POSITIVE AND NEGATIVE REGULATION OF IL-2 GENE EXPRESSION: ROLE OF MULTIPLE REGULATORY SITES

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Interleukin 2 (IL-2) is an important lymphokine required in the process of T cell activation, proliferation, clonal expansion and differentiation. The IL-2 gene displays both T cell specific and inducible expression: it is only expressed in $CD4^+$ T cells after antigenic or mitogenic stimulation. Several cis-acting regulatory sites are required for induction of the IL-2 gene after stimulation. In this study, we have analysed the function of these cis-acting regulatory sites in the context of the native IL-2 enhancer and promoter sequence. The results of this study suggest that the NFAT (−276 to −261), the distal octamer (−256 to −248) and the proximal octamer (−75 to −66) sites not only act as enhancers of IL-2 gene transcription in the presence of cellular stimulation, but also have a silencing effect on IL-2 gene expression in resting cells. Two other sites display disparate effects on IL-2 gene expression in different T leukemia cell lines: the distal purine box (−291 to −277) and the proximal purine box sites (−145 to −128). Finally, the AP-1 (−186 to −176) and the κB sites (−206 to −195) respond to different cellular activation in EL4 cells. The AP-1 site mediated the response to PMA stimulation while the κB site responded to IL-1 stimulation. These data suggest that the regulation of IL-2 gene expression is a complex process and multiple cis-acting regulatory sites interact to exert different effects in T cells representative of alternative stages of differentiation.

Interleukin 2 (IL-2) is a 15 kDa glycoprotein[1] produced by $CD4^+$ T lymphocytes after mitogenic or antigenic stimulation. Expression of the IL-2 gene is crucial to the function of the immune system. Binding of IL-2 to its receptor (IL-2r) on the cell surface is required for antigen-specific proliferation and differentiation of T cells, growth and differentiation of activated B cells into antibody-secreting cells, and activation of cytotoxicity by cytotoxic T cell clones.[2–4] Expression of the IL-2 gene is restricted to activated T cells. Previous studies have shown that the regulatory sequence for the IL-2 gene lie at the 5′ end of the gene, the region from −326 to −52 (with respect to the transcription initiation site) is sufficient to activate transfected IL-2 reporter plasmids in mitogen induced T cells.[5,6] DNA footprinting and electrophoretic mobility shift assays (EMSA) revealed that there are specific DNA-protein interactions at the NFAT site (−285 to −255), the proximal octamer site (−83 to −66), distal octamer sites (−256 to −248), and the distal purine box site (−291 to −271).[7–10] A κB site (−206 to −195) and an AP-1 site (−186 to −176) were also identified by sequence homology to the known consensus sequences. Figure 1 gives a simple representation of these sites and their location. In most of these studies, the functions of these cis-regulatory sites were determined through deletion experiments or by linking multiple copies of the site of interest to a heterologous promoter or enhancer. Although this kind of approach is simple and straightforward, the site is examined out of its native context, and the interpretation of these results may not reflect the true function of the regulatory site.

To elucidate the functions of cis-acting regulatory sites in the IL-2 enhancer region, each site has been mutated in its surrounding sequences in the present study. After transfecting these mutants into either Jurkat or EL4 cells, the basal or stimulated CAT activities are compared with those of native IL2-CAT plasmid to determine the role of each site in either the inhibition of the IL-2 gene expression in resting T cells

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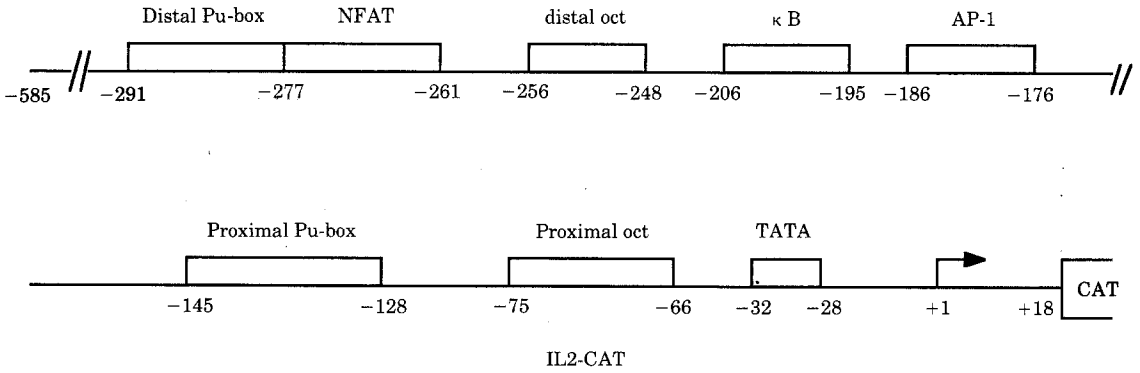

Figure 1. Schematic representation of the IL2-CAT plasmid.

Each box represents a cis-acting regulatory site with the position relative to transcriptional initiation site underneath it.

or the response to different extracellular stimulation signals. Unlike previous studies that used deletion plasmids or tandem copies of a single site, the present work uses site-directed mutagenesis to maximally preserve the DNA–protein or protein–protein interaction, making interpretation of each site's functions closest to its native ones. The results we report here show that all seven regulatory sites studied appear to be of some importance in the activation of the IL-2 gene in response to extracellular stimulation. The NFAT site, the proximal octamer site and the distal octamer site have a silencing effect on IL-2 transcription in untreated cells, and also act as enhancers of IL-2 gene transcription in the presence of stimulation. The proximal and distal purine box sites show different functions in different T cell lines, and the κB site and the AP-1 site respond to IL-1 or PMA stimulation, respectively. These results demonstrate that the regulation of the IL-2 gene is a complex process, involving the responses of multiple regulatory sites to different membrane signals.

RESULTS

Proximal Octamer, Distant Octamer and NFAT Sites

By sequence comparison to the octamer site in the Ig enhancer, two octamer sites had been identified in the IL-2 enhancer region; the proximal one (−75 to −66) has the same orientation as the octamer site in the Ig enhancer while the distal site (−256 to −248) is in the reverse orientation. Previous studies have shown specific protein-DNA interactions between the nuclear proteins from Jurkat cells and oligonucleotides containing either the proximal octamer or the distal octamer sequence, and one of the components in this protein complex is the ubiquitous transcription factor, Oct-1.[7,9,10] Also, these studies have shown that the proximal octamer site is a negative regulatory site and the distal octamer site is a positive regulatory site.

To investigate further the functions of the two octamer sites of the IL-2 enhancer in their native DNA sequence context, single or double octamer site mutants were made by site-directed mutagenesis and their transient expression studied in either Jurkat or EL4 cells. The basal and stimulated CAT activities of these mutants were compared to those of the native IL2-CAT plasmid. Similar results were obtained from both cell lines. CAT activity of each single mutant decreased 23% to 88% after cellular stimulation (Fig. 2), verifying that both octamer sites contribute to the positive regulation of IL-2 gene expression. In contrast, the basal activities of either of the single octamer site mutants increased by 6.6- to 16-fold (Fig. 2), indicating that both octamer sites are also important in the negative regulation of the IL-2 gene in resting cells. Additionally, when both octamer sites were mutated, the response to extracellular stimulation completely disappeared while the basal activity increased by 2.5- to 27.5-fold, further demonstrating the importance of both octamer sites in the negative and positive regulation of IL-2 gene expression. These data indicated that both the proximal and distal octamer sites contribute individually to the negative regulation of IL-2 promoter in resting cells, as well as the positive regulation of IL-2 gene in response to cellular stimulation. Mutation of both sites destroyed the inducible function entirely.

The NFAT site (−289 to −263) has been shown to interact with a T cell-specific factor NFAT-1 (nuclear factor of activated T cells) and confer T cell-specific, inducible and enhanced expression upon the

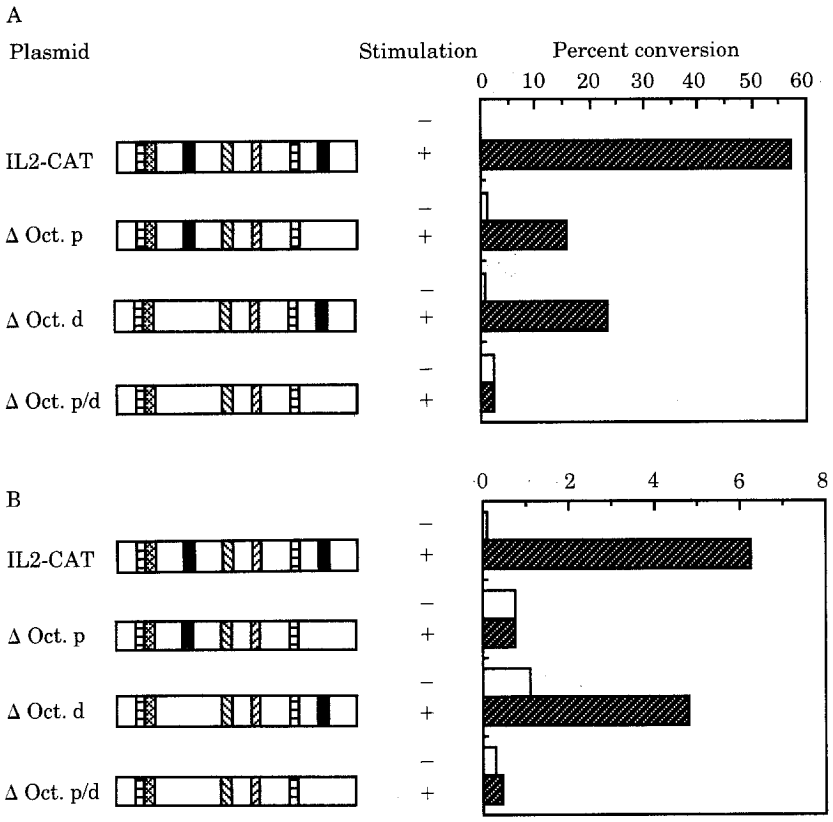

Figure 2. Effects of the single or double octamer site mutation on the transient expression of IL2-CAT.

Jurkat (A) or EL4 (B) cells were transfected with wild type plasmid (IL2-CAT), the single octamer site mutants (Δ Oct.p, or Δ Oct.d), or the double octamer site mutant (Δ Oct.p/d) in the absence (−) or presence (+) of stimulation. Cell treatment and CAT activity determination were performed as described in Materials and Methods. Each bar represents a cis-acting regulatory site in the order as indicated in Fig. 1.

normally uninduced fibrinogen promoter.[8] To determine the normal function of the NFAT site on the IL-2 promoter, a −277 to −261 mutant IL2-CAT-Δ NFAT was used in transfection experiments. Similar results to those of the double octamer site mutant were obtained in both Jurkat and EL4 cells (Fig. 3). The stimulated CAT activity in response to ionomycin and/or PMA stimulation completely disappeared, whereas the basal activity increased 5.3- to 6.2-fold. These data indicate that the NFAT site is also responsible for the low level of transcription from the promoter in unstimulated cells in addition to positive regulation after stimulation.

Purine Box Elements

The distal (−291 to −277) and proximal (−145 to −128) purine box sites were identified through their high purine content. Both purine box sites contain a consensus sequence for the Ets DNA binding site, AGGAA. A novel Ets family member, Elf-1, binds to both purine box sequences. The distal purine box is also the 5′ part of the DNase I protection sequence of NFAT.[11]

In Jurkat cells, mutation of the proximal purine box does not significantly alter expression compared to wild type IL2-CAT in the presence and absence of stimulation (Fig. 4A), suggesting that this site is not important in the aspects of IL-2 gene regulation studied here. In contrast, the proximal purine box mutant showed a decrease in both the basal and the stimulated CAT activities in EL4 cells (Fig. 4B), demonstrating the involvement of the proximal purine box site in the positive regulation of the IL-2 gene. The difference here may lie in the T cell lines, since Jurkat cells require two signals for the IL-2 gene induction, while EL4 cells require only one signal.[12,13] In the case of distal purine box mutant, the response of the mutant to extracellular stimulation is decreased in both Jurkat and EL4 cells (Fig. 4), showing that the distal purine box site is required for optimal activation of the IL-2 gene. However, the basal activity of the distal purine box mutant is increased in Jurkat cells (Fig. 4A), while decreased in EL4 cells (Fig. 4B).

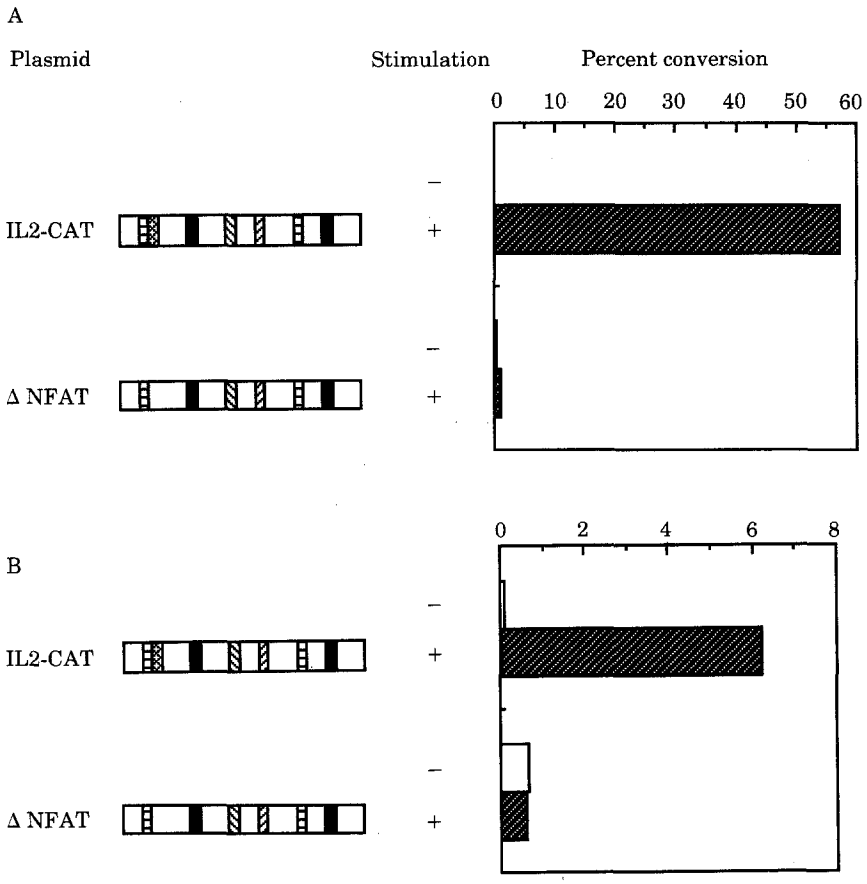

Figure 3. Effects of the NFAT site mutation on the transient expression of IL2-CAT.

Jurkat (A) or EL4 (B) cells were transfected with wild type plasmid (IL2-CAT) or the NFAT site mutant (Δ NFAT) in the absence (−) or presence (+) of stimulation. Cell treatment and CAT activity determination were performed as described in Materials and Methods. Each bar represents a cis-acting regulatory site in the order as indicated in Fig. 1.

Again, the difference may be explained by the different factors (possibly species-specific) in each cell lines.

IL-2 κB and AP-1 Sites

By sequence comparison with the k light chain enhancer of immunoglobulins, the IL-2 receptor a-chain promoter, and the HIV-1 LTR, a κB site (−206 to −195) within the IL-2 enhancer was identified. This κB site has been shown to form specific DNA-protein complexes with mitogen induced Jurkat nuclear extracts and is required for optimal mitogen-induced activation of IL-2 in both Jurkat cells and in non-transformed T cell clones.[14–16]

In contrast to these results, expression of the κB site mutant IL2-CAT-Δ κB was not significantly different from the wild type construct in the absence or presence of PMA in EL4 cells (Fig. 5B). In activated Jurkat cells, although the absolute CAT activity of the κB site mutant to ionomycin and PMA stimulation showed no difference to that of the IL-2-CAT plasmid, the induction of ionomycin and PMA decreased, since the basal activity increased 24.5-fold over IL2-CAT (Fig. 5A). Thus the κB site's function is minimal in EL4 cells following PMA treatment; however, the κB site appears to contribute more to the negative control than to the mitogenic induction of IL-2 expression in Jurkat cells.

The EL4 cell line used in this study expresses IL-1 receptors at the cell membrane and IL-2 production can be induced by a combination of IL-1 and ionomycin treatment (data not shown). In response to IL-1 and ionomycin stimulation, the κB site mutant showed a decreased CAT activity while an AP-1 site mutant showed no difference (Fig. 6), indicating the involvement of the κB site in the IL-1 pathway.

The IL-2 enhancer contains an AP-1 site (−186 to −176) by sequence comparison. This site is specifically recognized by purified AP-1, and confers IL-1 responsiveness on the IL-2 promoter in the mouse T cell line LBRM.[17] However, no response to IL-1

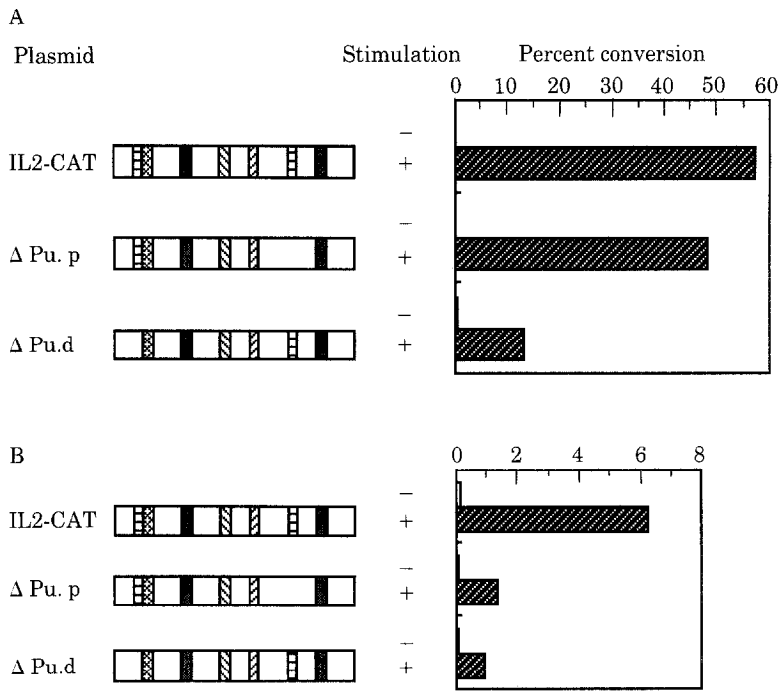

Figure 4. Effects of the purine box site mutation on the transient expression of IL2-CAT.

Jurkat (A) or EL4 (B) cells were transfected with wild type plasmid (IL2-CAT) or the purine box site mutants (Δ Pu.p, or Δ Pu.d) in the absence (−) or presence (+) of stimulation. Cell treatment and CAT activity determination were performed as described in Materials and Methods. Each bar represents a cis-acting regulatory site in the order as indicated in Fig. 1.

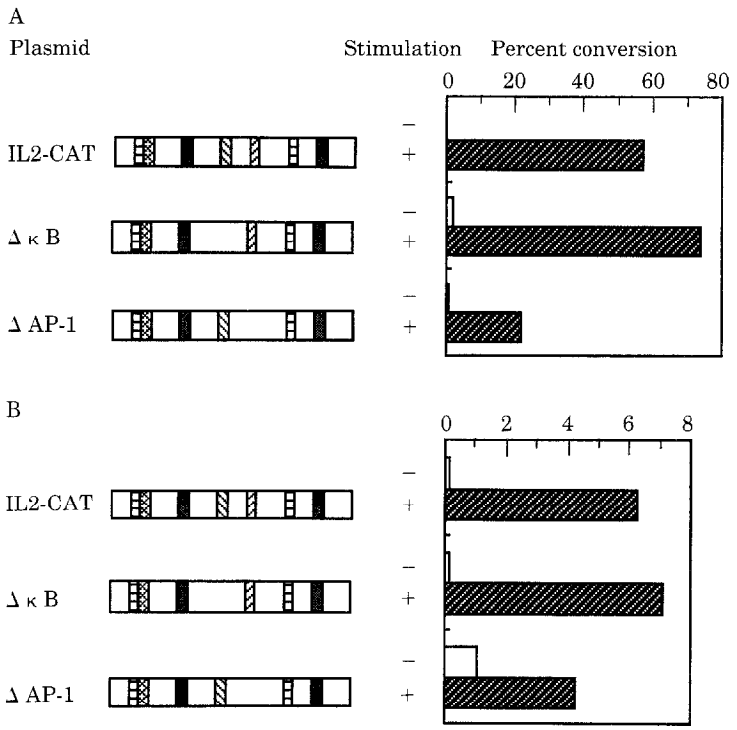

Figure 5. Effects of the κB site and the AP-1 site mutation on transient expression of the IL2-CAT.

Jurkat (A) or EL4 (B) cells were transfected with wild type plasmid (IL2-CAT), κB site mutant (Δ κB), or AP-1 site mutant (Δ AP-1) in the absence (−) or presence (+) of stimulation. Cell treatment and CAT activity determination were performed as described in Materials and Methods. Each bar represents a cis-acting regulatory site in the order as indicated in Fig. 1.

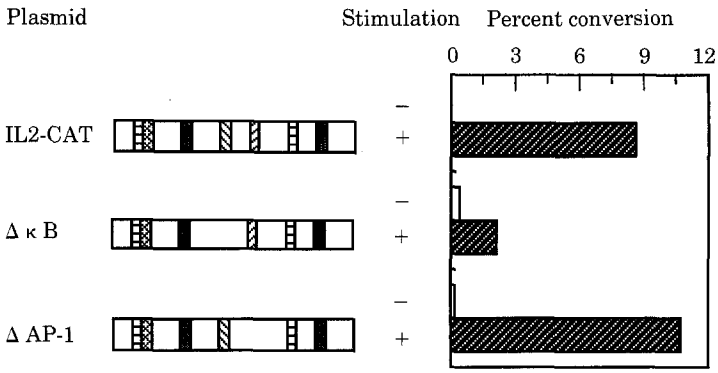

Figure 6. Responses of the κB site and the AP-1 site mutation to IL-1 and ionomycin stimulation.

EL4 cells were transfected with wild type plasmid (IL2-CAT), κB site mutant (Δ κB), and AP-1 site mutant (Δ AP-1) in the absence (−) or presence (+) of ionomycin and IL-1 stimulation. Cell treatment and CAT activity determination were performed as described in Materials and Methods. Each bar represents a cis-acting regulatory site in the order as indicated in Fig. 1.

stimulation in EL4 cells was observed through the AP-1 site in our studies (Fig. 6). In both Jurkat and EL4 cell lines, both an increase in the basal CAT activity of the AP-1 site mutant and a decrease in mitogen stimulated CAT activity were observed, although the extent of the decrease was not very large (Fig. 5). These data indicate that AP-1 site is a regulatory site in the IL-2 promoter but its effect is not as significant as the NFAT site or the octamer sites.

DISCUSSION

In this study, the function of the cis-acting regulatory sites in the IL-2 gene expression were elucidated by systematic mutation of these sites and testing their response to extracellular stimulation. Since the native IL-2 enhancer is relatively weak, almost all previous studies on IL-2 gene regulation had used a heterologous enhancer or promoter. Although the use of heterologous enhancer may elevate the signal level, it can also interfere with the action of the regulatory sites, making interpretation of the data difficult. This study used the native IL-2 enhancer and promoter to preserve maximally the interactions that occur at each site and between the DNA and protein(s).

Using this approach, we have found that the NFAT site, and the proximal and distal octamer sites were involved in the suppression of IL-2 gene expression in resting T cells, as well as the induction of IL-2 gene expression after mitogenic stimulation. Though the activity of wild type IL2-CAT plasmid in Jurkat cells is very low, the activity of the NFAT and each of the octamer mutant sites in resting cells is considerably above background level, highly reproducible, and therefore not likely due to variations in background of the CAT assay. Our results are consistent with previous findings that have shown the proximal octamer site negatively regulates IL-2 gene expression in resting T cells when linked to a heterologous enhancer;[9] however, in the same study, no positive function was found for the proximal octamer site, probably because a strong SV40 enhancer has overcome the deficit of the octamer mutation. A subsequent study, however, suggested a positive role for this element in the native enhancer[10] in mitogen-stimulated cells, also consistent with the findings presented here.

The NFAT site is crucial for the induction of IL-2 gene expression, since mutation of the NFAT site completely abolished the response to stimulation. Each of the two octamer sites contributes to both negative and positive regulation of the IL-2 gene expression, yet full induction of the IL-2 gene is achieved only with the presence of both octamer sites. These observations suggest that a single site can confer two different functions depending on the state of cellular activation. The changes in function of these regulatory sites may be due to changes in the protein complexes bound to these sites in response to stimulation. Although no difference was detected from specific DNA-protein binding at either the proximal or distal octamer sites before or after stimulation,[10] it remains possible that these protein complexes are modified post-translationally after stimulation. Even though the mobility of the complexes did not change, such modification (e.g. phosphorylation) may alter their function, resulting in repression before stimulation or induction after stimulation. Another explanation may involve a recently described inducible protein, OAP (Oct-1 associated protein), which binds 5′ to the proximal octamer site.[18–20] It is likely that octamer binding with the ubiquitous transcription fac-

tor, Oct-1, in resting cells is inhibitory to IL-2 transcription; after stimulation, the interaction of OAP alters the function of the octamer binding complexes. The appearance of NFAT-1 after T cell activation[7,8] correlates well with the induction of IL-2 gene through the NFAT site. Although no DNA-protein interactions were observed at the NFAT site in unstimulated cells, it is possible that in vitro DNA-protein binding may not reflect the in vivo interaction. We hypothesize that a low affinity DNA-protein interaction may occur at the NFAT site in unstimulated cells, which might contribute to the basal repression observed through this site.

The distal purine box site is also involved in the negative and positive regulation of the IL-2 gene. This purine box site is located just upstream of the NFAT site, and is seen as part of the DNA footprint of IL-2E.[7] The effect of the proximal purine box site alone is minor and differs between the two T cell lines tested. This may be because the factors needed for interactions with one of the two purine box sites are not present or not functional in one of the T cell lines, since the two T cell lines are representative of different stages of T cell maturation and require different stimuli to activate the IL-2 gene. Therefore, the regulation of IL-2 gene expression may be different at various stages of development.

The κB site is involved in the inducible expression of a large number of genes in different cell types.[21–23] The κB site in the IL-2 enhancer was initially defined through DNase I footprinting at this region with stimulated Jurkat cell nuclear extract,[23] but deletion analysis failed to detect any function in this site.[7] Other studies have shown that the κB site is important for IL-2 gene activation after mitogenic simulation.[14,15,24,25] Our results indicate that in Jurkat cells, the main function of the κB site is to keep the IL-2 gene silent in the absence of any stimulation. It is possible that this negative regulatory effect is conferred through NF-κB p50 homodimers since a previous study showed that the IL-2 gene expression parallels the decrease in the binding of NF-κB p50 homodimer to the κB site.[16] In EL4 cells, the κB site is not important in response to PMA stimulation, but is important in conferring the IL-1 responsiveness. It is therefore possible that redundancy of the enhancer, from the NFAT and/or other regulatory sites, can overcome the requirement for κB in EL4 cells in response to mitogen stimulation. In contrast, the AP-1 site responded to PMA to some extent in Jurkat and EL4 cells. This result is consistent with previous findings that the AP-1 site has only weak activity in Jurkat cells.[26] Contrary to previous reports that the AP-1 site is critical for the IL-1 pathway in LBRM cells,[17] our results showed that this AP-1 site does not respond to IL-1 stimulation in EL4 cells; the IL-1 responsiveness is conferred by the κB site. The difference may be due to the different factor(s) presented in these two cell lines. Another AP-1 site (−151 to −145) may also confer IL-1 responsiveness in EL4 cells, since this region of the mouse IL-2 enhancer is protected by an AP-1 like factor from EL4 cell extract in DNase I footprint experiment.[27]

In summary, our study indicates that all of the seven IL-2 regulatory sites studied participate to some extent in the activation of the IL-2 gene in response to extracellular stimulation. The NFAT, the distal and the proximal octamer sites having a silencing effect on IL-2 transcription in resting cells. After stimulation, however, these three sites all act positively to induce IL-2 gene expression. The distal and proximal purine box sites display different functions in the two T cell lines studied, Jurkat and EL4. For the κB and the AP-1 sites, these two sites are found to respond to IL-1 and PMA stimulation, respectively. These findings contribute to an understanding of the regulation of the IL-2 gene expression in different T cell subsets and during T cell progressive stages of development.

MATERIALS AND METHODS

Plasmid Construction

The IL2-CAT plasmid was made by inserting an *Rsa* I fragment from the IL-2 enhancer (−585 to +18, digested by Bal 31 to eliminate the translation initiation site) into the pUC-CAT plasmid. All the mutants were made using the site directed mutagenesis technique[28] and the sequence of the mutated regions were confirmed by dideoxy sequencing. The sequences of the oligonucleotides used in the construction of the mutants are: proximal octamer site, 5′-GTGTAATATGTCTCGAGTTTTGCACC-3′; distal octamer site, 5′-CATACAGAAGGCGCAGCGGATCCGAATTAGAGCTA-3′; NFAT site, 5′-A-AGGAGGAAAAATGTGCAGTCTGCAGCGGCGTTAATTGC-3′; proximal purine box site, 5′-GTCATCAGGGTCCTGCGTATGAAGGTAA-3′; distal purine box site, 5′-CCTTAAAGAAGATCTTCGTCACTGTTTCAT-3′; κB site, 5′-GTAACAAAGATTCTAGAT-CGATACATCCATTC-3′; AP-1 site, 5′-CTACATCCATTCGAAGCTTCTTTGGGGG-3′.

Cell Culture and Transfection

Jurkat and EL4 cells were maintained in RPMI 1640 media (GIBCO) containing 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin. b-mercaptoethanol, 50 mM, was also included in the media for EL4 cells. DNA transfection experiments were performed by the DEAE-dextran method as previously described.[29] Protein concentrations were determined and normalized during the CAT assay. With these conditions, co-transfection experiments using RSV-luciferase plasmid have shown that there is no significant variation among

transfection efficiency in a single experiment (Zhang and Nabel, unpublished). Plasmid, 10 μg, was used to transfect 10^7 cells. After the transfection, cells were maintained in RPMI 1640 media with 5% fetal bovine serum. Jurkat cells were treated with 10 ng/ml of PMA and 1.4 μg/ml of ionomycin for 20 h, and EL4 cells were treated with either 10 ng/ml of PMA for 12 h, or 1.4 μg/ml of ionomycin and 15 U/ml IL-1 for 16 h. Cells were harvested 44 h after transfection, and extracts were made after three rounds of freeze-thaw in ethanol/dry ice bath. The protein content of each extract was quantitated by Bradford assay and an equal amount of protein from each extract was used for CAT assay. Though the conversion of wild type IL2-CAT plasmid in Jurkat cells is near the limits of the linear range of the CAT assay, it nonetheless shows the difference in CAT activity among different protein extracts. The results shown from one experiment are representative of at least three independent replicates.

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