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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

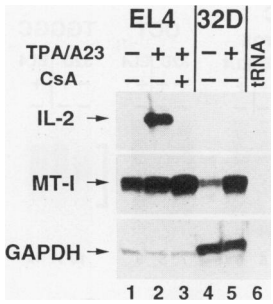

FIG. 1. RNase protection analysis of IL-2, MT-I, and GAPDH transcripts in RNA from the cell populations used in Fig. 2, 3, 4, and 7. RNA from unstimulated EL4 cells (lane 1), EL4 cells stimulated with TPA and A23187 for 7 h (lane 2), EL4 cells stimulated for 7 h in the presence of CsA (lane 3), unstimulated 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 CACCC (−72 to −52), gatcACATCGTGACACCCCCATATTgatc; octamer (−100 to −69), gatcTCTTTGAAAATATGTGTAATATGTAAAACATgatc; AP-1 (−161 to −143), AATTCCAGAGAGTCATCAG; TGGGC (−237 to −218), gatcCACCTAAGTGTGGGCTAACgatc; NF-κB (−211 to −192), AAAGAGGGATTTCACCTAAAT; distal NF-AT (−289 to −260), AAGAGGAAAATTTGTTTCATACAGAAGGCGAAT; and proximal NF-AT (−147 to −120), gATCAGAAGAGGAAAAACAAAGGTAATGCgatc.

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

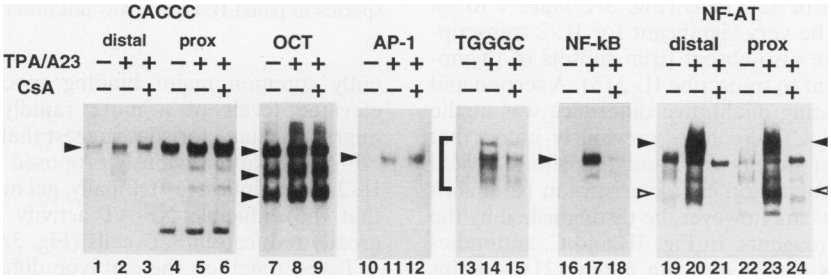

FIG. 2. Characterization of nuclear factors present in EL4 T-lymphoma cells by electrophoretic mobility shift assay. Nuclear extracts were prepared from the same populations used in the RNase protection and in vivo footprinting experiments. The ^{32}P-labeled oligonucleotide used in each experiment is 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).

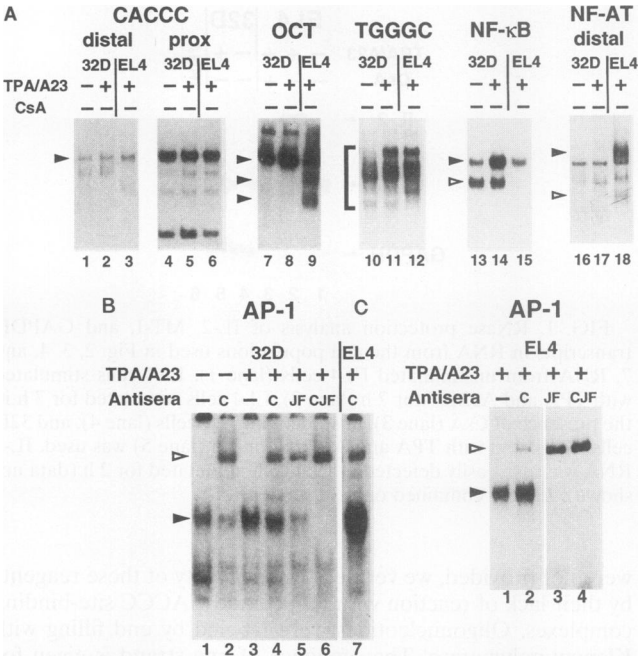

FIG. 3. Characterization of nuclear factors present in 32D premast cells by electrophoretic mobility shift assay as described in the legend to Fig. 2. To facilitate comparisons, nuclear extracts prepared from stimulated EL4 cells were examined in parallel with the 32D extracts. (A) Comparison of factors 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

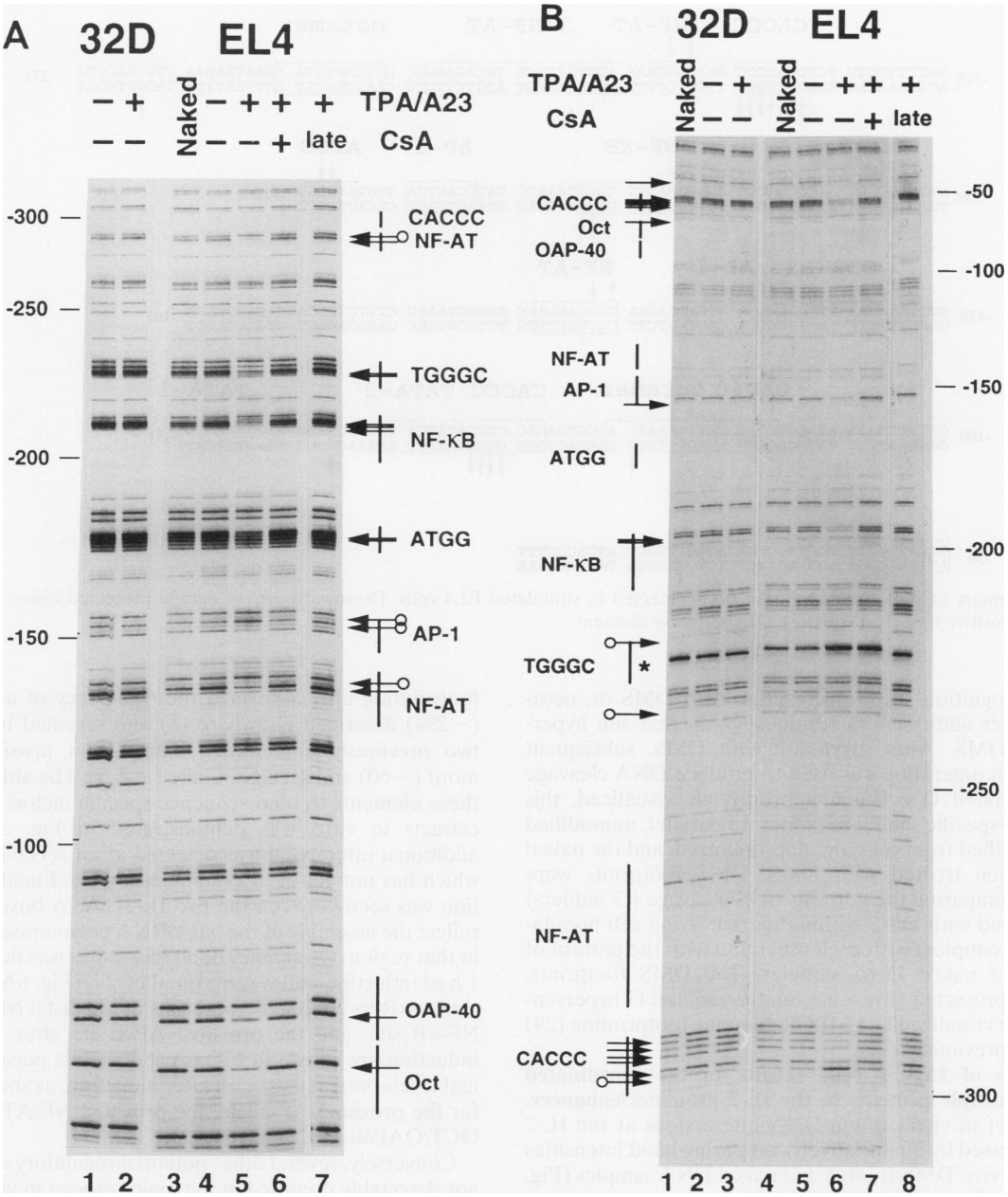

FIG. 4. DMS in vivo footprint of the IL-2 regulatory region. DNA from in vivo DMS-treated unstimulated 32D cells (panel A, lane 1; panel B, lane 2), stimulated 32D cells (panel A, lane 2; panel B, lane 3), unstimulated EL4 cells (panel A, lane 4; panel B, lane 5), stimulated EL4 cells (panel A, lane 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

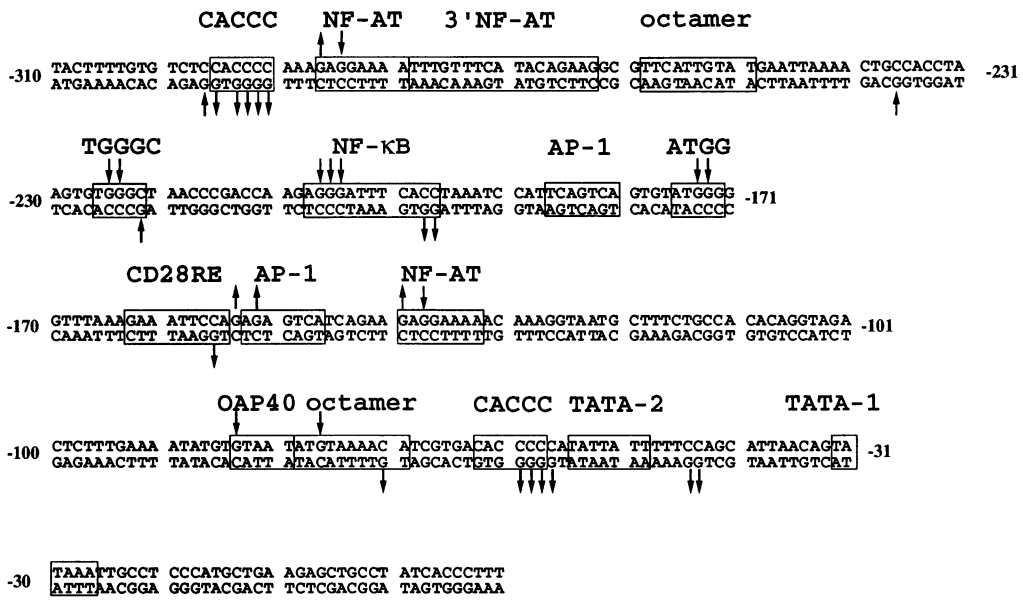

FIG. 5. Summary of protein-DNA interactions detected in stimulated EL4 cells. Downward arrows denote protected bases; upward arrows denote hypersensitive bases. CD28RE, CD28 response element.

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

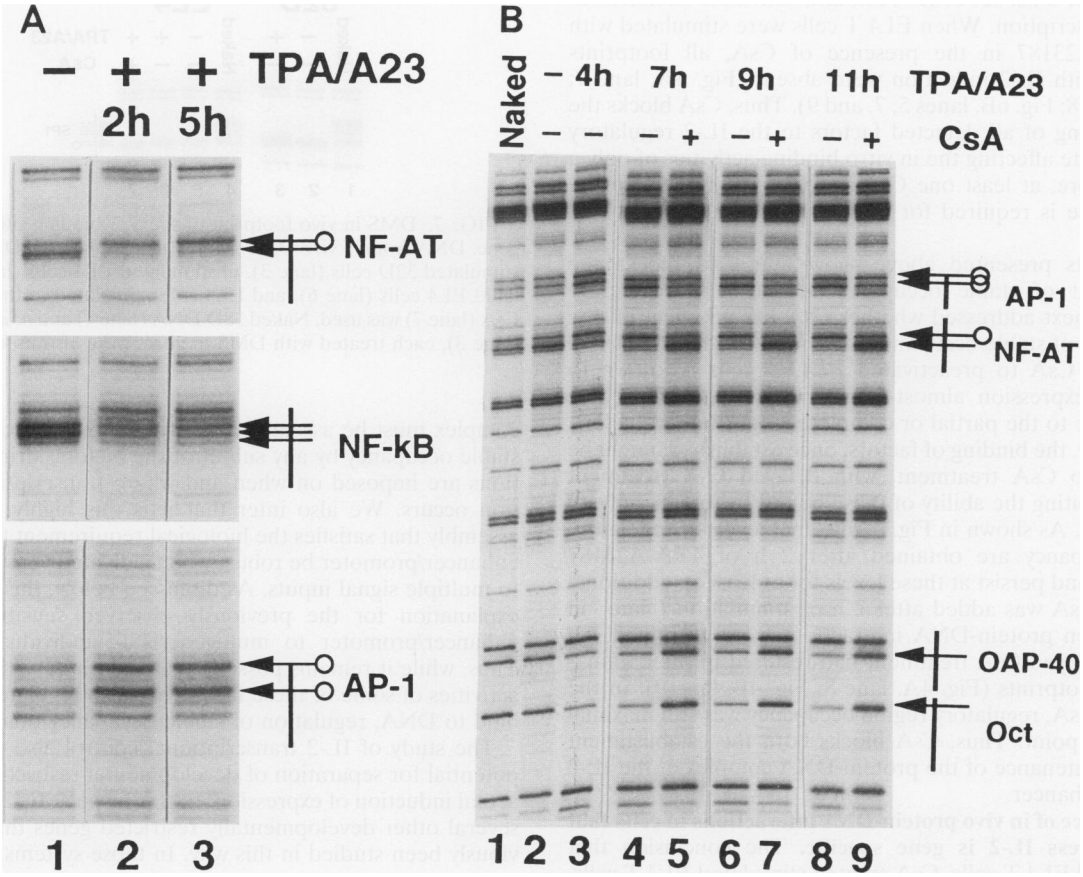

FIG. 6. (A) DMS in vivo footprints over representative binding sites at 2 and 5 h of induction. DNA from in vivo DMS-treated uninduced EL4 cells (lane 1), EL4 cells induced for 2 h (lane 2), and EL4 cells induced for 5 h (lane 3) was used. Lane 2 was exposed for 20% longer than lanes 1 and 3 to compensate 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).

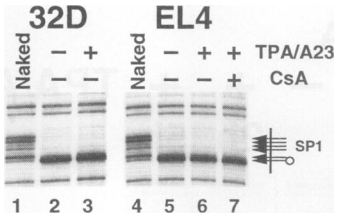

FIG. 7. DMS in vivo footprint of the Sp1-binding site in the MT-I gene. DNA from in vivo DMS-treated unstimulated 32D cells (lane 2), stimulated 32D cells (lane 3), unstimulated EL4 cells (lane 5), stimulated EL4 cells (lane 6), and EL4 cells stimulated in the presence of CsA (lane 7) was used. Naked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

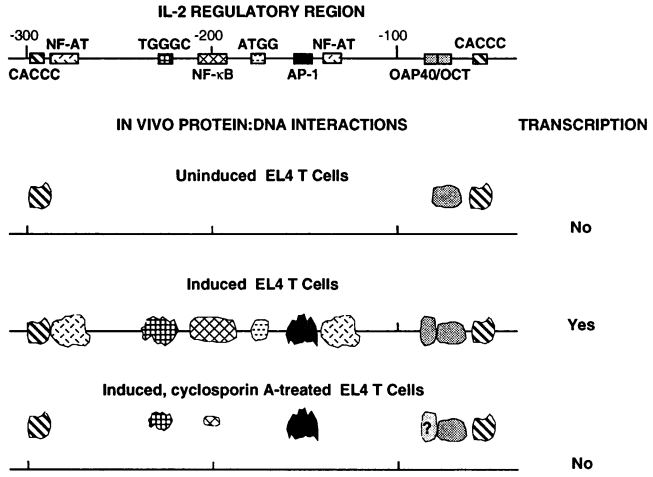

FIG. 8. Summary of the protein-DNA interactions detected in EL4 T lymphoma cells by in vivo footprint analysis and the presence of nuclear factors in these cells as determined by in vitro DNA-binding analysis. Decreased size of TGGGC- and NF-κB-binding activities in stimulated, CsA-treated EL4 cells 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 : 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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REFERENCES

1. **Brabletz, T., I. Pietrowski, and E. Serfling.** 1991. The immunosuppressive FK 506 and cyclosporin A inhibit the generation of protein factors binding to the two purine boxes of the interleukin 2 enhancer. Nucleic Acids Res. **19:**61–67.
2. **Briegel, K., B. Hentsch, I. Pfeuffer, and E. Serfling.** 1991. One base pair change abolishes the T cell-restricted activity of a κB-like proto-enhancer element from the interleukin 2 promoter. Nucleic Acids Res. **19:**5929–5936.
3. **Brorson, K. A., B. Beverly, S. M. Kang, M. Lenardo, and R. H. Schwartz.** 1991. Transcriptional regulation of cytokine genes in nontransformed-T cells: apparent constitutive signals in run-on assays can be caused by repeat sequences. J. Immunol. **147:**3601–3609.
4. **Brunvand, M. W., A. Schmidt, and U. Siebenlist.** 1988. Nuclear factors interacting with the mitogen-responsive regulatory region of the interleukin-2 gene. J. Biol. Chem. **263:**18904–18910.
5. **Chen, D., and E. V. Rothenberg.** 1993. Molecular basis for developmental changes in interleukin-2 gene inducibility. Mol. Cell. Biol. **13:**228–237.
6. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
7. **Durand, D. B., J.-P. Shaw, M. R. Bush, R. E. Replogle, R. Belagaje, and G. R. Crabtree.** 1988. Characterization of antigen receptor response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8:**1715–1724.
8. **Emmel, E. A., C. L. Verweij, D. B. Durand, K. M. Higgins, E. Lacy, and G. R. Crabtree.** 1989. Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science **246:**1617–1620.
9. **Ephrussi, A., G. M. Church, S. Tonegawa, and W. Gilbert.** 1985. B lineage specific interactions of an immunoglobulin enhancer with cellular factors *in vivo*. Science **227:**134–140.
10. **Flanagan, W. M., B. Corthesy, R. J. Bram, and G. R. Crabtree.** 1991. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature (London) **352:**803–807.
11. **Fraser, J. D., B. A. Irving, G. R. Crabtree, and A. Weiss.** 1992. Regulation of interleukin-2 gene enhancer activity by the T-cell accessory molecule CD28. Science **251:**313–316.
12. **Garrity, P. A., and B. J. Wold.** 1992. Effects of different DNA polymerases in ligation-mediated PCR: enhanced genomic sequencing and *in vivo* footprinting. Proc. Natl. Acad. Sci. USA **89:**1021–1025.
13. **Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. Biggs III, W. W. Vale, and M. R. Montminy.** 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature (London) **337:**749–752.
14. **Granelli-Piperno, A., and P. Nolan.** 1991. Nuclear transcription factors that bind to elements of the IL-2 promoter. J. Immunol. **147:**2734–2739.
15. **Hentsch, B., A. Mouzaki, I. Pfeuffer, D. Rungger, and E. Serfling.** 1992. The weak, fine-tuned binding to ubiquitous transcription factors to the IL-2 enhancer contributes to its T cell-restricted activity. Nucleic Acids Res. **20:**2657–2665.
16. **Herrera, R. E., P. E. Shaw, and A. Nordheim.** 1989. Occupation of the *c-fos* serum response element *in vivo* by a multiprotein complex is unaltered by growth factor induction. Nature (London) **340:**68–70.
17. **Jain, J., V. E. Valge-Archer, and A. Rao.** 1992. Analysis of the AP-1 sites in the IL-2 promoter. J. Immunol. **148:**1240–1250.
18. **Kamps, M. P., L. Corcoran, J. H. LeBowitz, and D. Baltimore.** 1990. The promoter of the human interleukin-2 gene contains two octamer-binding sites and is partially activated by the expression of Oct-2. Mol. Cell. Biol. **10:**5464–5472.
19. **Kang, S. M., A. C. Tran, M. Grilli, and M. J. Lenardo.** 1992. NF-κB subunit regulation in nontransformed $CD4^+$ T-lymphocytes. Science **256:**1452–1456.
20. **Kara, C. J., and L. H. Glimcher.** 1991. *In vivo* footprinting of MHC class II genes: bare promoters in bare lymphocyte syndrome. Science **252:**709–712.
21. **Kovary, K., and R. Bravo.** 1991. The Jun and Fos protein families are both required for cell cycle progression in fibroblasts. Mol. Cell. Biol. **11:**4466–4472.
22. **Kronke, M., W. J. Leonard, J. M. Depper, and W. C. Greene.** 1985. Sequential expression of genes involved in human T lymphocyte growth and differentiation. J. Exp. Med. **161:**1593–1598.
23. **Mattila, P. S., K. S. Ullman, S. Fiering, E. A. Emmel, M. McCutcheon, G. R. Crabtree, and L. A. Herzenberg.** 1990. The actions of cyclosporin A and FK506 suggest a novel step in the activation of T lymphocytes. EMBO J. **9:**4425–4433.
24. **McGuire, K. L., and E. V. Rothenberg.** 1987. Inducibility of interleukin-2 RNA expression in individual mature and immature T lymphocytes. EMBO J. **6:**939–946.
25. **McGuire, K. L., J. A. Yang, and E. V. Rothenberg.** 1988. Influence of activating stimulus on functional phenotype: interleukin-2 mRNA accumulation differentially induced by ionophore and receptor ligands in subsets of murine T cells. Proc. Natl. Acad. Sci. USA **85:**6503–6507.
26. **Miner, J. H., and B. J. Wold.** 1991. c-*myc* inhibition of MyoD and myogenin-initiated myogenic differentiation. Mol. Cell. Biol. **11:**2842–2851.
27. **Mueller, P. R., P. Garrity, and B. Wold.** 1992. Ligation-mediated single-sided PCR for genomic sequencing and footprinting, p. 15.5.1–15.5.26. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
28. **Mueller, P. R., S. J. Salser, and B. Wold.** 1988. Constitutive and metal-inducible protein:DNA interactions at the mouse metallothionein-I promoter examined by *in vivo* and *in vitro* footprinting. Genes Dev. **2:**412–427.
29. **Mueller, P. R., and B. Wold.** 1989. *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246:**780–786.
30. **Novak, T. J., D. Chen, and E. V. Rothenberg.** 1990. Interleukin-1 synergy with phosphoinositide pathway agonists for induction of interleukin-2 gene expression: molecular basis of costimulation. Mol. Cell. Biol. **10:**6325–6334.
31. **Novak, T. J., and E. V. Rothenberg.** 1990. cAMP inhibits induction of interleukin 2 but not of interleukin 4 in T cells. Proc. Natl. Acad. Sci. USA **87:**9353–9357.
32. **Novak, T. J., P. M. White, and E. V. Rothenberg.** 1990. Regulatory anatomy of the murine interleukin-2 gene. Nucleic Acids Res. **18:**4523–4533.
33. **Randak, C., T. Brabletz, M. Hergenrother, I. Sobotta, and E. Serfling.** 1990. Cyclosporin A suppresses the expression of the interleukin 2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer. EMBO J. **9:**2529–2536.
34. **Rigaud, G., J. Roux, R. Pictet, and T. Grange.** 1991. *In vivo* footprinting of rat TAT gene: dynamic interplay between the glucocorticoid receptor and a liver-specific factor. Cell **67:**977–986.
35. **Roeder, R. G.** 1991. The complexities of eukaryotic transcription

initiation: regulation of preinitiation complex assembly. Trends Biochem. Sci. **16:**402–408.
36. **Rothenberg, E. V.** 1992. The development of functionally responsive T cells. Adv. Immunol. **51:**85–214.
37. **Serfling, E., R. Barthelmas, I. Pfeuffer, B. Schenk, S. Zarius, R. Swoboda, F. Mercurio, and M. Karin.** 1989. Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin-2 gene in T lymphocytes. EMBO J. **8:**465–473.
38. **Shaw, J.-P., K. Meerovitch, R. C. Bleackley, and V. Paetkau.** 1988. Mechanisms regulating the level of IL-2 mRNA in T lymphocytes. J. Immunol. **140:**2243–2248.
39. **Shaw, J.-P., P. J. Utz, D. B. Durand, J. J. Toole, E. A. Emmel, and G. R. Crabtree.** 1988. Identification of a putative regulator of early T cell activation genes. Science **241:**202–205.
40. **Stein, B., H. J. Rahmsdorf, A. Steffen, M. Litfin, and P. Herrlich.** 1989. UV-induced DNA damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase, c-*fos*, and metallothionein. Mol. Cell. Biol. **9:**5169–5181.
41. **Tavtigian, S. V.** 1992. Thesis. California Institute of Technology, Pasadena.
42. **Thompson, C. B., C.-Y. Wang, I.-C. Ho, P. R. Bohjanen, B. Petryniak, C. H. June, S. Miesfeldt, L. Zhang, G. J. Nabel, B. Karpinski, and J. M. Leiden.** 1992. *cis*-acting sequences required for inducible interleukin-2 enhancer function bind a novel Ets-related protein, Elf-1. Mol. Cell. Biol. **12:**1043–1053.
43. **Travis, A., A. Amsterdam, C. Belanger, and R. Grosschedl.** 1991. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor α enhancer function. Genes Dev. **5:**880–894.
44. **Ullman, K. S., W. M. Flanagan, C. A. Edwards, and G. R. Crabtree.** 1991. Activation of early gene expression in T lymphocytes by Oct-1 and an inducible protein, OAP^{40}. Science **254:**558–562.
45. **Ullman, K. S., J. P. Northrop, C. L. Verweij, and G. R. Crabtree.** 1990. Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. Annu. Rev. Immunol. **8:**421–452.
46. **van de Wetering, M., M. Oosterwegel, D. Dooijes, and H. Clevers.** 1991. Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. EMBO J. **10:**123–132.
47. **Verweij, C. L., C. Guidos, and G. R. Crabtree.** 1990. Cell type specificity and activation requirements for NFAT-1 (nuclear factors of activated T-cells) transcriptional activity determined by a new method using transgenic mice to assay transcriptional activity of an individual nuclear factor. J. Biol. Chem. **265:**15788–15795.
48. **Waterman, M. L., W. H. Fischer, and K. A. Jones.** 1991. A thymus-specific member of the HMG protein family regulates the human T cell receptor Cα enhancer. Genes Dev. **5:**656–669.
49. **Weiss, A., I. Imboden, K. Hardy, B. Manger, C. Terhorst, and J. Stobo.** 1986. The role of the T3/antigen receptor complex in T-cell activation. Annu. Rev. Immunol. **4:**593–619.
50. **Williams, T. M., D. Moolten, J. Burlein, J. Romano, R. Bhaerman, A. Godillot, M. Mellon, F. J. Rauscher III, and J. A. Kant.** 1991. Identification of a zinc finger protein that inhibits IL-2 gene expression. Science **254:**1791–1794.