'
NF-κB (−211 to 192)	5' AAGAGGGATTTCACCT 3' 3' TTCTCCCTAAAGTGGATTTA 5'
NFAT-1 (−289 to 260)	5' AAGAGGAAAATTTGTTTCATACAGAAGGCG 3' 3' TTCTCCTTTTAAACAAAGTATGTCTTCCGCTTAA 5'
Sp1 (−300 to −278)	5' gatcTCTCCACCCCAAAGAGGAAAATT 3' 3' AGAGGTGGGGTTTCTCCTTTTAActag 5'
CD28RE (−174 to 156)	5' gatcGGGGGTTTAAAGAAATTCC 3' 3' CCCCCAAATTTCTTTAAGGctag 5'
NF-IL2A (−100 to −69)	5' gatcTCTTTGAAAATATGTGTAATATGTAAAACAT 3' 3' AGAAACTTTTATACACATTATACATTTTGTActag 5'

Lowercase letters indicate bases not found in the natural sequence. Oligonucleotides were annealed with their complements and labeled by polymerization with α-^{32}P-labeled deoxynucleoside triphosphates to fill in the 5' overhanging ends.

Gel mobility shift assay. The gel shift assays were performed as described previously (6), with minor modifications. Nuclear extracts (2.5 to 10 μg of protein) were preincubated with 0.5 to 2 μg of poly(dI · dC) at 25°C for 15 min and then incubated with 5 fmol of ^{32}P-end-labeled oligonucleotides (2×10^4 to 4×10^4 dpm) for another 15 min in the final volume of 10 to 20 μl. Reaction mixtures were electrophoresed in 6% acrylamide gels, using 0.5× Tris-borate-EDTA as running buffer. Gels were dried and ex-

posed to Kodak XAR-5 film overnight at −70°C with an intensifying screen. For competition or antibody supershifting experiments, the indicated molar excess of specific competitor DNA or 0.5 to 1 μl of antibody was added at the beginning of the preincubation, before addition of the labeled oligonucleotide. Under these conditions, the complexes scored here (indicated by arrows or brackets in the figures) were all sequence specific and able to be competed for by homologous oligonucleotides.

Data analysis. To compare the representation of a given DNA-binding protein in different extracts, as in Fig. 3 through 5, samples containing equal amounts of nuclear protein were analyzed in parallel by electrophoretic mobility shift assay with the same preparation of labeled oligonucleotide probe. The amounts of radioactivity in the shifted complexes and, where applicable, in the unbound fraction of the probe were determined by analysis with a Molecular Dynamics PhosphorImager. The levels of binding activity in the various cell types were compared after being normalized to the levels of Oct-1 protein determined in parallel in aliquots of the same extracts. Results reported were all based on at least three (usually six or more) analyses using at least two (usually four or more) independent cell preparations.

The introduction of antibodies to identify complexes containing CREB, Jun, and Fos significantly perturbs quantitation. In our hands, the antibody against CREB appears to enhance the DNA-binding affinity of the complexes that it reacts with, so that the total amount of complex (shifted plus supershifted by reaction with antibody) appears greater than the amount of complex formed in the absence of antibody. Conversely, the antibodies against Jun and Fos individually reduce the DNA-binding affinity of the complexes that they react with and appear to reduce the overall amount of complex formed when they are used together in supershifting experiments (data not shown). We have therefore estimated the relative amounts of AP-1 (Fos/Jun) complexes only from the level of complex formed that is not supershifted by anti-CREB.

TABLE 1. IL-2 inducibility in thymocyte subpopulations

Cell population[a]	IL-2 activity (U/ml/5 × 10^6 cells)[b]		
	Exp 1	Exp 2	Exp 3
Total thymus			
Unstim.	<4[c]		
Stim.	52		33
Stim. + IL-1	104		
$CD4^+$ $CD8^-$[d]			
Unstim.			
Stim.			323
$CD4^+$ $CD8^+$[e]			
Unstim.			<4
Stim.			3.5
TcR^- $CD4^-$ $CD8^-$[f]			
Unstim.		<4	
Stim.		<4	
Stim. + IL-1		355	
SCID			
Unstim.	<4		
Stim.	99	37	
Stim. + IL-1	1,581	848	

[a] Cells were prepared as described in the text and cultured as indicated for 20 h. Unstim., unstimulated; Stim., in the presence of 100 nM A23187 and 17 nM TPA; Stim. + IL-1, as for Stim. plus IL-2 at 20 U/ml for experiment 1 and 50 U/ml for experiment 2.

[b] Measured by bioassay on CTLL-2 cells. Units are defined in a given experiment as described in reference 39. Where no value is shown, that sample was not included in a particular experiment. Results from experiments with 3 independent cell preparations, representative of over 10 independent experiments, are given (see also references 24 and 38 to 40).

[c] Undetectable.

[d] Prepared by cytolysis with anti-CD8. This fraction also contained ~30% $CD4^-$ $CD8^-$ cells.

[e] Prepared by cytolysis with anti-H-$2K^b$. This fraction consisted of 92% $CD4^+$ $CD8^+$ cells with <5% each $CD4^+$ $CD8^-$, $CD4^-$ $CD8^+$, and $CD4^-$ $CD8^-$ cells.

[f] Prepared from normal mice.

RESULTS

Nuclear extracts from cells in defined stages of T-cell development. To define the IL-2-regulatory proteins present at various stages of thymocyte development, we prepared nuclear extracts from isolated subsets of thymocytes representing the immature ($CD4^-$ $CD8^-$ TcR^-), transitional (cortical $CD4^+$ $CD8^+$ TcR^{low}), and mature helper-type ($CD4^+$ $CD8^-$ TcR^{high}) stages of development. The nuclear extracts from these populations were then used in electrophoretic mobility shift assays to detect factors capable of binding to any of a panel of double-stranded synthetic oligodeoxynucleotide probes derived from the murine IL-2 5′ flanking sequence. The sequences of these probes are listed in Materials and Methods. Previous reports and our own preliminary studies had indicated that the appearance of certain critical binding activities depended on cellular activation. Therefore, we prepared nuclear extracts from thymocyte subsets that were cultured for 2 to 4 h with or without stimulation with the phorbol ester TPA (17 nM) plus the Ca^{2+} ionophore A23187 (70 to 120 nM). These stimuli mimic the second messengers induced by TcR ligands but do not require the presence of the TcR complex and therefore allow cells from a broad range of developmental stages to be assayed for responsiveness.

Each population of cells analyzed was prepared by two different methods to control for any perturbations in cell physiology that might be caused by a particular method of fractionation (see Materials and Methods). For any given thymocyte subset, the different methods of isolation in fact yielded essentially indistinguishable profiles of DNA-binding proteins. Thus, the differences between fractions discussed below reflect intrinsic cellular characteristics and not simply artifacts of the isolation procedure.

The immature $CD4^-$ $CD8^-$ TcR^- cells, cortical-type $CD4^+$ $CD8^+$ TcR^{low} cells, and mature $CD4^+$ $CD8^-$ TcR^{high} cells differ significantly in the ability to express IL-2 upon induction (Table 1). In all cases, IL-2 protein secretion (Table 1) agrees well with IL-2 RNA expression, as measured by in situ hybridization and by quantitative RNase protection assays, as described elsewhere (25, 39, 40). The $CD4^+$ $CD8^-$ TcR^+ cells (initially ~10% of total thymus) are about 10-fold enriched for the ability to express IL-2 relative to unfractionated thymocytes, whereas cells in the $CD4^+$ $CD8^+$ TcR^{low} fraction (initially 80% of total thymus) are at least 20-fold depleted of this ability. The immature cells from the SCID thymus produce modest amounts of IL-2 under normal stimulation conditions and very high levels if IL-1 is added. In this intense IL-1-dependent response, they resemble normal immature thymocytes, which have the capacity to express high levels of IL-2 but do so only when IL-1 is present (Table 1). By contrast, addition of IL-1 does not

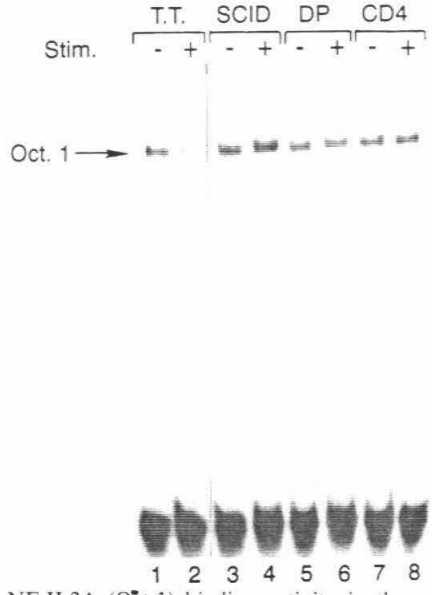

FIG. 1. NF-IL2A (Oct-1) binding activity in thymocytes. The NF-IL2A oligonucleotide (−100 to −69) was used as a probe to test the binding activity in nuclear extracts from unfractionated thymocytes (total thymocytes [T.T.]; lanes 1 and 2), SCID thymocytes (lanes 3 and 4), $CD4^+$ $CD8^+$ TcR^{low} cortical thymocytes (double positive [DP]; lanes 5 and 6), and a population enriched for mature $CD4^+$ $CD8^-$ TcR^{high} thymocytes (CD4; lanes 7 and 8). The cells were cultured in the absence (−) or presence (+) of A23187 plus TPA (Stim.) for 4 h prior to lysis. Aliquots estimated to represent equal amounts of nuclear protein from each sample were compared; in fact, the sample in lane 2 contained less protein than did the other samples. The electrophoretic mobility shift assay was performed as described in Materials and Methods.

reveal any cryptic IL-2 inducibility in the $CD4^+$ $CD8^+$ TcR^{low} cells (38; data not shown). These results agree well with previously published data (16, 24, 34, 38, 39) indicating that these fractions are functionally and phenotypically consistent.

DNA-binding factors present in developmental thymocyte subsets. The results of the electrophoretic mobility shift experiments revealed that the factors capable of binding to IL-2 gene flanking sequences can be divided into four classes on the basis of their developmental regulation.

(i) Constitutive activities. As shown in Fig. 1, all thymocyte fractions possess a nuclear protein with binding activity for the NF-IL2A element (−100 to −69), with or without induction by TPA plus A23187. Its mobility compared with those of the NFIL-2A-binding proteins in EL4.E1 cells suggests that this protein is Oct-1 (data not shown). In over 10 independent experiments, there was no consistent difference between fractions or between stimulated and unstimulated samples in the amount of this binding factor relative to total nuclear protein.

Another factor that appeared to be expressed constitutively was one that binds to the oligonucleotide containing a sequence upstream of the NF-AT site, namely, the region from −300 to −278 (Fig. 2a). Despite the lack of any conventional Sp1 sites in the IL-2-regulatory region (29), the −300/−278 oligonucleotide includes a sequence, CCAC CCC, which is similar to a reported variant Sp1-binding site (44). In fact, the −300/−278-binding factor binds with much higher affinity to a consensus Sp1 oligonucleotide than to the −300/−278 oligonucleotide itself, as demonstrated by the >10-fold difference between the molar efficiencies of these oligonucleotides as competitors (Fig. 2b). Binding of the thymocyte factor could not be significantly competed for by

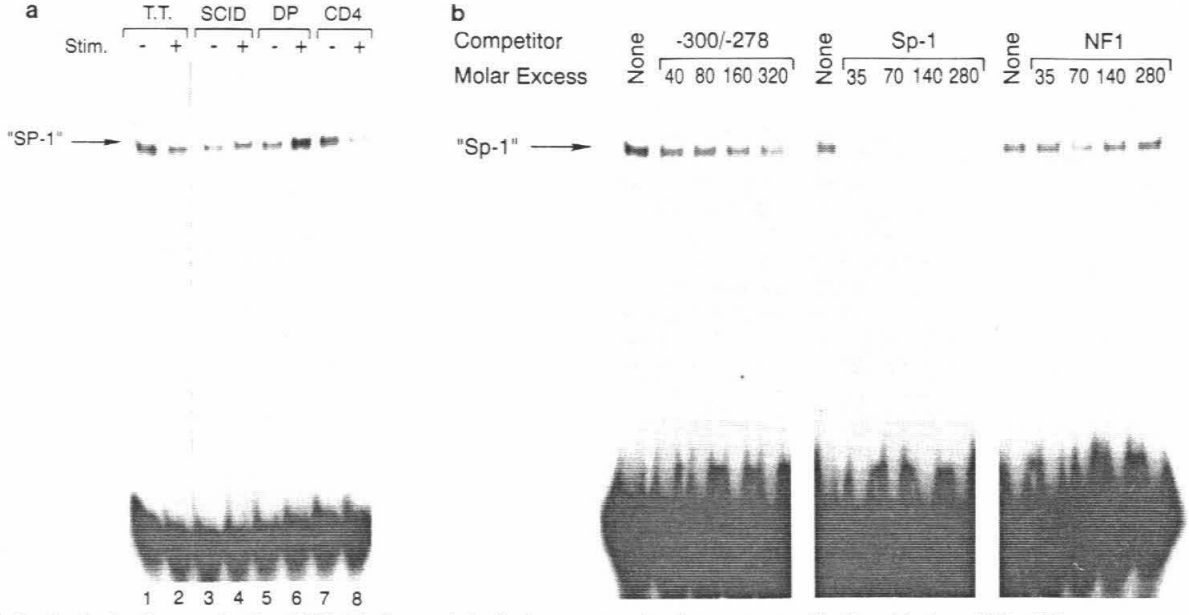

FIG. 2. Analysis of a constitutive DNA-binding activity in thymocytes that interacts specifically with the −300/−278 sequence. (a) Protein binding study. Nuclear extracts from samples of thymocytes that were cultured with (+) or without (−) stimulation were analyzed as for Fig. 1 to measure proteins binding to the −300/−278 oligonucleotide. Symbols and abbreviations are as defined in the legend to Fig. 1. The arrow indicates the prominent specific complex that may correspond to Sp1. (b) Results of a competition experiment suggesting that the major complex formed with the −300/−278 oligonucleotide may be closely related to Sp1. The indicated competitor oligonucleotides were added to an unfractionated thymocyte extract in the molar excesses over the labeled probe that are indicated above the lanes. The relatively poor self-competition by the −300/−278 oligonucleotide and the strong competition by the consensus Sp1 oligonucleotide indicate that the factor binds to the −300/−278 site with relatively low affinity. No competition is observed with the unrelated NF1 oligonucleotide.

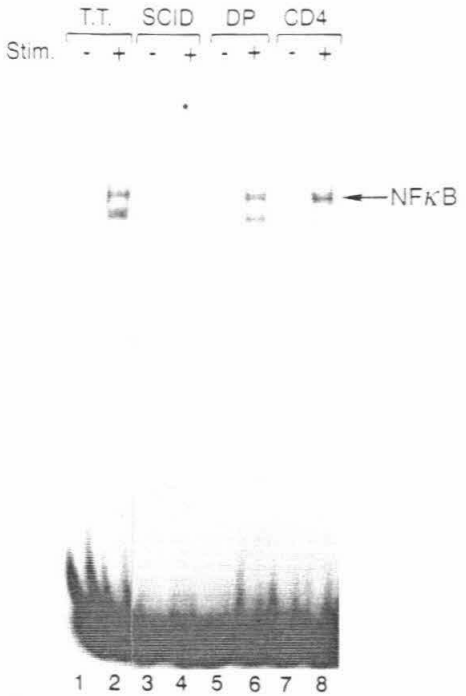

FIG. 3. Comparison of inducible NF-κB site-binding activity in different thymocyte subsets. Samples of the extracts analyzed in Fig. 1 and 2 were incubated with an oligonucleotide spanning from mouse IL-2 positions −211 to −192 to test for specific NF-κB site-binding activity. The specific inducible NF-κB-binding complex is indicated by the arrow. Abbreviations are as defined in the legend to Fig. 1.

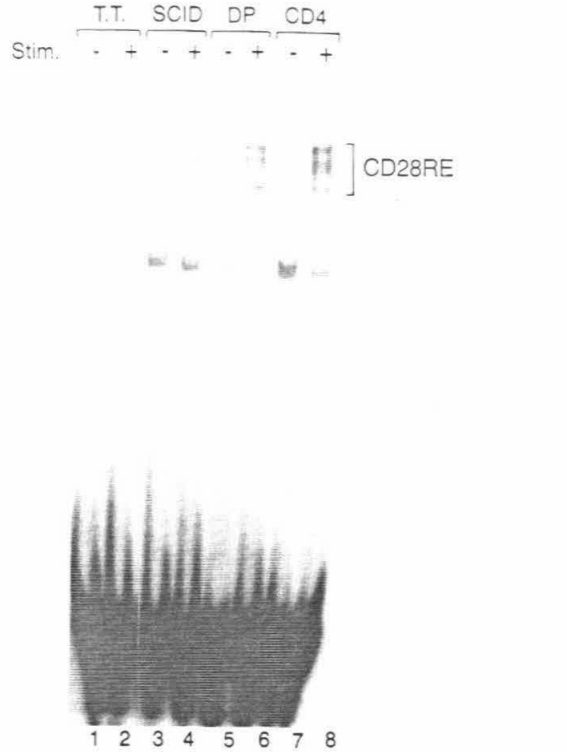

FIG. 4. Comparison of CD28RE-binding activity in different thymocyte subsets. The oligonucleotide spanning from IL-2 positions −174 to −156 was used in a binding assay with the panel of extracts analyzed in Fig. 1 through 3. The bracketed bands indicate inducible specific complexes. The complexity of this pattern, relative to that reported previously (11), may be due to the inclusion in our CD28RE oligonucleotide of five G residues that are omitted in the oligonucleotide used by Fraser et al. (11). Abbreviations are as defined in the legend to Fig. 1.

a consensus AP-2 oligonucleotide in excess (data not shown), even though in some cases AP-2 is reported to bind similar sequences (35). Thus, it is likely that Sp1 or a relative of Sp1 may bind to this site. Our in vivo genomic footprinting studies confirm that this candidate Sp1 site does become occupied as part of a coordinated protein-DNA binding complex during IL-2 induction (12).

(ii) Inducible factors correlated with developmental progression. The factors binding to the NF-κB site (−211 to −192) and to the region (−174 to −156) associated with a proposed CD28RE differ from the previous two in that their DNA-binding activities are highly dependent on induction. Both their induction dependence and their relative inducibilities in different thymocyte subsets define the factors that bind the NF-κB (Fig. 3) and CD28RE (Fig. 4) sites as members of a separate regulatory class.

Inducible factors binding the NF-κB and CD28RE oligonucleotides were observed in all thymocyte subsets. In the case of the NF-κB site, two well-defined complexes were seen, possibly representing the p65-p50 and p50-p50 forms of this transcription factor. As we have noted previously for the model IL-2-inducible cell line EL4.E1 (28), the appearance of the upper complex (putative p65-p50 form or T-cell-specific TCF; indicated by the arrow in Fig. 3) was more stringently induction dependent. We provisionally refer to this factor as NF-κB in the text that follows. The factors binding the CD28RE oligonucleotide formed multiple retarded complexes that were distinctively enhanced upon induction (bracket in Fig. 4). The specific type of complex reported to be induced in human Jurkat cells by CD28 engagement (11) may correspond to one of these bands but was not definitively identified in this study (see the legend to Fig. 4). However, in the aggregate, these complexes behaved as a cluster of inducible DNA-binding proteins whose activation, like that of NF-κB, could be seen as the outcome of a successful signal transduction cascade.

Although the $CD4^+$ $CD8^+$ TcR^{low} cortical thymocytes could make no IL-2, we found that they activate both the NF-κB upper complex and CD28RE-binding factors to levels at least as high, relative to Oct-1 or total nuclear protein levels, as in most preparations of SCID thymocytes (Fig. 3 and 4; compare lanes 4 and 6). The lower complex, which may represent a p50-p50 homodimer, has been associated with repression of IL-2 expression (22). Although it appears to be expressed in a high ratio relative to the upper complex in the $CD4^+$ $CD8^+$ cells illustrated in Fig. 3 (lane 6), more commonly the ratio of lower to upper complex in these cells was the same as in the other subsets of thymocytes.

(iii) Inducible factors correlated with IL-2 inducibility. Factors capable of binding to the NF-AT and AP-1p sites defined a third class of factors. The binding activities associated with these factors were highly induction dependent, like the factors in the second group. However, in contrast to the second group, the NF-AT and AP-1 (see below) factors appeared to be inducible mainly or exclusively in cells competent to express IL-2, and in both mature cells and immature thymocytes alike.

Figure 5 shows the results of assaying thymocyte extracts

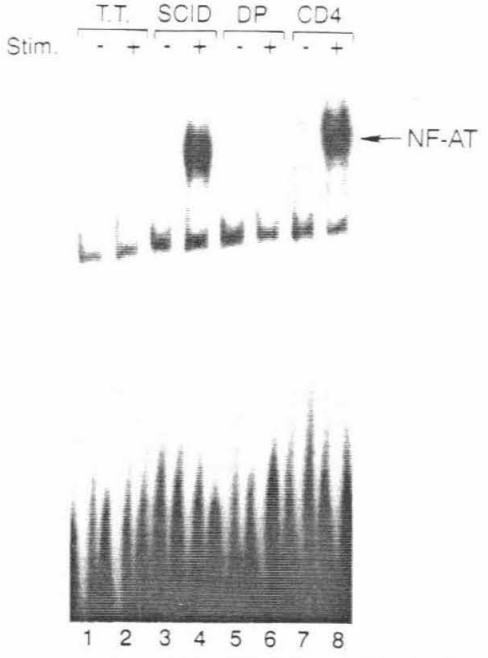

FIG. 5. Comparison of NF-AT-binding activity in different thymocyte fractions. The figure shows a typical result when the oligonucleotide from mouse IL-2 positions −289 to −260 was used in the binding assay with the panel of extracts used for Fig. 1 through 4. The inducible NF-AT complex is indicated by an arrow. Abbreviations are as defined in the legend to Fig. 1.

for NF-AT activity. NF-AT binding was highly inducible in mature $CD4^+$ cells but low or undetectable in unfractionated and $CD4^+$ $CD8^+$ cortical thymocytes (Fig. 5, lanes 2, 6, and 8). These results are in full agreement with the report by Riegel et al. (34). The inducibility of NF-AT-binding activity was not restricted to cells in the final stage of T-cell differentiation and maturation, however. SCID thymocytes (as well as $CD5^{low}$ $CD4^-$ $CD8^-$ TcR^- thymocytes isolated from normal mice; data not shown) were able to mobilize NF-AT-binding activity to levels quite comparable with those of mature $CD4^+$ $CD8^-$ TcR^{high} cells. A comparative titration of nuclear extracts from stimulated $CD5^{low}$ $CD4^-$ $CD8^-$ TcR^- immature cells from normal mice and stimulated $CD4^+$ $CD8^+$ TcR^{low} cortical cells showed that the immature cells contained at least 10 times more NF-AT DNA-binding activity than do the cortical cells (data not shown). Thus, NF-AT inducibility appears to be acquired at an early developmental stage.

(iv) Developmentally distinct factors binding to the AP-1 site. Our results indicate that the major AP-1 site at −161 to −143 in the IL-2 5′ flanking region ($AP\text{-}1_p$) can be engaged by at least two differentially regulated factors, one conventionally inducible factor consisting entirely of Fos and Jun family members (AP-1 complex) and one which includes at least one CREB subunit (CREB complex). The two complexes were similar in electrophoretic mobility and could be resolved only by use of antibodies.

All thymocyte subsets contained factors capable of binding to the putative AP-1 site, and surprisingly, most did so with or without in vitro stimulation (Fig. 6a). In total thymocytes overall and in isolated $CD4^+$ $CD8^+$ TcR^{low} cells, essentially all of the $AP\text{-}1_p$ site-binding activity was attributable to the CREB complex, whether or not the cells were stimulated (Fig. 6b, lane 2; Fig. 6c, lanes 1, 2, 7, and 8). CREB complexes were present in unstimulated cells of all subsets (Fig. 6c, lanes 1, 3, 7, and 9). Levels of the CREB complex appeared to be increased by exposure of the thymocytes to culture at 37°C (data not shown) but were not enhanced by TPA plus A23187. In $CD4^+$ $CD8^-$ TcR^{high} and SCID thymocytes specifically, however, activation with TPA plus A23187 led to strong induction of an AP-1 complex (Fig. 6c, lanes 3, 4, 9, and 10). Thus, we conclude that (i) all thymocytes can activate a leucine zipper family factor including at least one CREB subunit simply in response to stimuli such as a temperature shift and (ii) immature and mature thymocyte subsets alike can activate a canonical AP-1 factor in response to Ca^{2+} ionophore and protein kinase C stimulation, but (iii) cortical $CD4^+$ $CD8^+$ TcR^{low} thymocytes are specifically disabled in their activation of the inducible AP-1 factor despite their ability to activate other factors such as the CD28RE-binding complexes and NF-κB.

DISCUSSION

Summary of developmental changes in IL-2 DNA-binding factors. The pattern of inducibility of the DNA-binding proteins studied here is summarized qualitatively in Fig. 7. As a framework for the following discussion, the data are presented as a hypothetical developmental progression, in which the average cell types in each of the fractions studied here are assumed to be linked in a precursor-product continuum. The discontinuities in the inducibility of AP-1 (Fos/Jun) and NF-AT, both at the immature-to-cortical transition and, presumably, at the cortical-to-mature transition (see below) contrast sharply with the gradual, monotonic increases in the inducibility of NF-κB and at least the majority of the complexes that bind to the CD28RE oligonucleotide. Each thymocyte subset has its own characteristic profile of these factors. The combinatorial requirement for these factors in assembling IL-2 transcription complexes accounts for the ability of immature thymocytes and the inability of their cortical thymocyte descendants to express the IL-2 genes. The most interesting aspect of these results, however, is the light that they shed on the kinds of physiological changes imposed on developing thymocytes during intrathymic processing.

Reverse maturation of function in the immature-to-cortical thymocyte transition. The results presented here show that cells in the immature population can already deploy a set of transcription factors that includes all those known to be needed for IL-2 expression in mature cells. Even NF-AT, initially described as a mature T-cell-specific factor, is already highly inducible in these cells. Any developmental changes needed to turn on NF-AT expression must therefore already have occurred at this stage, before TcR gene rearrangement. Furthermore, the signaling pathways needed to activate NF-AT and other regulatory factors already appear to be effectively indistinguishable in immature cells from those in mature T cells. Both normal $CD4^-$ $CD8^-$ TcR^- populations (data not shown) and SCID thymocytes could activate NF-AT, the AP-1 complex, and other regulatory factors comparably to mature cells in response to A23187 plus TPA. Surprisingly, costimulation with IL-1 did not appear to be required for induction of any of these activities. The only difference in DNA-binding protein activation observed in the immature cell samples (normal or SCID) when IL-1 was added was a modest enhancement of the induction of the NF-κB binding activity (data not shown). The basis for the strong observed effect of IL-1 on IL-2 RNA and protein

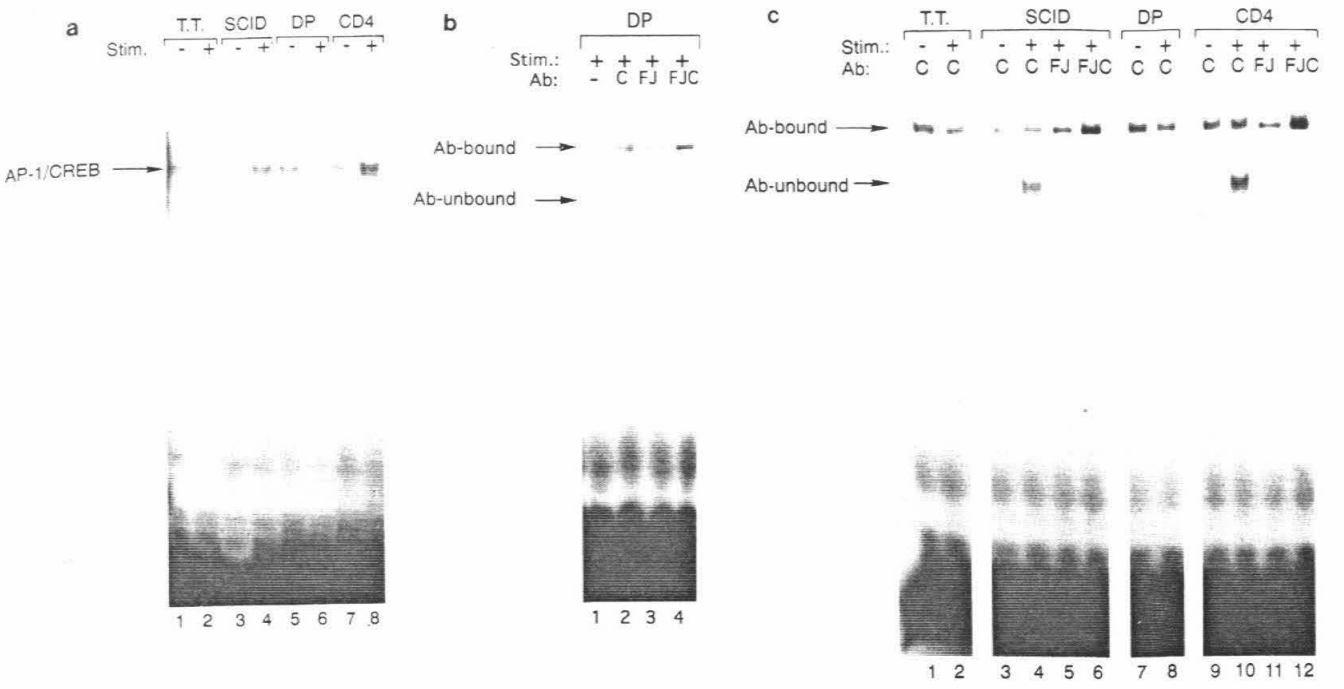

FIG. 6. AP-1/CREB proteins of thymocytes. (a) Comparison of AP-1_p site-binding activity in different thymocyte fractions. Nuclear extracts from the indicated thymocyte samples were analyzed for complex formation with the AP-1_p oligonucleotide. Abbreviations for thymocyte samples and culture conditions are as given in the legend to Fig. 1. (b) Reactivity of AP-1_p site-binding factors in cortical thymocytes with different antibodies. An extract from stimulated $CD4^+$ $CD8^+$ TcR^{low} thymocytes was used for electrophoretic mobility shift assays with the AP-1_p oligonucleotide, with the following antibodies (Ab) added: lane 1, none; lane 2, anti-CREB; lane 3, anti-Fos and anti-Jun family; lane 4, all three antibodies. Anti-CREB shifts a much larger fraction of the initial, unperturbed binding activity than does anti-Jun plus anti-Fos. The material reactive with anti-CREB defines the CREB complex. (c) Differential expression in thymocyte subsets of Fos/Jun and CREB complexes with binding activity for the AP-1_p site. Extracts from the thymocyte samples shown in panel a were analyzed for binding to the AP-1_p oligonucleotide in the presence of the following antibodies: anti-CREB (C), anti-Fos family plus anti-Jun family (FJ), and a cocktail of all three antibodies (FJC). As CREB-like factors are present in all samples, all C lanes contain supershifted (antibody-bound) complexes. The Fos/Jun or AP-1 complex referred to in the text is the material in the lanes treated with anti-CREB that remains in the antibody-unbound position. Lanes 6 and 12 verify that all this material is in fact reactive with anti-Jun and/or anti-Fos.

expression (9, 17, 39) must therefore reside elsewhere and remains under investigation. However, the results presented here clearly show that the molecular prerequisites for IL-2 producer function that we can score have already been achieved through extremely early events in T-cell development, in a process completely independent of T-cell recognition specificity.

In this context, the transition to $CD4^+$ $CD8^+$ TcR^{low} cortical thymocyte status is confirmed as a loss of response function. However, the loss is surprisingly specific. Previous work had shown that murine $CD4^+$ $CD8^+$ TcR^{low} thymocytes fail to express known response genes, like the IL-2 and IL-2 receptor alpha-chain genes, even when the TcR is bypassed with strong pharmacological stimuli (4, 8, 9, 16, 24). A priori, the cells might have been found to lack basic molecular transducers of signals such as calmodulin or appropriate isoforms of protein kinase C. Instead, the results described here show that these cells are capable of strong though selective transcription factor activation. For example, the inducibility of NF-κB in these cells makes it likely that a fully operational protein kinase C-dependent pathway is present. Thus, the dissociation of one ubiquitous phorbol ester-inducible response from another, in the selective loss of AP-1 induction while NF-κB induction is preserved, implies a precise, factor-specific mechanism rather than a cataclysmic, global change in cell physiology. There are two implications of these results. First, the reverse maturation process in which AP-1 and NF-AT inducibility are lost defines a novel transition in thymocyte development. Its possible timing and significance are discussed further below. Second, the cells in this functionally incompetent population remain highly responsive, with an ability, in principle, to integrate signals from protein kinase C, cyclic AMP/protein kinase A (50), and possibly also CD28 (14, 46) for transcription factor mobilization. Their potential to carry out efficient, if specialized, patterns of gene expression is further supported by our recent evidence that they can serve as preferential targets for transient expression of certain exogenous DNAs in transfection experiments (30). Thus, the cortical thymocytes need not be beyond rescue; in fact, the repertoire of inducible transcription factors that they preserve might participate in the poorly understood processes of positive and negative selection.

Selection and the selectable state. A most interesting possibility raised by these results is that changes in NF-AT and AP-1 inducibility may contribute to the mechanisms that (i) make thymocytes subject to selection and (ii) transform them into mature thymocytes via positive selection. Figure 7 indicates a possible temporal correlation between these important developmental transitions and the discontinuities in the use of NF-AT and AP-1. At present, this hypothesis is speculative, because it is not yet known whether the "typical" subset members depicted in Fig. 7 are truly related in a precursor-product continuum. However, the striking simi-

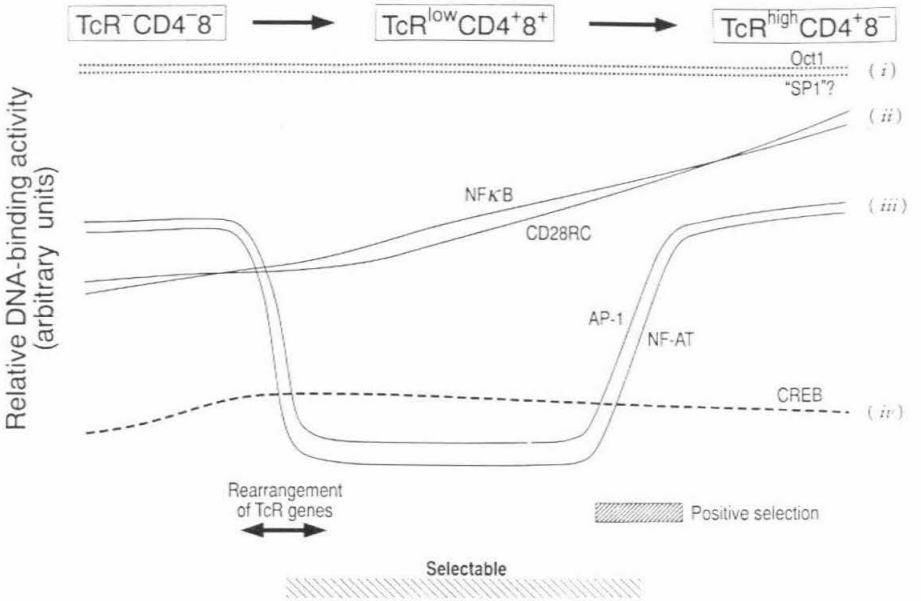

FIG. 7. IL-2 DNA-binding factors in thymocytes: a proposed developmental progression. This summary figure juxtaposes the results from Fig. 1 through 6 on the abundance of various IL-2 DNA-binding factors in three broad stages of thymocyte differentiation. The data are arranged along a horizontal axis that represents the differentiative progress of thymocytes toward maturity, with the key events that mark transitions and states of interest indicated. While the characteristics of the three stages are established by our results, the proposed connections between them are hypothetical, as discussed in the text. The vertical axis represents relative levels of DNA-binding activities of various factors, expressed on an arbitrary scale, with the results for different classes of factors offset. As the true copy numbers of different factors are not known, the values along the vertical axis in this figure cannot be used to compare one factor with another. (i), (ii), (iii), and (iv) refer to the four regulatory classes of factors distinguished in the text. · · · · , true constitutive factors. – – –, CREB, the binding activity of which is increased by culture but is not dependent on Ca^{2+}/protein kinase C activation; ——, factors whose DNA-binding activity is detectable only after Ca^{2+}/protein kinase C activation.

larities between the IL-2 DNA-binding activities available in immature and mature thymocytes suggest two possible kinds of linkages between transcription factor regulation and thymocyte fate determination. One, an alternative to the scheme shown in Fig. 7, is that the downregulation of NF-AT and AP-1 may occur only in cells that have failed positive selection and are committed to die. In this case, cortical thymocytes that remain susceptible to positive selection could be identified as a subset specifically protected from NF-AT and AP-1 downregulation, bypassing the typical cortical cell state. The other possibility, illustrated in Fig. 7, is that a relatively simple, reversible molecular switch may shut off AP-1 and NF-AT inducibility prior to any selection and restore it as a consequence of selection. In this case, the mechanism controlling NF-AT and AP-1 inducibility would define one of the molecular targets of the positive selection process. The choice between these alternative interpretations depends on the precise timing of NF-AT and AP-1 downregulation relative to TcR gene rearrangement and surface expression. As of this writing, the possibility depicted in Fig. 7 is favored by two kinds of evidence. We and others have already shown that thymocytes lose a variety of inducible gene expression responses at a stage well before their acquisition of a selectable TcR (3, 9, 37, 51). Also, our own preliminary results (7) indicate that NF-AT and AP-1 inducibility has already been lost by cortical thymocytes that are still actively in cycle, a population that clearly includes cells with full precursor activity that may yet be positively selected (15, 19, 31, 43). Thus, no evidence yet available suggests loss of activatability as a late event. If further investigation confirms the early timing of NF-AT and AP-1 downregulation, then this process must be reversible, as a direct or indirect consequence of positive selection. Work to define the biochemical basis of the changes in AP-1 and NF-AT availability is now under way.

In summary, the data from this study identify NF-AT and the AP-1 complex described here as potentially valuable probes to define the mechanisms regulating the varying responses of thymocytes to activation in the course of their development. A full understanding of their changes in activity must await the cloning of the genes encoding NF-AT components and the identification of AP-1 family members that actually participate in IL-2 regulatory complexes. However, the inducibility of these factors is at least correlated with the abrupt changes in cellular responses to signaling that define both onset of and rescue from the selectable state. Furthermore, their pleiotropic roles in response gene regulation can explain the functional effects of the regulatory changes. Whether or not their function is also implicated in the fate determination events per se, they provide likely molecular targets of the switch mechanism that transforms cell physiology to convert simple activation signals into the arbiters of irreversible developmental choice.

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Chapter 3

Characterization of AP-1 Induction in Developing Thymocytes: Differential Activation of fos Genes

Abstract

Thymocytes lose their interleukin-2 gene inducibility upon transition from $CD4^-CD8^-TCR^-$ stage into the $CD4^+CD8^+TCR^{lo}$ stage. This inducibility is restored once the double positive cells are positively selected to become mature T cells. Our previous studies showed that nuclear extracts from the stimulated $CD4^+CD8^+TCR^{lo}$ cortical thymocytes have decreased AP-1-and NF-AT-binding activities in comparison to the immature $CD4^-CD8^-TCR^-$ precursor cells and postively selected mature $CD4^+TCR^{hi}$ cells. To investigate whether the reduced nuclear factor binding activity is due to an absolute reduction of AP-1 components at the transcriptional level, we analyzed jun and fos mRNA accumulation upon stimulation in thymocytes representing different stages of development. The appearance of all three jun mRNAs is stimulation-dependent and there is no consistent variation in the levels of their expressions across development. However, the accumulation of c-fos, fosB and fra-2 mRNAs are significantly lower in stimulated cortical cells than in stimulated mature cells. Although immature $CD4^-CD8^-TCR^-$ cells also have similar low levels of c-fos expression, they can transcribe high levels of fosB and fra-2 mRNAs which are equivalent to those of mature thymocytes. There is no detectable fra-1 expression in any population of thymocytes 1 hr after stimulation. These results strongly suggest that the lack of AP-1 and NF-AT binding activities in cortical thymocytes is due to a deficiency of Fos proteins.

Introduction

The AP-1 transcription factor is composed of two families of leucine zipper proteins, the Jun family and the Fos family (Vogt and Bos, 1990; Angel and Karin, 1991). Proteins within each family share a high degree of homology at the amino acid level. Genes encoding them are categorized as immediate-early genes, since they are readily induced by growth or differentiation signals and are independent of protein synthesis. In addition to the control at transcriptional level, they are also controlled post-translationally.

As a *trans*-acting factor, AP-1 in combination with other *trans*-acting factors regulates a number of genes including c-jun through binding to the AP-1 site (TGAC/GTCA) at the regulatory sequence. Dimerization via the leucine zipper motif is required for DNA binding (Sassone-Corsi et al., 1988). The Jun family proteins can form either Jun-Jun intrafamily dimers or Jun-Fos heterodimers with Fos family proteins, while the latter can only heterodimerize with the Jun family. The dimer complex formed by different combinations of Jun-Jun and Jun-Fos may have similar binding specificities but different affinities and different *trans*-activation activities (Ryder and Bravo, 1991; Halazonetis et al., 1988). For a given Jun-Jun or Jun-Fos dimer, both DNA-binding acivities and *trans*-activation activities are subject to regulations by post-translational modification (Hunter and Karin, 1992; Smeal et al., 1991). Differential regulation of AP-1 proteins has been shown during different stages of the cell cycle (Kovary and Bravo, 1991 and 1992).

The interleukin-2 gene is one of the target genes which are subjected to AP-1 regulation along with other *trans*-activators. AP-1 activation is a target of perturbation not only in developing $CD4^+CD8^+TcR^{lo}$ thymocytes, but also in glucocorticoid-treated T cells, in anergized T cells, and in IL-1 treated T cells (Northrop et al., 1992; Vacca et al., 1992; Kang et al., 1992; Chapters 1 and 2). The 300 bp IL-2 gene 5' regulatory sequence contains at least four sites which interact with AP-1 factor, which are one AP-1 site, the two NF-AT sites and the octamer/OAP40 site. The attempt to identify which dimer combination of Jun and Fos proteins binds to each site has not been satisfactory. It has reported that c-Jun, c-Fos, FosB, Fra-1, and Fra-2 are able to bind the AP-1 site in the IL-2 promoter (Jain et al., 1992; 1994). For the NF-AT site, c-Jun, JunD, JunB, c-Fos, and Fra-1 have shown to be able to form complexes with this site *in vitro*. With the exception of JunB, they have all been shown to enhance NF-AT site driven transcription and to eliminate the TPA stimulation requirement in *in vivo* cotransfection experiments (Northrop et al., 1993; Jain et al., 1992; Hentsch et al., 1992; Boise et al., 1993). JunD and c-Jun form complexes with the octamer protein to bind Oct/OAP40 site. In addition,

JunD, c-Jun, and c-Fos, but not JunB, can contribute to the enhancer activity of a concatamerized Oct/OAP40 site when cells are stimulated with ionomycin alone without PMA (Ullman et al., 1992 and 1993). Therefore, all members have been implicated in binding to one site or another or to all of them, suggesting a possible functional redundancy.

In chapter 2, we reported the finding that $CD4^+CD8^+TcR^{lo}$ cortical thymocytes have decreased inducible AP-1 and NF-AT binding activities. It is not known which Jun or Fos proteins are physically or functionally deficient in these cells. As an initial approach to address this problem, we examined the AP-1 mRNA accumulation upon stimulation in these IL-2-noninducible cortical cells as compared with the IL-2-inducible immature $RAG2^{-/-}$ thymocytes or SCID thymocytes and mature $CD4^+TcR^+$ and $CD8^+TcR^+$ thymocytes. Similar to other types of cells, expression of jun and fos genes is induction dependent in all fractions of thymocytes. Uninduced thymocytes express no or very low levels of jun or fos mRNA. In this study, we showed that mRNA of jun family members are equally inducible in either IL-2 inducible and non-inducible thymocytes, but that three of the fos genes, c-fos, fra-2, and fosB, are differentially regulated. c-fos mRNA is poorly inducible in both immature and cortical cells, but it is significantly more inducible in mature thymocytes. More interestingly, the fosB and fra-2 mRNA are much more highly inducible in both immature and mature IL-2-producing thymocytes than in IL-2-nonproducing cortical cells. Therefore, the decreased AP-1 and NF-AT binding activities could be a result of the low fos gene activation.

Materials and Methods

Cells. Thymocytes were prepared mostly from 3 to 5-week old mice, except RAG2-/- mice which were 2-month old. Immature $CD4^-CD8^-TCR^-$ thymocytes were prepared from either $RAG2^{-/-}$ mice (From F. W. Alt) or Balb/C or C57BL/6 SCID mice. Unfractionated thymocytes were prepared from C57BL/6 mice. Mature TCR^{hi} thymocytes

were prepared from C57BL/6 mice two days after 2.5 mg hydrocortisone i.p. treatment. Cells in suspension were then cultured in RPMI1640 with 6% fetal bovine serum with supplements and antibiotics, with or without TPA and A23187 as stimuli for 1 hr.

RNA analyses. Total cellular RNA samples were prepared by guanidinium isothiocyanate/phenol-chloroform extraction methods (Chomczynski and Sacchi, 1987) and 2 μg of RNA was used for RNAse probe protection analyses as described before (McGuire and Rothenberg 1987; Miner and Wold 1991). For Northern analysis 10 μg of RNA was denatured with glyoxal and DMSO and was electrophoresed in NaH_2PO_4 buffered agarose gel. The RNA was blotted onto nylon membranes (Nytran; Schleicher and Schuell) with 7.5 mM NaOH and was fixed to the membrane by baking at 80°C for 60 min. Hybridizations were performed as described in chapter 1 (Novak et al., 1990). The c-jun and junD templates were generated by PvuII and ApaIII digestion, respectively, of the appropriate cDNA constructs (from ATCC). The junB template was made by subcloning a 5'-end EcoRI and SmaI fragment of junB cDNA (ATCC) into the corresponding sites of pSP73 (Promega). This was linearized with EcoRI. The fosB template was prepared by subcloning a PvuII/PstI fragment of fosB cDNA (a gift from Schölthal) into the same sites of pSP72 (Promega), and linearizing with XhoI. All jun family and fosB templates were transcribed with T7 polymerase to make antisense probes. The fra-1, the c-fos and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probes were generated and described by Sean Tavitigian. (Tavtigian, 1992). The fra-2 probe was an EcoRI human fra-2 cDNA fragment (a gift from Schölthal) and was labeled with random priming for Northern blot analysis.

Reagents. Both tetradecanoyl phorbol acetate (TPA) and the calcium ionophore A23187 are from Sigma (St. Louis, MO). They were used at concentrations of 10ng/ml and 200nM respectively. Interleukin-1 (Genzyme) was used at 50u/mL and added to the culture simutanously with TPA and A23187. Forskolin (Sigma) was used at 10 μg/mL and

added 15 to 30 min prior to the addition of TPA and A23187. Cortisone acetate (Merck Sharp Co.) (25mg/ml) was administered at 2.5mg/mouse, by i.p. injection.

Results

To test the possibility that the lack of inducible AP-1 binding activities in cortical $CD4^+CD8^+TcR^{lo}$ thymocytes is regulated at the transcriptional level of AP-1 components, we decided to examine the fos and jun mRNA accumulation in different populations of thymocytes. Our previous experiments indicated that both immature and mature thymocytes can be induced to express high levels of IL-2 *in vitro*. The intermediate cortical thymocytes, on the other hand, cannot be induced to do so. Therefore, RNA was prepared from the following samples: thymocytes from $RAG2^{-/-}$ or SCID mice, representing immature thymocytes, from C57BL/6 mice representing cortical thymocytes, and from hydrocortisone treated C57BL/6 mice, respresenting mature single positive $CD3^{hi}$ cells. Unfractionated normal thymocytes could be used to approximate the behavior of cortical cells because cortical type thymocytes represent the majority (~85%) and dominate the behavior of the unfractionated population (Chapter 2). These cells were collected and cultured, with or without TPA and ionomycin A23187 treatment. Most AP-1 mRNA peaks around 30 min to 2 hr (Reed et al., 1986), so we used 1 hr stimulation for our analyses. Total RNA was extracted from the cultured thymocytes treated with or without TPA/A23187 for 1 hr. RNase probe protection analyses and Northern blot analysis were used to detect the expression of jun and fos mRNA.

Similar experiments were also done with RNA extracted from thymocytes stimulated with TPA/A23187 in the presence of IL-1 or forskolin. As shown in the previous two chapters, IL-2 expression is subjected to the regulation of various signals, including cytokines like interleukin-1 and pharmacological agents like cAMP-elevating agent forskolin. IL-1 in particular is a stage-specific enhancer of IL-2 expression for immature cells, and has been shown to induce c-jun expression in a T-cell line (Mueggge et

al., 1989). It is possible that the synergistic effect of IL-1 with signals mediated through TCR to augment the IL-2 expression can be attributed to the increase in both NF-κB and AP-1 DNA-binding activities. The cAMP-elevating agent forskolin, on the other hand, exerts an antagonistic effect on signals activating IL-2 gene. Although the DNA-binding activity of AP-1 is not changed in the presence of forskolin, junB expression is increased, while the expression of c-jun and junD is reduced in EL4 cells. It is possible that the unchanged AP-1-DNA complex varies its AP-1 components by activating cAMP-dependent protein kinases. It has been shown in cotransfection experiments that junB failed to replace the TPA signal requirement to induce NF-AT or OAP40/OCT enhancer activities (Northrop et al., 1993; Ullman et al., 1992; 1993). Therefore, it is important to see whether increasing intracellular cAMP in thymocytes would have a similar effect as seen in EL4 cells or some other T-cell lines. The results are summarized below.

Levels of different jun mRNA induction are similar in all subsets of thymocytes. First, junD mRNA is equally inducible in all thymocytes. As shown in figure 1C, two specific bands, possibly due to incomplete digestion, are seen after probing with a 3' cDNA fragment . There are no significant variations in the level of mRNA accumulation among different fractions of cells (Fig. 1C, compare lanes 2, 5, and 8), nor is there any increase or decrease of induced junD expression upon stimulation in the presence of IL-1 (Fig. 1C, compare lanes 2 and 3, 5 and 6, and 7 and 8). Similarly, junB expression is also inducible in all fractions of thymocytes (Fig. 1B). However, stimulation in the presence of forskolin can further enhance junB induction in all the fractions tested (data not shown). The accumulation of c-jun mRNA is also observed in immature, cortical, and mature thymocytes after 1 hr of stimulation (Fig. 1A). Costimulation in the presence of IL-1 superinduces c-jun mRNA accumulation, which is more obvious in $RAG2^{-/-}$ and cortical thymocytes (Fig. 1A, compare lanes 2 and 3, 8 and 9). Interestingly, the $RAG2^{-/-}$ thymocytes express relatively higher levels of c-jun mRNA in comparsion to other populations. Such IL-1 induced higher expression of c-jun, however, is not observed in

SCID thymocytes, another preparation of immature thymocytes. Since c-jun mRNA is least abundant and is roughly 10 to 20-fold less than that of junD and junB mRNA, if c-jun is differentially regulated, it might likely be regulated by post-transcription modification (Smeal et al., 1991).

Differential regulation of fos family mRNA in thymocytes during development. Upon examination of c-fos mRNA accumulation, we observed highly inducible c-fos mRNA induction in mature single positive TCR^{hi} cells and only barely detectable c-fos induction in either $RAG2^{-/-}$ (data not shown), SCID or unfractionated total thymocytes (Fig. 2A). The difference is about 2 to 4-fold. Addition of either IL-1 or forskolin does not change the level of c-fos expression in mature cells, but they slighly decrease c-fos in SCID cells and in one sample of unfractionated thymocytes (Fig. 2A, compare lanes 2 and 3, 8 and 9, 11 and 12). Thus, c-fos appears to be mature cell-specific. Induction of fosB mRNA is seen in all fractions of thymocytes, and is not affected by forskolin addition (Fig. 2A and data not shown). However, there are some differences among different populations of cells when comparing the level of fosB expression. The unfractionated total thymocytes have a barely detectable basal level of fosB expression, while both immature $RAG2^{-/-}$ or SCID thymocytes and mature thymocytes have a relatively high basal level expression of fosB (Fig. 2A, compare lanes 1 and 10 with 4 and 7, and data not shown). Upon induction, immature and mature cells express roughly 3 times more fosB mRNA than total cells (Fig. 2A. compare lanes 2, 5, 8, and 11). Addition of IL-1 to the stimulation does not change fosB accumulation in most populations examined except for one preparation of unfractionated thymocytes in which decreased c-fos expression is observed (Fig. 2A, compare lanes 8 and 9). Since mature cells have consistently lower levels of GAPDH expression compared to other fractions (2.5-fold less), c-fos and fosB expression in mature cells was normalized with the average level of GAPDH in immature and unfractionated thymocytes, and similar normalization was done for fra-2 expression.

Bar graphs accompaning each fos protection analysis is used to show the relative fold of induction.

Fra-2 has been reported to be one of the immediate early genes which may be involved in IL-2 gene activation (Jain et al., 1992 and 1994). The murine fra-2 gene has not been cloned, but there is a high degree of homology between human and chicken fra-2 cDNA (Matsui et al., 1989; Nishina et al., 1990). To analyze its induction in murine thymocytes, a human fra-2 cDNA was used as the probe for Northern analysis. As shown in figure 2B, the 6 kb species represents the mouse fra-2 mRNA. The expression pattern is similar to fosB, with both immature and mature cells able to express relatively high levels of fra-2 1 hr after stimulation, while unfractionated thymocytes can only express very low levels of fra-2 (Fig. 2B, compare lanes 2, 4, and 5). Data from two independent experiments showed that there is between 3 to 8-fold higher fra-2 expression in immature thymocytes than in unfractionated thymocytes. There is no change in fra-2 induction with or without IL-1 addition (data not shown).

We also examined fra-1 mRNA expression in these cells. Unlike other AP-1 mRNA which can be induced by TPA/A23187 treatment, there is no inducible fra-1 mRNA accumulation even after 6 hr stimulation (data not shown).

Discussion

In current studies, three populations of thymocytes were prepared to represent T cells at three intrathymic developmental stages. Thymocytes from either SCID or $RAG2^{-/-}$ mice were used to represent immature $CD4^{-}CD8^{-}TCR^{-}$ thymocytes. Phenotypic analyses have shown that T cell arrest point in SCID mice is not beyond the immature stage of normal thymocyte development. Cells from $RAG2^{-/-}$ mice have also been shown to arrest at similar point (Rothenberg et al., 1993; Shinkai et al., 1992; SA Diamond & K Makabi, unpublished data). Functional analyses further confirmed that SCID cells and immature cells from normal mice are indistinguishable. Both populations can be induced to express

IL-2 in the presence of IL-1 (Rothenberg et al., 1993). In chapter two, we also showed equivalent DNA-binding activities of a set of transcription factors in both SCID and normal immature cells. Thus, we expect that normal immature cells would behave similarly to cells from SCID and RAG2-/- mice. Unfractionated thymocytes from C57BL/6 mice were used to represent cortical cells. It has been shown many times that these cells contain over 85% of cortical $CD4^+CD8^+TCR^{lo}$ cells. Besides, experiments in chapter two clearly showed that the ability to mobilize a set of transcription factors are very comparable in both induced and non-induced states. Nonetheless, the 10 to 15% non-cortical cell contamination in unfractionated total thymocytes might affect the intepretation of the result. Especially in cases where we see decreases in particular transcripts, contaminated immature or mature cells might actually account for the residue mRNA detected. In fact, if we have used enriched cortical cells for the RNA analysis, the difference seen in the expression of certain fos genes might be even bigger. Finally, for mature T cells we used cells from mice treated with cortisone, which selectively induces the apoptosis of cortical cells. FACS analysis clearly demonstrated that over 90% of cells are either $CD4^+TCR^{hi}$ or $CD8^+TCR^{hi}$ (data not shown). We did not use anti-CD8 antibody plus complement to eliminate $CD4^+CD8^+$ cortical cells, because cells obtained in this way still contain small percentage of immature cells. However, by injecting hydrocortisone we may inadvertantly vary the responsiveness of these cells to induction. But circumstantial evidence supports the validity of the results. This is because although glycocorticoid induced apoptosis in cortical cells has been shown in rat thymocytes to be accompanied by increased c-jun and c-fos induction, the response is very rapid and transient (Grassilli et al., 1992). Our cortisone treatment lasted two days, and if there is any residual induction effect left on AP-1 it would be likely seen in cells freshly isolated in the absence of further activation. However, cells in that condition do not have higher levels of either jun family gene expression when compare to other populations. In addition, these cells do not have higher levels of inducible jun family gene expression either. The higher basal levels of some fos genes in these cells are also seen in immature

cells which were not treated with cortisone. Therefore, cells from cortisone treated mice can be used to represent mature cells. Ideally, a direct comparison of mature thymocytes obtained by different methods should be done to verify the data.

So far three jun family genes and four fos family genes have been found in human, murine, and chicken systems. In the current study we examined the mRNA accumulation of these AP-1 components at different stages of developing thymocytes. The results showed that three jun mRNAs are similarly inducible in thymocyte populations representing different developmental stages, but that c-fos, fosB and fra-2 are differentially inducible in various populations of thymocytes as summarized in Table 1. It is unlikely that the reduced accumulation of fos mRNA might be due to its different induction kinetics in certain populations of thymocytes, because most of these genes are protein synthesis independent immediate early genes whose accumulations peak around 30 min to 1 hr after induction (Reed et al., 1986). The 1 hr time point we chose is a proper time to examine most of them except fra-1 which takes 6 h to induce. If the mRNA accumulation reflects the level of AP-1 proteins, the possible Jun/Fos heterodimer combinations would contain all three Jun proteins with FosB and Fra-2 in immature cells and three Jun proteins with c-Fos, FosB and Fra-2 in mature thymocytes. However, for cortical thymocytes, the low level of inducible c-fos, fosB and fra-2 mRNA may indicate the deficiency for those Fos proteins as well. Thus, it leaves only c-Jun, JunB, and JunD to form homodimers or interfamily heterodimers, which have been shown to have a lower DNA binding affinity than Jun/Fos heterodimers (Ryseck and Bravo. 1991; Halazonetis et al., 1988). The current data clearly show that the reduced AP-1 and possibly NF-AT DNA-binding activities in nuclear extracts from stimulated cortical cells can be attributed to their inability to transcribe high levels of fos mRNA upon induction. The subsequent deficiency in Fos protein production predicted from these data would result in the lack of Jun/Fos heterodimer formation, and any resulting Jun-Jun homodimers may not be sufficient to bind nor to activate the IL-2 gene. In this study we did not rule out the possibility that

AP-1 could also be regulated at the level of transcription and/or post-translational modifications. However, our findings provide a strong indication that Fos protein levels might be reduced in cortical cells. The fact that c-jun's *trans*-activation activity depends on the phosphorylation of serine residues indicates that further characterization of AP-1 proteins themselves is necessary.

The ability of c-fos to participate in IL-2 gene activation was actually demonstrated in H-2K^{b}-c-fos transgenic mice, in which T cells express constitutively high level of c-fos mRNA. Upon stimulation with anti-CD3 antibody, those T cells showed increased AP-1 site binding acitivity and produced higher level of IL-2 both at RNA and protein level than T cells from non-transgenic littermates (Ochi et al., 1994). Overexpressing Fos in cotransfection studies not only enhances the NF-AT-dependent transcription but also eliminates the dependency on TPA stimulation (Northrop et al., 1993). In our experiment, even though immature cells also lack of c-fos expression, the relative higher level of fosB and Fra-2 expressions might compensate the c-fos deficiency, and this compensation cannot be achieved in cortical cells. It is also possible that fra-2 or fosB is the actual AP-1 component which is used in both immature and mature thymocytes to activate IL-2 gene. Recent experiments on c-fos knockout mice confirmed the ability of these products to drive IL-2 expression in the absence of c-fos. Spleen T cells from c-fos deficient mice are able to make IL-2 in a level similar to T cells from wild-type mice. Biochemical analyses revealed that activation of these T cells can induce AP-1 binding, which contains FosB and Fra-2; while the induced AP-1 binding complex from wild-type T cells contains c-Fos, FosB and Fra-2 (Jain et al., 1994). Another experiment showed that c-jun is not necessary and is dispensible for T-cell development and activation. The chimeric mice generated from RAG2$^{-/-}$ blastocytes injected with c-jun mutant ES cells have normal thymocyte development and normal IL-2 production in response to stimulation (Chen & Alt, personal communication). This need not be abnormal, because it is likely that these Jun and Fos proteins have redundant functions and are interchangable, especially in the context of

lymphocyte development. The redundancy of myoD family proteins has been shown in muscle development (Rudnicki et al., 1993). As of yet there has not been an experiment to address the outcome of disrupting more than one fos or jun member. The multiple defects with osteogenesis, gametogenesis, and neurological responses seen in c-fos knockout mice and the lethality in c-jun knockout mice should not hinder the investigation, because the manipulation of blastocysts from RAG2$^{-/-}$ mice has proven to be an excellent method to analyzing genes responsible for lymphocyte development (Johnson et al., 1992; 1993; Wang et al., 1992; Chen et al., 1993). To study whether reduced levels of Fos protein in cortical cells alone causes the lost IL-2 gene inducibility, a complementation experiment can be performed by either transfecting fos genes driven by a strong promoter into cortical cells or by directly examining those cells in H-2K-c-fos transgenic animals.

How is the change in AP-1 inducibility achieved? Detailed dissection of the thymic developmental stages further subdivides immature CD4$^-$CD8$^-$TcR$^-$ thymocytes into different populations according to their surface expression of CD44, IL-2Rα, and HSA. Roughly, the progression proceeds from CD44$^+$IL-2Rα$^-$ to CD44$^+$IL-2Rα$^+$ to CD44$^-$ IL2Rα$^+$ to CD44$^-$/IL-2Rα$^-$. Functional analyses actually showed that the loss of IL-2 gene inducibility occurs at the transition from CD44$^-$IL-2Rα$^+$ to CD44$^-$IL-2Rα$^-$ (Godfrey and Zlotnik, 1993; Rothenberg et al., 1993; Rothenberg & Diamond, 1994). TcR β chain gene rearrangement occurs at the CD44$^+$IL-2Rα$^+$/CD44$^-$IL-2Rα$^+$ stages prior to loss of IL-2 gene inducibility. It is certain that successful β chain rearrangement is required for the immature to cortical transition, since the thymocytes from both SCID and RAG-1$^{-/-}$ or RAG-2$^{-/-}$ mice which are unable to rearrange their TCR or Ig genes are arrested at immature stage (Rothenberg et al., 1993; Mombaerts et al., 1992; Shinkai et al., 1992). As the loss of IL-2 gene inducibility is correlated with the TcR β rearrangement, the possibility remains that TcRβ/gp33 surface expression not only signals the cell to stop β gene and start α gene rearrangement (Groettrup et al., 1993), but also induces other changes including reduced fosB and fra-2 expression in response to activation signals. The positive selection signal,

however, may reverse the fosB and fra-2 levels and restore the AP-1 binding activity in mature T cells. Correlation of lineage differentiation with differential AP-1 expression has been observed in hematopoietic cell differentiation. In human promeylocytic HL-60 cells, differentiation toward macrophages by addition of TPA is accompanied by upregulation, while differentiation toward granulocytes by treatment of DMSO is accompanied by downregulation of AP-1 expression and its DNA-binding activity (Mollinedo et al., 1993).

It has been shown that tyrosine kinase lck is the downstream signalling molecule of TCR β chain during early thymocyte development. Increasing dosage of a dominant-negative lck transgene results in decreased differentiation of immature triple negative thymocytes to cortical double positive thymocytes (Anderson et al., 1992 and 1993; Levin et al., 1993). By crossing a TCR β chain transgenic mouse with a dominant-positive lck transgenic mouse, animals could be generated in which the otherwise immature $CD4^-CD8^-TCR^-$ cells would prematurely express TCR β and have high levels of lck activity. If the activation of fos genes in these immature cells is downregulated when compared to the cells from single transgenic mice, a direct correlation between signals mediated by TCR β-lck and the inducibility of fos genes can be established. Meanwhile, *in vitro* systems which allow double positive thymocytes bearing transgenic TCR to be positively selected in response to the target antigen and antigen-presenting cells can be used to study whether and how positive selection signals could reverse the activation of fos genes (Kaye and Ellenberger, 1992).

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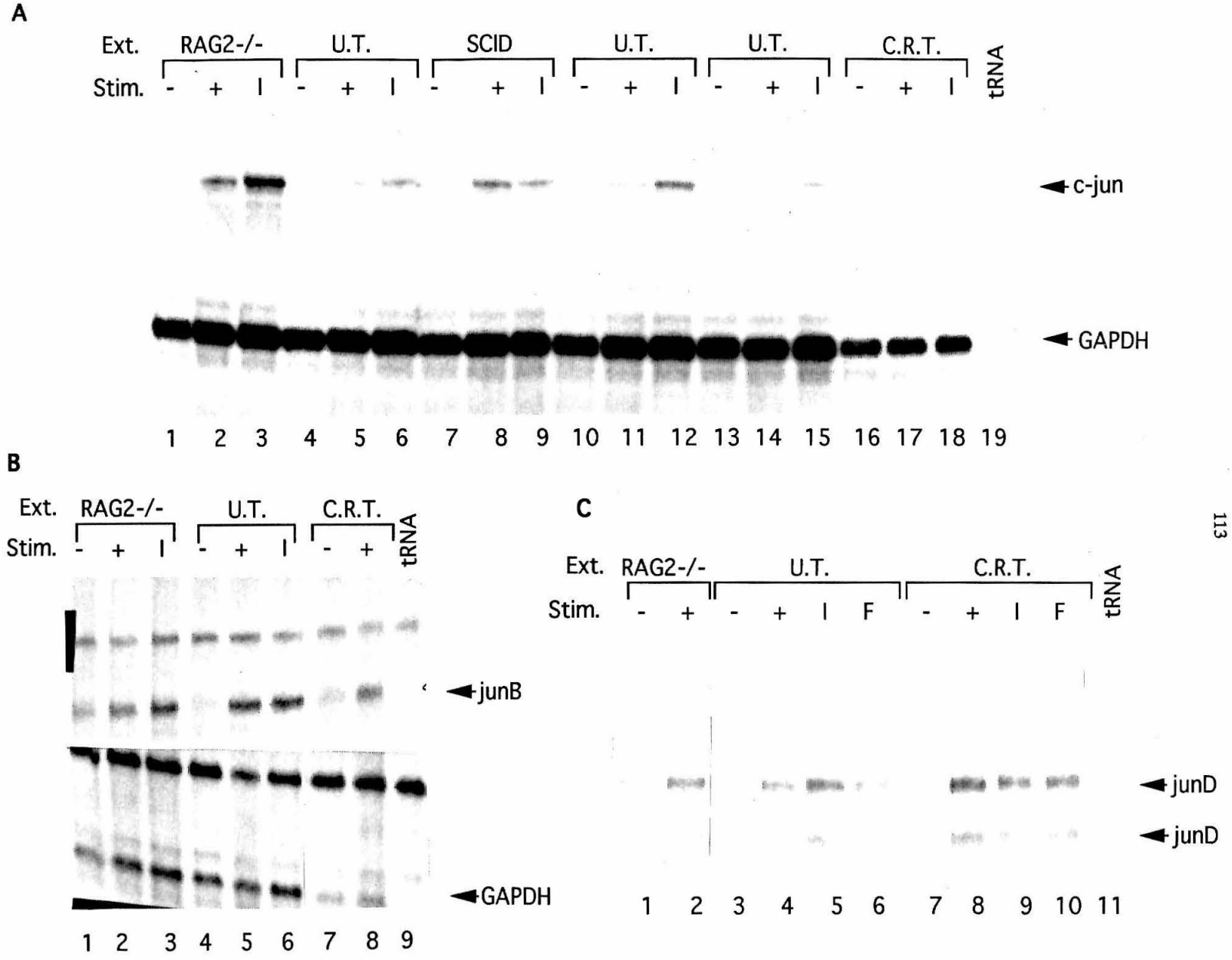
	Stimulated thymocytes		
	Immature	Cortical	Mature
IL-2 inducibility	+	-	+
AP-1 binding	++	+/-	++
c-fos induction	+	+	+++
fosB induction	+++	+	+++
fra-2 induction	+++	+	+++
c-jun induction	++/+	+	+
junB induction	++	++	++
junD induction	++	++	++

Table 1. Summary of fos and jun RNA induction in different populations of thymocytes in correlation with their IL-2 gene inducibility and the AP-1 binding activity in their nuclear extracts. Number of "+" is determined by amounts of corresponding RNA or DNA-binding acitvity relative to each population of thymocytes.

Figure legends:

Figure 1. RNase protection analysis of jun transcripts in RNA from different populations of thymocytes. A. Thymocyte RNA from RAG2-/- (lanes 1-3), from SCID (lanes 7-9), from different preps of normal C57BL/6 mice (lanes 4-6, 10-12, 13-15), and from hydrocortisone treated C57BL/6 mice (lanes 16-18) were hybridized with the c-jun and GAPDH probes. Each lane represents RNA from cells cultured without stimulation (-), with TPA/A23187 stimulation (+), or with TPA/A23187 and IL-1 stimulation (I). Lane 19 is a negative control sample with 30 μg of tRNA. B. and C. Similar RNA samples hybridized with junB and junD probes respectively. Two specific bands resolved with junD protection may be caused by incomplete digestion.

Figure 2. A. RNase protection analysis of c-fos and fosB mRNA in different populations of thymocytes. B. Northern blot analysis of fra-2 mRNA with a human fra-2 cDNA probe. A 6 kb species as indicated in the figure represents the murine fra-2 RNA. Each lane is similarly labelled as in figure 1. Bar graphs on the side show the quantitiative results from phosphoimager scanning. In the fra-2 graph, another set of SCID and U.T. data from another independent experiment is also shown.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Ext.
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U.T.
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- + I - + I - + I - + I - + I - + I
c-jun
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
B
Ext.
RAG2-/-
U.T.
C.R.T.
tRNA
Stim.
- + I - + I - + tRNA
junB
GAPDH
1 2 3 4 5 6 7 8 9
C
Ext.
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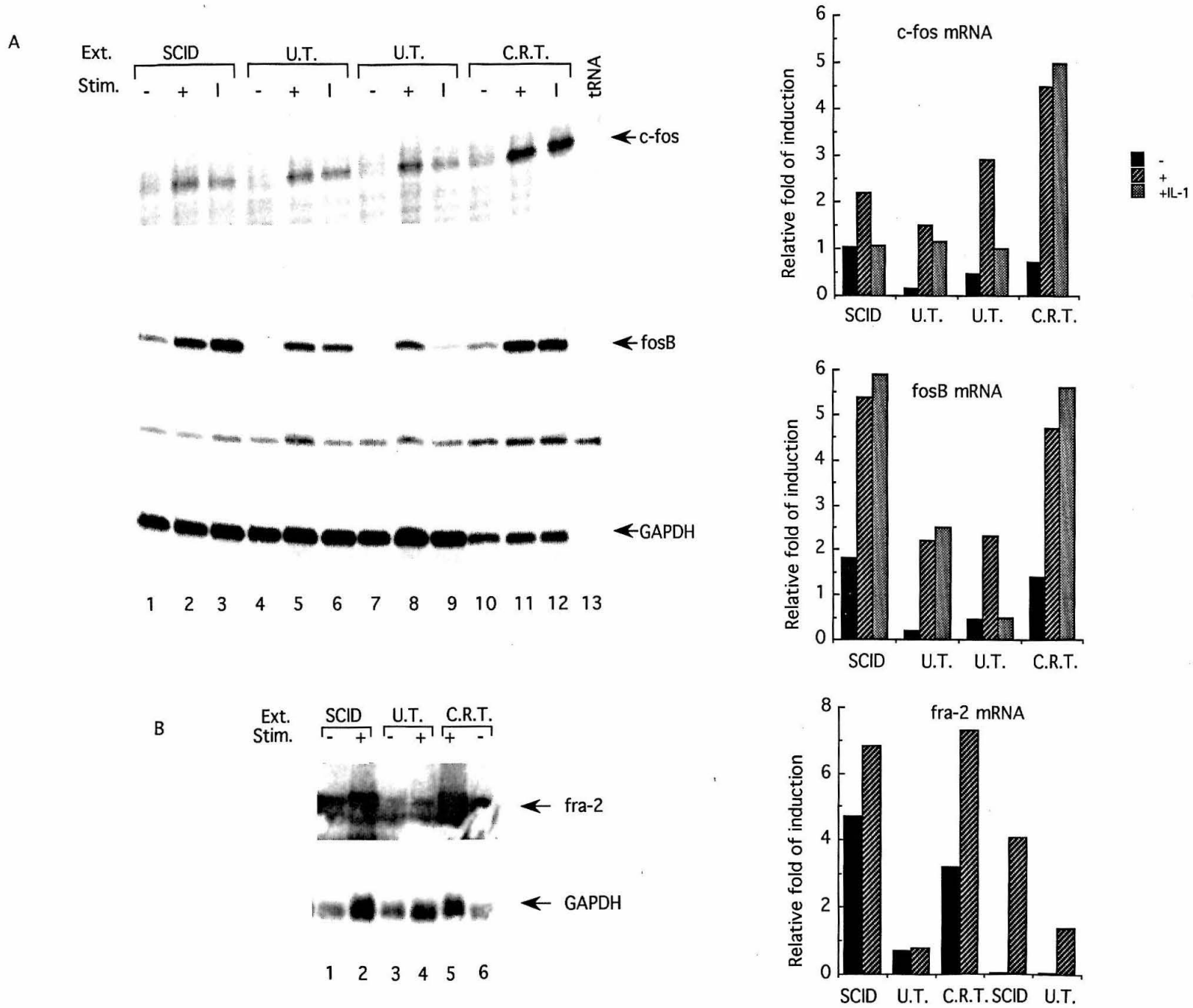
A
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- + I - + I - + I - + I
tRNA
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GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13
c-fos mRNA
Relative fold of induction
SCID U.T. U.T. C.R.T.
-
+
+IL-1
fosB mRNA
Relative fold of induction
SCID U.T. U.T. C.R.T.
B
Ext.
Stim.
SCID
U.T.
C.R.T.
- + - + + -
fra-2
GAPDH
1 2 3 4 5 6
fra-2 mRNA
Relative fold of induction
SCID U.T. C.R.T. SCID U.T.

Chapter 4

IL-2 Transcription is Regulated *in vivo* at the Level of Coordinated Binding of Both Constitutive and Regulated Factors

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Interleukin-2 Transcription Is Regulated In Vivo at the Level of Coordinated Binding of Both Constitutive and Regulated Factors

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Interleukin-2 (IL-2) transcription is developmentally restricted to T cells and physiologically dependent on specific stimuli such as antigen recognition. Prior studies have shown that this stringent two-tiered regulation is mediated through a transcriptional promoter/enhancer DNA segment which is composed of diverse recognition elements. Factors binding to some of these elements are present constitutively in many cell types, while others are signal dependent, T cell specific, or both. This raises several questions about the molecular mechanism by which IL-2 expression is regulated. Is the developmental commitment of T cells reflected molecularly by stable interaction between available factors and the IL-2 enhancer prior to signal-dependent induction? At which level, factor binding to DNA or factor activity once bound, are individual regulatory elements within the native enhancer regulated? By what mechanism is developmental and physiological specificity enforced, given the participation of many relatively nonspecific elements? To answer these questions, we have used in vivo footprinting to determine and compare patterns of protein-DNA interactions at the native IL-2 locus in cell environments, including EL4 T-lymphoma cells and 32D clone 5 premast cells, which express differing subsets of IL-2 DNA-binding factors. We also used the immunosuppressant cyclosporin A as a pharmacological agent to further dissect the roles played by cyclosporin A-sensitive factors in the assembly and maintenance of protein-DNA complexes. Occupancy of all site types was observed exclusively in T cells and then only upon excitation of signal transduction pathways. This was true even though partially overlapping subsets of IL-2-binding activities were shown to be present in 32D clone 5 premast cells. This observation was especially striking in 32D cells because, upon signal stimulation, they mobilized a substantial set of IL-2 DNA-binding activities, as measured by in vitro assays using nuclear extracts. We conclude that binding activities of all classes fail to stably occupy their cognate sites in IL-2, except following activation of T cells, and that specificity of IL-2 transcription is enforced at the level of chromosomal occupancy, which appears to be an all-or-nothing phenomenon.

Interleukin-2 (IL-2) is an important cytokine that acts on both T cells and B cells. Its synthesis is developmentally restricted to a subset of T-helper cells; in these cells, IL-2 is exclusively expressed as a transient response to stimulation. In vivo, the appropriate combination of signals to elicit IL-2 expression is usually triggered by antigen recognition (reviewed in references 36 and 49). Because much of the regulation of IL-2 is transcriptional (3, 22), the IL-2 gene has been extensively studied to learn how multiple signal transduction pathways are integrated to evoke a specific transcriptional response (45). In this study, we have focused on the mechanisms that control cell type restriction and signal dependence of IL-2 transcription.

In vivo interactions between sequence-specific DNA-binding proteins and their cognate DNA regulatory elements have been described for only a small number of genes. Even within this limited data pool, substantially different strategies are used for different genes. At one extreme, a protein-enhancer complex can be preassembled on the DNA but be inactive until receipt of an appropriate stimulatory signal or signals. This describes the activation of c-*fos* transcription in fibroblasts upon epidermal growth factor treatment (16). At the other extreme are cases like the myocyte-specific enhancer of the muscle creatine kinase gene, which is not occupied by any of the DNA-binding factors present in the nonexpressing myoblast precursors (29). Only when differentiation is triggered by decreasing levels of growth factors such as fibroblast growth factor or transforming growth factor beta do multiple regulatory factors bind to the enhancer to activate muscle creatine kinase transcription. Similar diversity can be imagined for the mechanisms controlling transcription in a cell-type-specific manner, although in the cases studied so far, including the immunoglobulin heavy-chain enhancer (9), no protein-DNA interactions have been detected in nonexpressing cell types. Since IL-2 expression is governed by both lineage restrictions that confer cell type competence and transient mechanisms that depend on activation signals, this gene might employ either mechanism or elements of both.

The 300 bp immediately upstream of the IL-2 transcriptional start site contains a minimal promoter/enhancer region which, as a whole, can drive expression in a stimulation-dependent, T-cell-restricted fashion (45). It is composed of multiple individual regulatory elements which, when assayed on their own, have strikingly different behaviors. The NF-AT recognition element binds a complex factor assembled in the nucleus only in activated T cells (38). When multimerized, this element drives expression that is largely restricted to T cells and is activation dependent (47). The NF-κB and AP-1 elements also drive expression in stimulated but not unstimulated T cells (1); however, these elements are also active and signal responsive in other cell types (15). Two CACCC motifs likely act as

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constitutive, general elements (5). Finally, the IL-2 regulatory region contains a site, octamer (OCT)/OAP40, which binds the relatively ubiquitous octamer family proteins (15, 18), adjacent to the more restricted, stimulation-dependent OAP40 protein (44). The OCT/OAP40 unit functions as a T-cell-specific and stimulation-dependent activator site, though the octamer factor component is capable of binding to DNA constitutively (1, 18, 44). We and others have previously shown that different subsets of these factors are mobilized in T cells as a function of the use of different combinations of stimuli or in T-lineage cells of different developmental states (5, 8, 14, 19, 23, 30, 33). In general, conditions that permit activation of only a subset of factors do not allow IL-2 expression. The importance of interactions between multiple sites to create the IL-2 transcriptional pattern is also evident from mutational studies. For example, disrupting individual *cis* elements, such as the NF-AT, AP-1, or OCT/OAP40 site, leads to 4- to 20-fold decreases in expression (7, 17, 32). These data suggest extensive functional collaboration between regulatory elements of diverse character, but the mechanism of their collaboration is not known. This functional collaboration could be at the level of enhancer occupancy by these factors or at the level of factor activity or a combination of the two.

In this work, we investigate the mechanistic basis of IL-2 regulation by determining the pattern of protein-DNA interactions at the IL-2 locus in living cells. For simplicity, we have focused on a few well-defined cellular environments in which the presence of distinct but overlapping spectra of IL-2 DNA-binding activities are associated with dramatic all-or-none effects on IL-2 expression. Three different cell types were compared: lineally related cells which can or cannot be induced to transcribe IL-2 as well as an unrelated cell type which does not make IL-2. We also make use of cyclosporin A (CsA) to test how specific factors affect both establishment and maintenance of an active transcriptional complex. The results indicate that diverse DNA-binding activities that participate in controlling IL-2 expression collaborate at the level of stable occupancy of IL-2 regulatory sequences.

MATERIALS AND METHODS

Cell culture. EL4.E1.F4 (EL4) thymoma cells and 32D clone 5 (32D) premast cells were grown as described in reference 31, and L cells were grown as described in reference 12. EL4 cells and 32D cells were induced with 10 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml and 180 nM A23187. When inductions were performed in the presence of CsA, CsA was used at a concentration of 0.5 μg/ml.

RNA preparation and measurements. Total RNA was prepared by the guanidinium isothiocyanate-organic extraction method of Chomczynski and Sacchi (6), and RNase protection analysis was performed as previously described (24, 26). RNA probes were as previously described for IL-2 (24), metallothionein I (MT-I) (28), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (41). Five micrograms of total RNA was used in the IL-2 analysis, 1 μg was used in the MT-I analysis, and 1 μg was used in the GAPDH analysis.

In vivo and in vitro DMS-piperidine treatment of DNA. Adherent L cells were treated as described in reference 28. EL4 and 32D suspension cells in medium (10^7 to 10^8 in 50 ml) were pelleted at 500 × *g* for 5 min at room temperature. Sufficient medium was left behind to allow resuspension of the cell pellet in a final volume of 1 ml. Cells were transferred to a 1.5-ml microcentrifuge tube and placed in a 37°C water bath; 10 μl of a freshly made 10% dimethyl sulfate (DMS)–90% ethanol solution was added, and the sample was mixed by gentle inversion. After incubation at 37°C for 1 min, cells were transferred to a tube containing 49 ml of ice-cold phosphate-buffered saline (PBS) and centrifuged at 500 × *g* for 5 min at 4°C. The cell pellet was resuspended by gentle pipetting in 1 ml of ice-cold PBS, 49 ml of additional ice-cold PBS was added, and the sample was centrifuged at 500 × *g* for 5 min at 4°C. The cell pellet was resuspended in 0.3 ml of ice-cold PBS and then added to 2.7 ml of lysis solution (300 mM sodium chloride, 50 mM Tris [pH 8.0], 25 mM EDTA [pH 8.0], 200 μg of proteinase K per ml, 0.2% sodium dodecyl sulfate). DNA was prepared as previously described (27). Naked DNA preparation and in vitro DMS treatment were done as described in reference 28 except that 0.125% DMS for 2 min at room temperature was used. Subsequent piperidine cleavage was performed as previously described (27).

LMPCR visualization of genomic footprints. Ligation-mediated PCR (LMPCR)-aided DMS in vivo footprinting was carried out as detailed previously (12). Note that the activity of each unit of Vent polymerase as provided by the manufacturer (New England Biolabs) has increased in the time since the experiments in reference 12 were done, and thus 0.5 U of Vent polymerase is now used in the first-strand synthesis, 1.0 U is used in the PCR amplification step, and 1.0 U is used in labeling step. Oligonucleotides used in LMPCR to detect interactions on the noncoding strand of IL-2 were primer 1 (CTATCTCCTCTTGCGTTT-GTCCACC), primer 2 (TGTC CACCACAACAGGCTGCTTACAGGT), and primer 3 (CA CCACAACAGGCTGCTTACAGGTTCAGGATG). Coding-strand IL-2 oligonucleotides were primer 1 (GGACTTGAGG TCACTGTGAGGAGTG), primer 2 (CAAGGGTGATAGG CAGCTCTTCAGCATG), and primer 3 (CAAGGGTGATA GGCAGCTCTTCAGCATGGGAG). LMPCR hybridization temperatures for both primer sets were as follows: primer 1, 60°C; primer 2, 69°C; and primer 3, 72°C. The coding-strand MT-I oligonucleotides were primer 1 (CGGAGTAAGTG AGCAGAAGGTACTC), primer 2 (GGAGAAGGTACTC AGGACGTTGAAG), and primer 3 (GAAGGTACTCAGG ACGTTGAAGTCGTGG). LMPCR hybridization temperatures were as follows: primer 1, 60°C; primer 2, 66°C; and primer 3, 69°C.

Quantitation of band intensity was performed by using an LKB UltroScan XL laser densitometer and recording peak heights. Fixed and dried gels were exposed to XAR-5 film without an intensifying screen. Multiple exposures of two independent in vivo footprint experiments were quantitated, using three scans per lane. Protections and hypersensitivities indicated in Fig. 4 ranged in intensity from 25 to 65% protection and from 25% to twofold hypersensitivity in the induced EL4 cell samples compared with both naked DNA samples and uninduced EL4 cell samples; these were all observed in both experiments. Comparison of band intensities among in vivo samples from nonexpressing cells indicated that all were within 7%. Not marked in Fig. 4 are several G residues on the coding strand near −100 and −200 which were between 20 and 25% hyperreactive in all in vivo samples from nonexpressing cells compared with naked DNA (see text).

Electrophoretic mobility shift assays. Nuclear extracts were prepared as described previously (40) from the same cell populations as used in the in vivo footprinting experiments. The mobility shift assay was performed as described previously (5), using 5 μg of nuclear extract, 0.5 μg of poly(dI-dC), and approximately 5 fmol of the appropriate ^{32}P-labeled oligonucleotide. Antibody supershift experiments were performed as described previously (5) with anti-CREB serum provided by Marc Montminy and anti-Fos family and anti-Jun family sera provided by Rodrigo Bravo. As preimmune serum controls

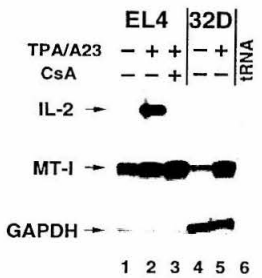

FIG. 1. RNase protection analysis of IL-2, MT-I, and GAPDH transcripts in RNA from the cell populations used in Fig. 2, 3, 4, and 7. RNA from unstimulated EL4 cells (lane 1), EL4 cells stimulated with TPA and A23187 for 7 h (lane 2), EL4 cells stimulated for 7 h in the presence of CsA (lane 3), unstimulated 32D cells (lane 4), and 32D cells stimulated with TPA and A23187 for 2 h (lane 5) was used. IL-2 RNA was also easily detected in EL4 cells stimulated for 2 h (data not shown). Lane 6 contained only 30 μg of tRNA.

were not provided, we verified the specificity of these reagents by their lack of reaction with NF-κB and CACCC site-binding complexes. Oligonucleotides were labeled by end filling with Klenow polymerase. The sequence of one strand is given for each oligonucleotide used (bases not present in the IL-2 gene are in lowercase): distal CACCC (−300 to −278), gatcTCTCCACCCCAAAGAGGAAAATTgatc; proximal CA CCC (−72 to −52), gatcACATCGTGACACCCCCATATTg atc; octamer (−100 to −69), gatcTCTTTGAAAATATGTGT AATATGTAAAACATgatc; AP-1 (−161 to −143), AATT CCAGAGAGTCATCAG; TGGGC (−237 to −218), gatcC ACCTAAGTGTGGGCTAACgatc; NF-κB (−211 to −192), AAAGAGGGATTTCACCTAAAT; distal NF-AT (−289 to −260), AAGAGGAAAATTTGTTTCATACAGAAGGCG AAT; and proximal NF-AT (−147 to −120), gATCAGAAG AGGAAAAACAAAGGTAATGCgatc.

RESULTS

To examine the mechanisms used to activate IL-2 transcription in T cells as well as mechanisms that may be used to keep it inactivated in nonexpressing cells, we determined the pattern of protein-DNA interactions at the IL-2 locus in three different types of cells. EL4 thymoma cells were used as a model IL-2 producer cell type, as 70 to 80% of these cells can be induced to express IL-2 synchronously (24). Upon stimulation with the phorbol ester TPA and the calcium ionophore A23187, which mimic many of the physiological effects of antigen stimulation, EL4 cells show many of the same gene induction events as antigen-activated T-helper cells do. As in normal T cells, IL-2 transcriptional induction in EL4 cells is sensitive to CsA treatment (33). To examine why the IL-2 gene is not expressed in other hematopoietic cell types, 32D premast cells were chosen because they respond to TPA and ionophore stimulation with some of the same gene induction responses as EL4 cells but fail to induce IL-2 (31, 32). Finally, L-cell fibroblasts were studied to examine mechanisms that may be used to keep the IL-2 gene inactivated in nonhematopoietic cells (data not shown). RNase protection analysis was performed to confirm the expression pattern of IL-2 in the various cell populations with and without stimulation (Fig. 1), using the same cell preparations analyzed in the in vitro DNA binding and in vivo footprinting experiments described below.

Multiple factors that bind sites in the IL-2 promoter/enhancer in vitro are present in the nuclei of cells that do and cells that do not express IL-2. The presence of nuclear factors that can bind to the IL-2 promoter/enhancer is a prerequisite for the assembly of a transcription complex on the IL-2 gene in vivo. Previous studies using in vitro DNA footprinting have already established that large regions of the IL-2 enhancer can be bound by nuclear factors from cells that cannot make IL-2, such as HeLa cells (4, 37). The identity, abundance, and relevance to IL-2 expression of the factors responsible for such binding are unknown. To establish the availability of specific factors implicated in the control of IL-2 expression, nuclear extracts were prepared from the same EL4 T cells and 32D premast cell populations examined above. These nuclear extracts were then tested to monitor and identify the factors capable of binding sites in the IL-2 promoter/enhancer in vitro by using gel mobility shift assays. The binding activities detected in EL4 T cells (Fig. 2) can be placed into three categories: constitutive, stimulation dependent (inducible), and stimulation dependent but CsA sensitive. Oligonucleotides

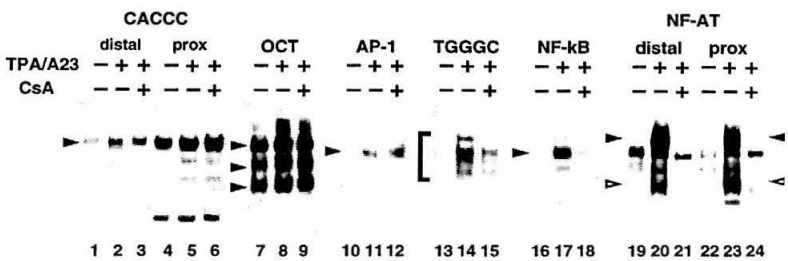

FIG. 2. Characterization of nuclear factors present in EL4 T-lymphoma cells by electrophoretic mobility shift assay. Nuclear extracts were prepared from the same populations used in the RNase protection and in vivo footprinting experiments. The ^{32}P-labeled oligonucleotide used in each experiment is indicated at the top. Arrowheads and brackets mark the major binding activities, as discussed in the text. The major complex for either CACCC motif can be competed for by the oligonucleotide containing the other CACCC motif or, even more efficiently, by a consensus SP1 oligonucleotide (5) (data not shown). The unmarked, inducible lower complex in lane 2 can be competed for by the distal NF-AT oligonucleotide and represents binding of factors to the portion of the distal NF-AT site present in the distal CACCC oligonucleotide (data not shown). Additional, faster-migrating complexes were detected in lanes 4 to 6. None were CsA sensitive, and the most prevalent species was constitutively expressed. In lanes 7 to 9, OAP40-binding activity could not be detected in these experiments, probably because of the abundance of octamer-binding species in EL4 cell extracts. In lanes 16 to 18, a faster-migrating, minor NF-κB site-binding species which was inducible and partially CsA sensitive like the major species was detected. In lanes 19 to 24, in addition to the well-characterized, inducible, CsA-sensitive NF-AT complex (filled arrowheads), a faster-migrating species with similar regulatory characteristics (open arrowheads) was observed for both NF-AT sites. Additional, constitutive NF-AT motif-binding activities were also detected in these EL4 cells. prox, proximal.

spanning the CACCC sites in the IL-2 promoter bound nuclear factors in extracts from all cell preparations (Fig. 2, lanes 1 to 6). The most prominent CACCC activities were neither stimulation dependent nor CsA sensitive. Similarly, the octamer (NF-IL2A)-binding activities in these cells were neither stimulation dependent nor CsA sensitive (lanes 7 to 9). The proximal AP-1 site was bound by factors which were inducible but were not CsA sensitive (lanes 10 to 12). By contrast, both the distal and proximal NF-AT-binding sites, as seen previously by others (8, 33), showed stimulation-dependent binding activity that was highly CsA sensitive (lanes 19 to 24). The NF-κB site bound inducible factors whose binding activities, under our conditions, were also much reduced by CsA (lanes 16 to 18). Finally, a newly identified TGGGC element (see below) bound inducible activities with partial CsA sensitivity (lanes 13 to 15). Thus, multiple factors capable of binding regulatory elements in IL-2 DNA are present in the nuclei of unstimulated, stimulated, and CsA-treated stimulated EL4 T cells.

Similar assays were done to determine whether factors recognizing IL-2 regulatory elements were present in the 32D premast cells before or after excitation of signal transduction pathways (Fig. 3). 32D cell nuclear extracts contained constitutive activities capable of binding the CACCC and octamer motifs at levels similar to those in EL4 cells (Fig. 3A, lanes 1 to 9; see below). Both constitutive and signal-responsive factors in 32D cell extracts bound to the NF-κB and TGGGC sites (lanes 10 to 15). At both sites, the most prominent of the inducible complexes were at least as prevalent as those in EL4 cell extracts. 32D cells also contained diverse constitutive and inducible AP-1-binding activities (Fig. 3B; see below). Thus, 32D cell nuclei contain multiple, sequence-specific factors, some of which are inducible, that bind elements within the IL-2 regulatory region in vitro.

Although many sites have binding activities in both cell types examined here, previous studies have shown qualitative distinctions among protein complexes at some individual IL-2 regulatory elements. Moreover, expression of IL-2 has been correlated with the relative prevalences of these different activities (5, 19). Here, comparison of 32D cell factors with EL4 cell factors revealed such differences in the complexes binding at four sites. First, the two more rapidly migrating octamer-binding activities found in EL4 cells, apparently corresponding to the Oct-2 species observed in other cell types (18), were not observed in 32D cells (Fig. 3A, lanes 7 to 9). However, this may not be very significant for IL-2 transcription, as these species are also absent from subsets of thymocytes which are competent to transcribe IL-2 (5). A second and potentially more interesting qualitative difference was at the AP-1 site (Fig. 3B and C). We have previously noted that although several distinct factors can bind this site, all yield complexes with similar migration characteristics in band shift assays (5). These factors can, however, be distinguished by the use of specific immune reagents. In Fig. 3B and C, antibodies against CREB (13), Fos family, and Jun family (21) proteins were used to characterize the complexes binding at this site. As in thymocytes, the majority of activity in uninduced 32D cells is immunologically related to the cyclic AMP-responsive CREB factor (Fig. 3B, lanes 1 and 2, and data not shown). Upon 32D cell stimulation, an activity that reacted with Fos and Jun family-specific antisera was induced (Fig. 3B, lanes 3 to 6). By contrast, extracts from stimulated EL4 cells contained relatively little of the CREB-related factor (Fig. 3C, lane 2) and higher levels of Fos and Jun family activities (Fig. 3B, lane 9; Fig. 3C, lanes 3 and 4). NF-κB site-binding activities also differed between 32D and EL4 cells. In addition to an apparently common major binding species, 32D cells contained elevated levels of a more rapidly migrating complex. Its migration characteristics suggest that it may correspond to the NF-κC activity previously proposed to play a negative role in IL-2 transcription (19). Finally, gel mobility shift assays showed that the inducible NF-AT activity observed in EL4 cells is greatly reduced in 32D cells (Fig. 3A, lanes 16 to 18).

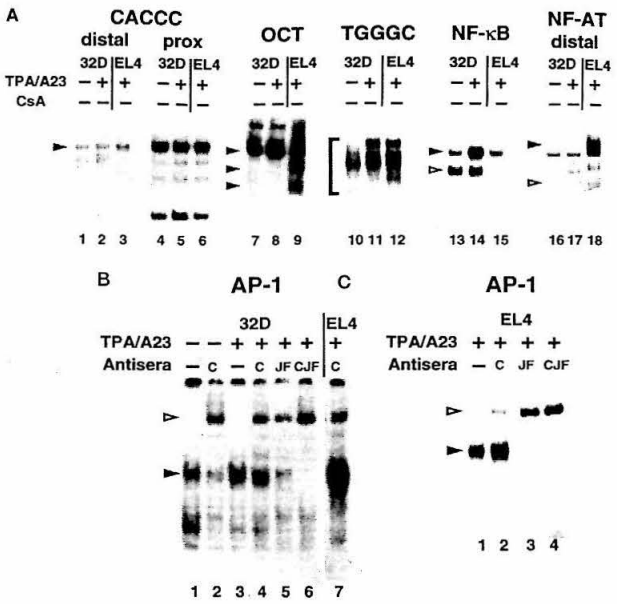

FIG. 3. Characterization of nuclear factors present in 32D premast cells by electrophoretic mobility shift assay as described in the legend to Fig. 2. To facilitate comparisons, nuclear extracts prepared from stimulated EL4 cells were examined in parallel with the 32D extracts. (A) Comparison of factors in unstimulated 32D cell nuclear extracts, stimulated 32D cell nuclear extracts, and stimulated EL4 cell nuclear extracts. Arrowheads and brackets mark the major binding activities, as discussed in the text. prox, proximal. (B and C) Perturbation of complexes formed at the AP-1 site with specific antisera. For perturbation of gel shift complexes with antibodies against CREB (C), Fos family (F), and Jun family (J) proteins, samples of nuclear extracts were preincubated with the appropriate antibodies (13, 21) exactly as described by Chen and Rothenberg (5). Nuclear extracts prepared from unstimulated 32D cells (panel B, lanes 1 and 2), stimulated 32D cells (panel B, lanes 3 to 8), and stimulated EL4 cells (panel B, lane 9; panel C, lanes 1 through 4) were used. Filled arrowheads denote the major binding species in extracts not exposed to antisera, and open arrowheads denote the major antibody-reactive supershift complexes. Neither supershift complexes nor inhibition of binding was observed when these antibodies were assayed for perturbation of complexes with the NF-κB site (data not shown). The prevalent, faster-migrating species in panel B, lane 1, was not observed reproducibly.

Taken together, the cell type differences and similarities in individual binding activities emphasize several points. First, although the difference in NF-AT-binding activity correlates nicely with the demonstrated importance of its sequence element for IL-2 transcription, there are numerous other quantitative and qualitative differences in the sets of binding factors in 32D cells compared with EL4 cells. Second, these differences involve not only regulatory factors thought to act positively but also potential negative regulators. Finally, the multiplicity of factors suggests that only a subset of the factors detected in vitro are assembled in the active complex in vivo. Moreover, the factors that are relevant in vivo may, at least in

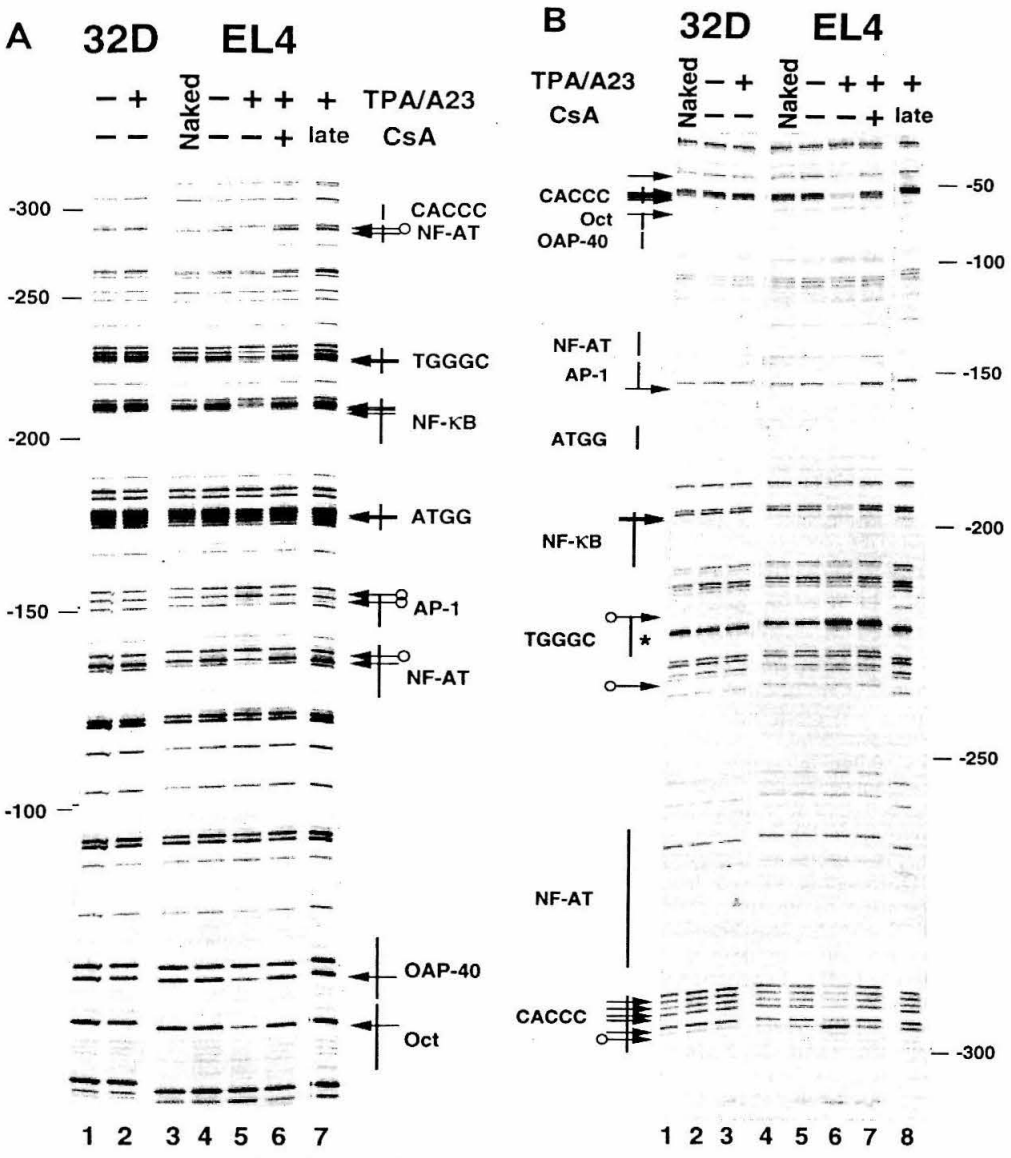

FIG. 4. DMS in vivo footprint of the IL-2 regulatory region. DNA from in vivo DMS-treated unstimulated 32D cells (panel A, lane 1; panel B, lane 2), stimulated 32D cells (panel A, lane 2; panel B, lane 3), unstimulated EL4 cells (panel A, lane 4; panel B, lane 5), stimulated EL4 cells (panel A, lane 5; panel B, lane 6), EL4 cells stimulated in the presence of CsA (panel A, lane 6; panel B, lane 7), and EL4 cells stimulated for 2 h prior to the late addition of CsA (panel A, lane 7; panel B, lane 8) was used. Naked 32D DNA (panel B, lane 1) and naked EL4 DNA (panel A, lane 3; panel B, lane 4), each treated with DMS in vitro, were also used. The in vivo footprint patterns of the coding (A) and noncoding (B) strands are shown. Location in the sequence ladder with respect to the major start site of IL-2 transcription is indicated at the inside edge of each panel. Bands marked with arrows were reproducibly more than 25% protected (plain arrow) or more than 25% hypersensitive (arrow with circle) in induced EL4 cells compared with uninduced EL4 cells and naked EL4 DNA. Weak interactions apparent in the experiment shown but not reproducibly observed over the multiple independent experiments performed (e.g., a protection near −100 on the coding strand) are not marked. An artifactual band not predicted from the sequence of IL-2 (asterisk) was seen when the noncoding-strand primer set was used. It became approximately 10-fold more intense than any other band in the ladder under LMPCR conditions which gave generally high background (e.g., with use of excess Vent DNA polymerase; data not shown). Its intensity correlated in no way with the identity of the DNA sample (data not shown).

some cases, be species other than the quantitatively dominant ones observed in vitro.

Determining the pattern of protein-DNA interactions at the IL-2 locus in vivo. To detect in vivo protein-DNA interactions, in vivo footprinting was performed by treating intact cells with the membrane-permeable alkylating agent DMS (9). DMS detects proteins bound to DNA at G residues in the major groove. Protein occupancy can protect individual G residues

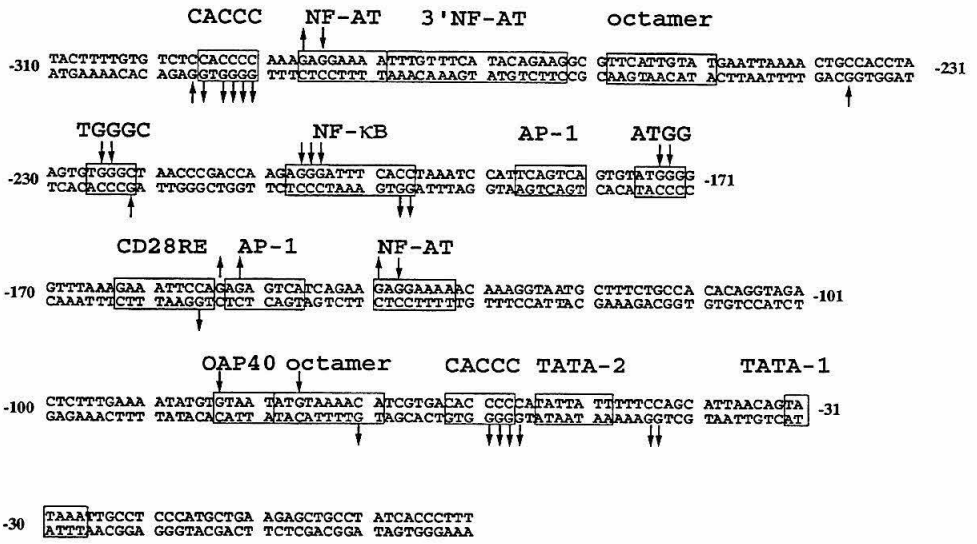

FIG. 5. Summary of protein-DNA interactions detected in stimulated EL4 cells. Downward arrows denote protected bases; upward arrows denote hypersensitive bases. CD28RE, CD28 response element.

within its recognition site from reaction with DMS or, occasionally, render additional G residues within that site hypersensitive to DMS. After alkylation with DMS, subsequent treatment with piperidine was used to produce DNA cleavage at the methylated G's. When appropriately visualized, this results in a G-specific sequence ladder. In parallel, unmodified DNA was purified from cells and deproteinized, and the naked DNA was then treated with DMS. DMS footprints were revealed by comparing the patterns of G cleavage (G ladders) of DNA treated with DMS within different living cell populations (in vivo samples) with each other and with the pattern of G cleavage of naked DNA samples. The DMS footprints, consisting of protected G residues and associated G hypersensitivities, were visualized by LMPCR genomic footprinting (29) as described previously (12).

Stimulation of EL4 T cells results in the coordinated binding of multiple proteins to the IL-2 promoter/enhancer. The pattern of in vivo protein-DNA interactions at the IL-2 locus was assessed by quantitatively comparing band intensities in different in vivo DMS-treated and naked DNA samples (Fig. 4). First, note that many sites in the IL-2 promoter/enhancer showed footprints in EL4 T-lymphoma cells upon induction. Comparison of the G ladder generated from IL-2-transcribing, induced EL4 T cells (Fig. 4A, lane 5; Fig. 4B, lane 6) with the G ladder from the same cells prior to induction (uninduced EL4 T cells; Fig. 4A, lane 4; Fig. 4B, lane 5) revealed many differences in DMS reactivity. Each interaction, indicated at the margins of Fig. 4 and summarized in Fig. 5, involves just a few specific G residues, as expected for a DMS footprint. These footprints were highly reproducible in multiple independent experiments.

Each interaction corresponds to a sequence-specific binding activity that can be detected in vitro in an electrophoretic mobility shift assay, with the exception of one site not yet tested. In vivo footprints were found at previously identified NF-AT (both −280 and −135), AP-1 (−150), OCT/OAP40 (−70 to −85), and NF-κB (−200) recognition sites, all of which have been shown to be functionally important by *cis*-element mutagenesis experiments (2, 7, 17, 37, 44). In vivo footprinting also confirmed the occupancy of a CACCC site (−294) described elsewhere (5) and revealed interactions at two previously unidentified elements, a proximal CACCC motif (−60) and a TGGGC site (−225). The ability of each of these elements to bind sequence-specific factors from nuclear extracts in vitro was demonstrated in Fig. 2 and 3. An additional interaction was detected at an ATGG site (−175), which has not yet been examined in vitro. Finally, an interaction was seen between the two IL-2 TATA boxes, which may reflect the assembly of the basic RNA polymerase II machinery in that region. Occupancy of all these sites was detectable after 1 h of induction and was maximal by 2 h (Fig. 6A and data not shown). Representative footprints at the distal NF-AT site, the NF-κB site, and the proximal AP-1 site after 2 and 5 h of induction are shown in Fig. 6A. Footprints persisted at maximal levels until at least 11 h after induction, as shown in Fig. 6B for the proximal AP-1 site, the proximal NF-AT site, and the OCT/OAP40 site.

Conversely, several other potential regulatory elements were not detectably occupied in the major groove in vivo. We failed to detect contacts at the G residues in the distal octamer motif at −255, the distal AP-1 motif at −180, and the region 3′ of the distal NF-AT core at −280 (3′ NF-AT site), whose counterparts in the human gene, though not perfectly homologous to the murine elements, have been shown to contribute to the function of the human IL-2 gene (17, 18, 42). In these cells, no clear evidence was found for the binding of factors to the CD28 response element either, although protection was seen at the junction of this site with the proximal AP-1 site. This is consistent with observations that the CD28 response element plays no role in IL-2 induction in response to TPA-A23187 induction in the human Jurkat T-cell line (11). Though in vivo footprints were performed up to 24 h after induction (data not shown), no major groove interactions were detected at the G residues in the putative negative element at −105, proposed to decrease induced transcription of the human IL-2 gene by functioning to shut off IL-2 transcription at later time points (50). Thus, for several potential regulatory elements, we

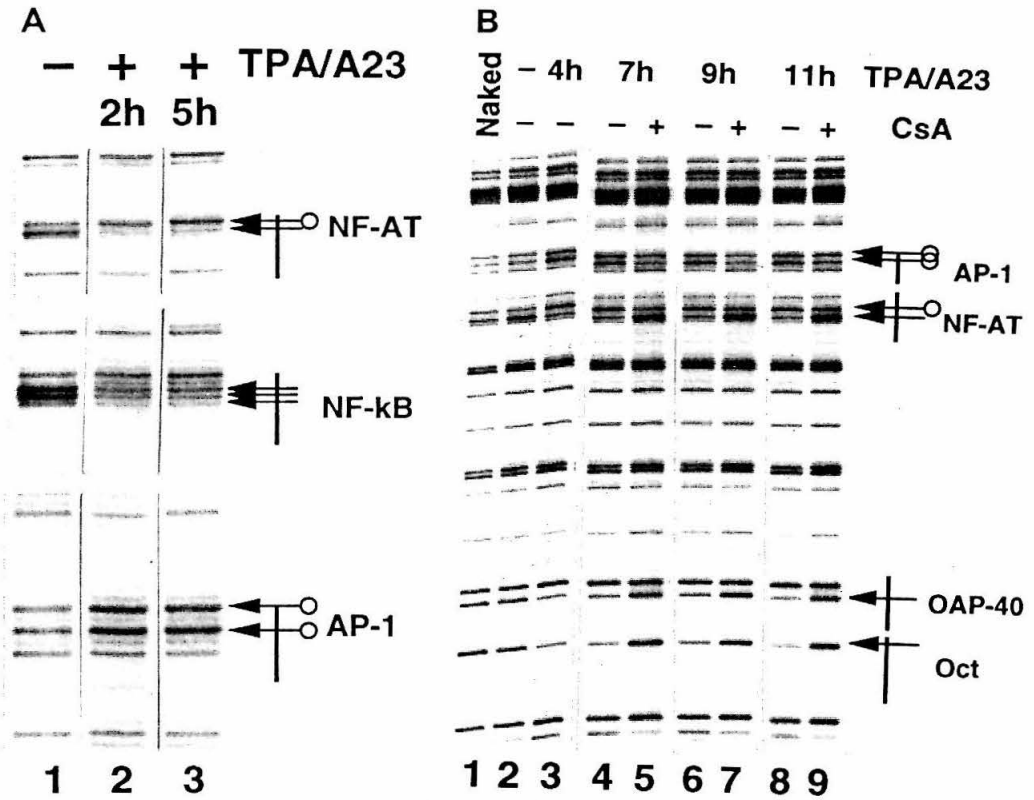

FIG. 6. (A) DMS in vivo footprints over representative binding sites at 2 and 5 h of induction. DNA from in vivo DMS-treated uninduced EL4 cells (lane 1), EL4 cells induced for 2 h (lane 2), and EL4 cells induced for 5 h (lane 3) was used. Lane 2 was exposed for 20% longer than lanes 1 and 3 to compensate for underloading of that lane. (B) DMS in vivo footprints over representative binding sites between 4 and 11 h of stimulation. DNA from in vivo DMS-treated unstimulated EL4 cells (lane 2), EL4 cells stimulated for 4, 7, 9, and 11 h (lanes 3, 4, 6, and 8), and EL4 cells stimulated in the presence of CsA for 7, 9, and 11 h (lanes 5, 7, and 9) was used. Naked EL4 DNA (lane 1), treated with DMS in vitro, was also used. The entire set of interactions shown in Fig. 4 was seen in these experiments. All were of equal relative intensity in the induced samples, but only a subset are shown here.

see no in vivo evidence that they play a role in IL-2 transcriptional regulation in mouse EL4 T cells.

No protein-DNA interactions were detected in unstimulated EL4 T cells, 32D premast cells, or L cells. In principle, cells that are not transcribing IL-2 might have stably bound repressors at the IL-2 locus or partially assembled enhancer complexes. Furthermore, a developmentally committed but as yet uninduced IL-2 producer (EL4 T cell) might reveal its committed status in the form of a pattern of protein-DNA interactions different from that of an IL-2 nonproducer (32D cell or L cell). Finally, an activated 32D cell might in principle assemble partial protein-DNA complexes at the IL-2 locus, reflecting its partial content of inducible IL-2 DNA-binding activities. However, we detected no in vivo protein-DNA interactions in any of the nonexpressing cells examined, including unstimulated EL4 T cells, 32D cells, and L cells (data not shown), as well as stimulated 32D cells. This conclusion was drawn by comparing the G ladders derived from naked DNA samples with those from in vivo samples. The naked DNA samples (Fig. 4A, lane 3; Fig. 4B, lanes 1 and 4) were very similar to the in vivo samples from cells not actively expressing IL-2 (Fig. 4A, lanes 1, 2, and 4; Fig. 4B, lanes 2, 3, and 5). The only changes in DMS reactivity between naked DNA samples and these in vivo DMS-treated samples were observed on the coding strand near −100 and −200, where several G's were 20 to 25% hyperreactive in all the in vivo samples. However, the complete absence of associated protections is not typical of most protein-DNA interactions, and no interactions of any sort were detected at adjacent bases on the noncoding strand. Such isolated hypersensitivities have sometimes been encountered in comparisons of naked DNA versus in vivo DMS-treated DNA for other genes, and their significance remains unknown (20, 29). More importantly, all elements in the pattern of interactions seen in the induced EL4 T cells are entirely absent in the nonexpressing cells. For example, the NF-κB site is unoccupied even in stimulated 32D cells, though ample levels of NF-κB-binding activities (including putative NF-κC) are present in nuclear extracts from these cells. Furthermore, no interactions are apparent elsewhere in the IL-2 regulatory region in nonexpressing cells. This absence of detectable in vivo binding contrasts with the presence of corresponding DNA-binding activities in the nuclei of these cells.

CsA treatment entirely blocks protein-DNA interactions in stimulated EL4 T cells. IL-2 transcription can be completely inhibited by CsA treatment, although individual IL-2 regulatory elements show diverse sensitivities to the drug. This could result from CsA totally blocking the binding of both CsA-sensitive and -insensitive factors to the DNA or through the

elimination of a subset of DNA-bound activators sufficient to prevent transcription. When EL4 T cells were stimulated with TPA and A23187 in the presence of CsA, all footprints associated with IL-2 induction were absent (Fig. 4A, lane 7; Fig. 4B, lane 8; Fig. 6B, lanes 5, 7, and 9). Thus, CsA blocks the in vivo binding of all detected factors to the IL-2 regulatory region, despite affecting the in vitro binding activities of only a few. Therefore, at least one CsA-sensitive component of the EL4 response is required for the coordinated binding of all factors.

The results presented above show that CsA blocks the establishment of stable occupancy at the IL-2 regulatory region. We next addressed whether CsA has an effect on the maintenance of stable occupancy after it has been established. Addition of CsA to preactivated EL4.E1 cells is known to abort IL-2 expression almost immediately (38). This effect might be due to the partial or complete loss of bound factors. Alternatively, the binding of factors, once established, might be insensitive to CsA treatment, with delayed CsA treatment instead inhibiting the ability of these bound factors to activate transcription. As shown in Fig. 6, maximal levels of regulatory region occupancy are obtained after 2 h of TPA-A23187 stimulation and persist at these levels for at least an additional 9 h. Thus, CsA was added after 2 h of stimulation ("late" in Fig. 4). When protein-DNA interactions were examined 7 h later, the delayed CsA treatment had resulted in the elimination of all footprints (Fig. 4A, lane 8; Fig. 4B, lane 9). In the absence of CsA, regulatory region occupancy was still maximal at this time point. Thus, CsA blocks both the establishment and the maintenance of the protein-DNA complex at the IL-2 promoter/enhancer.

The absence of in vivo protein-DNA interactions in cells that do not express IL-2 is gene specific. The conclusion that unstimulated EL4 T cells, CsA-treated stimulated EL4 T cells, and 32D cells show none of the in vivo interactions characteristic of induced T cells required a positive control to show that the absence of IL-2 footprints is gene specific and not a simple artifact of toxicity, cell handling, or footprinting manipulations. This issue was addressed by using the same DNA preparations to footprint another gene, the MT-I gene, which is expressed in all of these cell populations (Fig. 1). The characteristic in vivo footprint at the MT-I locus Sp1 site was present in all samples (Fig. 7 and data not shown).

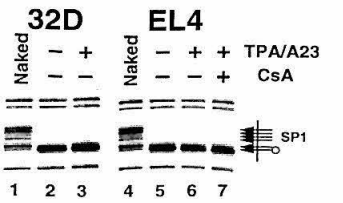

FIG. 7. DMS in vivo footprint of the Sp1-binding site in the MT-I gene. DNA from in vivo DMS-treated unstimulated 32D cells (lane 2), stimulated 32D cells (lane 3), unstimulated EL4 cells (lane 5), stimulated EL4 cells (lane 6), and EL4 cells stimulated in the presence of CsA (lane 7) was used. Naked 32D DNA (lane 1) and naked EL4 DNA (lane 3), each treated with DMS in vitro, were also used.

DISCUSSION

Regulation of the IL-2 gene is interesting on several levels. First, the stringency of its transcriptional regulation is extreme with respect to its developmental specificity and its rapid response to specific environmental signals. The well-established complexity of IL-2 *cis* elements and of their cognate binding activities demands the integration of many less specific activities. How is this integration accomplished, and what prevents low-level, leaky expression? To address these issues, we have determined the nature of in vivo protein–IL-2 gene complexes and compared them with the sets of nuclear factors present in an informative group of cell lines. These cell lines represented different developmental lineages and were studied under different signal transduction states. A central result was that the level of regulation which directly correlated with active IL-2 transcription was enhancer occupancy by all factor types involved. This appeared to be an all-or-nothing, reversible phenomenon. Insults to subsets of the binding activities totally abolished all interactions by those factors that remained (summarized in Fig. 8). This places the burden of specificity on stable chromosomal occupancy. If the whole protein-enhancer complex must be assembled on the DNA in order to achieve stable occupancy by any subset of the factors, stringent limitations are imposed on when and where transcriptional activation occurs. We also infer that it is this highly coordinated assembly that satisfies the biological requirement that the IL-2 enhancer/promoter be robust when activated yet very sensitive to multiple signal inputs. As discussed below, this provides an explanation for the previously observed sensitivity of this enhancer/promoter to mutagenesis of individual elements. Thus, while it remains possible that the transcription-inducing activities of some of these factors are also regulated after they bind to DNA, regulation of stable occupancy dominates.

The study of IL-2 transcriptional control also presents the potential for separation of developmental restriction from the actual induction of expression. In this respect, IL-2 differs from several other developmentally restricted genes that have previously been studied in this way. In those systems, differentia-

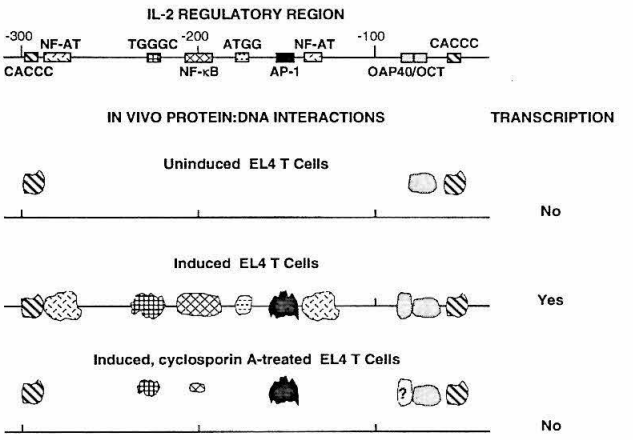

FIG. 8. Summary of the protein-DNA interactions detected in EL4 T lymphoma cells by in vivo footprint analysis and the presence of nuclear factors in these cells as determined by in vitro DNA-binding analysis. Decreased size of TGGGC- and NF-κB-binding activities in stimulated, CsA-treated EL4 cells depicts the quantitative decrease in in vitro DNA-binding activity in Fig. 2. OAP40 activity could not be detected in Fig. 2, but its presence in stimulated EL4 cells was inferred because of the in vivo methylation protection of a G residue adjacent to the octamer motif which specifically disrupts OAP40 binding when methylated (44). Stimulation-dependent OAP40 binding activity has been detected in the human Jurkat T-cell line, but its CsA sensitivity was not examined (44). The ATGG site was not assayed for in vitro binding activity.

tion of the cell was temporally and physiologically coupled to expression of the gene; for example, muscle creatine kinase is expressed upon differentiation of myoblasts into myocytes. Because differentiation of T-helper cells is separated from their activation to express IL-2, it seemed possible that in this system, there might be factor-DNA interaction at the IL-2 locus in T cells prior to activation that would distinguish them from non-T cells, thereby setting the stage for the induction event. Alternatively, such developmental marking might take the form of specific repressing interactions present in non-T lineages and then removed in T cells. We tested these possibilities by comparing in vivo protein-DNA interactions in T cells with those in other hematopoietic and nonhematopoietic cells prior to induction. No evidence was found for stable, lineage-dependent molecular commitment at the IL-2 locus. Thus, the cell-specific competence of the IL-2 locus to be induced could not be detected at the level of protein-DNA contacts in the major groove. In addition, even a potent activation cascade could not trigger any protein-DNA contacts in the nonpermissive developmental environment of the 32D cells. It is important to recognize that these observations do not rule out the possibility of repressive or permissive interactions to which DMS was insensitive, such as binding of proteins in the minor groove or to recognition sites devoid of G residues. Thus, the possible involvement of factors such as TCF-1 and TCF-1α/LEF-1 (43, 46, 48) remains to be investigated. However, the compelling result is that there was no evidence, in any nonexpressing cell examined, of in vivo occupancy of those sites that ultimately do become occupied in IL-2-expressing T cells. This finding argues strongly against straightforward competition between activator and repressor proteins for binding site contacts in the major groove as a way of enforcing cell-type- and signal-dependent restriction of IL-2 in these cells.

CsA was used as a pharmacological tool to probe separately the sensitivity of complex establishment and complex maintenance to the presence of CsA-sensitive factors. One or more CsA-sensitive factors were required for the stable binding of all factors, a finding consistent with the functional requirement for CsA-sensitive regulatory elements such as NF-AT determined by *cis*-element mutagenesis studies (7, 32, 42). But such mutational analyses cannot separate a transient need for an activity from a sustained requirement for it. The possibility that establishment of an active enhancer-protein complex can be functionally separated from its ongoing maintenance has precedent. For example, a "hit-and-run"-style mechanism has been proposed for the tyrosine aminotransferase gene whereby interaction of the glucocorticoid receptor is apparently transient, being needed only to initiate hormonally responsive expression, with other elements driving ongoing maintenance and activity of the complex (34). By analogy, CsA, which has been shown to block initial translocation of NF-AT components to the nucleus (10), could act on the initiation of occupancy but not interfere with its maintenance. However, CsA treatment of cells containing preassembled complexes clearly showed that these complexes are not stable over the time course tested. Thus, one or more CsA-sensitive factors are required for the persistence of stable binding by all other factors. Furthermore, unless an unknown effect of CsA is the active disassembly of protein-DNA complexes, the complexes that we observe in footprints are not static but are instead in a dynamic equilibrium with nuclear factors. Mechanistically, the loss of in vivo binding shows that either a CsA-sensitive factor(s) functions through cooperative binding interactions with CsA-insensitive factors or, alternatively, if CsA-sensitive factors regulate the region's accessibility, this opening is readily reversible and depends on the CsA-sensitive factors for its persistence.

Within the IL-2 enhancer, we identified new TGGGC and proximal CACCC elements whose functions can be addressed in part by reexamining previously published deletion mutant studies. A nested series of 5′ deletion constructs of the mouse IL-2 gene displayed a fivefold drop in inducibility in EL4 T cells when 36 bp including the TGGGC motif was eliminated (37). This region contains no other element which showed an in vivo interaction in our analysis (Fig. 4). An internal deletion across the proximal CACCC motif in the human IL-2 gene decreased activity of the otherwise intact enhancer fivefold, though this deletion includes the distal TATA box, and alterations in spacing could have contributed to the effect (7). Thus the protein-DNA contacts at the newly identified elements, like those at the previously described elements, appear to contribute to IL-2 expression.

Either of two models, which are not mutually exclusive, could explain the coordinated binding observed at the IL-2 locus: cooperative binding or limited site accessibility. Cooperative binding interactions between the multiple DNA-binding proteins that can interact with individual *cis* elements, perhaps anchored by a few key factors, would stabilize the binding of each member of the complex. Such cooperativity could operate through direct interactions of the DNA-binding factors with each other as well as through additional, as yet unidentified proteins which would act through protein-protein interactions with the DNA-binding factors. At a minimum, inducible, T-cell-specific, CsA-sensitive factors would be limiting for stable complex assembly, but other factors, not limiting in any case studied here, could be equally essential.

One cooperative interaction has been already documented for the IL-2 regulatory region at the OCT/OAP40 element, where co-occupancy by OAP40 and Oct-1 stabilizes the binding of Oct-1 (44). Additional evidence also suggests that the binding of factors to the IL-2 NF-κB element is stabilized when the adjacent AP-1 site is included in the target DNA sequence (31). It will now be important to look for such interactions more broadly between the multiple elements in IL-2. Evidence consistent with cooperativity as a substantial component of IL-2 transcriptional control also comes from *cis*-element mutational analysis, in which individual alteration of any of a number of different sites strongly decreases overall promoter/enhancer activity (7, 17, 18, 37, 42, 44). However, those experiments did not address whether the collaborative effect was at the level of DNA binding or at the level of transcriptional activation. An additional feature of the IL-2 regulatory region that may especially favor dependence on cooperative interactions is that, as noted by Hentsch et al. (15), the multiple sites in IL-2 for general factors, such as NF-κB and AP-1, deviate significantly from their respective consensus sequences. These changes decrease the binding affinity of the major species detected in gel shifts and may render them especially dependent on interactions with nearby factors to bind in vivo.

By contrast, regulated site accessibility is envisioned to involve the masking of potential binding sites near IL-2 in a repressed chromatin configuration. In this case, the action of inducible factors could serve to open the region, thus allowing all of the proteins to interact with their binding sites. These two models differ in the roles that they ascribe to activation-dependent, T-cell-specific, CsA-sensitive factors like NF-AT. In one case, they are needed as architectural elements that act strictly in concert with the other factors; in the other case, they have a unique role which all other interactions depend on and follow. The disappearance of existing complexes upon the

delayed addition of CsA is consistent with either model. However, it does require that if chromatin accessibility alone is used to regulate protein-DNA contacts at IL-2, the increase in accessibility must be fully reversible. Further examination of the relative contributions of binding cooperativity and site accessibility should reveal the molecular details of the observed coordinated assembly controlling IL-2 transcription.

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Chapter 5

Interleukin 2 Transcription Factors as Molecular Targets of cAMP Inhibition: Delayed Inhibition Kinetics and Combinatorial Transcription Roles

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Interleukin 2 Transcription Factors as Molecular Targets of cAMP Inhibition: Delayed Inhibition Kinetics and Combinatorial Transcription Roles

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Summary

Elevation of cAMP can cause gene-specific inhibition of interleukin 2 (IL-2) expression. To investigate the mechanism of this effect, we have combined electrophoretic mobility shift assays and in vivo genomic footprinting to assess both the availability of putative IL-2 transcription factors in forskolin-treated cells and the functional capacity of these factors to engage their sites in vivo. All observed effects of forskolin depended upon protein kinase A, for they were blocked by introduction of a dominant negative mutant subunit of protein kinase A. In the EL4.E1 cell line, we report specific inhibitory effects of cAMP elevation both on NF-κB/Rel family factors binding at −200 bp, and on a novel, biochemically distinct "TGGGC" factor binding at −225 bp with respect to the IL-2 transcriptional start site. Neither NF-AT nor AP-1 binding activities are detectably inhibited in gel mobility shift assays. Elevation of cAMP inhibits NF-κB activity with delayed kinetics in association with a delayed inhibition of IL-2 RNA accumulation. Activation of cells in the presence of forskolin prevents the maintenance of stable protein–DNA interactions in vivo, not only at the NF-κB and TGGGC sites of the IL-2 enhancer, but also at the NF-AT, AP-1, and other sites. This result, and similar results in cyclosporin A–treated cells, imply that individual IL-2 transcription factors cannot stably bind their target sequences in vivo without coengagement of all other distinct factors at neighboring sites. It is proposed that nonhierarchical, cooperative enhancement of binding is a structural basis of combinatorial transcription factor action at the IL-2 locus.

Activation of T helper cells results in secretion of diverse lymphokines. The precise combination of lymphokines produced is variable, depending on the circumstances of the activation process. Different effector mechanisms are triggered by different lymphokines. Locally and systemically, the relative levels of different lymphokines with respect to one another strongly affect in vivo outcomes. Thus, the type of immune response that is mobilized ultimately depends on the differential transcriptional regulation of lymphokine genes. The major stimulatory signal for lymphokine production is delivered by engagement of the TCR with antigen in the context of MHC on APCs. However, engagement of accessory molecules on the T cell with additional ligands, either on APCs or in the microenvironment, may also deliver other kinds of signals that affect lymphokine gene expression differentially. Expression of the T cell growth factor, IL-2, is particularly susceptible to this kind of modulation. Glucocorticoids inhibit IL-2 induction preferentially (1–3), and contact of a T cell with antigen in the absence of a CD28 ligand can drive the T cell into an anergic state in which IFN-γ can be expressed, but not IL-2 (4, 5). Agonists that elevate cAMP levels, such as prostaglandin E_1 and E_2, give especially clear evidence for selective inhibition of IL-2 expression (6, 7), as they can inhibit IL-2 expression in cases where there is no inhibition of IL-4 expression at all (8–10). The effects of cAMP on T cells can be exerted at both cell- and gene-specific levels, depending on the cells and stimuli used. In certain IL-2–producing cells, cAMP elevation can uncouple the TCR/CD3 complex from its downstream signaling mediators, preventing the generation of Ca^{2+} and protein kinase C signals (11–13). On the other hand, even when Ca^{2+} and protein kinase C signals are provided pharmacologically, cAMP elevation can still interfere selectively with induction of IL-2 gene expression, yet allowing other responses to proceed. It is the mechanism of this second, gene-specific effect that we investigate here.

The IL-2 gene regulatory region consists of compact, clustered binding sites for an assortment of transcription factors that are disparately regulated (14). In most or all cases studied, IL-2 expression is induced only when all the known factors are present, and the IL-2 promoter only works at maximal efficiency when all the major binding sites for these factors are intact. However, in physiological situations, data seem to indicate that certain factors play more critical roles than

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others. Two of them, AP-1 and NF-AT, appear repeatedly to be rate limiting for IL-2 expression. When selective down-regulation of IL-2 production is caused by exposing T cells to glucocorticoids, the possible mechanisms are a protein–protein interaction that sequesters AP-1 by dimerization of AP-1 components with the glucocorticoid receptor (2), or a specific inhibition of interaction between AP-1 and NF-AT (3). When IL-2–producing cells have been anergized and become unable to express IL-2, their failure appears to be due to either a general block of NF-AT, NF-κB, and AP-1 activation (15), or a selective inability to activate AP-1 (16). The potent immune-suppressive drugs cyclosporin A (CsA)[1] and FK 506 completely block IL-2 transcription by blocking the nuclear translocation of one component of NF-AT (17). Whereas this is an example of strictly pharmacological inhibition, defects in both NF-AT and AP-1 mobilization also appear to be responsible for a normal developmental block in the ability to express IL-2, namely in cortical thymocytes (18). A similar phenotype is observed in a pre-mast cell line that can express IL-4 but not IL-2 (19). Thus, an important question is whether all physiological regulation of IL-2 expression is exerted through effects on AP-1 or NF-AT, or whether cAMP-elevating agonists might inhibit IL-2 expression through effects on different factors.

To address these questions, we have chosen EL4.E1 thymoma cells as a model T cell system, and the Ca^{2+} ionophore A23187 and the phorbol ester PMA (TPA) as stimulants that can bypass any effects of cAMP on signal generation at the cell membrane. Using this system previously, we showed that cAMP could inhibit IL-2 transcription (9). We also obtained initial indications that elevation of cAMP caused a slight decrease in the binding activity of NF-κB, but did not inhibit NF-AT or AP-1 site binding activities (20). In our present studies, we have clearly defined two specific effects of cAMP on factor-binding activities and examined the resulting protein–DNA interactions at the IL-2 locus in vivo. Our results confirm that cAMP, acting via activation of protein kinase A (PKA), selectively affects the in vitro DNA binding activities of NF-κB and a newly described TGGGC binding factor, and that it does so with novel and distinctively delayed kinetics. No evidence was found for any negative effect on NF-AT or AP-1 binding. However, cells stimulated in the presence of forskolin failed to sustain stable protein-DNA contacts at a broad array of sites spanning the entire IL-2 enhancer region. The lack of occupancy may be evidence for a new role for NF-κB and TGGGC binding activities, as required partners in transcription complex assembly.

Materials and Methods

Cells. EL4.E1 cells (EL4) were grown in RPMI-1640 in the presence of 2 mM L-glutamine, 50 mM β-ME, antibiotics, and 6% fetal bovine serum (Hyclone, Logan, UT). Cells were cultured to confluence before stimulation and were stimulated at 1–2 × 10^6 cells/ml using 17 nM TPA and 120 nM A23187 in the presence or absence of 10 μM of forskolin (Sigma Chemical Co., St. Louis, MO), which raises the cAMP level by directly activating adenylate cyclase. Recombinant human IL-1α (Genzyme Corp., Cambridge, MA) was used at 50 U/ml.

IL-2 Analysis. RNase probe protection analysis to quantitate IL-2 RNA was performed as previously described (19, 21). For each sample, 10 μg of RNA was used to hybridize with the IL-2 probe (22), and 1 μg RNA was used to hybridize with either an actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (23).

Cyclic AMP Assay. Levels of cAMP were measured in EL4 cells as described previously using an Amersham dual-range cAMP kit (9), except that nonacetylated standards and samples were used.

Gel Mobility Shift Assays. Nuclear extracts of EL4 cells were prepared as before (18). Double-stranded oligonucleotide corresponding to portions of the mouse IL-2 gene regulatory region were labeled by end filling with α-[^{32}P]deoxynucleoside triphosphates. The detailed sequences and corresponding sites relative to the transcription start site were described elsewhere (18–20). Oligonucleotides used for this experiment were synthesized in the California Institute of Technology Biology Microchemical Facility. The binding reaction and gel electrophoresis procedures were exactly as reported before (18). Note that these conditions for detection of NF-AT site binding activity differ from those we used in initial studies (20), as poly (dI-dC) is used as a nonspecific competitor in place of poly (dA-dT). In our hands, this substitution yields a spectrum of gel-shift complexes more consistent with NF-AT complexes described elsewhere, and less dominated by highly protease-sensitive complexes. However, the insensitivity of NF-AT site binding factors to forskolin reported in our earlier study (20) is confirmed here.

In vivo Footprinting Analysis. The genomic footprinting analysis was done as before with the same set of IL-2 gene primers previously reported (19). Briefly, 3 × 10^7 cells were concentrated into 1 ml medium, and treated with 0.1% of dimethylsulfate at 37°C for 1 min. The reaction was stopped by transferring the cells to a tube containing 49 ml of ice-cold PBS, and the cells were pelleted and washed once more with 50 ml PBS. The cell pellet was then resuspended in 3 ml of lysis buffer (300 mM NaCl, 50 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, 200 μg/ml proteinase K, and 0.2% SDS), and incubated at 37°C overnight. Nucleic acids were obtained by phenol/chloroform extraction and isopropanol precipitation, then subjected to piperidine cleavage and subsequent ligation-mediated PCR (24). The final labeled PCR products were separated on a 6% polyacrylamide sequencing gel.

Transfection and Selection. 10 μg of linearized HL-REV$_{AB}$pKOneo (designated pΔPKA in the text) (a gift from G. S. McKnight, University of Washington, Seattle, WA) was electroporated at 960 μF and 320 V into 10^7 EL4.E1 cells in 1 ml of serum-free RPMI-1640 medium with supplements and 14.4 mM β-ME. The cells were then diluted into 50 ml of medium and plated in two 24-well plates (1 ml/well). 2 d after plating, G418 (GIBCO BRL, Gaithersburg, MD) was added to the wells at 400 μg/ml. Positive wells were identified 7–10 d after selection and the cells were expanded in 200 μg/ml G418 for further analysis. The plasmid DNAs, pBR322 and pSV2neo (25), were used in transfection as negative and positive controls. Integration of the mutant PKA construct in established transfectant lines was confirmed by Southern blot analysis and its expression was further confirmed by Northern blot analysis. One mutant PKA transfectant line, IIA4, and two pSV2neo control lines were used in the subsequent experiments.

[1] *Abbreviations used in this paper:* CsA, cyclosporin A; DMS, dimethyl sulfate; EMSA, electrophoretic mobility shift assay; PKA, protein kinase A; TPA, 12-O-tetradecanoyl phorbol 13-acetate (PMA).

Results

Forskolin Affects DNA-binding Activities of a Specific Subset of Factors. To investigate the effects of cAMP on IL-2 transcriptional control, EL4 cells were stimulated with TPA+A23187 in the presence or absence of 10 μM of forskolin, and the IL-2 mRNA level in the cells was examined 5 h after induction (Fig. 1 *A*). Addition of forskolin to the TPA+A23187 stimuli significantly decreased the level of IL-2 mRNA (Fig. 1 *A*, lane *3*). In previous work (9), we reported that forskolin could also inhibit inducible expression of a reporter gene under the control of the minimal 321-bp IL-2 promoter/enhancer. This indicated that at least part of the effect of forskolin was mediated through the 5′ regulatory sequence of the IL-2 gene, presumably at the level of transcriptional initiation, and that the ~300 bp minimal promoter/enhancer sequence was sufficient to produce this response. Therefore, to determine the mechanism of the forskolin inhibition effect, we focused on protein–DNA interactions in this 300-bp region.

To ascertain whether forskolin inhibits the binding of particular factors to the IL-2 regulatory region, we compared the levels of most DNA binding activities known to be correlated with IL-2 gene activation in nuclear extracts from stimulated cells and from cells stimulated in the presence of forskolin. Nuclear extracts were prepared from EL4 cells incubated under different conditions for 4–5 h, and these extracts were analyzed for sequence-specific binding activities by electrophoretic mobility shift assays (EMSAs) (Fig. 1, *B* and *C*). Double stranded oligonucleotide probes were chosen to span all the major sites of protein–DNA contact in IL-2–producing EL4 cells, as previously identified by in vivo footprinting (19). As shown in Fig. 1 *C*, the DNA binding activities of factors that are constitutively present, such as the

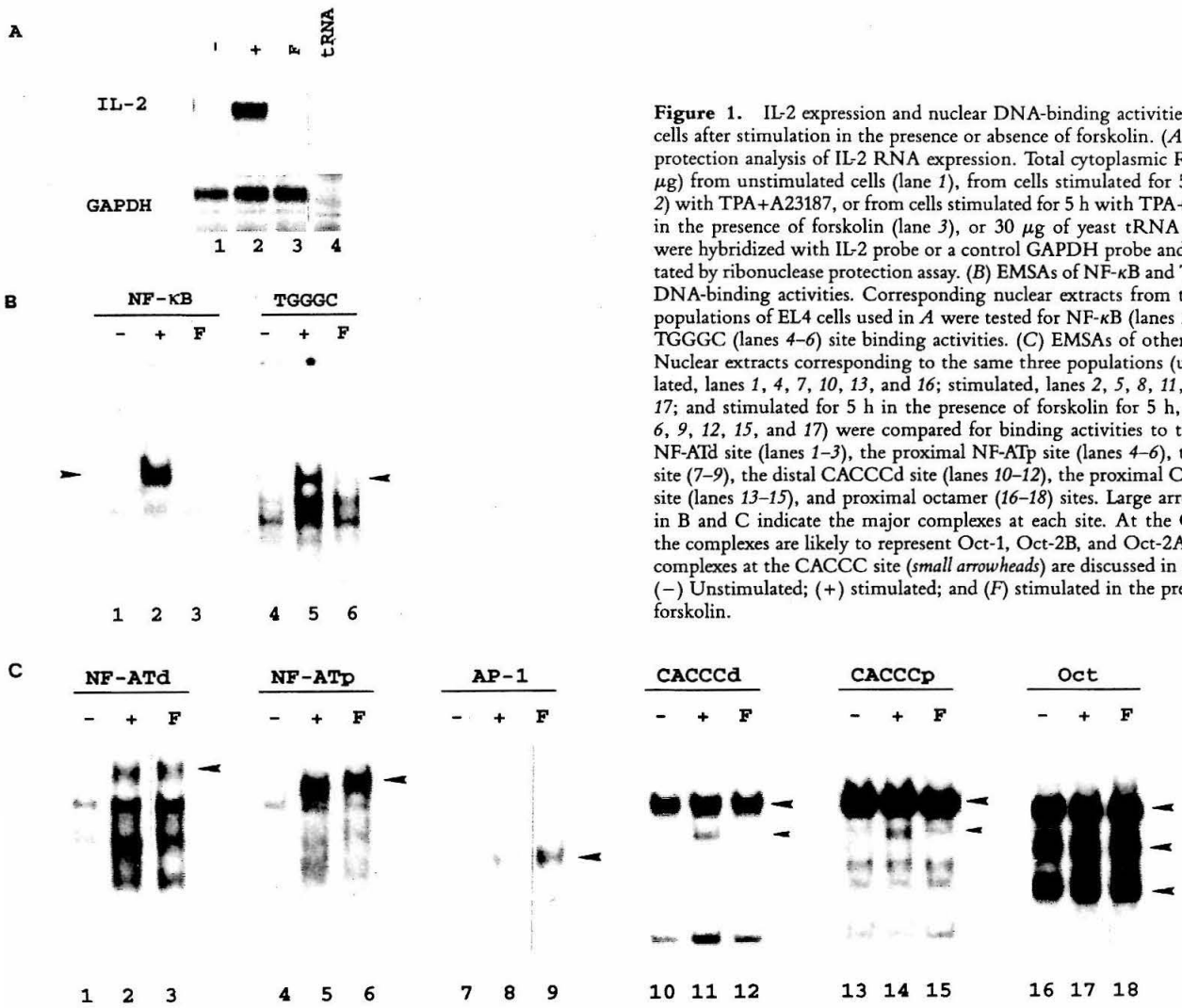


Figure 1. IL-2 expression and nuclear DNA-binding activities in EL4 cells after stimulation in the presence or absence of forskolin. (*A*) RNase protection analysis of IL-2 RNA expression. Total cytoplasmic RNA (10 μg) from unstimulated cells (lane *1*), from cells stimulated for 5 h (lane *2*) with TPA+A23187, or from cells stimulated for 5 h with TPA+A23187 in the presence of forskolin (lane *3*), or 30 μg of yeast tRNA (lane *4*) were hybridized with IL-2 probe or a control GAPDH probe and quantitated by ribonuclease protection assay. (*B*) EMSAs of NF-κB and TGGGC DNA-binding activities. Corresponding nuclear extracts from the same populations of EL4 cells used in *A* were tested for NF-κB (lanes *1–3*) and TGGGC (lanes *4–6*) site binding activities. (*C*) EMSAs of other factors. Nuclear extracts corresponding to the same three populations (unstimulated, lanes *1*, *4*, *7*, *10*, *13*, and *16*; stimulated, lanes *2*, *5*, *8*, *11*, *14*, and *17*; and stimulated for 5 h in the presence of forskolin for 5 h, lanes *3*, *6*, *9*, *12*, *15*, and *17*) were compared for binding activities to the distal NF-ATd site (lanes *1–3*), the proximal NF-ATp site (lanes *4–6*), the AP-1 site (*7–9*), the distal CACCCd site (lanes *10–12*), the proximal CACCCp site (lanes *13–15*), and proximal octamer (*16–18*) sites. Large arrowheads in B and C indicate the major complexes at each site. At the Oct site, the complexes are likely to represent Oct-1, Oct-2B, and Oct-2A. Minor complexes at the CACCC site (*small arrowheads*) are discussed in the text. (−) Unstimulated; (+) stimulated; and (*F*) stimulated in the presence of forskolin.

major binding factors for the CACCC sites (18, 19), and the proximal octamer site, were not affected by forskolin (Fig. 1 *C*, lanes *10–18*, *large arrowheads*). Similarly, some induction-dependent activities, such as the major inducible complexes binding the NF-AT and AP-1 sites, were unaffected or even slightly enhanced by stimulation in the presence of forskolin (Fig. 1 *C*, lanes *1–9*, *large arrowheads*). These results are in agreement with our previous conclusions from more limited studies (20). However, as shown in Fig. 1 *B*, binding activities of several other inducible factors were significantly reduced in nuclear extracts from cells that were activated in the presence of forskolin. Most prominent among these were the upper complexes binding to the NF-κB and TGGGC sites (Fig. 1 *B*, lanes *2*, *3* and *5*, *6*). In addition, we noted effects of forskolin on minor complexes binding the two CACCC sites, which in both cases were of faster mobility than the major CACCC complex (Fig. 1 *C*, lanes *11*, *12*, and *14*, *15*, *small arrowheads*). The identities of the lower inducible complexes bound to the CACCC sites are not known. They could not be competed by a consensus Sp-1 oligonucleotide as could the major CACCC binding complexes (18, and data not shown). The nature of the TGGGC binding factor is also not known. As it appeared to show a similar electrophoretic mobility to that of the upper NF-κB complex, it was subjected to some further characterization.

Although both NF-κB and TGGGC binding activities were coinhibited by forskolin, the protein components involved were clearly different. This was confirmed by the demonstration, (*a*), that the TGGGC and NF-κB binding sites could not cross-compete in mobility shift assays, and (*b*), by antibody supershift experiments in which anti-p65, anti-c-rel, and anti-p50 antibodies that could supershift or inhibit the NF-κB complexes were all completely unable to affect the TGGGC complexes (data not shown). Furthermore, whereas TNF-α could "rescue" NF-κB binding activity in cells stimulated in the presence of forskolin, there was no rescue of the TGGGC factor (data not shown). These results, and data presented below, show that the regulation of activation of these two binding activities was different.

The presence of forskolin during stimulation appeared to result in a reduction in the total amount of NF-κB complexes per cell, not simply a sequestration of NF-κB activity (26). This was shown both by Western blotting of nuclear and cytoplasmic extracts with antibodies against p65, p50, and c-rel, and by using deoxycholate to dissociate NF-κB/IκB complexes, thus revealing previously masked NF-κB binding activities (27). Stimulation for 4 h led to a sharp increase of p65/p50 and c-rel/p50 complexes in the nucleus, and this increase was profoundly antagonized by the presence of forskolin. However, there was no compensatory increase in NF-κB components or binding activities in the cytoplasm (data not shown). Thus, forskolin may inhibit NF-κB synthesis or promote NF-κB degradation.

Delayed Inhibition of IL-2 Expression Via Delayed Inhibition of NF-κB. The effects of forskolin on NF-κB appear to be paradoxical, for it has been reported that PKA can activate NF-κB, and our own earlier study (20) did not show consistent reduction of NF-κB binding at 2 h of forskolin treatment. To clarify this contradiction, we did kinetic studies to see whether the reduction we observed here is an early event or a possible secondary effect. First, we examined the kinetics of cAMP elevation after forskolin treatment. A significant increase in cAMP levels could be seen 15 minutes after administration of forskolin, reaching a maximum within an hour. High levels of cAMP were then retained for up to 6 h of forskolin treatment (Fig. 2 *A*). The addition of TPA+A23187 did not affect the initial elevation or the later accumulation of cAMP (Fig. 2 *B*). Pretreatment of cells for 2 h with TPA+A23187 before addition of forskolin also did not significantly affect the subsequent elevation of cAMP (Fig. 2 *C*). The high levels of cAMP sustained for many hours made it possible that cAMP-dependent mechanisms could exert effects at late time points, not just immediately after stimulation.

In fact, the effects of forskolin on NF-κB and TGGGC binding factor induction showed strikingly different time courses. When cells were activated in the presence of forskolin, mobilization of the factors binding to the TGGGC

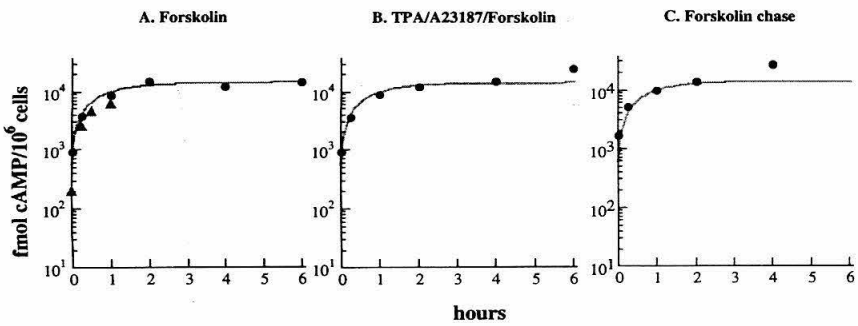
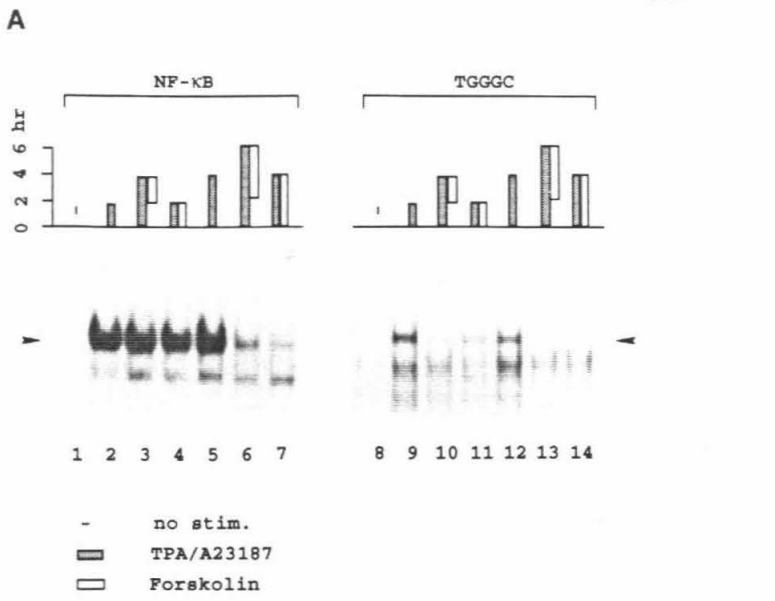


Figure 2. Sustained elevation of cAMP as a function of duration of forskolin treatment. The figure shows cAMP levels in EL4 cells that were cultured in the presence of forskolin alone (*solid and dotted curve*, *A*; *solid curve*, *B* and *C*); with TPA+A23187+ forskolin added simultaneously (*dots*, *B*); or with forskolin added (time 0) after 2 h of pretreatment with TPA+A23187 (*dots*, *C*). Levels of cAMP are also shown for IIA4 transfectants (see Fig. 4) that were cultured with forskolin alone (*triangles*, *A*). The incubation was stopped at different time points and cAMP was measured in aliquots of 10^5 cells. As a reference for the data in *A–C*, the data from EL4 cells treated with forskolin alone (*A*) were used to calculate the solid curve shown in all three panels. Individual data points were plotted as triangles or dots to compare with the solid curve. This comparison emphasizes the similarity in the kinetics of cAMP accumulations with or without stimulation. The experimental data for forskolin-treated EL4 cells (*solid curve*) are well-represented by the equation $C_{(t)} = K_s/K_d - (K_s/K_d - C_0)\exp(-K_d t)$, where $C_{(t)}$ is the amount (fmol) of cAMP/10^6 cells at time t (h). C_0 (=568 fmol/10^6 cells) is the amount of cAMP at time 0, K_s (=1.6 × 10^4 fmol/10^6 cells h) is the calculated cAMP synthesis rate constant in the presence of forskolin. K_d (=1.14 h^{-1}) is the cAMP decay rate constant, from which we compute $t_{1/2}$ = 0.79 h.



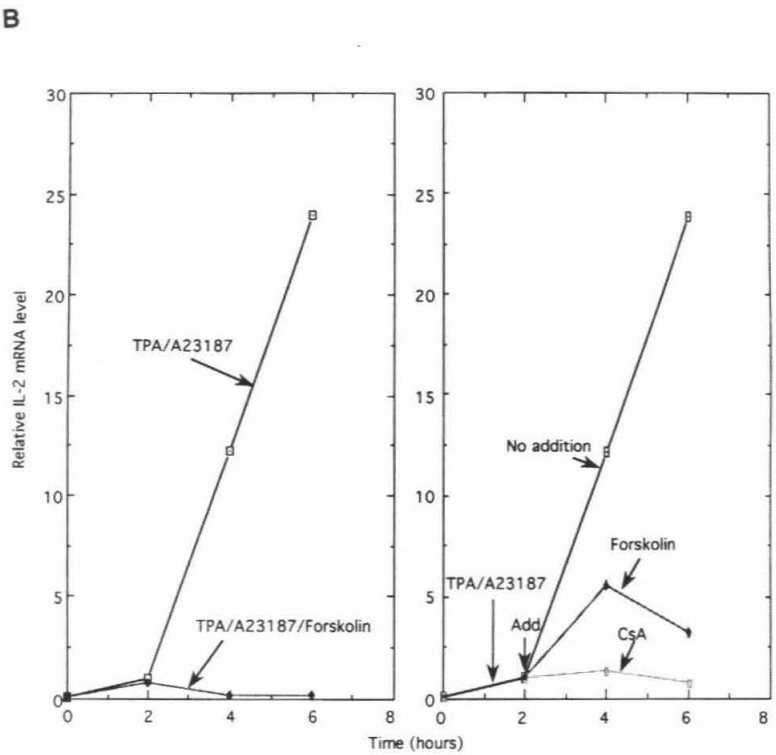


Figure 3. Delayed inhibition of IL-2 RNA accumulation correlated with delayed inhibition of NF-κB binding activity. (*A*) NF-κB and TGGGC site binding activities were measured at different intervals of stimulation (▨), in the presence (▭), or absence of forskolin, as indicated in the bar graph over each lane. Note that it took 4 h of forskolin treatment to reduce NF-κB binding activity, whether forskolin addition was delayed or not (lanes *6* and *7*), but only 2 h to reduce TGGGC binding activity significantly (lanes *10* and *11*). (*B*) IL-2 mRNA levels at various times of stimulation were measured by RNase protection analysis. Data were collected from two independent experiments. (*Left*) Cells stimulated for the indicated lengths of time in the absence (⊡) or presence (◆) of forskolin from time 0. (*Right*) Forskolin and CsA chase experiments. All samples were stimulated from time 0. Forskolin (◆) or CsA (⊡) was added to the culture 2 h after initiating the stimulation with TPA+A23187.

site was inhibited both at 2 and at 4 h of treatment (Fig. 3 *A*, compare lanes *9* and *11*, *12*, and *14*), indicating that this inhibition is an early event. However, when analyzed after 2 h of stimulation, NF-κB binding activities appeared to be induced equally well whether forskolin was present or not (Fig. 3 *A*, compare lanes *2* and *4*). Only after at least 4 h of stimulation in the presence of forskolin did we see the characteristic decrease of the NF-κB site binding activity (Fig. 3 *A*, compare lanes *5* and *7*). These findings indicated that elevation of cAMP does not inhibit the initial dissociation of NF-κB from IκB or its subsequent nuclear localization. They also suggested either that the effects of forskolin on NF-κB were intrinsically subject to delay, or that NF-κB proteins only became susceptible to forskolin-dependent inhibition at some point at least 2 h after initial exposure to TPA+A23187. To test these possibilities, we first stimulated the cells with TPA and A23187 for 2 h, to make all the necessary *trans*-acting factors available and initiate transcription of IL-2 RNA, then we introduced 10 μM forskolin to the culture and continued the culture for another 2 or 4 h. As shown in Fig. 3 *A* (lanes *3* and *6*), forskolin "chasing" for 2 h still had little effect on NF-κB binding, but chasing for 4 h greatly

reduced the binding. These results suggested that PKA activation had an intrinsically delayed effect on NF-κB binding activity.

The delayed and asynchronous effects of cAMP elevation on the NF-κB and TGGGC site binding factors were associated with delayed inhibition of IL-2 mRNA expression, as shown in Fig. 3 *B*. IL-2 mRNA accumulation in the stimulated control cells followed biphasic kinetics with a relatively low rate of accumulation in the first 2 h, which then shifted to a higher rate that was sustained for the next 4 h. When forskolin was present at the start of stimulation, IL-2 mRNA initially accumulated almost as fast as in the control cells without forskolin, but then declined to baseline between 2 and 4 h of stimulation (Fig. 3 *B*, *left*). When the addition of forskolin was delayed until after 2 h of stimulation, the rate of IL-2 mRNA accumulation was significantly reduced as compared with the control samples without forskolin, and inhibitory effects were evident within the first 2 h of forskolin chasing (Fig. 3 *B*, *right*). However, this inhibition was slow as compared with the immediate shut-off of IL-2 mRNA accumulation when CsA, instead of forskolin, was added after 2 h of stimulation (Fig. 3 *B*, *right*). Thus, some early effects of forskolin which inhibit IL-2 activation may be exerted before its effects on NF-κB, suggesting that a lack of factors other than NF-κB, including the TGGGC binding factor, may be sufficient to hinder the transcription but not to block it fully. The reduced NF-κB binding activity seen at relatively later time points would then be associated with the complete arrest of IL-2 mRNA accumulation.

Effects of Forskolin Are Mediated by PKA. Forskolin elevates intracellular cAMP by directly activating adenylate cyclase, and cAMP in turn activates cAMP-dependent protein kinases (PKA). To ascertain whether all the effects of forskolin depend on the activation of PKA, we introduced a dominant negative mutant PKA regulatory subunit cDNA construct, driven by the Harvey sarcoma virus LTR, into EL4 cells (28). The product encoded by this construct has two point mutations at cAMP-binding sites which block its binding to cAMP but still allow it to associate with the catalytic subunits in competition with endogenous regulatory subunits. Once bound, the mutant regulatory subunit will not dissociate from the catalytic subunit in response to cAMP and therefore acts as a dominant negative regulator of PKA activity. One stable transfectant line showing high expression of the mutant subunit, IIA4, and two pSV2neo transfectant control lines, were tested to compare their forskolin responses. As shown in Fig. 2 *A* (*triangles*), the introduction of the mutant subunit had no effect on the ability of the cells to elevate their cAMP levels in response to forskolin. However, the mutant subunit completely protected the induction of IL-2 mRNA from forskolin inhibition in the IIA4 line, in contrast to the significant inhibition of IL-2 expression by forskolin in control lines as measured 4 h after stimulation (Fig. 4 *A*). In agreement with its effects on IL-2 mRNA expression, expression of the dominant negative mutant PKA also relieved the inhibitory effects of forskolin on NF-κB and TGGGC binding activity (Fig. 4 *B*). The NF-κB and TGGGC

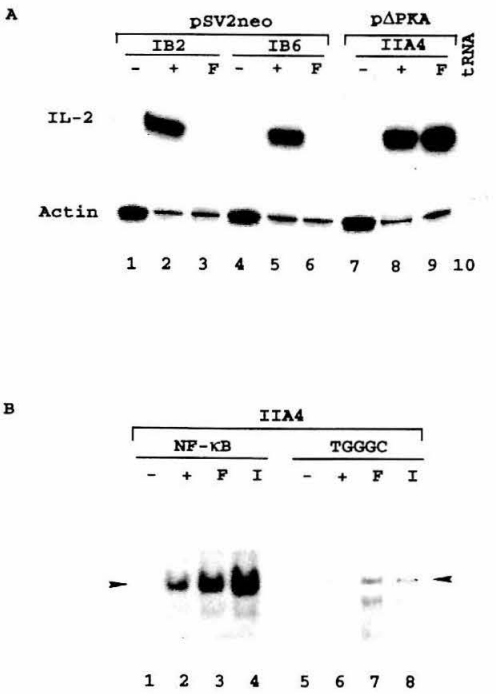


Figure 4. Dominant negative mutant PKA transfected cells are resistant to the forskolin inhibitory effect. (*A*) Levels of IL-2 mRNA induced in two control lines of pSV2neo transfectants, IB2 and IB6, and in one line of mutant PKA transfectants, IIA4, were compared by RNase protection analysis with 10 μg of RNA from uninduced cells (−), or cells after 4-h stimulations (+) in the absence or presence (*F*) of forskolin. 1 μg of RNA from each sample was hybridized with actin probe to verify comparability and integrity of the RNA. (*B*) Nuclear extracts from IIA4 cells were tested for NF-κB and TGGGC binding activities. All stimulations were for 4 h. (−) Uninduced; (+) induced with TPA and A23187; (*F*) induced with TPA and A23187 in the presence of forskolin; (*I*) induced with TPA and A23187 in the presence of 50 U/ml IL-1.

binding complexes, and the two lower inducible CACCC binding complexes, were fully inducible in the mutant PKA-containing cell line IIA4 whether forskolin was added or not (compare Figs. 4 *B* and 1 *B*; and data not shown). The binding activities of other factors in forskolin-treated IIA4 cells remained the same as in cells stimulated without forskolin (data not shown). The mutant cells were not simply refractory to modulation of NF-κB activity, for they remained capable of upregulating their NF-κB site binding activity upon stimulation in the presence of 50 U/ml IL-1 (Fig. 4 *B*, lane *4*) (20). Therefore, it is clear that PKA is a mediator of the inhibitory effects of forskolin on DNA-binding activities.

Forskolin Inhibits Stable In Vivo Assembly of the IL-2 DNA–Protein Complex. The highly selective effects of forskolin on particular DNA-binding activities raised the question of whether the binding activities that remain are capable of forming stable contacts with IL-2 regulatory DNA in the nuclei of forskolin-treated, stimulated cells. To resolve this issue, living cells were treated with dimethyl sulfate (DMS), which methylates G residues (met-G) in the major groove.

Intimate protein contacts with these residues can either protect them from methylation or make them hypersensitive to methylation. The subsequent piperidine cleavage of the extracted DNA at met-Gs followed by ligation-mediated PCR amplification generates a G-specific sequence ladder. In fact, this technique has revealed specific major groove contacts at most or all of the sites known to be required for IL-2 expression (19). In agreement with the results we have shown previously (19), there is no obvious difference in the G ladder pattern between the DNA from DMS-treated, uninduced cells and naked control DNA which was DMS treated after purification (Fig. 5 *A*, lanes *1*, *2*, and *5*). However, multiple footprints spanning almost the entire 300-bp upstream sequence were found in DNA from cells stimulated for 4 h with TPA+A23187 in the absence of forskolin, which corresponded to the well-characterized *cis* elements noted previously (Fig. 5, compare lanes *2* and *3* in *A* and lanes *1* and *2* in *B*). Whereas these DMS footprinting results do not rule out some form of protein–DNA associations at the IL-2 locus in uninduced cells, such associations clearly do not include the diagnostic, specific contacts that are formed at regulatory sites in induced cells. As noted above, these specific sites of occupancy define the oligonucleotide probes used in Fig. 1. Each site was revealed in vivo by protection (○►), hypersensitivity (→), or a characteristic pattern of both; examples are shown in Fig. 5 *C*. As previously discussed (19), G residues in the footprint are never completely protected from cleavage, and this effect is exacerbated by the asynchrony of IL-2 transcription in the stimulated population, in contrast with the more complete protection seen in the regulatory sequence of a constitutively expressed gene such as metallothionein-I (19, and data not shown). However, the pattern of protections and hypersensitivities at the IL-2 locus in stimulated cells was still highly reproducible.

When examining the DNA from cells induced for 4 h in the presence of forskolin, pronounced differences from the characteristic induced pattern were noted. First, at almost every *cis* element, signs of occupancy were absent (Fig. 5, *A*, lane *4* and *B*, lane *3*). Not only were the NF-κB and TGGGC sites empty, but the two NF-AT sites (Fig. 5 *C*), the AP-1 site and the proximal CACCC site also remained unoccupied, even though the factors binding these latter sites were present in the nucleus and were able to bind to their sites individually as shown by in vitro mobility shift studies (see above, Fig. 1). The global blockade of stable footprint formation is reminiscent of the CsA effect, which tightly shuts off IL-2 transcription and completely eliminates all evidence for protein–DNA interaction in footprinting analysis (19 and see below). Only one sign of protein–DNA interaction was consistently seen in the cells stimulated in the presence of forskolin: a persistent hypersensitivity at the 5′-most G in the distal CACCC element. The significance of this isolated feature, at the border of the known IL-2 enhancer, is unknown.

The elimination of protein–DNA contacts in cells that had been stimulated in the presence of forskolin was dependent on PKA activity, as shown with DNA samples from mutant PKA transfectants. Like the parental cells, IIA4 cells established a full range of protein–DNA contacts in response to TPA+A23187 (Fig. 5, *A*, lane *6*; *B*, lane *5*). In contrast to the wild-type parental EL4 cells, treatment of IIA4 cells with forskolin did not affect any of the TPA+A23187-induced protections and hypersensitivities on the DNA sequence ladder (Fig. 5, compare lanes *4* and 7 in *A* and lanes *3* and *6* in *B*). Thus the disruption of protein–DNA contacts at the IL-2 locus was a specific effect of PKA activation.

The effect of cAMP elevation on protein–DNA contacts differed from that of CsA, described previously (19), in that the initial establishment of footprints proceeded normally in the presence of TPA+A23187+forskolin, for example as measured after 2 h of stimulation (data not shown). The absence of footprints at the later time points thus reflected the net disassembly of preexisting complexes. To confirm the ability of forskolin treatment to block continuation of occupancy, we examined DNA samples from two independent forskolin chasing experiments (Fig. 6). In agreement with the binding studies and RNA analysis, the results showed that 2 h of forskolin chasing after 2 h of stimulation did not change the preexisting footprints formed after 2-h stimulation (Fig. 6, lane *3*). By contrast, the same 2-h chase with CsA completely reversed the stimulated footprint pattern to an "uninduced" one (Fig. 6, lane *4*). When forskolin chasing was extended to 4 h, however, a gradual weakening of protein–DNA interactions was seen (Fig. 6, lane *9*). Notably, footprints weakened at all sites concomitantly, with the unstimulated pattern reemerging as quickly at the NF-AT site as at the NF-κB site. Thus, even preformed IL-2 DNA–protein complexes are not stable in vivo in activated cells without the continued contribution of forskolin-sensitive components.

Discussion

Agonists that elevate intracellular cAMP are likely modulators of immune responses in vivo (6–10). We and others have previously shown that they selectively interfere with IL-2 expression. Here we also report that the inhibition can follow a distinctively delayed time course, allowing an initial limited burst of IL-2 expression before transcription ceases. These qualitative and kinetic effects on cytokine gene expression resulting from the elevation of cAMP could be used in vivo to play a role in the shaping of a Th2 vs. Th1 response.

In this work, we present molecular evidence for the way elevation of cAMP downregulates expression of the IL-2 gene in a model cell line. At least two transcription factors associated with IL-2 activation, including NF-κB and the newly described TGGGC-binding factor, were specifically reduced in nuclear extracts from cells that were activated in the presence of forskolin. This pattern of transcription factor inhibition differed markedly from the patterns of inhibition by CsA treatment or anergy induction, in that neither NF-AT nor AP-1 binding activities were detectably reduced. With a closer look at protein–DNA interactions by in vivo footprinting, we discovered that the loss of the NF-κB and TGGGC binding activities was correlated with the disappearance of stable pro-

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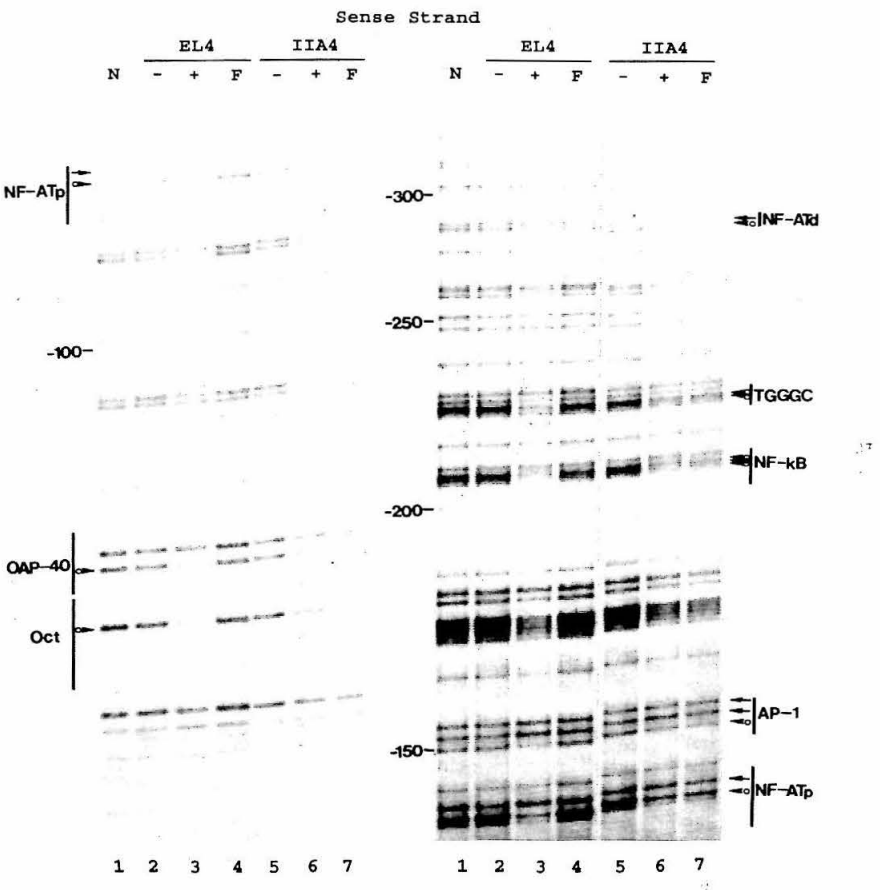
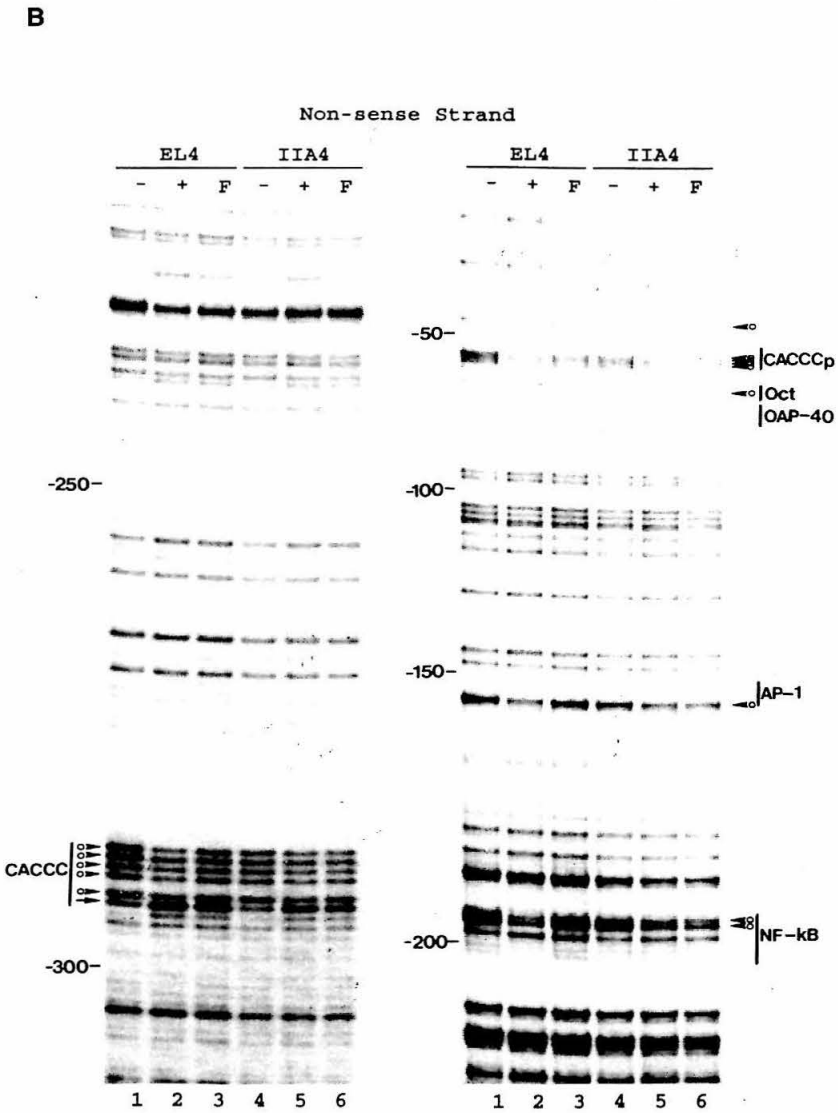


tein–DNA contacts at virtually all detectable sites in the IL-2 enhancer. Thus, cAMP-sensitive transcription factors appear to be as necessary as the CsA-sensitive factors described previously (19) for the maintenance of transcription factor complexes at the IL-2 locus.

Mechanism of Forskolin Effects on NF-κB. All the inhibitory effects of forskolin treatment studied here were executed by cAMP-dependent protein kinases, since cells expressing a dominant negative PKA mutant were completely resistant to forskolin effects. These cells preserved a high level of IL-2 mRNA accumulation, full NF-κB and TGGGC binding activities in their nuclear extracts, and persistent high affinity protein–DNA interactions at the IL-2 locus even in the presence of forskolin. In some cellular contexts, NF-κB can be activated by PKA (26, 29–31), but in stimulated EL4 cells, the net role of PKA is to inhibit NF-κB availability. Because it was not a complete failure of NF-κB induction but rather an inability to sustain NF-κB activation that was observed, it remains possible that early PKA activation might initially synergize with PKC to activate NF-κB through immediate IκB phosphorylation (27, 29–31). However, recent work has described additional pathways for PKA that provide a basis for delayed effects. It was reported that it takes 30 min for the catalytic subunit of PKA to translocate to the nucleus, and phosphorylation of the cAMP response factor CREB and CREB-dependent gene activation occur only afterwards (32). Thus a delayed effect of cAMP elevation on NF-κB levels would be expected if the inhibition were dependent on de novo transcription of some target gene. Preliminary experiments did not reveal qualitative differences between the types of NF-κB/c-Rel complexes induced at 2 h, at 4 h, and at 2 h in the presence of forskolin (data not shown). However, any sustained NF-κB activation, as observed in stimulated EL4 cells, requires both new synthesis and ongoing nuclear translocation of NF-κB molecules (33). Forskolin could interfere with this by (*a*) causing direct cAMP-dependent repression of ongoing NF-κB subunit transcription; (*b*) causing cAMP-dependent overexpression of an appropriate form of IκB; or (*c*) directly or indirectly stimulating NF-κB proteolysis. The degree to which each of these mechanisms contributes to the lagged inhibition of NF-κB binding activity remains to be determined.

How Are Reduced NF-κB and TGGGC Binding Activities Related to the Inhibition of IL-2 Gene Expression? In our



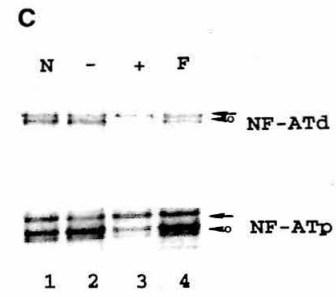


Figure 5. Forskolin affects the formation of specific DNA binding complexes at multiple sites of the IL-2 promoter/enhancer region. In vivo genomic footprinting on IL-2 300-bp promoter/enhancer regions was performed with DNA samples from EL4 (lanes *2–4*, *A* and lanes *1–3*, *B*) and IIA4 cells (lanes *5–7*, *A* and lanes *4–6*, *B*) cultured under different conditions. Resulting ladders for the coding strand are shown in *A*, and for the noncoding strand in *B*. (*C*) A close view of the effects of forskolin treatment on footprints at the distal and proximal NF-AT sites. (*A* and *B*) Specific sites and the distance relative to the transcription start site are as indicated. (*N*) Naked DNA sample from EL4 cells; (−) unstimulated cells; (+) induced with TPA+A23187 for 4 h; (*F*) induced with TPA+A23187+forskolin for 4 h. Comparing lanes between the uninduced and induced samples, multiple footprints throughout the whole 300-bp region are clearly observed, consisting of reproducible partial protections (○►) and hypersensitivities (►). In the forskolin-treated EL4 lane, the sequence ladder generally resembles the uninduced one, with the exception of the hypersensitive G in the distal CACCC site (*non-sense* strand), as noted in the text. However, as seen in lanes *5–7* for both strands, forskolin did not affect the footprint formation in IIA4 cells. This subline exhibited relatively weak footprints in general, but there was no significant difference between the footprints in induced samples of the mutant cells whether forskolin was present or not.

previous studies (19), we described three extreme situations: when the IL-2 gene was silent, fully activated, or suppressed by CsA. Each condition was associated with the availability of a different spectrum of IL-2 DNA binding proteins as assayed in nuclear extracts. However, in vivo footprints revealed a simpler dichotomy, with either no detectable occupancy or a fully occupied footprinting pattern (Fig. 7). This indicated that in vivo, transcription factors interact with IL-2 regulatory DNA in a highly coordinated, all-or-none fashion. The effects of forskolin treatment on in vivo footprints at the IL-2 locus enhance the previous conclusion in three ways.

First, the key finding is that exposure to forskolin during stimulation blocks the formation of stable footprints at virtually all the sites of protein–DNA contact that we detect in the IL-2 enhancer. In nuclear extracts from forskolin-treated cells, almost all the major binding activities for these sites appeared to be available at normal levels; only two major complexes, the upper NF-κB and TGGGC complexes, were affected. Yet in the nuclei of living, forskolin-treated cells, none of the binding activities could in fact engage their target sites in the context of the full IL-2 enhancer (Fig. 7). In vivo, the binding of forskolin-insensitive factors like NF-AT was therefore contingent upon the binding of forskolin-sensitive factors like NF-κB. This finding parallels the effect of CsA on the establishment of stable contacts (19, and Fig. 6). However, as summarized in Fig. 7, a different subset of factors is prominently affected by each inhibitor. The effect of CsA treatment, considered alone, made it possible to speculate that a single CsA-sensitive factor, like NF-AT, might play a unique role in opening the IL-2 chromatin structure for transcription. However, considered in light of the similar effects of forskolin treatment, such a model is much less likely. A probable inference from the results summarized in Fig. 7 is that the assembly of stable transcription factor complexes at the

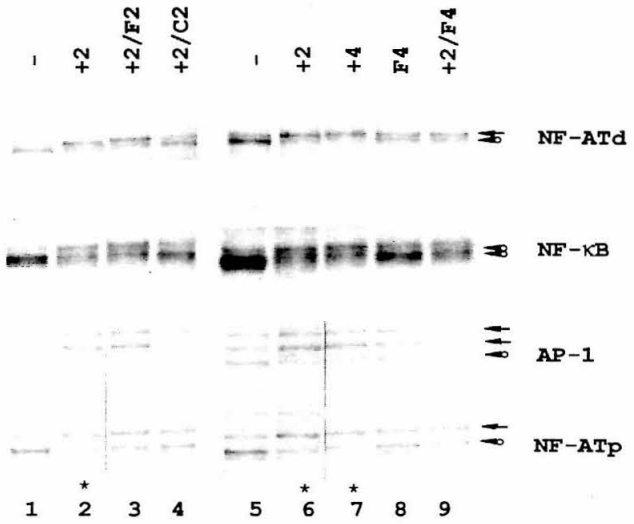


Figure 6. Forskolin has delayed effects on the stability of preformed protein–DNA complexes. In vivo footprints at three regions of the IL-2 regulatory sequence (*sense* strand) are shown. The data in lanes *1–4* and in lanes *5–9* are from two independent experiments. Lanes *1* and *5* contain DNA from uninduced cells. Lanes *2* and *6* contain DNA from cells induced with TPA+A23187 for 2 h. In lanes *3* and *4*, cells were stimulated for 2 h, and then forskolin (lane *3*) or CsA (lane *4*) were added for another 2 h. In lane *3*, the inducible footprints seen in lane *2* remain identifiable. However, in lane *4*, those inducible footprints seen in lanes *2* and *3* disappear and the overall sequence ladder approaches the uninduced state seen in lane *1*. In lane 7, cells were stimulated for 4 h without inhibitor. In lane *8*, cells were stimulated for 4 h with forskolin added from time 0. In lane *9*, cells were prestimulated for 2 h, then incubated with forskolin for another 4 h. Stimulated samples without inhibitors (lanes *2*, *6*, and 7) are indicated with asterisks.

IL-2 locus requires multivalent protein–protein interactions as well as the protein–DNA interactions at the individual sites. Thus, a major component of the combinatorial action of these transcription factors may be the cooperativity required for their binding.

Second, a further clue to support the need for cooperative binding in IL-2 regulation may be provided by the persistence of a sole residual site of activation-associated protein–DNA contact in nuclei of forskolin-treated cells, long after other footprints have disappeared. The transcription factor or nucleosomal structure responsible for this feature is unknown. The isolated DMS hypersensitivity at the distal CACCC site is a structure that is not only distinguished from the resting state and the fully activated state of the IL-2 locus, but also distinguished from the state of the IL-2 locus in cells activated in the presence of CsA. The equivalence of the CsA-treated pattern with the resting (uninduced) pattern had not allowed us to distinguish whether limited chromatin accessibility or a requirement for cooperative binding actually restricted occupancy. However, it is less likely that "accessibility" is limiting after PKA activation, since residual CACCC occupancy is still permitted in forskolin-treated cells.

Finally, the effect of forskolin differs from that of CsA (19) in that the presence of forskolin does not block transcription factor binding initially. In fact, the time course of its effect on in vivo protein–DNA contacts is delayed in parallel with the effect on NF-κB availability. This indicates that a key role is played by NF-κB or by a factor(s) inhibited with a similar time course. The transient assembly and disassembly of complexes in forskolin-treated cells suggests that "stable" complex formation could be a misnomer in general. Instead, the accessibility of the IL-2 gene to sharply discontinuous regulation may result from rapid cycling of transcription factors between disassembled and reassembled states. The rate of transcriptional initiation, by this model, would depend on the rate with which the most limiting component could be engaged. Such a model would explain how forskolin could first inhibit IL-2 expression to a modest degree, through reduction in the TGGGC factor, and then more severely as NF-κB complexes also became rare. Further support for this interpretation is provided by the results of the experiments in which cells were stimulated normally, then subjected to a inhibitor chase. Forskolin and CsA work through completely different mechanisms, resulting in blocking the availability of different subsets of factors, yet both can shift the equilibrium to dissociation of active IL-2 transcription complexes.

This study adds a third set of transcription factors to those already known to be critical for physiological regulation of IL-2. As discussed in the introduction, inhibition of NF-AT and inhibition of AP-1 separately can block IL-2 expression. Neither of these factors is detectably inhibited by forskolin. Our results suggest a similar need for NF-κB, the TGGGC factors, and/or other factors that are sensitive to PKA activation. In vivo footprinting data are not yet available for any of the cases where AP-1 is selectively inhibited. However, generalizing from the effects of forskolin and the effects of CsA, we could predict that selective AP-1 inactivation would also destabilize the whole protein–DNA assembly at the IL-2 enhancer. This would confirm a nonhierarchical requirement for the various IL-2 regulatory factors. Such a mechanism vividly illustrates how, as a cell-specific and activation-dependent response, IL-2 expression is rendered vulnerable to multiple signaling events.

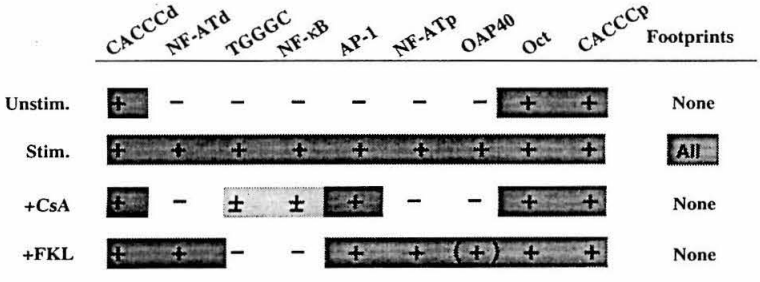


Figure 7. Summary of the availability and in vivo binding to the IL-2 enhancer of different transcription factors, as measured by gel mobility shift assays and in vivo footprinting in EL4 cells under different stimulation conditions. (*Stim.*) TPA+A23187; (*+CsA*) TPA+A23187+CsA; (*+FKL*) TPA+A23187+forskolin. The pattern shown represents results obtained when stimulation, in the presence or absence of the indicated agents, is carried out for at least 4 h.

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CONCLUSION

The ultimate goal for T helper cells during an immune response is to be activated to secrete lymphokines to boost the functions of T killer cells and B cells. The regulation of lymphokine secretion is under the control of multiple different kinds of signals. T cells normally keep cytokine genes silent as much as possible and, if the stimulation is inappropriate, the cells simply become anergized. Only when the T cell receptor matches antigen/APC and the appropriate accessory signals are present can T cells be activated. In the present study, we selected IL-2 as an example of a set of lymphokines specific to T cells to investigate the molecular mechanisms of developmentally controlled IL-2 gene inducibility, specifically the mechanisms of how different signals affect IL-2 expression at the level of protein-DNA interaction.

First, we showed that one reason that T cells at a critical developmental stage, i.e., $CD4^+CD8^+TCR^{lo}$, transiently lose the ability to make IL-2 is due to the fact that they cannot activate fos genes, which results in a significant reduction in AP-1 binding activity in the nuclei. Furthermore, we showed that these cells in parallel with a reduction of AP-1 binding activity, also have a reduced NF-AT binding activity. Both may be attributable to the lack of fos proteins, although it is also possible that there is a blocking of NF-ATc nuclear localization. Another developmental stage dependent control of the IL-2 gene is in the immature $CD4^-CD8^-TCR^-$ cells. In addition to 'TCR'-like activation signals, they also require IL-1 to make IL-2. Stimulation of immature cells in the absence of IL-1 only activates limited amount of NF-κB, which is lower than in mature T cells. We showed that IL-1 can potentiate the NF-κB binding activity in these nuclei (Rothenberg et al., 1994). The compensation of the intrinsic, developmental stage related deficiency of NF-κB in activated cells by IL-1 could be the reason why immature cells require IL-1 to produce IL-2.

Second, upon examing the IL-2 promoter region with in vivo footprinting analysis, two forms of the promoter are seen. One shows a complete occupancy of the promoter by transcription factors, the other is no occupancy by sequence-specific transcription factor at

all. The footprinting patterns parallel directly with the activation state of the IL-2 gene. Under conditions when all the sites in the promoter region are bound by transcription factors, IL-2 gene transcription is detected; while under conditions when there are no footprints on the promoter, no IL-2 transcription is observed. These results indicate that sequence-specific protein-DNA interaction and subsequent IL-2 gene activation require the presence of all the necessary transcription factors.

Our studies went one step further from previous work toward an understanding of both the developmental control and signalling regulation of IL-2 gene expression. We especially focused on the point which connects the final targets of signal transduction pathways and initiation of transcription activation, and demonstrated how trans-acting factors connects these two processes. The results not only help us understand gene regulation at a new level but also shed light on the issue of T-cell development.

Implications of gene regulation

Requirement for combinatorial and coordinated protein-DNA interaction. The all-or-none footprinting pattern seen in IL-2 producing T cells and IL-2 non-producing cells at the IL-2 proximal promoter region means that both in T cells in the resting state and in non-T cells, the induction independent transcription factors, such as octamer and the factor for the CACCC site, are unable to bind their cognate sites. Yet in T cells in the stimulated state, the induction independent and dependent factors assemble a protein-DNA complex. In several cases where stimulated T cells are unable to express the IL-2 gene, a limited number of transcription factors has been implicated as the cause. For example, CsA treatment results in decreased NF-κB and especially NF-AT binding activities, anergized T cells are specifically unable to activate the AP-1 factor, glucocorticoid stimulation sequesters the AP-1 factor by dimerizing it with GR, and in $CD4^+CD8^+TCR^{lo}$ thymocytes both NF-AT and AP-1 binding activities are reduced. All these cases seem to imply that either NF-AT or AP-1 or both may be the key factor which may either be involved in

loosening up the chromatin structure or initializing the coordinated binding. However, we showed that NF-AT and AP-1 are not the only factors which are required for the coordinated binding. Forskolin treatment, which reduced the TGGGC and NF-κB bindings but not NF-AT nor AP-1, also led to no protein-DNA interaction. Thus, the maintenance of the protein-DNA complex may not be an ordered process as is the assembly process in which NF-AT is presumbly the pre-requisite, rather it is an interdependent process requiring the presence of every activator protein.

The coordinated assembly and maintanence of the protein-DNA complex may have two purposes. First, it may intiate a high affinity cooperative binding to form a stable nucleoprotein-DNA structure. Because some of the binding sites for inducible factors like AP-1 and NF-κB, are not 100% homologous to the consensus sequences, the individual binding affinities may be many fold lower than they are to the concensus site. Second, the low affinity binding sites may be required to maintain the cell specificity of the IL-2 gene. It has been demonstrated that mutating the NF-κB site to a higher affinity κB site resulted in a loss of T-cell specificity of the concatamerized IL-2 κB promoter (Briegel et al., 1991). Therefore, in addition to providing the inducibility of the gene, the combinatorial and coordinated fashion of binding may also contribute to its cell specificity.

The protein-DNA interaction is dynamic. Once the regulator is bound to its cognate site and the footprint is observed, the interaction is not static but dynamic. The maintenance of the interaction requires continued protein synthesis or modification. It has been demonstrated that blocking protein synthesis with cycloheximide leads to loss of the protein-DNA contact in vivo (Weih et al., 1990). In CsA and forskolin chase experiments, we showed that the disappearance of footprints correlated proportionately with a decrease in IL-2 transcription, indicating that the DNA-binding activity of CsA and forskolin sensitive factors are subject to turnover in the nuclei. More importantly, this finding provides strong evidence for a dynamic equilibrium on-and-off interaction of the transcription factors with DNA. Addition of forskolin gradually reduced the TGGGC and

NF-κB binding activities, which shifts the equilibrium toward the "off" direction. In contrast to the relatively slow onset of the forskolin effect, CsA blocking is kinetically more efficient than forskolin. This could be related to its immediate inactivation of the Ca^{2+} signalling pathway, which is required for the nuclear localization of the cytoplasmic component of NF-AT and the trans-activation activity of the octamer factor. The coordinated binding basically demands the presence of all the binding factors. Decreasing nuclear concentrations of certain factors in turn shifts the equilibrium towards dissociation. The equilibruim reaction could also shift toward the "on" direction by increasing the nuclear concentration of certain factors. This is best illustrated in activated T cells in the presence of IL-1. With the rapid increase in AP-1 and NF-κB factor binding activities, the initial increase in association rate shifts the onset and peak of IL-2 transcription earlier kinetically. Later during activation, when factor-DNA interactions reach equilibrium, transcription levels off. The conclusion, which indicates that formation of stable protein-DNA complex requires sustained high level of transcription factors, is also in agreement with the interpretation for the threshold phenomenon (Fiering et al., 1990) .

According to our conclusions, it can be argued that mutating one of the binding sites should abolish any binding and prevent transcription. But in fact, mutagenesis experiments did not give 100% reduction of transcription. This is because first, mutating a site is not equivalent to knocking out the activator protein physically. Mutated sites may have reduced binding activity when analyzed individually in *in vitro* assays. In the *in vivo* situation, where all the necessary factors are available upon stimulation, the protein-protein interaction may synergize in binding and overcome the low affinity association to the mutated site. Beside, there may be multiple sites for one factor, especially in the natural gene, and reducing one factor may affect the binding at multiple sites. Mutating one site, on the other hand, does not affect the binding to other sites. Second, the high affinity binding of factors for mutated sites may not be absolutely required but protein-protein interactions including interactions with transcription factors or with general transcription

machinery, like the interaction of NF-κB and TATA-binding protein may be sufficient (Kerr et al., 1993). Third, studies on MMTV promoter showed that transiently introduced template is free of nucleosome and has hormone-independent NF1 factor binding, while stably integrated MMTV template has phased nuclesomes and requires hormone induction to form NF1 binding. In both cases, transcription is hormone-dependent, but the stable template has 3 to 4-fold greater enhancement by the hormone. This suggests that transient template may be derepressed (Archer et al., 1992). Similarly, transiently transfected IL-2 templates in mutagenesis assays may have no or improperly phased nucleosome assembly, which may change the accessibility of the DNA and the transfected gene may be less stringently regulated compared to the endogenous nucleosome-phased IL-2 gene.

How is the protein-DNA complex assembled? It may take two steps to finish the assembly. Since a DNAase I hypersensitive site in this region (~-100) is induction-dependent, the assembly of the protein-DNA complexes may be secondary to the alteration of chromatin structure. An inducible, T-cell specific factor may be responsible for such an alteration, similar to the role which the glucocorticoid receptor plays in hormone induction of the liver-specific TAT gene and the MMTV promoter (Rigaud et al., 1991; Cordingley et al., 1987). This factor may be among a subset of factors sensitive to cyclosporin A inhibition and may bind either to the -300bp or -100 bp DNase I hypersensitive site. Once the DNA is accessible, cooperative binding is initiated which can be visualized as stable footprints covering almost the entire IL-2 promoter. Such cooperativity could increase the binding affinity 3 to 6 orders of magnitude in certain cases (Xia et al., 1991). These two steps are sequential events in the activation process.

Different types of IL-2 non-expression can be enforced by different mechanisms. As for non-T cells, like muscle cells or fibroblasts, the inaccessibility of DNA to nuclease indicates a closed chromatin structure at the IL-2 locus. In addition to the deficiency of inducible transcription factors, the chromatin inaccessibility also acounts for the IL-2 gene repression. In resting T cells, which have an activatable chromatin structure characterized

by the presence of nuclease hypersensitive sites, the lack of inducible factors to further open up the chromatin and initiate cooperative binding may be attributed to their inability to express the IL-2 gene. This mechanism may also apply to some T cell related hematopoietic cells which also have an activatable chromatin configuration. In activated T cells, withdrawing factors during activation by cyclosporin A or forskolin treatment destablizes the coordinated binding of all transcription factors along the entire promoter region and results in interruption of IL-2 transcription. However, it is not clear whether chromatin resumes the resting state.

The assumption that T-cells committed to make IL-2 may have been imprinted at the IL-2 locus is not disproven yet. This is because the further upstream 300 bp region is not examined and, together with the proximal 300 bp, is highly homologous to the human IL-2 gene and contains an induction-independent DNAase I hypersensitive site specific for T cells and some hematopoietic cell lines. It remains possible that T-cell commitment or hematopoietic-lineage commitment may be characterized by a distinct pattern of protein-DNA interactions in this region and at the other two induction-independent hypersensitive sites as well.

Implications of T lineage specification

IL-2 gene inducibility is known to be specific to $CD4^+$ T cells, but does it correlate only with the determination of the $CD4^+$ T lineage or does it correlate with even earlier developmental events? Recent progress in understanding the divergence of $CD4^+$ and $CD8^+$ cells demonstrated that positive selection is not when double positive cells make their lineage choices; rather, that decision is made prior to the selection processes (Chan et al., 1993; David et al., 1993). The functional competency required for either CD4 or CD8 cells has been shown to be present in immature $CD4^-CD8^-TCR^-$ cells prior to TCR gene rearrangement and prior to the selection process. For example, these imature cells can be induced to make T helper-specific genes like the IL-2 gene and T killer-specific genes like

perforin (Rothenberg et al., 1993; G. Reynard's unpublished data). It is not known whether these cells are either still multi- or bi-potential or whether they have been determined to be either CD4 or CD8 cells; the distinction requires the identification of whether each gene is expressed individually in only a subset of immature cells or if that are co-expressed in all cells. Lineage tracing would also answer this question. Several lines of evidence, however, may actually indicate that acquisition of IL-2 gene inducibility might even occur earlier and that it is possibly maintained in cells which differentiate to the CD4 lineage but conditionally lost in cells directed towards the CD8 lineage. First, both $CD8^+$ thymocytes and $CD8^+$ splenic T cells can be induced to make IL-2 under certain conditions (McGuire & Rothenberg, 1987; McGuire et al., 1988). Second, certain cells in day 14 fetal thymus and $\gamma\delta$ T cells in the gut and skin are making IL-2 in vivo, suggesting that hematopoietic stem cells from different developmental stages are able to generate IL-2 producing T cells, and that IL-2 producing cells are not limited to cells derived from stem cells that give rise to adult thymic-derived $CD4^+$ $\alpha\beta$ T cells (Yang-Snyder & Rothenberg, 1993; Zúñiga-Pflücker et al., 1993). Third, some B cell lines or B lymphomas can also be induced to make IL-2 (Amigorena et al., 1992; Bonnerot et al., 1992). Splenic B cells from transgenic mice bearing 3x(NF-AT sites)-TK promoter-SV40 large T antigen construct also make T antigen, although with kinetically delayed expression compared to splenic T cells (Verweij et al., 1990). Some T-lineage specific transcription factors for activating the expression of TCR genes are also present in pre-B cells and mature B cells, more interestingly, some even express in cells beyond the prothymocytes stage (Clevers et al., 1993; and later discussion). These data may suggest that IL-2 gene inducibility occurs earlier before the divergence of T and B lymphocytes. Fourth, chromatin structure analysis revealed that the three DNase I hypersensitive sites observed in resting T cells are also present in two related hematopoietic cell lines, but not in two non-hematopoietic cell lines, including an epithelial Hela cell line (Siebenlist et al., 1986). Although the presence of nuclease-sensitive sites may indicate the potential to make IL-2, it has not been shown that

non-lymphoid hematopoietic cells can make IL-2. Thus, based on these observations it can be speculated that the IL-2 gene may be methylated and has a repressed chromatin structure. Generation of hematopoietic stem cells from blood island, fetal liver, and adult bone marrow might be accompanied by demethylation and changes in chromatin structure of the IL-2 locus. Differentiation of hematopoietic cells to non-lymphoid cells might be accompanied by maintaining or losing nuclease-sensitive sites. Finally, differentiation to lymphoid cells might be accompanied by acquisition of lymphoid cell-specific transcription factors which enable them to make IL-2. However, further differentiation to B cells, $CD8^+$ T cells, and to T helper type 2 cells probably down-regulates these factors, as has been shown for the regulation of cortical cells, which make them less competent than T helper type 1 cells. The latter point is supported by the observation that exogenously introduced IL-2 promoter-CAT gene constructs cannot be activated in a series of cell lines representing B-cells, pre-mast cells, macrophages, Th2 type helper cells, thymoma cells, and CTLs (Novak et al., 1990). It is also supported by the fact that increasing certain transcriptional activators by over expressing eIF-4E in Th2 cells can activate the IL-2 gene (Barve et al., 1994).

What are the transcription factors that potentiate IL-2 gene inducibility? One candidate is NF-AT, which has been shown to be a T-cell specific transcription factor by in vitro gel shift studies and by transgenic studies which showed a T-cell restricted expression pattern of a reporter gene driven by a heterologous promoter plus a multimerized NF-AT site (Verweij et al., 1990). NF-AT is composed of two components; one is Ca^{2+} signalling sensitive cytoplasmic protein, the other is a PKC sensitive general transcription factor AP-1 (Flanagan et al., 1991; Jain et al., 1992). The lack of this cytoplasmic NF-AT could block IL-2 gene activation as shown in this study and other studies (Flanagan et al., 1991), it is very important to find out whether this factor is developmentally regulated and whether it is the factor which endows lymphoid or T lymphoid cells with the competence to make IL-2. The second candidate is AP-1. It is not simply because it is the nuclear component of

NF-AT, but also because it is co-regulated with NF-AT in cortical thymocytes. Since AP-1 is a general transcription factor and regulates multiple genes, it is difficult to envision it as a regulator involving T cell differentiation and mediating IL-2 gene activtion. However, we showed that the inducible transcription of three fos genes is reduced in cortical thymocytes, and the AP-1 factor formed in these cells would only be Jun-Jun intrafamily dimers which have greatly reduced DNA binding affinity and may have reduced transcriptional activity. A similar up and down regulation of AP-1 has been reported during meyloid and granulocyte lineage differentiation (Mollinedo et al., 1993). The B cell developmental defect in c-fos knockout animals is another example of how this seemingly general transcription factor can actually fine tune lineage differentiation (Okada et al., 1994). This regulation of transcription factor level is mediated by developmental signals. Signals mediated by receptors other than the TCR have been shown to regulate c-fos and c-jun expression. For example, signals transmitted through the IL-2 receptor induce c-myc, c-fos, and c-jun transcription, and IL-2 receptor γ chain has been implicated for this function (Asao et al., 1993). Since the γ chain is down-regulated during the transition to cortical cell type, there might be a connection between γ chain expression and c-fos expression. It is highly possible that both tissue or lineage specific factors and general transcription factors may function in cooperation to control differentiation processes. The NF-AT and AP-1 are more likely involved in not only the acquisition and maintenance of the competence to make IL-2, but they may also take part in the lineage commitment process.

Recently, a number of T-cell specific or lymphocyte specific transcription factors were discovered which may also correlate with or contribute to early T-cell commitment and IL-2 gene activation. Among these factors are a group of minor groove binding proteins which are difficult to visualize by the DMS *in vivo* footprinting technique used in our assays. These factors were discovered during an attempt to identify TCRα and CD3-ε gene trans-acting factors. Three T-cell specific transcription factors (TCF-1, LEF-1, and

Sox-4) were cloned; all of them contained a motif for high mobility group protein (HMG) and are required for enhancer activity in CD3 and CD4 gene expression (reviewed by Clevers et al., 1993). The HMG proteins are a family of proteins containing a region of homology to HMG-1 proteins, and can be categorized as either those that recognize DNA in a relatively non-specific fashion, like HMG I(Y), or those that display high sequence specificity like TCF-1/LEF-1 and Sox-4, etc (Laudet et al., 1993; Bustin et al., 1990). The former has been implicated in the transcription of lymphotoxin genes and the IFN-β gene (Fashena et al., 1991; Thanos & Maniatis, 1992). T cell factor 1 (TCF-1) and lymphoid enhancer-binding factor 1 (LEF-1) do not have any transactivation activity by themselves, but Sox-4 can activate a reporter gene driven by concatamerized Sox-4 sites (van de Wetering et al., 1993). However, LEF-1 has been shown to bend DNA and facilitate assembly of functional nucleoprotein structures in a LEF-1 and a bacterial integration host factor bend-swap experiment (Giese et al., 1992). It is likely that TCF-1 and LEF-1 may function as architectural proteins which bend DNA and facilitate the interaction of proteins bound at the distant 5' and 3' sites in the enhancer. Sequence inspection reveals that the IL-2 promoter region contains many AT-rich islands and two perfect TCF-1/LEF-1 sites are at -130 bp and -95 bp respectively. The site (AACAAAG) at -130 is immediately downstream of the NF-AT site, while the -95 site (CTTTGAA) is immediately upstream of the Oct/OAP40 site. More interestingly, no DNA occupancy or any known binding sites was identified between -130 to -95 bp, and the in vivo footprinting results suggest a lack of protein-DNA interaction in this segment in the major groove. It is likely that in addition to increasing the affinity of NF-ATd and Oct/OAP40 bindings, these minor groove binding proteins may also participate in IL-2 gene regulation by inducing a bending in the region and bringing the proteins bound upstream or downstream of the HMG region closer and facilitating protein-protein interactions. Similar to TCF-1/LEF-1, HMG-I and HMG-Y have been demonstrated to be required for IFN-β gene regulation. Although like TCF-1/LEF-1 they do not have trans-activation activity, they are able to bind to the minor

grooves which fully or partially overlap the NF-κB and ATF-2 sites in the IFN-β promoter and can increase their DNA binding activities by 30 and 4 fold respectively. It has also been shown that there is a direct physical association between HMG-I(Y) and NF-κB or ATF-2 (Thanos and Maniatis, 1992; Du et al., 1993). Therefore, it can be hypothesized that upon stimulation the HMG-domain-containing T cell specific factors will initiate and stabilize the protein-DNA interaction at the IL-2 promoter/enhancer by cooperatively interacting with their adjacent factors like NF-AT and Oct/OAP40. Such increased binding affinity of these transcription factors is crucial for the IL-2 promoter, since the binding sites are not canonical and have relatively low affinity. One experiment showed that conversion of NF-κB, AP-1, and Oct sites to canonical sites resulted in an increase of IL-2 enhancer constitutive and inducible activity in both T cells and non-T cells (Hentsch et al., 1992). Non-T cells do not have TCF-1, LEF-1, or Sox-4 and therefore could not increase the binding affinities of other inducible trans-acting factors and also could not induce bending of the DNA to facilitate protein-protein interaction. There has not been any evidence which shows that these minor groove binding proteins are involved in IL-2 gene regulation. However, the presence of the consensus sites in the promoter and their similar cell-specificity make it possible that these factors may direct early lymphoid and T lineage differentiation; and one of their target genes would be IL-2.

In short, current studies suggest that activation or repression of transcription factors involved in T-cell specific gene regulation may also control lineage specification. A number of T-cell specific transcription factors have been found by studying the regulation of T-cell specific genes (Reviewed by Clevers et al., 1993). As I was writing this up, the murine form of cytoplasmic NF-AT was reported to be cloned (McCaffrey et al., 1993). It seems that now is a good time to test whether any of these factors or sets of factors, in addition to regulating any cell-specific gene expression, can actually initiate T-lineage commitment or regulate a particular stage of T-lineage differentiation in response to developmental signals.

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