T-Cell-Specific Expression of Interleukin 2: Evidence for a Negative Regulatory Site

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T-cell-specific expression of interleukin 2: Evidence for a negative regulatory site

(repression/DNA-binding protein/transcription/T-cell activation)

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ABSTRACT **To understand the basis for T-cell-specific induction of interleukin 2 (IL-2), we have analyzed nuclear factors from the Jurkat T-lymphoid leukemia cell, which can be induced to secrete IL-2. We have used an electrophoretic mobility shift assay to examine binding of proteins to the upstream regulatory region, before and after activation with mitogens. We find two types of binding sites. One resembles an inducible enhancer element, but the protein that recognizes it is found in non-T cells and is unlikely to determine T-cell-specific expression of IL-2. A second site negatively regulates expression in resting T cells. A complex that binds to a DNA fragment containing this site is modified only when IL-2 is expressed, and it lies near a specific inducible DNase hypersensitive region. We suggest that negative regulation at this site, mediated by its associated protein(s), may contribute to the cell-specific expression of IL-2.**

Activation of inducer T cells by mitogens or antigens leads to the synthesis of several cell-specific gene products; among the newly activated genes is that for interleukin 2 (IL-2). This factor is encoded by four exons spanning ≈6.5 kilobases (1–3). Its expression is restricted to T cells (4), and its induction is dependent on increased rates of transcription (5–7). By using an electrophoretic mobility shift assay (8–12), we have analyzed binding to regulatory regions of IL-2. Several regulatory regions have been defined (7, 13). One positive regulatory element lies between positions −319 and −264. It is contained in a larger region (positions −319 to −127) that may function as an inducible enhancer (7, 13), similar to other such elements (14–17). A second region, more proximal to the "TATA box" (positions −81 to +51), may serve as a promoter for a heterologous enhancer (7). A T-cell-specific inducible DNase I hypersensitive region has been defined near this promoter element at position −85 (18).

To identify the proteins that regulate IL-2 transcription and define their mechanism of activation, we have analyzed binding of proteins from resting and activated Jurkat cell nuclear extracts to radiolabeled DNA probes derived from the IL-2 regulatory region. We have defined several binding regions. The nucleotides required for binding to one site have been identified by methylation interference analysis. Mutations that disrupt binding to this site lead to an increase in IL-2 promoter-dependent gene expression in resting T cells. Because of its proximity to an inducible T-cell-specific hypersensitive site, we suggest that this site may be associated not only with negative regulation of IL-2 transcription but also with the determination of its T-cell-specific expression.

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MATERIALS AND METHODS

Cell Lines and Extracts. Jurkat and EL4 cells were maintained in RPMI 1640 medium containing 5% (vol/vol) heat-inactivated fetal bovine serum, penicillin at 50 units/ml, and streptomycin at 50 μg/ml at cell densities between 0.2 and 1.5 × 10^6 cells per ml. Jurkat cells were activated by incubation with 1 nM phorbol myristate acetate (PMA) (Sigma) and phytohemagglutinin (PHA) at 2 μg/ml (Pharmacia). These agents were added 24 hr after transfection and maintained for an additional 20 hr in culture. EL4 cells were incubated in a similar fashion with 1 nM PMA to activate IL-2 secretion.

Nuclear extracts were prepared from uninduced or activated Jurkat cells as described by Dignam *et al.* (19), and protein concentration was determined by using the Bradford assay with bovine serum albumin standards. Extracts were prepared also from the following cell lines: MEL, mouse erythroleukemia cells; L cells, mouse fibroblast line; PD31, mouse Abelson leukemia virus transformant that represents a pre-B cell line, because it contains a rearranged (variable/diversity/joining; VDJ) heavy chain locus; BW5147, mouse AKR strain thymoma; RL♂-11, mouse radiation leukemia induced thymoma; W7, mouse thymoma; 70Z, mouse pre-B cell leukemia. None of these cell lines secretes detectable IL-2.

Plasmid DNA and Probes. Genomic IL-2 plasmid, pURg-HIL2-1(2), kindly provided by W. Fiers (State University of Ghent, Belgium), was digested with *Sau*3A and cloned into the *Bam*HI site of pUC13. A 632-base-pair *Rsa* I fragment was then subcloned into the *Sma* I site of p106CAT (20). The DNA probe for the A region was prepared from a fragment digested with *Nla* III and *Alu* I (positions −355 to −238) and ligated into the *Sph* I and *Sma* I site of pUC18. The DNA probe for the B region was prepared from an *Mnl* I fragment (positions −144 to +39) of the IL-2 upstream sequence subcloned into the *Sma* I site of pUC13.

SV-IL2-CAT was prepared as follows: The B probe, subcloned into the pUC13 plasmid was digested with *Xba* I and *Sac* I, isolated, and cloned into *Xba* I- and *Sac* I-digested p106CAT plasmid (20) [kindly provided by M. Gilman (Cold Spring Harbor Laboratory)], treated with calf intestinal phosphatase. This plasmid was digested with *Hin*dIII, treated with the Klenow fragment of DNA polymerase I, isolated, treated with calf intestinal phosphatase, and ligated to an isolated fragment containing the simian virus 40 enhancer removed from a *Bam*HI site that had been incubated with Klenow fragment of DNA polymerase I.

Abbreviations: CAT, chloramphenicol acetyltransferase; IL-2, interleukin 2; PHA, phytohemagglutinin; PMA, phorbol myristate acetate.

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Gel Binding Analysis and Methylation Interference. Nuclear extracts were prepared and incubated with radiolabeled probes as described (8–12, 14). For reactions with probe A, 1 μg of poly[d(IC)] was used as competitor, and 4 μg was included with probe B and immunoglobulin promoter (octamer) probes (12).

Methylation interference was performed as described (21) by using extracts from Jurkat cells activated for 24 hr with PMA (1 nM) and PHA (2 μg/ml).

Chloramphenicol Acetyltransferase (CAT) Assay. Cell extracts were prepared for CAT assay 44 hr after DEAE-dextran transfection and after activating cells for the last 20 hr of culture. CAT activity was determined as described (14) and expressed as the percent conversion of chloramphenicol to its acetylated forms.

DNA Transfection. Plasmid DNAs (10 μg) were transfected into Jurkat or EL4 cells (10^7 cells) by using DEAE-dextran as described (14).

RESULTS AND DISCUSSION

To identify regulatory sites recognized by DNA-binding proteins, radiolabeled DNA probes were prepared from ≈600 base pairs of sequence upstream from the IL-2 transcriptional start site. Nuclear proteins were extracted from Jurkat cells—either untreated or activated with PHA and PMA—and incubated with these probes. The three regions that showed alterations in amount or mobility of DNA–protein complexes after activation were studied further. The first probe (probe A, Fig. 1) was derived from a region shown to contain a sequence required for induction of IL-2 transcription by deletion mapping (7, 13). A DNA-binding protein appeared in nuclear extracts after activation with PMA and PHA that bound to probe A (Fig. 2*A*). Similar to other inducible enhancer binding proteins, this activity was barely detectable in uninduced cells. The specificity of binding was confirmed by competition studies. Addition of an unlabeled DNA fragment representing the A site specifically inhibited formation of the complex with probe A (Fig. 2*E*, lanes 2 and 3), whereas an immunoglobulin promoter fragment had no effect (Fig. 2*E*, lanes 6 and 7). Inclusion of unlabeled B site fragments also prevented complex formation (Fig. 2*E*, lanes 4 and 5), suggesting that the related A-site motif in the B probe (Fig. 1) may also compete with the A probe.

By using a radiolabeled fragment from the second region (probe B, Fig. 1), we found a qualitative change in the pattern of binding after activation. Two slowly migrating complexes in uninduced extracts disappeared as a new faster migrating band appeared after activation (Fig. 2*B*). A third probe (probe B′, Fig. 1) showed a pattern of binding similar to that seen with probe B (Fig. 2*C*). In contrast, minimal difference in binding to an immunoglobulin promoter fragment was observed in these nuclear extracts (Fig. 2*D*). The binding specificity of these radiolabeled probes was confirmed by competition studies. When unlabeled probe B was included in the reaction mixture with radiolabeled probe B, the slowly migrating complexes were not seen (Fig. 2*F*, lanes 2 and 3), suggesting a specific interaction. Similarly, incubation with a fragment derived from the B′ region prevented formation of complexes with probe B (Fig. 2*F*, lanes 4 and 5), suggesting that the fragments contain related binding sites. A fragment derived from immunoglobulin promoter did not inhibit complex formation (Fig. 2*D*, lanes 6 and 7). We determined the binding sequences at the B site by using a methylation interference assay (21) and extracts from induced cells to define bases critical for contact (Fig. 2*G*). The sequence of the B site shown in Fig. 1 is based on the critical contact residues detected and homology with the upstream B′ sequence.

To determine which factors might determine T-cell specificity, we analyzed binding to A and B sites in non-T cells. Nuclear extracts were prepared from several cells types and analyzed by using the electrophoretic mobility shift assay. Although the level of expression varied, complexes that comigrated with the A-site-binding factor were found in several cell types, most notably in PD31, a pre-B cell (Fig. 3*A*, lane 3), and the non-IL-2-producing early T cell RLδ-11 (Fig. 3*A*, lane 5). While nuclear factors that recognized the B site were found in non-T cells, all produced the slowest migrating band seen in uninduced Jurkat cells (Fig. 3*B*, lanes 1–7). This complex disappeared in Jurkat cells activated to secrete IL-2 (Fig. 3*B*, lane 8). To determine whether the changes in binding to these sites occurred following activation in a non-T cell, we prepared nuclear extracts from the

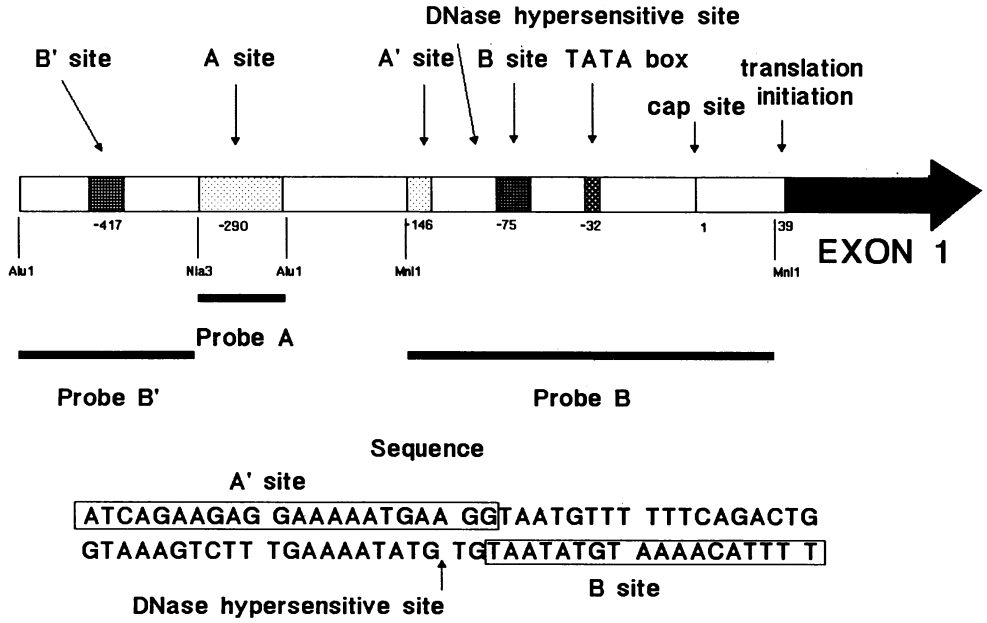

FIG. 1. Definition of DNA probes and the location and sequence of protein-binding sites in the IL-2 upstream region. Sequence and position of several IL-2 regulatory sites have been described (7, 13). Probes A and B were prepared as described. Site A′ is deduced from deletion mapping studies (7), and site B was determined as described in Fig. 2*G*.

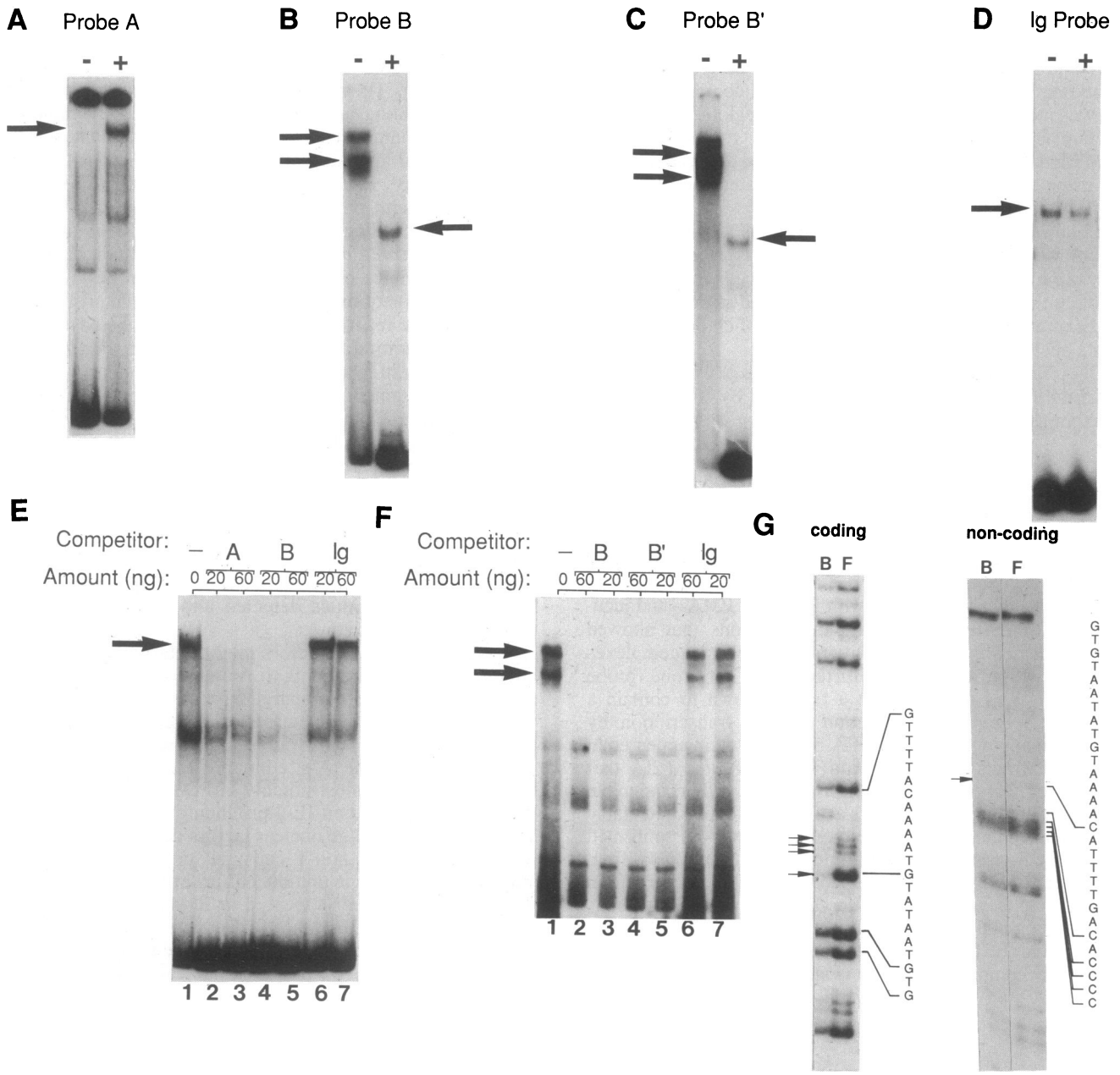

FIG. 2. Induction and specificity of IL-2-binding factors in Jurkat cells. Analysis of binding of nuclear factors to IL-2 regulatory regions by using electrophoretic mobility shift assay. Nuclear extracts (14, 19, 22) from uninduced (–) or induced (+) Jurkat cells were incubated with radiolabeled probe A (*A*), probe B (*B*), probe B' (*C*), or immunoglobulin (Ig) promoter probe (*D*). The specificity of binding to the A site was determined by competition with known DNA fragments (*E*). Nuclear extracts from induced Jurkat cells were incubated with radiolabeled probe A (see Fig. 1) alone (*E*, lane 1), in the presence of the indicated amounts of unlabeled fragment A (*E*, lanes 2 and 3), unlabeled fragment B (*E*, lanes 4 and 5), or an immunoglobulin promoter fragment (*E*, lanes 6 and 7). Specificity of binding to the B site (*F*), was assessed with nuclear extracts from uninduced Jurkat cells incubated with probe B alone (*F*, lane 1) or in the presence of the indicated amounts of unlabeled B site fragment (*F*, lanes 2 and 3), B' site fragment (*F*, lanes 4 and 5), or Ig promoter fragments (*F*, lanes 6 and 7). Analyses of sequences required for binding to the B sites were performed (*G*) in induced Jurkat cells labeled on the coding and noncoding strand by the methylation interference assay (21). Preparative binding reactions were performed with partially methylated B probe by using extracts from activated Jurkat cells. The bound (lanes B) and free (lanes F) fragment bands were eluted from a low-ionic-strength polyacrylamide gel, treated with piperidine and analyzed on an 8% sequencing gel. Jurkat cells were activated with PMA and PHA for 2 hr or 24 hr before preparation of nuclear extracts (14, 19, 22). Extracts from activated cells prepared at 2 hr were used to detect complexes bound to radiolabeled probe A, and those made at 24 hr were used with radiolabeled probes B and Ig promoter.

pre-B cell line 70Z that expresses immunoglobulin light chain but not IL-2 after stimulation with PMA or lipopolysaccharide. Stimulation of 70Z cells leads to the appearance of another DNA-binding protein, NF-κB, that is central to the activation of immunoglobulin light chain transcription (15, 21). As with NF-κB, activation of 70Z cells led to the appearance of factor binding to the A site in <2 hr after treatment (Fig. 3*C*). Similar to NF-κB, appearance of binding activity is insensitive to cycloheximide (data not shown) and binding disappears by 20 hr. In contrast, no change was observed in binding to the B probe in the same extracts from activated 70Z cells (Fig. 3*D*), even after 24 hr. This result, the cellular distribution of the B site (Fig. 3*B*), and the definition of a specific inducible hypersensitive site in this region (18) suggest that it may play a role in the T-cell-specific induction of IL-2.

To evaluate function of the IL-2 B site, we prepared a plasmid, SV-IL2-CAT, containing the CAT gene linked to

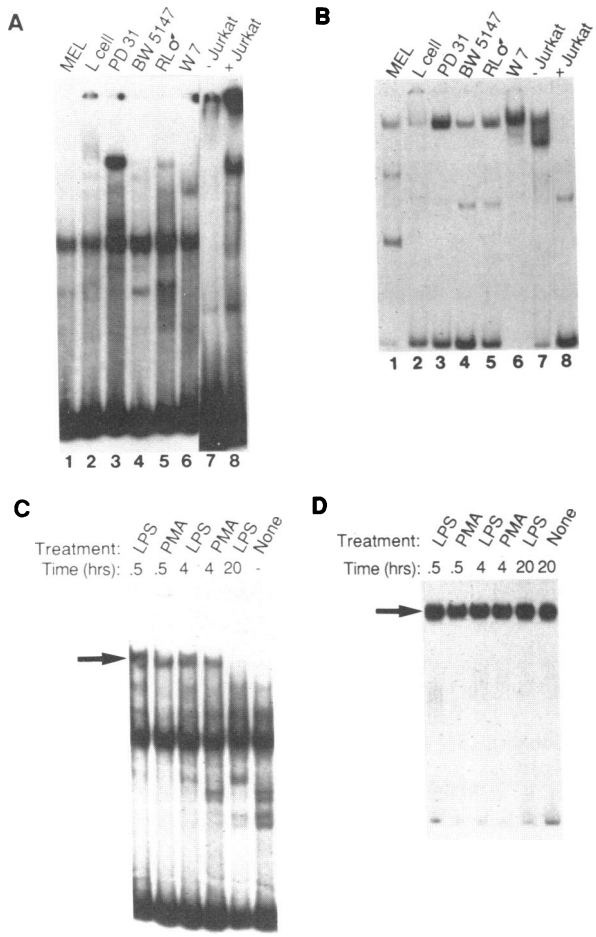

FIG. 3. Analysis of binding to A and B sites. Nuclear extracts from the indicated cell lines were incubated with radiolabeled probe from the A region (*A*) or B region (*B*) in the electrophoretic mobility shift assay. To assess induction following activation of a non-T cell, nuclear extracts from line 70Z were prepared after treatment with lipopolysaccharide (LPS at 10 μg/ml) or PMA (1 nM) for the indicated times and incubated with a radiolabeled DNA probe from the A region (*C*) or B region (*D*). The arrows indicate the position of the relevant complex.

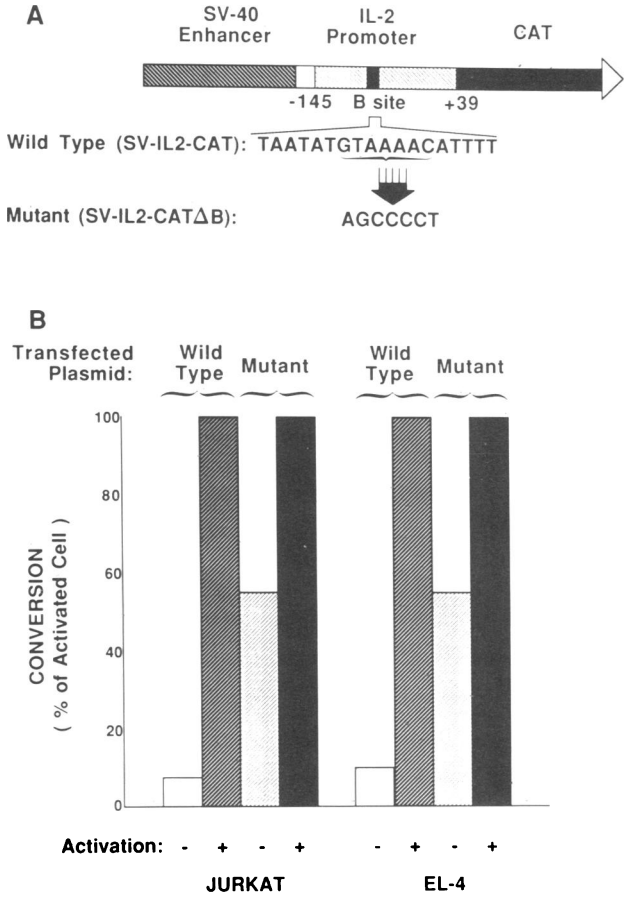

FIG. 4. Function of the IL-2 B site. (*A*) To assess the function of the B site, plasmid SV-IL2-CAT was constructed. Mutant SV-IL2-CATΔB, derived by site-directed mutagenesis (14), was identical except for the indicated base changes at the B site that inactivate binding. SV-40, simian virus 40. (*B*) SV-IL2-CAT (10 μg) or SV-IL2-CATΔB (10 μg) were transfected into the indicated cell lines by using DEAE-dextran (23) and incubated at 37°C for 44 hr. Activated Jurkat cells were treated with PHA-P (2 μg/ml) and PMA (1 nM) for the final 20 hr before preparation of extract for CAT assay. Induced EL4 cells were treated with PMA (1 nM) for the final 20 hr before assay. CAT expression was normalized to the level of expression in activated cells and represents the average of at least three independent transfections. The level of expression in activated cells by using SV-IL2-CAT and mutant plasmid did not differ significantly (<10%) when transfections were performed simultaneously with the same DEAE-dextran solution and ranged from 5 to 30% CAT conversion. −, Unstimulated; +, stimulated.

the first 145 base pairs of the IL-2 upstream region and simian virus 40 enhancer (Fig. 4*A*). This enhancer was included so that significant CAT activity could be detected in uninduced Jurkat cells. Another plasmid, SV-IL2-CATΔB, made by site-directed mutagenesis, was altered only at bases critical for binding to the B site (Fig. 4*A*). These plasmids were transfected independently into Jurkat or mouse EL4 T-lymphoma cells. In both T cells, after activation by PMA and PHA, the mutant and wild-type plasmids were equally active (Fig. 4*B*), implying that the B site does not control induced activity levels. By contrast, the wild-type construct showed a much lower activity in nonactivated cells than did the mutant construct (Fig. 4*B*). This result implies that the B site is important for repression of IL-2 gene transcription in nonactivated T cells.

We have described several nuclear factors that recognize binding sites upstream of the IL-2 gene. It is likely that these DNA-binding proteins are factors that regulate transcription of the gene. The sequences they recognize contain regulatory regions, defined by deletion mapping (7, 13). In addition, chromatin changes have been detected near these sites *in vivo*. Two upstream DNase hypersensitive sites have been described in Jurkat cells (18): one is constitutive and localized near the A site at position −306, and the second is inducible at position −86 (near the B site). Whereas several sites are necessary for expression of the IL-2 gene (7, 13), our data show that changes in binding to the B probe correlate with IL-2 production in T cells. One protein that binds to this probe recognizes a potential negative regulatory site near the T-cell-specific inducible DNase hypersensitive site (18), suggesting that this factor may play a crucial negative role in determining tissue-specific expression of IL-2.

The presence of the B-site-binding protein is associated with decreased IL-2 promoter-dependent expression in nonactivated T cells. Several elements are present near the B region. Fujita *et al.* (7) have shown that sequences downstream of position −81, containing only part of the B site described here (see Fig. 1), may serve as a promoter for a heterologous enhancer. They have also described an element between positions −145 and −127 (the A′ site, Fig. 1) required for upstream sequence to function as an inducible enhancer. This sequence is highly homologous to a consensus binding site described by sequence comparison in the upstream regulatory region (7), suggesting that the A′ site

may positively regulate IL-2 transcription and is necessary but not sufficient for activation.

It has become apparent that the enhancers of several inducible genes, including β interferon (24, 25), c-*myc* (26), c-*fos* (27), and possibly the sea urchin histone 2B gene (28) may be composed of distinct subregions that contain positive and negative regulatory elements. The activation of IL-2 synthesis requires the presence of positive regulatory enhancer-like sequences in the upstream region (7, 13). We have found an inducible DNA-binding factor that binds to this region, but its presence and induction are not restricted to T cells. Changes in binding to the B region, however, are correlated with T-cell-specific activation (Fig. 3, and see also ref. 18), raising the possibility that this site, associated with negative regulation, may play a role in determining T-cell-specific expression of IL-2.

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