

This paper was presented at a colloquium entitled "Biology of Developmental Transcription Control," organized by Eric H. Davidson, Roy J. Britten, and Gary Felsenfeld, held October 26–28, 1995, at the National Academy of Sciences in Irvine, CA.

A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin 2 gene regulation

(T lymphocytes/*in vivo* footprinting/NF-AT/NF- κ B/AP-1)

ELLEN V. ROTHENBERG* AND SUSAN B. WARD

Division of Biology 156–29, California Institute of Technology, Pasadena, CA 91125

ABSTRACT The interleukin 2 (IL-2) gene is subject to two types of regulation: its expression is T-lymphocyte-specific and it is acutely dependent on specific activation signals. The IL-2 transcriptional apparatus integrates multiple types of biochemical information in determining whether or not the gene will be expressed, using multiple diverse transcription factors that are each optimally activated or inhibited by different signaling pathways. When activation of one or two of these factors is blocked, IL-2 expression is completely inhibited. The inability of the other, unaffected factors to work is explained by the striking finding that none of the factors interacts stably with its target site in the IL-2 enhancer unless all the factors are present. Coordinate occupancy of all the sites in the minimal enhancer is apparently maintained by continuous assembly and disassembly cycles that respond to the instantaneous levels of each factor in the nuclear compartment. In addition, the minimal enhancer undergoes specific increases in DNase I accessibility, consistent with dramatic changes in chromatin structure upon activation. Still to be resolved is what interaction(s) conveys T-lineage specificity. In the absence of activating signals, the minimal IL-2 enhancer region in mature T cells is apparently unoccupied, exactly as in non-T lineage cells. However, in a conserved but poorly studied upstream region, we have now mapped several novel sites of DNase I hypersensitivity *in vivo* that constitutively distinguish IL-2 producer type T cells from cell types that cannot express IL-2. Thus a distinct domain of the IL-2 regulatory sequence may contain sites for competence- or lineage-marking protein contacts.

Dual Regulation of the Interleukin 2 (IL-2) Gene: Activation Dependence and Cell-Type Specificity

T cells control the magnitude of the immune response by their highly regulated secretion of cytokines. IL-2, which drives the proliferation of T cells, B cells, and natural killer cells, is one of the best characterized of these cytokines. The expression of IL-2 is controlled almost entirely at the transcriptional level, and the control is stringent: non-T cells have rarely if ever been reported to express this gene, and even T cells do not transcribe the IL-2 gene except in immediate response to an appropriate activating signal. When the IL-2 gene is induced, its expression is transient; the kinetics and magnitude of this response are highly influenced by modulating signals from the environment. Thus the mechanism controlling transcription of the IL-2 gene

uses at least two different kinds of information: one, whether or not the cell is a T cell; and the other, exactly what kinds of signaling pathways are currently activated in that cell.

To explain the mechanism through which IL-2 transcription is regulated, we need to define how these two kinds of information—one stable and heritable through cell lineage, and the other transient and subject to constant physiological revision—are conveyed to the gene. Several possibilities can be envisioned. (i) One is that IL-2 expression depends on at least one transcription factor that is only present in T-lineage cells. Such a factor might be a constitutive T-cell lineage identifier. If this is the case, then the way a specific factor conveys lineage information to the IL-2 gene depends on the way in which it interacts with factors that respond to transient activation pathways. (ii) A second possibility is that there is a factor that is only present or activated in response to a T-cell-specific activation pathway. In this case, the IL-2 gene might in principle be regulated by a single factor, but that factor's own availability would be the resultant of both lineage-specific and activation-specific mechanisms. (iii) A third possibility is that cell-type specificity and activation dependence are mediated by temporally separate mechanisms: the IL-2 locus may be "opened" in a lineage-specific way that is necessary, but not sufficient, for transcription to occur; then activation-dependent factors execute transcriptional initiation in response to immediate physiological conditions. In this last possibility, the activation-dependent factors that control transcriptional initiation need not be T-cell specific at all, so long as the repression of the locus in non-T cells is sufficiently tight. There are also versions of each of these mechanisms in which the critical element is the absence of a negative regulator, instead of the presence of a positive regulator.

It is relevant, in thinking about these mechanisms, that the "lineage-specification" and the actual expression of IL-2 appear to be widely separated in developmental time. Normal resting T cells express no baseline IL-2 transcripts at levels detectable by *in situ* hybridization. The overwhelming majority of T-cell precursors, differentiating in the thymus, also express no detectable IL-2 mRNA (1–4). However, the capacity to express IL-2 in response to an artificial inducing signal appears very early in T-cell development. T cells differentiate from hematopoietic stem cells throughout life, in a programmed series of events including ordered rearrangement of the genes that encode the T-cell receptor for antigen and selection of cells with appropriate recognition specificities (5, 6). Developing T cells are already competent to express IL-2 in response to induction before undergoing any T-cell receptor gene

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Abbreviations: IL-2, interleukin 2; CSA, cyclosporin A; DMS, dimethyl sulfate.

*To whom reprint requests should be addressed. e-mail: evroth@cco.caltech.edu.

rearrangement events (7) (R. A. Diamond, S.B.W., K. Owada-Makabe, and E.V.R., unpublished observations), and it even appears that excellent potential IL-2 producers are among the most primitive cell types in the thymus (7–9), in a population that includes cells that are not yet fully committed to a T-lineage fate (10, 11). This responsiveness contrasts with the inability of non-T bone marrow hematopoietic cells to make IL-2 generally, and the complete lack of IL-2 expression by any somatic non-hematopoietic tissue whatsoever. Thus, some alteration at a molecular level confers inducibility on the IL-2 locus at a particular, early point in the specification of T-lineage cells. This inducibility is then a heritable characteristic of cells in the T lineage, separated from IL-2 expression *per se*, which can remain latent indefinitely or be used at any time in response to stimulation.

While this developmental picture may favor a model of IL-2 regulation with separate mechanisms controlling activation and cell-type specificity, there is evidence that sets of information complex enough to include cell-type specificity can be integrated in the response of the gene to activating signals alone. IL-2 expression, although readily inducible in most mature T cells, is also readily inhibited by any of a variety of developmental or physiological conditions (Fig. 1). Some of these, like exposure to glucocorticoid hormones or elevation of intracellular cAMP, are common occurrences in T cells *in vivo*, and thus are likely to play significant roles in determining when and where IL-2 is actually made. The expression of IL-2 mRNA can also be significantly enhanced under some physiological circumstances, for example by costimulation of the T cell through the CD28 cell-surface receptor along with the T-cell receptor for antigen, and in immature T cells by costimulation with the macrophage-derived cytokine IL-1 (1). Whereas it remains controversial how much of the effect of CD28 is transcriptional and how much posttranscriptional (12–14), it is clear that IL-1 can enhance transcription from the IL-2 promoter (15). Although not all the biochemical mediators in the modulatory pathways are yet defined, enough is known to indicate that at least three separate pathways are involved (discussed further below). Thus activation stimuli are modulated by combinatorial interactions among different signaling pathways in determining the rate and duration of IL-2 transcription.

Regulatory Sequences and Transcription Factors for IL-2 Induction

All the effects of the inducing agents and interfering agents described above appear to be mediated through an ≈ 300 -bp

minimal enhancer region immediately upstream of the transcriptional start site (rev. in ref. 16). This conclusion has emerged from an extensive set of transient transfection experiments introducing IL-2 promoter constructs into tissue culture T-cell lines and assaying their responses to activating stimuli. This region is highly conserved between mouse and human (86% identical) (17, 18), and essentially the whole extent of this minimal enhancer is packed with binding sites for multiple transcription factors, as shown by *in vitro* footprinting (18–20) and electrophoretic mobility shift experiments with nuclear extracts (reviewed in refs. 21 and 22). Many of these sites are bound by transcription factors that are related or identical to well-known factors that mediate activation responses in other cell types—e.g., AP-1 and NF- κ B. Others are bound by ubiquitous factors that are also constitutive, such as Oct-1. There are also factors that were novel when first reported, some of which have subsequently been better characterized: an important example is NF-AT. Other factors have remained controversial [“CD28RC” (14, 20, 23, 24)], while others play roles which remain to be fully elucidated [“Sp1-like” (25, 26) and “TGGGC factor” (27, 28)]. In spite of the multiplicity of these factors, their sites are not functionally redundant. Point mutations or small deletions at almost any one of the known binding sites throughout the minimal enhancer can cause severe effects on transcription. Thus induction of the IL-2 promoter in response to activating agents depends on the binding of multiple distinct factors at multiple sites.

Different Signaling Pathways Differentially Mobilize Induction-Dependent IL-2 Transcription Factors

A notable feature of the factors used to drive IL-2 induction is that they each respond to somewhat different conditions of signaling. Some are constitutive: for example, Oct-1 (29, 30) and a Sp1-like factor (25, 26) that binds at least twice at the borders of the minimal enhancer (27). The others require “activation” to exhibit their binding activities in nuclear extracts, but the activation pathways involved are different. Three important factors have been studied in some detail.

(i) **NF-AT.** NF-AT plays a role that we can paraphrase as the key transducer of the information that the T-cell receptor for antigen, the generator of Ca^{2+} signals, has been engaged. NF-AT is expressed constitutively but can only enter the nucleus after release, via the Ca^{2+} -dependent phosphatase calcineurin, from a cytoplasmic tethering site (rev. in ref. 21). NF-AT is actually a family of factors with divergent Rel domains, each of which is expressed in a somewhat different cell-type distribution (31–35), but all of which appear to have their activities regulated in the

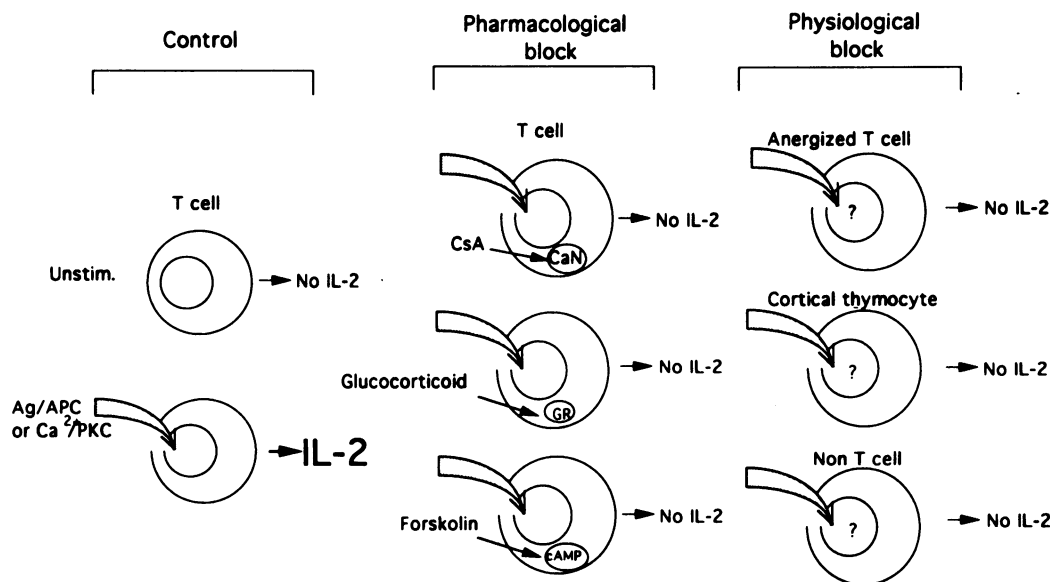


FIG. 1. Sensitivity of IL-2 induction to inhibition through diverse inhibitory pathways. The figure shows the ability of distinct pharmacological and physiological conditions to inhibit the response of cells to strong IL-2-inducing stimuli. All cells represent mature T cells except where otherwise indicated. Ag/APC, antigen plus “professional” antigen-presenting cells. Ca^{2+}/PKC , direct pharmacological stimulation of signaling pathways downstream from T-cell receptor-associated components. Both types of stimulation induce IL-2 expression in T cells, but not in other cell types. CSA, cyclosporin A; CaN, calcineurin; GR, glucocorticoid receptor; PKC, protein kinase C.

same ways. Any condition that interferes with Ca^{2+} flux and/or with calcineurin activity specifically prevents NF-AT from participating in IL-2 expression. CSA or FK506, which inhibit calcineurin, strongly inhibit NF-AT availability but with much less severe effects on other factors (36). However, NF-AT DNA binding activity is apparently unaffected by cAMP, glucocorticoid hormones, or by the changes in signaling that occur in anergized (paralyzed) T cells (28, 37–39).

(ii) **NF- κ B.** The role of NF- κ B can be paraphrased as a messenger of stress: a situation where an immune response is needed to stave off danger. NF- κ B consists of any of a variety of canonical Rel-domain family dimers, with p50 (NFKB1) homodimers, p50-p65 (relA) heterodimers, and p50-c-Rel heterodimers most often found in T cells. Of these, only p50-p65 and p50-c-Rel play an activating role in IL-2 gene regulation. NF- κ B activity can be induced through a variety of pathways, including two Ca^{2+} -independent ways (40, 41): (a) by phorbol esters presumably activating Ras via protein kinase C, or (b) by a “stress-induced” sphingomyelinase-dependent pathway. Part of the activation of NF- κ B is due to the posttranslational release of pre-formed factor from a cytoplasmic tethering component, I κ B, while part may be due to *de novo* synthesis (reviewed in ref. 40). A strong inhibition of the activating forms, p65-p50 or c-Rel-p50, results when intracellular cAMP is elevated (28, 37, 42), although the mechanism for this inhibition is not yet understood. Under some conditions, also, calcineurin inhibitors like CSA can inhibit optimal quantitative release of NF- κ B from inhibition (43, 44). The ability of these inhibitors to block NF- κ B availability depends on the path used to stimulate NF- κ B; however, since the blockade can be overridden by adding the inflammatory cytokine tumor necrosis factor- α (28), which activates NF- κ B through the separate sphingomyelinase-dependent cascade.

(iii) **AP-1.** AP-1, another complex factor family, has a more subtle role, which incorporates both information about the activation conditions facing the cell and information about the developmental status of the cell itself. In T cells, a combination of Ca^{2+} and protein kinase C signals induces AP-1 DNA-binding activity better than protein kinase C alone (15, 45), and either cAMP elevation or IL-1 costimulation (in susceptible cells) leads to a further enhancement in AP-1 binding activity (15, 28, 37). Under various physiological conditions of cellular nonresponsiveness, AP-1 DNA-binding activity is not available to the cell at all [anergy (39, 46) and cortical thymocytes (25, 47)]. However, the ability of AP-1 to activate transcription is regulated not only by its capacity to bind DNA, but also by its subunit composition (dimers composed of any of three different Jun family members and/or any of four or five different Fos family members). Different stimulation conditions activate the synthesis of different Jun and Fos family members (45, 48, 49), and although the binding affinities of most Fos/Jun complexes are very similar, their effects on transcription are distinct.

The DNA-binding activities of these diverse factors are necessary but not sufficient for their transcriptional activity [e.g., NF-AT (37, 50) and NF- κ B (51)]. This is particularly true for AP-1, since the ability of c-Jun and JunD to activate transcription once bound to DNA depend on their N-terminal phosphorylation status. The key activating kinase, Jun N-terminal kinase, itself responds to combinations of signaling pathways (52, 53) in ways that mimic much of the regulation of IL-2 expression. This is likely to explain the fact that transcription from a concatemer of optimal AP-1 binding sites displays activation requirements similar to those of transcription from the whole IL-2 promoter, even though strong AP-1 binding activity is induced in a much broader set of conditions (45). Thus AP-1 binding activity is limiting in some physiological circumstances, while only its trans-activating activity is limiting in others. Finally, and critically, AP-1 appears to bind to the IL-2 enhancer in close association with other factors in at least three and possibly as many as five cases: with Oct-1 at one site (30, 48, 54), with NF-AT at two other sites (reviewed in

ref. 55), and possibly with additional NF-AT complexes at two additional sites (20). In each of these cases, the interaction between AP-1 and the other factor enhances DNA-binding affinity for both. It is not yet clear how much Fos/Jun family member selectivity is required for these protein-protein interactions as seen on the full promoter, as distinct from their ability to bind to isolated AP-1 site oligonucleotides.

The novel factors involved in IL-2 regulation may have still other sets of activation requirements. An example is the novel “TGGGC” factor, which binds to a site that could represent a nonconsensus form of a Su(H) site or a PEBP2 α site. The DNA-binding activity of this factor is induction-dependent and only partially CSA sensitive like NF- κ B, and it is fully inducible in cortical thymocytes, which can mobilize NF- κ B but not AP-1 (27, 56) (D. Chen and E.V.R., unpublished results). Also, like NF- κ B, it is inhibited by elevation of cAMP. However, it is inhibited with more rapid kinetics than NF- κ B in this case, and unlike NF- κ B, its synthesis is not restored by costimulation with tumor necrosis factor α (28). Thus this additional factor exhibits a distinct pattern of regulation by signaling pathways.

Coordinated Binding as a Basis of the Requirement for Multiple Transcriptional Activators

Fig. 2 presents a summary of the availabilities of different transcription factor binding activities observed in nuclear extracts from cells under different conditions of stimulation. As shown in Fig. 2, in each of the cases where IL-2 induction is blocked, at least one of the transcription factors with sites in the minimal enhancer is missing. In each of these cases, however, there are many factors that are present and unaffected. As with the question of why any of a number of different single-site mutations should block expression of the IL-2 gene, this observation raises the question of how IL-2 gene expression can be so dependent on the presence of all of these factors.

One possibility is that the factors that are present are not really active. This might be the case if modifications of all the factors are needed to endow them with transcriptional activation capability, as in the case of AP-1. This would then require that these modifications are all executed correctly only under conditions that coincide with the presence of all the transcription factors. In this case, all “signal integration” would really be carried out by the modifying enzyme(s). Another possibility is that factors of diverse types really must be bound to the enhancer to make it work correctly, because their distinct activation domains deliver qualitatively different, complementary interactions with the TATA-binding protein-associated factor (TAF) components of the basal transcriptional apparatus (as suggested in refs. 58 and 59). However, if such a requirement were enforced stringently, it would be hard to explain the repeated observation that multimers of a single factor binding site from the IL-2 enhancer can also work well to drive reporter expression in an IL-2-like way. A third possibility is that the factors need to be present together to stabilize each other's interactions with the DNA. In this case, assembly of the transcription complex itself would be a mechanism for integrating contributions from factors at the termini of different signaling pathways, and the requirement for multiple factors would be based on an inability of the key factors to bind stably on their own.

Whereas the first and second possibilities can only be tested by an extensive biochemical analysis of individual transcription factors and their interactions with the basal apparatus, the third possibility can be tested directly by analysis of the actual protein-DNA contacts formed on the IL-2 enhancer in activated T cells. We have done this in a series of studies using *in vivo* footprinting of the IL-2 locus in living T-lineage cells treated with the DNA-alkylating agent, dimethyl sulfate (DMS), which reacts with G residues in the major groove (27, 28). In these studies we compared nonactivated T-lineage cells,

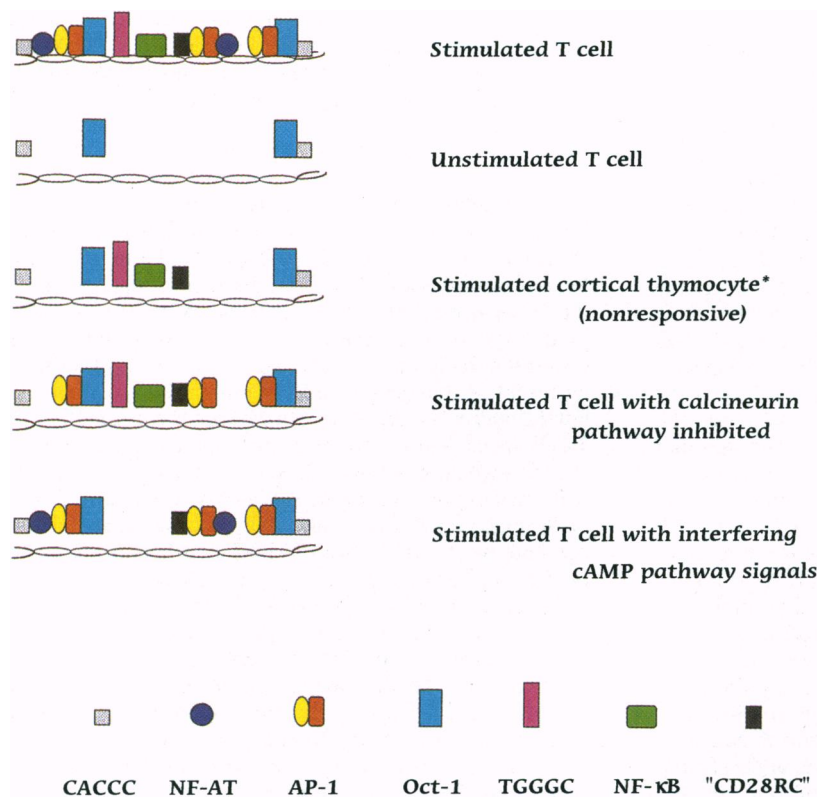


FIG. 2. Failure to express IL-2 is correlated with an absence of distinct subsets of IL-2 DNA-binding activities. The figure summarizes the effects of conditions from Fig. 1 on the presence of specific transcription factors in nuclear pools, as measured by *in vitro* binding activities. (See text for details.) In this figure, the number of NF-AT sites is given as two (57), pending confirmation of a recent report that there are more (20). In the case of one putative new NF-AT site (CD28RE) (20), we have found a discrepancy in cortical thymocytes between the binding activities for that site and for conventional NF-AT sites (25), suggesting that the main factor binding at this site is not NF-AT.

T cells activated under optimal conditions for IL-2 expression, and T cells activated under conditions in which the binding capacities of different subsets of transcription factors *in vitro* are known to be inhibited. The question we asked was whether the occupancies of individual transcription factor binding sites in the endogenous IL-2 regulatory region depended only on the amount of binding activity of the relevant factor, as assayed in nuclear extracts, or alternatively, whether binding itself *in vivo* might depend on activities other than those that we could measure with these isolated sites.

The results of these studies show that IL-2 transcription factors are acutely interdependent for their binding to the DNA *in vivo*. Regardless of the activation requirements of the individual factors, the sites they bind in the IL-2 minimal enhancer are either all occupied *in vivo*—if and only if all factors are present; or all empty—in every other case (27, 28). Furthermore, whether the inhibitor used is CSA, which preferentially inhibits the induction of NF-AT DNA-binding activity, or forskolin, which preferentially inhibits the mobilization of NF- κ B and TGGGC factor DNA-binding activities, the result is the same: the footprints at all sites, including the AP-1 sites and the sites of constitutive factor binding, are abolished. This shows that neither NF-AT nor any of the other factors can bind to the IL-2 minimal enhancer DNA autonomously *in vivo*, however well they bind to their isolated target sites *in vitro*.

There are two possible explanations for these results. First, as we have proposed elsewhere (28), a straightforward interpretation is that there is no single “master regulator” for the IL-2 gene; instead, multiple types of factors, each responsive to different signaling pathways, must simultaneously bind to the DNA and interact to stabilize each other's binding. This interpretation envisions that stable transcription factor binding at the IL-2 minimal enhancer occurs only in the context of a multifactor complex with direct or indirectly mediated protein–protein interactions linking each factor with multiple partners (60, 61). This would provide an architectural mechanism for “checking off” the occupancies of sites over most of the regulatory region DNA.

Second, the experiments we have done leave open the possibility that there is a kind of “master regulator,” if this

master regulator is AP-1. As discussed above, AP-1 is differentially modified or composed of different subunits under the different stimulation conditions we have tested, either via effects on Jun N-terminal kinase or via preferential induction of JunB versus c-Jun. The key is that these modifications, at least in response to CSA and forskolin, do not decrease the efficiency with which AP-1 binds to its isolated target DNA sequence. However, for the protein–protein interactions leading to enhancement of Oct-1/AP-1 and NF-AT/AP-1 DNA-binding affinities (31, 54), there is some evidence that the exact composition of the AP-1 can strongly influence the outcome (48). Thus, the form of AP-1 available after stimulation in the presence of CSA or forskolin might fail both to stabilize Oct-1 (at one site at least) and to stabilize NF-AT (at two to five sites). Note that even if AP-1 is a master regulator in this sense, it still cannot bind its DNA target sites autonomously *in vivo*: it controls expression through its interactions with other factors. Thus, in both of these scenarios, the integration of signals to control IL-2 expression depends on factor–factor interactions as well as on factor–DNA interactions.

Complex Assembly and Disassembly Cycles for Dynamic Signal Integration

A striking feature of the *in vivo* footprinting pattern is the apparent lack of evidence for protein–DNA contacts over the minimal enhancer in unstimulated, resting T cells. Footprints only appear at any site when they appear at every site—namely under optimal conditions of induction. If the appearance of these stable DNA–protein contacts is the outcome of a multifactor assembly process, then in principle the nucleation of initial subassemblies might be separable from the addition of later, stabilizing components. However, it has not yet been possible to detect any partial complexes that might be kinetic intermediates. Similarly, even once formed, the multifactor complexes remain unstable. When an inhibitor such as CSA or forskolin (to elevate cAMP) is added to cells that are already responding to stimulation, we find that the footprints over the whole IL-2 minimal enhancer disappear rapidly, with a time-

course that closely follows the timecourse of disappearance of the free target factor from the nucleus (NF-AT or NF- κ B, respectively) as measured by binding activity (28). Thus, not only do individual protein-DNA contacts appear to be too weak to form autonomously, they also appear to be readily reversible even after a full transcription complex has assembled (28).

This mechanism contrasts with the stable occupancy observed at sites of constitutive transcription factor binding—e.g., in the IL-2 receptor α -chain and metallothionein-I genes (62, 63). However, by contrast with other well-studied genes, it is important to realize that the IL-2 gene is normally designed to remain silent, even in cells of the right lineage to express it, and to return to silence quickly after its expression has been induced. Structural characteristics of the binding sites themselves support this interpretation. Of all the sites for known factors in the IL-2 minimal enhancer, none match the consensus for optimal binding (64). Instead, base changes that critically weaken the DNA binding affinities of these known factors are conserved perfectly between the murine and human versions of the enhancer (17). When any of these sites is mutated to match the consensus, it damages the induction-dependence and/or cell-type specificity of the IL-2 promoter (64). Taken alone, this finding could mean that there are T-cell-specific members of the AP-1, NF- κ B, and Oct families that bind most tightly to the IL-2 versions of these sites, whereas the “consensus” versions are preferred by the ubiquitous (and canonical) members of these families. However, our data on the weakness *in vivo* of these contacts in activated T cells argue that it is not the need for novel high-affinity interactions, but rather for low-affinity interactions *per se*, that is important for correct IL-2 regulation. We can think of the systematically weakened protein-DNA contacts throughout the minimal enhancer, and the resulting requirement for simultaneous factor binding, as one mechanism for maintaining the lack of background IL-2 expression.

The apparently “empty” status of the enhancer in resting T cells appears inconsistent with several lines of evidence suggesting that particular transcription factors act as negative regulators of IL-2 transcription. Candidate negative regulators include the p50₂ form of NF- κ B, which presumably antagonizes p65-p50 and c-Rel-p50 forms at the NF- κ B site (51); Nil-2A, a zinc finger protein that binds to a distinct site at about -110 (65); Ets-1, which like other Ets family proteins may bind at or near the NF-AT site(s) (66, 67); and a less characterized but intriguing 45-kDa factor that also binds to the NF-AT site (68). We have seen no evidence for DMS footprints *in vivo* at any of these sites, either in resting EL4 cells or in certain cell types that maintain high levels of p50₂ complexes constitutively (27). Thus our data make it seem unlikely that these factors work by stable occupation of their sites and competitive exclusion of positive factors. This conclusion is provisional, of course, because the readily stimulated EL4 cells that we have had to study may lack some of the regulatory apparatus of normal T cells. However, the requirement for continual reassembly of positive transcription complexes may indicate the way these negative factors could work: to interact with their sites just long enough to provide kinetic, rather than equilibrium, interference with the interactions needed to stabilize the multifactor complexes in the minimal enhancer.

In any case, the absence of any long-term “locking-in” of the activating transcription factors, once they do bind to the minimal enhancer, means that the probability of a transcription complex being present on the gene is closely linked kinetically to the instantaneous concentration(s) of whichever factor(s) are most limiting. This is a mechanism that makes the IL-2 transcriptional initiation rate continuously sensitive to immediate changes in the signaling physiology of the T cell. The structure of the enhancer thus provides a way that the amount of IL-2 produced can be precisely modulated in the course of an ongoing immune response. At another level, the use of this mechanism emphasizes

the continuous accessibility of the IL-2 locus to equilibrate with nuclear pools of transcriptional activators in T cells. As discussed further below, this establishes constraints on the mechanisms making IL-2 expression T-cell specific.

The Problem of T-Cell Lineage Specificity

With this picture of the mechanism that governs IL-2 transcription in response to stimulation, we can return to consider how IL-2 expression is rendered T-cell specific. Three general kinds of models were described at the beginning of this manuscript, and these are summarized diagrammatically in Fig. 3. In the first model, the IL-2 locus is “open” everywhere, but can only be transcriptionally activated if at least one T-lineage-specific factor is present. In the second model, again the locus is open in all cells, but the ability of factors—which may not themselves be absolutely T-cell specific—to activate transcription depends on a T-cell-specific signaling pathway. In the third model, the T-cell specificity resides not solely in the factors used to drive IL-2 expression or in their posttranslational modifications, but rather resides in the state of accessibility of the IL-2 locus in chromatin, which is opened only as cells enter the T-cell lineage.

For now, the second model (T-lineage-specific activation of factors) is difficult to test except by excluding the alternatives. However, we can consider evidence relevant to the first and third models. To distinguish among them, we can ask: Are there unique T-cell-specific trans-acting factors that play the rate-limiting role in IL-2 gene expression, and is there any stable T-lineage-specific cis-alteration in the state of the IL-2 chromatin prior to induction?

Many of the factors that participate in IL-2 induction are clearly ubiquitous, including AP-1, NF- κ B, and Oct-1, and therefore are eminently excluded as sources of T-lineage specificity. The initial discovery of NF-AT was thought to provide a T-cell-specific factor, as NF-AT activity was found only in activated T cells (69). Subsequent characterization, cloning, and analysis of the expression of the NF-AT genes have shown that the binding activity is contributed by a multi-gene family, and that members of this family are expressed in many tissues other than T cells (31–34, 70). Thus the expression of NF-AT (even with access to AP-1, NF- κ B, and Oct-1) is not sufficient to make a cell into an IL-2 producer. To date, none of the factors needed to drive IL-2 expression from the minimal enhancer are clearly shown to be T-cell specific.

The suggestion that lineage specificity may be regulated at the level of chromatin opening might also appear unlikely. *In vivo* DMS footprinting has not shown any pattern of occupancy of G residues in the minimal enhancer that distinguishes T cells, prior to stimulation, from cells of any other lineage (27). The minimal enhancer region not only appears “empty” in unstimulated T cells; it is equally empty in non-IL-2 producing hematopoietic cells, such as 32D clone 5, and non-IL-2 producing cells of other types altogether, such as L cells and 10T_{1/2} cells. There is no evidence for constitutive repression in non-IL-2 producing cell types, at least not through a mechanism of stable contacts with enhancer region G residues in the major groove. The difference is that in the T cells activation results in occupancy of these sequences, whereas in the non-T cells the IL-2 enhancer remains unoccupied even after stimulation (27) (S.B.W., unpublished results). Taken alone, these observations could point to a mechanism of lineage specificity that is exercised entirely through T-cell-specific signaling pathways.

The point is important enough to deserve a better test, however. Exclusive use of DMS would not allow us to detect minor groove binding factors, such as the T-cell-specific high mobility group box factors Sox-4 and TCF-1, and high mobility group box proteins can have either positive or negative effects on expression (61, 71). The inability to detect contacts on anything but G residues is particularly unsatisfying for a sequence that is as highly A/T-rich as the IL-2 5'-flanking region. In fact, using an altered protocol that visualizes effects at A as well as G residues,

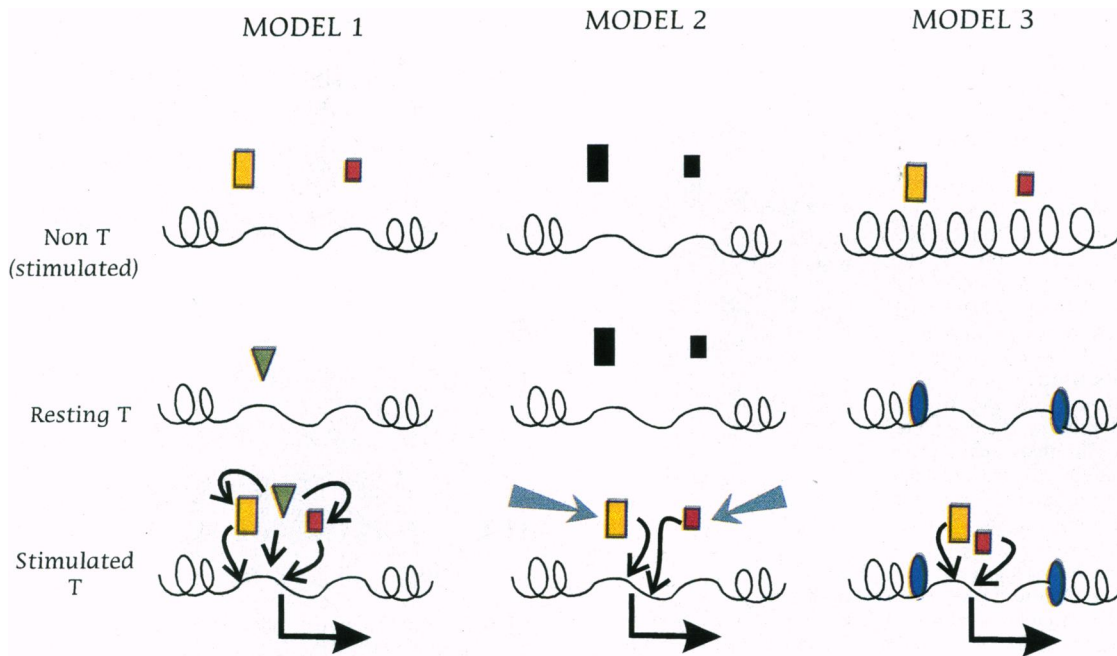


FIG. 3. Three models for combining stable T-cell lineage specificity with rapid, transient activation dependence of IL-2 expression. As a "worst case" scenario, the non-T cells are represented as activated, so that any ubiquitous activation-dependent factors (rectangles) are present in those cells as well as in activated T cells. The T-cell-specific components differ as described in the text. In model 1, a trans-acting factor (triangle) is uniquely present in T cells, which is required to interact with the activation-dependent factors to activate transcription (black right-angled arrow). In model 2, the activation-dependent factors are of the wrong composition or possess the wrong posttranslational modification for use at the IL-2 promoter (black rectangles) unless activated by T-cell-specific signaling pathways (open arrows). In model 3, the IL-2 gene is not accessible in non-T cells, but only in T cells, due to a stable cis-modification of chromatin (border-forming factors represented by ovals) which precedes activation.

Brunvand *et al.* (72) have reported that some contacts may occur near the TATA boxes of the human IL-2 gene even prior to stimulation. Furthermore, although the ≈ 300 -bp minimal enhancer is sufficient to control expression in transient expression experiments, it is not the only region likely to be important for IL-2 regulation *in vivo*. An early survey of the IL-2 gene by Siebenlist *et al.* (73) reported regions of DNase I hypersensitivity outside of the minimal enhancer in resting T cells. There is evidence for evolutionary conservation of sequences beyond the minimal enhancer as well. Noncoding sequences from approximately -600 to beyond $+45$ with respect to the transcriptional start site are all conserved to $>80\%$ identity between mouse and human, significantly better conservation than within the coding regions themselves (17). Finally, it is noteworthy that in numerous attempts to generate transgenic mice in which a reporter gene is driven from the IL-2 promoter, none have succeeded using less than the ≈ 600 -bp version of the 5'-flanking region (74–76). In recent work, therefore, we have compared IL-2 regulatory sequence footprints in T and non-T cells over ≈ 1 kb from -600 to $+350$, and we have supplemented the DMS analysis with two additional *in vivo* footprinting reagents, DNase I and KMnO_4 .

This broadened search has provided new evidence for constitutive differences at the IL-2 locus between the protein-DNA interactions in resting T cells and those in non-T cells. The new reagents confirm the analysis with DMS, insofar as the minimal enhancer region (-300 to -45) still appears virtually unoccupied in T cells prior to stimulation. One exception is that we confirm some of the contacts seen by Brunvand *et al.* (72) in A/T rich regions close to the TATA boxes. Upon stimulation, the DNase reactivity increases sharply over several regions of the minimal enhancer (unpublished data), in parallel with the appearance of DMS footprints. However, outside the minimal enhancer region, a new pattern of occupancy is seen. In the region from -300 to -600 , no contacts on G residues are detected by DMS footprinting in stimulated or unstimulated cells. Even so, a series of specific residues (between about -350 and -460) are maintained in a DNase hypersensitive state in the resting T cells, as shown in Fig.

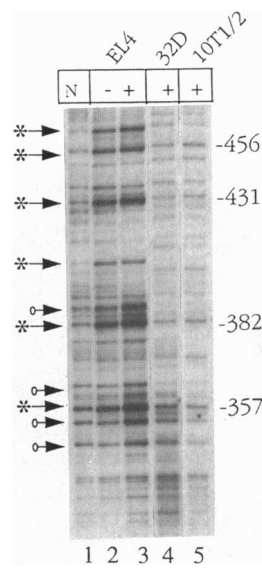
4 (compare lanes 1 and 2). Upon stimulation of the cells, the degree of DNase hypersensitivity of these sites increases (Fig. 4, compare lanes 2 and 3), and in fact one stimulation-dependent alteration of an A residue at -410 can be detected by DMS as well (data not shown; unpublished data). KMnO_4 also detects a structural distortion of the DNA in this region. Thus the precise conformation of DNA and protein-DNA complexes at these sites may change with activation. However, the most important feature of these novel DNase hypersensitivities is that they are not seen in any cells of non-IL-2 producing lineage that we have examined (Fig. 4, lanes 4 and 5).

The initial identification of these sites provides specific candidate sequences to test for interaction with a potential lineage-marking protein or protein complex at some discrete stage of T-cell development. Thus far, it is not certain what proteins might be playing a role. The general lack of DMS footprints at these sites indicates that the factors which bind here constitutively are very unlikely to be the same ones that occupy easily footprinted sites in the minimal enhancer upon induction. Serfling *et al.* (22) have pointed out that induction-dependent Ets-like binding proteins (GABP) and other factors might occupy sites between -500 and -420 . These sites are not footprinted with DMS, but fall within the range of DNase hypersensitive sites seen in both stimulated and unstimulated cells. The ≈ 40 -bp region centered at -410 contains two palindromes and binding sites for a large number of factors (unpublished results), and the full identification of these factors is in progress. Clearly a factor that associated constitutively with the conserved region of this gene in a T-cell-specific way would be of great interest as a factor implicated in a T-lineage identification function.

Architectural Integration of Lineage and Activation Information in IL-2 Induction

The characterization of potential stable lineage-identifier factors is at a very early stage, and ideas about their mode(s) of action must remain speculative for the time being. Of course

FIG. 4. Constitutive DNase I hypersensitive sites in the upstream IL-2 enhancer are tissue specific. Hypersensitive sites in the upstream enhancer are only found in EL4 T cells and not in two non-T cell lines. Stimulated (+) or unstimulated (-) cells were treated with DNase I *in vivo*. Genomic DNA was subsequently purified and analyzed by a modification of the ligation-mediated PCR method (ref. 77; and unpublished data), using primers that amplify the region from -200 to -500. PCR products were resolved on a 6% sequencing gel. To determine the inherent DNase sensitivity of a region, purified genomic DNA was treated with DNase I *in vitro* (lane N; i.e., naked DNA) and then analyzed in parallel with the *in vivo* treated samples. DNase I sensitivities are then determined by comparing the intensity of a particular residue to its corresponding site in lane 1 (lane N). Arrows with stars denote residues that are hypersensitive in both unstimulated and stimulated EL4 cells (lanes 2 and 3) and represent constitutive hypersensitive sites. Arrows with circles denote residues that are hypersensitive to DNase I only upon stimulation. Note that upon stimulation certain constitutively sensitive residues become even more sensitive to DNase I (residues -357 and -382). In contrast, the non-T cell lines 32D clone 5 (pre-mast cells, lane 4) and 10T_{1/2} (fibroblasts, lane 5), although stimulated, do not display DNase I hypersensitive sites in this region.



the confirmation of a locus-marking model must await the identification of discrete factors that are shown to be essential for opening the IL-2 locus in ontogeny. However, two features of the DNase and KMnO₄ footprinting data suggest how these stably bound elements might cooperate with the fluid, dynamic assembly and disassembly of activation mediators at the minimal enhancer.

One feature is the spatial separation between the sites of constitutive DNA perturbation in the upstream region (as inferred from DNase and KMnO₄ footprints distinct from those of naked DNA) and the whole minimal enhancer. In addition to the sites in the upstream region, there is also evidence for a stable KMnO₄ footprint in an intronic region (unpublished data), but no obvious "marking" within the minimal enhancer itself. These specific assignments are in agreement with the estimated location of constitutive DNase hypersensitive sites around the human IL-2 gene, as derived initially by Siebenlist *et al.* (73).

The second feature of the footprints is the breadth of the changes in DNase sensitivity that occur throughout the IL-2 regulatory DNA upon stimulation (unpublished data). The constitutively hypersensitive sites become even more sensitive, and there are further changes. Not only do specific bases at the borders of known transcription factor footprints in the minimal enhancer become hypersensitive, but whole regions of DNA, 10–40 bases at a stretch, are also affected. On the coding strand, the bases from -110 to -120, -250 to -260, and -300 to -320 become highly sensitive (unpublished data); on the other strand, a region from -350 to -390 becomes particularly sensitive (for example, see the bracketed regions in Fig. 5). These broad tracts of hypersensitive DNA are striking in their strand asymmetry. An attractive hypothesis is that they represent local distortions of the DNA between transcription factor binding sites, induced by the interaction of factors binding upstream of the hypersensitivity with factors (or with the basal transcriptional apparatus) binding downstream of the hypersensitivity. The longer tracts may represent larger distortions or regions of chromatin that become free of canonical nucleosomes.

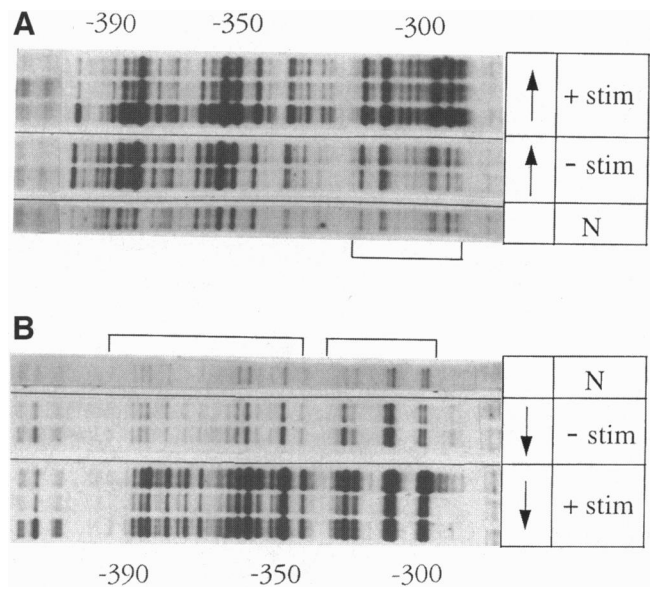


FIG. 5. Stimulation-dependent increases in DNase I sensitivity *in vivo* of extended regions of the IL-2 upstream regulatory region. The figure shows the DNase I footprints over the coding (A) and noncoding (B) strands of IL-2 DNA from -300 to -400, in stimulated EL4 cells (+ stim) and unstimulated EL4 cells (- stim) treated with DNase I *in vivo*, as compared with EL4 naked DNA treated with DNase I *in vitro* (lane N). Samples were processed and analyzed as in Fig. 4. Arrows in the legend (on the right) indicate the order of samples treated with increasing amounts of DNase. The figure is a composite of data from different gels, in which the same samples were electrophoresed for different lengths of time to obtain a relatively even spacing of bands as a function of length. All lanes in A were generated by using primers that direct polymerization toward the distal end of the enhancer, while those in B were generated by using primers that direct polymerization toward the promoter-proximal end. To facilitate comparisons of hypersensitivities of corresponding regions of the sequence, the coding and noncoding strand gels are arranged with their origins at opposite sides of the figure. Brackets indicate regions of the DNA that increase significantly in DNase sensitivity in stimulated cells. In some cases this increased sensitivity is evident on both strands—e.g., the region indicated by the brackets near -300. In other regions, the increase is asymmetrically strand-specific (see bracketed region from -350 to -390).

It seems likely, therefore, that cell-type-specific identifiers bind stably to the distal parts of the conserved IL-2 regulatory region in T-lineage cells without immediately affecting protein–DNA contacts over the minimal enhancer itself. Even so, this occupancy can "sensitize" the minimal enhancer, between 50 and 300 bp away, to transcription factor binding at any time that sufficient components are present to bind cooperatively. There are several ways that this can happen, based on precedents in the literature. Sensitization can occur through effects on local and neighboring nucleosomes, promoting a more open chromatin configuration (78–82), or through effects on other transcription factors, recruiting them or stabilizing their binding (60, 83, 84). The distortions of the DNA that we detect in the upstream region can play a role in either type of mechanism (61, 85). In this case, because the DNase hypersensitivity in the upstream region (-300 to -600) itself increases on activation, it is possible that the interactions between the factors binding in the upstream region and those recruited to the minimal enhancer proper may be reciprocal. Not only may the upstream factors facilitate transcription factor loading at the minimal enhancer, but they may also be looped around and drawn directly into the complex that activates IL-2 transcription (see refs. 86 and 87). The identification of specific, lineage-stable protein–DNA contact sites outside of the minimal enhancer provides us with a new key to discover exactly how the architecture of the IL-2 gene transcription complex may

bring together the mediators of physiological and developmental information.

We wish to thank Drs. Edgar Serfling and Mark Brunvand for communication of valuable work prior to publication, Eric Davidson and Michael Levine for challenging discussion and stimulating criticism, and Dan Chen, Paul Garrity, Paul Mueller, and Barbara Wold for continuing interest and advice. We thank the members of the Rothenberg laboratory for support, and James Staub and Richard Gomez for careful help with the photography. This work was supported by grants from the Public Health Service (AI34041 and AG13108) and from the State of California Tobacco-Related Disease Research Program, and aided by a donation from the Golden West Co.

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