2134–2142 Nucleic Acids Research, 1994, Vol. 22, No. 11

Cyclosporin A sensitivity of the NF-κB site of the IL2Rα promoter in untransformed murine T cells

Patricia G.McCaffrey[1,2,*], Peter K.Kim[1], Viia E.Valge-Archer[1,3], Ranjan Sen[4] and Anjana Rao[1,2]
[1]Division of Tumor Virology, Dana-Farber Cancer Institute, Boston, MA 02115, [2]Department of Pathology, Harvard Medical School, Boston, MA 02115, [3]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139 and [4]Rosenstiel Center and Department of Biology, Brandeis University, Waltham, MA, USA

Received January 19, 1994; Revised and Accepted May 2, 1994

ABSTRACT

We have investigated the characteristics of IL2Rα gene induction in untransformed murine T cells. Induction of IL2Rα mRNA by TCR/CD3 ligands in a murine T cell clone and in short-term splenic T cell cultures was inhibited by protein synthesis inhibitors and by CsA. This result was contrary to previous observations in JURKAT T leukemia cells and human peripheral blood T cells, suggesting a difference in the mechanisms of IL2Rα gene induction in these different cell types. The CsA sensitivity of IL2Rα mRNA induction represented a direct effect on the TCR/CD3 response, and was not due to CsA-sensitive release of the lymphokines IL2 or tumour necrosis factor α (TNFα) and consequent lymphokine-mediated induction of IL2Rα mRNA. The NF-κB site of the IL2Rα promoter was essential for gene induction through the TCR/CD3 complex, and the induction of reporter plasmids containing multimers of this site was significantly inhibited by CsA. Northern blotting analysis indicated that while the p65 subunit of NF-κB was constitutively expressed and not appreciably induced upon T cell activation, mRNA for the p105 precursor of p50 NF-κB was induced in response to TCR/CD3 stimulation and this induction was sensitive to CsA. Electrophoretic mobility shift assays and antiserum against the p50 subunit of NF-κB indicated that p50 was a component of the inducible nuclear complex that bound to the IL2Rα κB site. Appearance of the kB-binding proteins was insensitive to CsA at early times after activation (~15 min), but was partially sensitive to CsA at later times. Based on these results, we propose that the NF-κB site of the IL2Rα promoter mediates at least part of the CsA sensitivity of IL2Rα gene induction in untransformed T cells, possibly because *de novo* synthesis of p105 NF-κB is required for sustained IL2Rα expression.

INTRODUCTION

Activation of T cells through the T cell receptor (TCR)/CD3 complex results in induction of a large number of activation-associated genes (reviewed in [1]). Among these are the genes encoding the lymphokine Interleukin 2 (IL2) and the α subunit of the high affinity IL2 receptor (IL2Rα), which are coordinately involved in the control of T cell proliferation (reviewed in [2]). Both genes are transcriptionally induced by 1–6 h following exposure to stimulus [3,4]. However induction of the IL2 gene requires protein synthesis [5,6], and is inhibited by the immunosuppressive agents cyclosporin A (CsA) and FK506 [4,7,8]. In contrast, the IL2Rα gene is induced in JURKAT human T leukemia cells even in the presence of protein synthesis inhibitors [6,9], and its induction in JURKAT cells and in human peripheral blood lymphocytes (PBL) is not sensitive to CsA and FK506 [4,7].

A potential explanation for the insensitivity of IL2Rα gene induction to protein synthesis inhibitors was suggested by examination of the human IL2Rα promoter. Mutational and deletion analysis of the promoter showed that a consensus binding site for the ubiquitous transcription factor NF-κB, located at about −270 bp relative to the transcription start site, was required for promoter induction by phorbol 12-myristate 13-acetate (PMA), phytohaemagglutinin (PHA) and PMA, the HTLV-1 *tax* gene product, Interleukin-1, and tumour necrosis factor a (TNFα) [10–14]. NF-κB sites bind rel proteins, a family of related DNA-binding proteins whose primary mode of activation involves modification of a preexisting complex rather than *de novo* synthesis (reviewed in [15]). Prior to stimulation, NF-κB proteins exist in inactive cytosolic complexes with inhibitory IκB proteins; activation results in release of active DNA-binding rel dimers which subsequently translocate into the nucleus [16,17]. Several rel family members (p50, p65, c-rel) capable of binding to the IL2Rα κB site are induced in JURKAT T cells stimulated with PHA and PMA [9,18]; as expected, induction of these factors is insensitive to protein synthesis inhibitors [9]. However the importance of the IL2Rα κB site in PMA-stimulated JURKAT cells has been questioned by other workers [19,20], and its role in T cells stimulated through the TCR/CD3 complex has not yet been established.

Here we show that induction of IL2Rα mRNA in untransformed murine T cells stimulated through the TCR/CD3 complex was unexpectedly sensitive to protein synthesis inhibitors and to CsA. The NF-κB site of the IL2Rα promoter was required for IL2Rα gene induction in these cells, and induction of a

*To whom correspodence should be addressed at: Vertex Pharmaceuticals, 40 Allston St., Cambridge, MA 02139,USA

reporter plasmid containing a multimer of this site was significantly sensitive to CsA. Moreover induction of mRNA for the p105 precursor of p50 NF-κB was also CsA-sensitive. Based on these results, we propose that the CsA sensitivity of the IL2Rα promoter is at least partly mediated through the NF-κB site, and that *de novo* synthesis of p105 NF-κB is required for sustained IL2Rα expression.

MATERIALS AND METHODS

Cell lines and antibodies

The murine T cell clone Ar-5 [21] was cultured in IL2-containing T cell medium [22,23] and was used for all gel-retardation and transfection experiments. Short-term splenic T cell cultures were derived by stimulation of (BALB/c×C57BL/6)F1 spleen cells with Concanavalin A (Con A) and culture of the resulting T cell blasts in IL2-containing medium [24]. TA3 B lymphoma cells were used as antigen-presenting cells [25]. 145-2C11 hybridoma cells were used as a source of hamster monoclonal antibody against murine CD3ε [26]. Supernatants from the rat hybridoma cells 7D4 [27] and PC61 [28], which produce monoclonal antibodies against the murine IL2 receptor α chain, were concentrated and used in blocking experiments as previously described [29].

T cell activation

T cells were activated with Con A (1−2 μg/ml), with anti-CD3ε (1% 145-2C11 hybridoma supernatant) crosslinked either via Fc receptors on 2×10^6 TA3 cells or on flasks coated with 300 μg/ml rabbit-anti-hamster antibody (Cappel), with TNFα (Genzyme, 400 U/ml), or with 10−20 U/ml IL2 (partially purified from rat conditioned medium; CR-TCGF, Collaborative Research). The biological activity of the TNFα obtained from Genzyme was tested in an L cell killing assay by Dr. Anne E. Goldfeld, DFCI, and was shown to be equivalent to that claimed by the manufacturer. Proliferation and mRNA production by Ar-5 cells in response to CR-TCGF is mediated only by IL2; identical results were obtained with human recombinant IL2 [29,30]. Where indicated, cells were treated with 1 μM CsA, 40 μM cycloheximide (CHX) and 10 μM anisomycin (ANI), or a cocktail of the 7D4 and PC61 anti-IL2Rα antibodies [29], 10−30 min prior to addition of the activating stimulus. For kinetic experiments, T cells were placed in second-antibody-coated flasks, anti-CD3ε was added, and the cells were centrifuged onto the coated surface in the cold to ensure synchronous activation. Activation was monitored in all experiments by measuring proliferation or IL2 secretion [22,23].

Northern analysis

Ar-5 cells were activated for the indicated times with Con A or anti-CD3ε crosslinked with immobilised second antibody. Preparation of cytoplasmic RNA, electrophoresis on formaldehyde-agarose gels, transfer to nylon membranes, and hybridisation to ^{32}P-labelled cDNA probes were performed as previously described [22]. Probes for p50 and p65 subunits of NF-κB were kindly provided by Dr. S. Ghosh. Probes for the murine IL2Rα gene and for the constant region of the TCR α chain (Cα) have been described [22].

Plasmids used for transfections

The −417, −317, −271, and −248 human 5′IL2Rα-CAT promoter deletion mutants, and the −317 5′IL2Rα-CAT mutants M3 (NF-κB site) and M4 (3′ of NF-κB site) were kindly provided by Dr. W. C. Greene [12]. The 3×IL2Rακ B CAT plasmid was obtained by isolation of a trimer of the IL2Rα κB site oligonucleotide (see below) from a low-melt agarose gel after self-ligation, and subcloning into the BamHI site of pBLCAT2 [31]. The 5×TCEd plasmid, containing 5 copies of the IL2 NF-κB (TCEd) site in pBLCAT2, was kindly provided by Dr. E. Serfling [32]. Plasmids were sequenced to confirm their identity.

A plasmid containing ~2000 bp of the murine IL2Rα promoter cloned into pUC18 was kindly provided by Dr. T. Honjo [33]. A *Pvu*II/*Eco*RI fragment containing the region from −572 to +161 of the murine IL2Rα promoter was cloned into a Bluescript vector (Stratagene KS+) cut with *Sma*I and *Eco*RI to generate the pBS-mIL2Rα plasmid. To make the −572 murine 5′IL2Rα-CAT plasmid, the *Spe*I/*Hin*dIII fragment containing the RSV promoter in pRSVCAT [34] was replaced with a 733 bp *Spe*I/*Hin*dIII fragment from pBS-mIL2Rα containing the IL2Rα promoter [35]. Transfections of Ar-5 cells were performed as previously described [36].

Electrophoretic mobility shift assays

Preparation of small-scale nuclear extracts from activated and unactivated Ar-5 cells, labelling of oligonucleotide probes, and conditions for formation of nuclear complexes and gel-shift analysis have been described [24]. Unless otherwise stated, cells were activated for 2 h with anti-CD3ε antibody which was crosslinked using immobilised second antibody. Protein concentration of the extracts was measured by the method of Bradford [37]. The amount of DNA-binding activity was quantitated by direct counting of radioactivity in the dried gels using a Betascope. The p50 antiserum, raised in rabbits against recombinant p50 protein [38] was a kind gift from Dr. Michael J. Leonardo. For antibody reactivity studies, immune or preimmune sera was incubated with nuclear extracts on ice for 30 minutes, followed by addition of binding mix and ^{32}P-labeled oligonucleotide. The following oligonucleotides were annealed with their complements and used as probes in the gel-shift assays:

Human IL2Rα κB site: 5′gatcGAGAGGGAGATTCCCCTGCCG;
Mutated human IL2Rα κB site: 5′gatcGAGATCTAGATTCCCCTGCCG;
Murine IL2Rα κB site: 5′gatcGAGAGGGAGATTCCCCTGCCG;
Murine Ig κ enhancer κB site [39]: 5′gatcCAGAGGGGACTTTCCGAGA;
3′Ig κB mutant: 5′gatcCAGAGGGGACTTGAAGAGA;
5′Ig κB mutant: 5′gatcCAGAATTCACTTTCCGAGA;
IL-2 κB site; gatcACCAAGAGGGATTTCACCTAAATCC;
NFAT site: gatcGCCCAAAGAGGAAAATTTGTTTCATACAG.

RESULTS

Induction of IL2Rα mRNA is sensitive to protein synthesis inhibitors and to CsA in untransformed murine T cells

We used the murine T cell clone Ar-5 [21,22] to examine the sensitivity of IL2Rα mRNA induction to protein synthesis inhibitors and to the immunosuppressive agent cyclosporin A (CsA). Cytoplasmic RNA from Ar-5 cells, unstimulated or stimulated for 6 h with crosslinked aCD3ε antibody, was analysed by hybridisation to a cDNA probe for the IL2Rα gene. As previously described [22], steady-state levels of IL2Rα mRNA were increased severalfold in stimulated over unstimulated Ar-5 cells (Figure 1a, compare lanes 1 and 3). This increase is due primarily to transcriptional induction of the IL2Rα gene [3,4,30], and is henceforth referred to as mRNA induction. Surprisingly, induction of IL2Rα mRNA was sensitive to treatment of Ar-5 cells with either the protein synthesis inhibitors cycloheximide

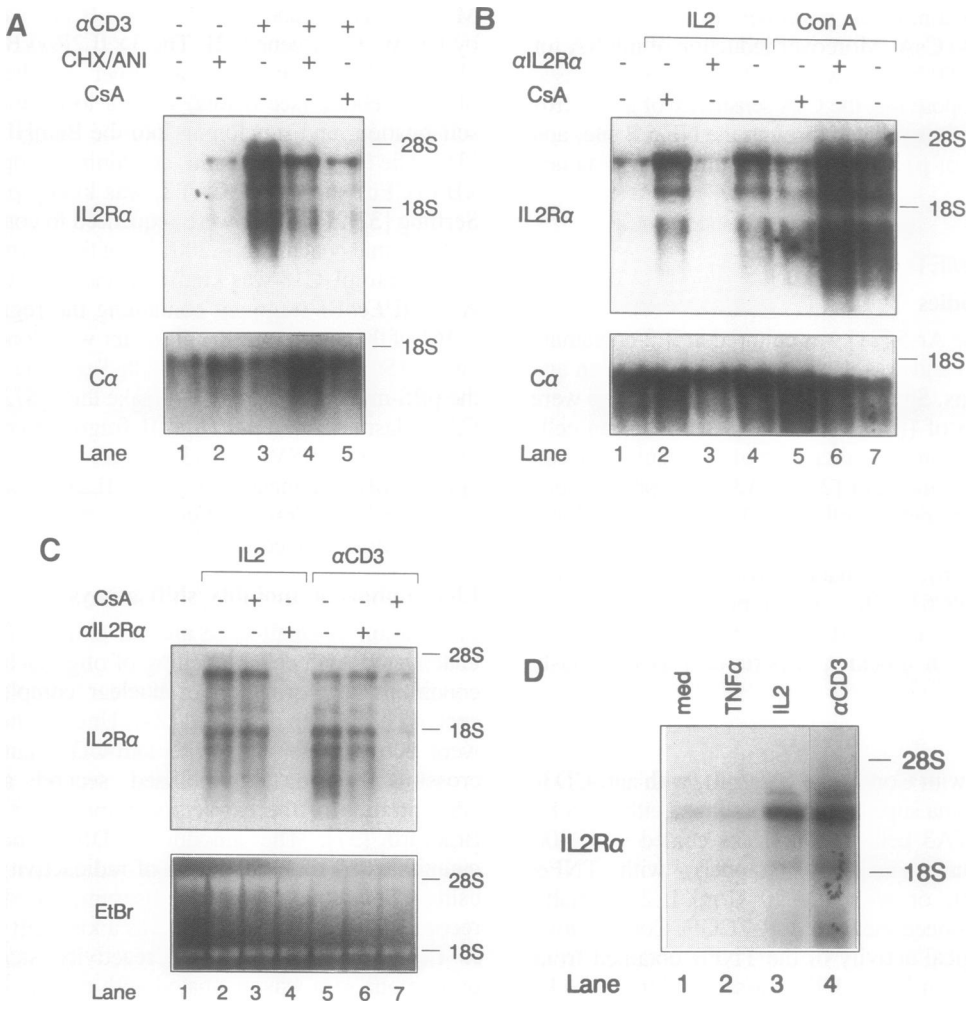

Figure 1. Decreased induction of IL2Rα mRNA upon T cell activation in the presence of protein synthesis inhibitors or CsA is not due to inhibition of IL2 or TNFα production. **A**, Ar-5 T cells were treated for 30 minutes with 40 μM cycloheximide and 10 μM anisomycin (CHX/ANI) or for 10 minutes with CsA (1 μM) as indicated, and then stimulated for 6 hours with crosslinked anti-CD3ε. Cytoplasmic RNA was isolated and IL2Rα mRNA levels were determined by Northern blotting (upper panel). RNA loading was assessed by probing the same blot with a murine T cell receptor a constant region cDNA (lower panel). **B**, Ar-5 cells were treated for 10−30 minutes with 1 μM CsA or with anti-IL2Rα antibodies as indicated, followed by 10 U/ml IL2 (lanes 2−4) or 1 μg/ml ConA (lanes 5−7) for 6 hours. IL2Rα and TCRα mRNA were analyzed as in part A. **C**, T cells derived from short-term splenic cultures were treated as in part B with CsA or anti-IL2Rα antibodies as indicated, followed by stimulation with IL2 (lanes 2−4) or crosslinked anti-CD3ε (lanes 5−7) for 6 hours. IL2Rα mRNA was detected by Northern blotting (upper panel); equivalent loading of RNA in each lane was verified by ethidium bromide staining (lower panel). **D**, TNFα does not induce IL2Rα mRNA in Ar-5 T cells. Ar-5 cells were stimulated for 6 h with medium (lane 1) 400 U/ml TNFα (lane 2), 10 U/ml IL2 (lane 3), or crosslinked anti-CD3ε (lane 4). IL2Rα was analysed by Northern blotting as in part A. Equivalent loading of RNA in each lane was verified by ethidium bromide staining (not shown).

(CHX) and anisomycin (ANI) (Figure 1a, compare lane 3 with lane 4), or with CsA (Figure 1a, compare lane 3 with lane 5).

Because CsA inhibits production of IL2 [4,7], which itself induces IL2Rα mRNA [22,40], the apparent CsA sensitivity of IL2Rα gene induction might have been indirectly due to the CsA sensitivity of IL2 production in Ar-5 cells. We tested this possibility using blocking antibodies to the IL2Rα chain [28,29] which completely inhibited proliferation [29,30] and IL2Rα mRNA induction (Figure 1b, compare lanes 1,4 with lane 3) in response to IL2. The same concentrations of anti-IL2Rα antibodies did not affect IL2Rα mRNA induction in Ar-5 cells stimulated with Con A (Figure 1b, compare lane 7 with lane 6) or anti-CD3ε (data not shown), indicating that IL2Rα mRNA induction in response to TCR ligands was not dependent on secretion of IL2. Again, treatment with CsA abolished IL2Rα gene induction in response to Con A (Figure 1b, compare lane 7 with lane 5; >90% decrease); as expected, it had no effect on IL2Rα gene induction in response to IL2 (Figure 1b, compare lane 4 with lane 2). Thus the CsA sensitivity of IL2Rα mRNA induction in response to TCR stimulation is not due to CsA-sensitive release of IL2 and IL2-mediated stimulation of IL2Rα mRNA induction.

The above experiments were performed with the Ar-5 T cell clone, which has been maintained for several years in culture [21,22]. To ensure that the CsA sensitivity of IL2Rα mRNA induction was not an artefact of using an established T cell clone, we repeated the experiments using short-term splenic T cell cultures (Figure 1c). The results were similar to those seen with Ar-5 cells. IL2Rα mRNA induction in response to TCR stimulation of splenic T cells was not mediated by secreted IL2, since blocking antibodies to the IL2Rα chain which abolished induction in response to IL2 (Figure 1c, compare lanes 2 and

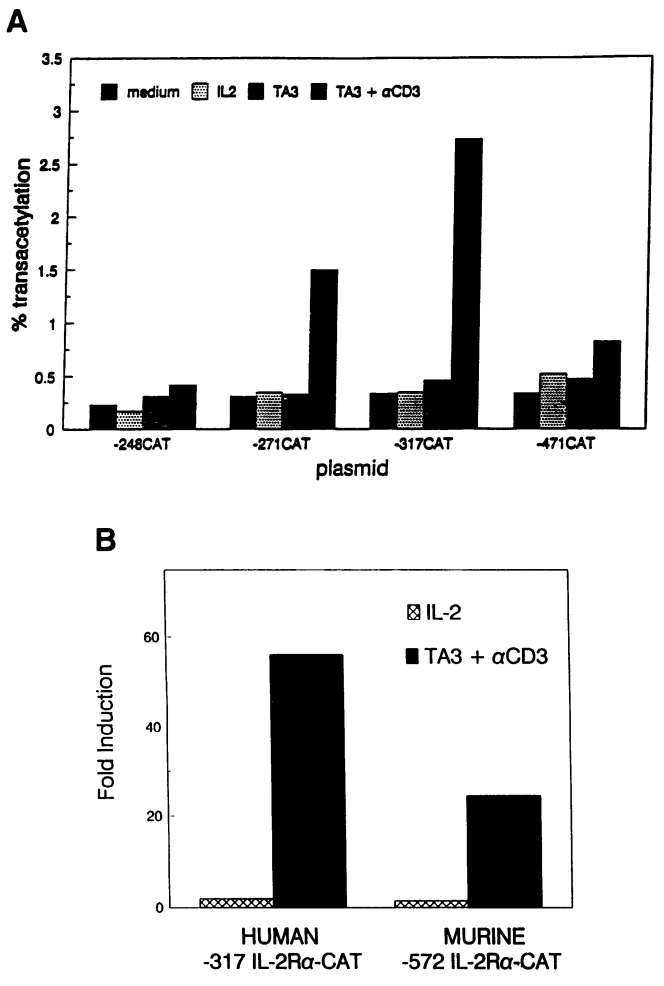

Figure 2. Induction of proximal promoter regions from the human and murine IL-2Rα genes by TCR ligands but not IL-2. **A**, A series of 5′ deletion mutants of the human IL2Rα promoter with endpoints at −248, −271, −317, and −471 relative to the major transcription intiation site was transfected into Ar-5 cells using DEAE-dextran (see Methods). The cells were stimulated 24 hours after transfection with 10 U/ml IL2 or with anti-CD3ε antibody presented on the surface of TA3 B lymphoma cells. The cells were harvested 24 hours later and extracts were assayed for CAT activity. The data are presented as percent of the input ^{14}C-chloramphenicol converted to acetylated forms, adjusted for the protein concentration of the cell extracts. A representative experiment, of at least three similar experiments, is shown. **B**, Activity of human −317 IL2Rα promoter-CAT plasmid and murine −572 IL-2Rα promoter-CAT plasmid in Ar-5 cells stimulated with anti-CD3ε antibody or IL-2. The activity of each plasmid is expressed relative to the activity in unstimulated cells. The data presented for the human promoter construct are the average of 9 independent experiments (range= 25−121 fold for anti-CD3e and 1−3 fold for IL-2); the data for the murine construct are the average of 5 experiments (range = 3.6−29 fold for anti-CD3ε and 1.3−2.0 fold for IL-2).

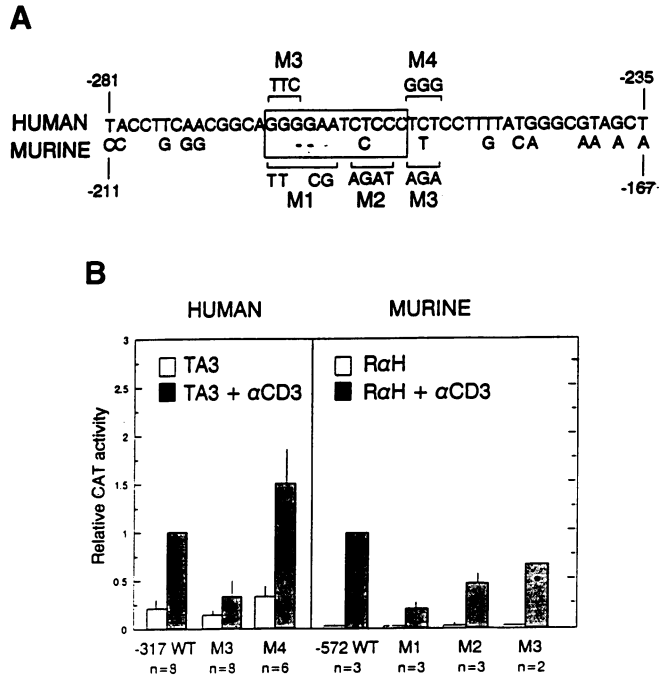

Figure 3. Effect of mutations in the NF-κB site on induction of the human and murine IL-2Rα promoter after TCR stimulation. **A**, Comparison of sequences from the human and mouse IL2Rα promoters in the region of the NF-κB site (boxed). Single base differences between human and mouse sequences are indicated. The dashes represent two bases in the NF-κB site which are deleted in the mouse promoter. Specific mutations introduced into the human -371 IL2Rα CAT or the murine -572 IL2Rα CAT plasmids are indicated with brackets. **B**. Activity of the human −317 IL-2Rα promoter with the mutations indicated was measured as described in Figure 2. Transfection of the murine −572 IL-2Rα promoter plasmids was identical except that the cells were stimulated with aCD3 antibodies cross-linked with an immobilized second antibody (RaH) rather than TA3 cells. The activity of each plasmid is expressed relative to the activity of the wild-type plasmid in stimulated cells, which was set to 1. CAT activity in cells transfected with the wild-type −317 IL-2Rα promoter construct ranged from 0.2 to 3.1 percent conversion for unstimulated cells, and 1.0 to 9.5 percent conversion for stimulated cells in 8 independent experiments; the murine promoter showed similar levels of activity. The bars show the average and standard deviation of values from n transfections of each plasmid, except for the murine promoter mutant 3 where the bar represents the average of two independent trials (solid circles).

4) had no effect on induction of IL2Rα mRNA in response to anti-CD3ε (Figure 1c, compare lanes 5 and 6). Hence the ability of CsA to diminish IL2Rα mRNA induction in response to anti-CD3e (Figure 1c, compare lanes 5 and 7; ~70% decrease) was also observed in short-term splenic T cell cultures.

The lymphokine TNFα has also been reported to induce IL2Rα gene expression in both normal and leukemic human T cells [12−14]. Since Ar-5 cells produce TNFα upon stimulation through the T cell receptor, and since induction of TNFα mRNA is sensitive to CsA (41), we tested whether this lymphokine could induce IL2Rα mRNA expression in Ar-5 T cells as well. Surprisingly, TNFα had no effect on the level of expression of IL2Rα mRNA expression in Ar-5 T cells (Figure 1d). We conclude that the CsA sensitivity of IL2Rα mRNA induction in response to TCR stimulation is not due to CsA-sensitive release of TNFα.

Identification of an NF-κB site in the human and murine IL2Rα promoters required for induction by TCR ligands in murine T cells

To identify the site(s) of CsA sensitivity in the IL2Rα promoter, we transfected Ar-5 cells with a series of 5′IL2Rα-CAT plasmids containing 5′ deletions or site-specific mutations in the human or murine IL2Rα promoters [12,13]. Figure 2a shows induction of a panel of deletion mutants of a human 5′IL2Rα-CAT plasmid, containing 471, 317, 271 and 248 bp respectively of 5′ flanking sequences [12]. The pattern of induction of these plasmids by anti-CD3ε antibodies, crosslinked using either TA3 cells (Figure 2a) or immobilised second antibody (not shown), was consistent with previous observations in JURKAT and YT-1 cells [13,42]

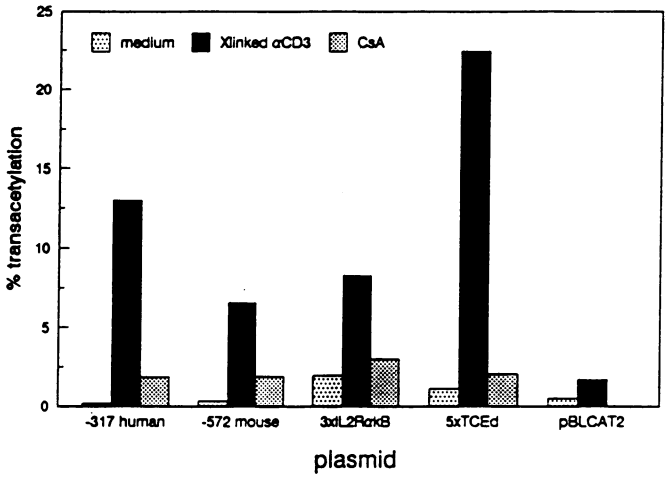

Figure 4. The effect of CsA on induction of IL2Rα promoter-CAT plasmids and on multimers of the NF-κB sites from the IL2Rα and IL2 promoters. Ar-5 cells were transfected with the indicated plasmids and stimulated as described in Methods. CsA (1 μM) was added to the indicated cultures 30 minutes before the anti-CD3ε antibody. The plasmids used were the −317 human IL2RαCAT plasmid (−317 human), a murine IL2RαCAT plasmid (−572 mouse), a trimer of the human IL2Rα κB site in pBLCAT2 (3×IL2RακB), a pentamer of the human IL2 κB site in pBLCAT2 (5×TCEd), and pBLCAT2 with no additional promoter sequences (pBLCAT2). The results shown are representative of at least 2 independent transfections for each plasmid.

and in PMA-stimulated human PBL [12]. In particular, the −271 plasmid was induced by anti-CD3ε stimulation (albeit less effectively than the −317 plasmid), while the −248 plasmid was inactive (Figure 2a), suggesting that sequences 3′ of −271 were necessary for activation in Ar-5 cells. Moreover the −317 plasmid was consistently more strongly induced by anti-CD3ε stimulation than the −471 plasmid (Figure 2a), indicating that the negative regulatory element postulated to exist between −471 and −317 [43] was also functional in Ar-5 cells. Interestingly, none of the plasmids was induced by IL2 (Figure 2a and see below).

There are significant differences (16/47 bp) between the murine and human IL2Rα promoter sequences in the region thought to be important for induction of the human 5′IL2Rα-CAT plasmid ([33]; see Figure 3a). Therefore, we also tested a 5′IL2Rα-CAT plasmid containing 572 bp of the murine IL2Rα promoter linked to the CAT gene for induction by crosslinked anti-CD3ε or IL2 in transfected Ar-5 cells. The average induction of the −572 murine 5′IL2Rα-CAT plasmid in five experiments was 25-fold in response to crosslinked anti-CD3ε, but only 1.5-fold in response to IL2 (Figure 2B). For comparison, the average induction of the −317 human 5′IL2Rα-CAT plasmid was 56-fold in response to crosslinked anti-CD3ε, but only 2-fold in response to IL2 (9 experiments, Figure 2B). These results indicate that TCR ligands and IL2, which both cause transcriptional activation of the IL2Rα gene [3,4,40], use different promoter regions and intracellular mechanisms for their effect.

The NF-κB site at −256 to −267in the human IL-2Rα promoter has been shown to be required for IL2Rα induction in Jurkat cells [20,44−46]. The murine IL-2Rα promoter contains a potential NF-κB binding site at −188 to −197 in a region of sequence similarity between the human and murine promoters. Due to the deletion of a GG dinucleotide, the NF-κB site in the murine IL-2Rα promoter is oriented in the opposite direction from that of the human promoter (Figure 3a). We used site-directed mutations in the −317 human 5′IL2Rα-CAT plasmid and the −572 murine IL-2Rα-CAT plasmid to determine whether these sites were functional in Ar-5 T cells. Ar-5 cells were transfected with 5′IL2Rα-CAT plasmids containing site-specific mutations in each of the above elements and stimulated with anti-CD3e crosslinked using TA3 cells or immobilized second antibody (Figure 3b). In agreement with results using JURKAT cells and human PBL [12,13,45], we observed greatly reduced induction of the human promoter containing a mutation in the 5′ GGG of the NF-κB site that abrogates binding of NF-κB proteins (M3, Figure 3b). M4, which contains a mutation outside of the NF-κB site, did not show a decrease in induction of the promoter, and in fact showed a slight increase in inducibility. In the case of the murine promoter, two mutations (M1 and M2) within the putative NF-κB site decreased induction of the −572 plasmid (Figure 3b), suggesting that both the 5′GG-AA and CCCC3′ sequences in this site are important for inducibility, consistent with this site functioning as an NF-κB site. A mutation outside of the putative NF-κB site (M3) did not show a significant decrease in inducibility. These results indicate that the NF-κB site is required for full induction of the human or murine IL2Rα promoter in T cells stimulated through the T-CR/CD3 complex.

Induction of IL2Rα promoter or multimerized NF-kB sites is sensitive to CsA in untransformed murine T cells

Since neither the −317 human 5′IL2Rα-CAT plasmid nor the −572 murine 5′IL2Rα-CAT plasmid was induced by IL2, we were able to test whether their induction by TCR ligands was sensitive to CsA. Cyclosporin A (1 mM) considerably diminished, but did not abolish, induction of either plasmid by anti-CD3ε, crosslinked using either TA3 cells (not shown) or immobilised second antibody (Figure 4). Induction of the −317 human 5′IL2Rα-CAT plasmid in CsA-treated, stimulated T cells was 11−42% of its induction in untreated, stimulated cells (range of 4 experiments); the corresponding value for induction of the −572 murine 5′IL2Rα-CAT plasmid was 25−35% (range of 2 experiments).

Our results with mutated 5′IL2Rα-CAT plasmids (Figure 3b) indicated that the NF-kB site of the human and murine promoters was a major site required for induction of the IL2Rα promoter in Ar-5 cells. To determine whether the isolated IL2Rα NF-κB site was sensitive to CsA, we constructed a pBLCAT-based plasmid containing three copies of the human IL2Rα κB site cloned upstream of the minimal thymidine kinase promoter (see Materials and Methods). This plasmid (3×IL2RακB) was induced by activation of Ar-5 cells with crosslinked anti-CD3ε, and its induction was significantly sensitive to CsA (Figure 4). In 3 experiments, the average induction of the 3×IL2RακB plasmid was 5.3-fold, and induction in the presence of 1 mM CsA was 37% of induction in the absence of CsA.

These results with the 3×IL2RακB plasmid were surprising in light of the reported insensitivity to CsA of a plasmid containing 5 copies of the NF-κB site from the IL2 promoter (5×TCEd, [47,48]). Since this plasmid was tested in EL4 cells, it was possible that factors constitutively present in transformed EL4 cells, but inducible in a CsA-sensitive manner in untransformed T cells, accounted for the difference. We therefore tested the 5×TCEd plasmid in Ar-5 cells for induction and CsA sensitivity. In contrast to results in EL4 cells, we found that the 5×TCEd

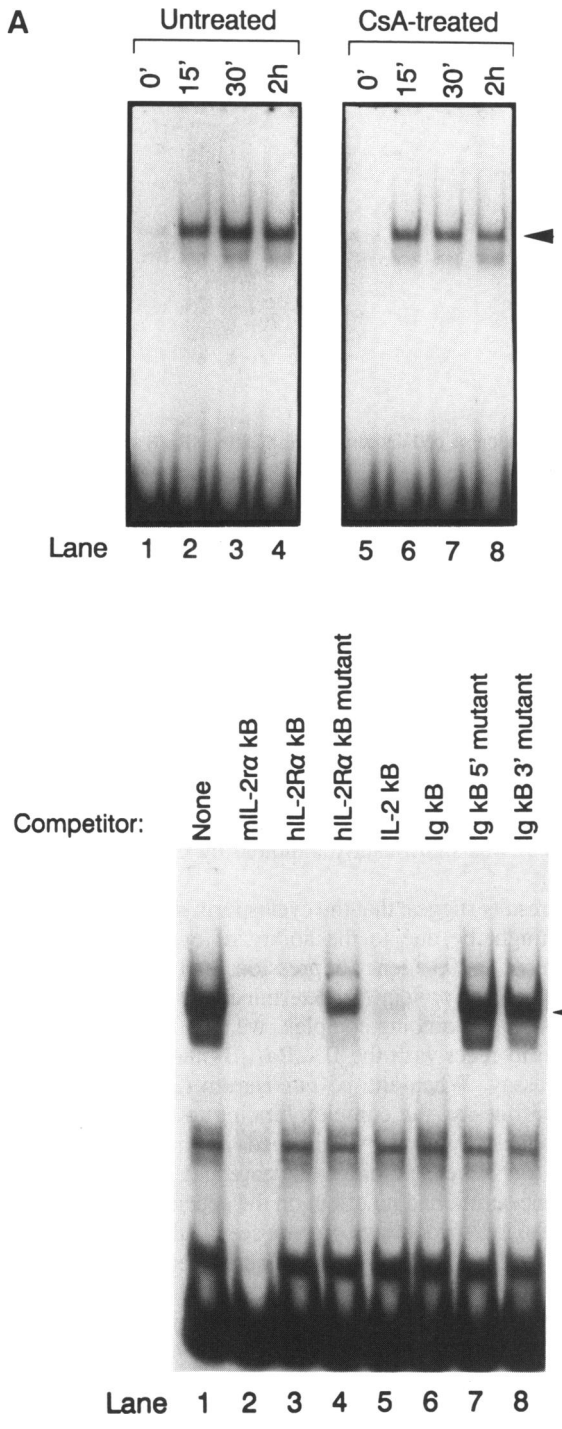

Figure 5. Gel shift analysis of inducible factors binding to the IL2Rα κB site. **A**, Nuclear extracts were prepared from Ar-5 cells that had been activated for the indicated times with crosslinked anti-CD3ε in the absence (lanes 1–4) or presence (lanes 5–8) of 1 μM CsA. For lanes 1 and 2 ('0 min') cells were incubated with CsA for 40 min in the absence of any stimulus. Nuclear proteins (5 μg per lane) were analysed by mobility shift assay for binding to an oligonucleotide comprising the human IL2Rα κB site. The arrow indicates the mobility of the major inducible complex observed under these conditions. **B**, Nuclear proteins from Ar-5 cells activated for 2 h with crosslinked anti-CD3ε were analysed for binding to the murine IL2Rα κB oligonucleotide alone (lane 1) or in the presence of 20 ng of unlabeled competitor oligonucleotide containing the murine (lane 2) or human (lane 3) IL-2Rα κB site (lane 2), a mutated form of the human IL2Rα κB site (lane 4), the IL2 NF-κB site (lane 5), the κ enhancer κB site (lane 6) or mutated forms of the κ enhancer κB site (lanes 7 and 8).

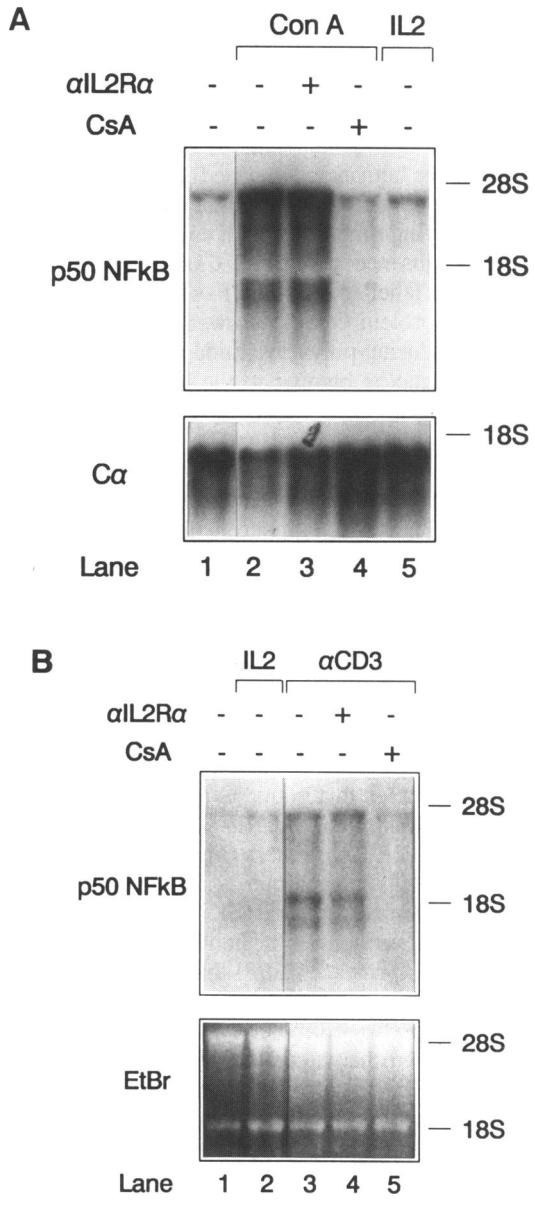

Figure 6. The increase in NF-κB p105 mRNA levels upon T cell activation is inhibited by CsA. **A**, RNA from Ar-5 cells was analysed for NF-κB p105 mRNA by Northern blotting analysis with a p105 cDNA probe (upper panel, p50 NF-κB). The filter shown is the same as that shown in Figure 1B, and contains RNA from unstimulated Ar-5 cells (lane 1), cells activated with Con A (lanes 2–5; inhibitors as indicated), or cells activated with 10 U/ml IL2 (lane 5) for 6 hours. An autoradiograph of the same blot after probing with a murine T cell receptor a constant region cDNA is shown to compare RNA loading (lower panel). **B**, RNA from splenic T cells was analysed for NF-κB p105 mRNA by Northern blotting analysis with a p105 cDNA probe (upper panel, p50 NFκB). The filter shown is the same as that shown in Figure 1C, and contains RNA from unstimulated cells (lane 1), cells stimulated with IL-2 (lane 2), or cells stimulated with anti-CD3ε (lanes 3–5; inhibitors as indicated) for 6 hours. Equivalent loading of RNA in each lane was verified by ethidium bromide staining (lower panel).

plasmid was highly inducible and almost completely CsA-sensitive in Ar-5 cells (Figure 4). In 2 experiments, the average induction of the 5×TCEd plasmid was 22-fold, and induction in the presence of 1 mM CsA was 8% of induction in the absence of CsA.

Gel-shift analysis of inducible factors binding to the IL2Rα κB site

To determine whether an inducible, cyclosporin-sensitive factor bound to the NF-κB site of the IL2Rα promoter, we performed gel retardation experiments with labelled oligonucleotides corresponding to the human and murine IL2Rα κB sites. Nuclear extracts were prepared from Ar-5 cells, either unstimulated or stimulated for varying times with with crosslinked anti-CD3ε in the presence or absence of 1 mM CsA. The extracts were incubated with the labelled IL2Rα NF-κB site oligonucleotide, and bound DNA-protein complexes were separated from free probe by non-denaturing polyacrylamide gel electrophoresis. A nuclear protein complex binding to the human IL2Rα NF-κB oligonucleotide was induced within 15 minutes in stimulated T cells (Figure 5a, compare lanes 1 and 2), and persisted until at least 2 h after stimulation (lanes 3 and 4). CsA treatment had no effect on induction of the complex at early times (15 min) after stimulation (Figure 5a, compare lanes 2,6) but diminished appearance of the complex at later times (compare lanes 3,7; 4,8). Identical results were obtained whether the NF-κB site from the human or murine promoter was used as the probe. In 4 independent experiments, induction of NF-κB binding factor after two hours was reduced by cyclosporin treatment to 50 to 75% of that observed in the absence of CsA. Similar results were obtained when protein synthesis inhibitors were used (data not shown).

The inducible complex that bound to the murine IL-2R4α κB site was efficiently competed by NF-κB sites from either the murine or human IL-2Rα promoters (Figure 5b, lanes 2 and 3), from the human IL-2 promoter (lane 5) or from the immunoglobulin κ enhancer (lane 6). The complex was not competed by mutated versions of the human IL-2Rα κB site (lane 4) or the κ enhancer (lanes 7 and 8) that had been changed in bases critical for NF-κB binding. Identical results were obtained when the human IL-2Rα promoter NF-κb site was used as a probe. These results further suggested that the kB-binding factor was not related to NFAT, since The human IL2Rα κB site has been shown to bind several rel family heterodimers from nuclear extracts of activated T cells, including p65 homodimers and p50/p65 or p50/c-rel heterodimers [18]. Our results suggest that α similar factor or factors also binds to the murine IL-2Rα kB site.

CsA-sensitive induction of mRNA for the p105 precursor of p50 NFκB in activated T cells

It was possible that CsA inhibited induction of NF-κB, and of the 3×IL2Rακ B and 5×TCEd plasmids, by inhibiting induction of mRNAs encoding one or more components of NF-κB. To test this hypothesis directly, we prepared cytoplasmic mRNA from Ar-5 cells induced in the presence and absence of CsA. Hybridisation of Northern blots with a labelled p65 probe [49] indicated that p65 mRNA was constitutively expressed in Ar-5 cells, was not significantly induced by TCR ligands, and was unaffected by CsA (data not shown). In contrast, mRNA for the p105 precursor of p50 NF-κB [50] was significantly induced in Ar-5 cells over the background present in resting cells (Figure 6a, compare lanes 1 and 2). Induction did not occur in response to IL2 (lane 5) and TCR/CD3-mediated induction was not inhibited by anti-IL2R antibodies (lane 3); however it was completely inhibited by CsA (lane 4; ~90% decrease). Similar results were obtained using short-term splenic T cell cultures

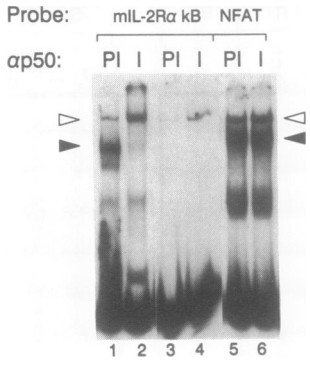

Figure 7. Reaction of p50 antiserum with IL2Rα NF-κB binding complexes. Nuclear extracts from Ar-5 cells that had been stimulated for 2 hours with anti-CD3ε antibodies were incubated with pre-immune serum (lanes 1,5) or immune serum against p50 (lanes 2,6) and then analyzed for binding to the murine IL-2Rα κB oligonucleotide (lanes 1,2) or to an oligonucletide comprising the distal NFAT site from the murine IL-2 promoter (lanes 5,6). The closed arrow indicates the specfic NF-κB complex, while the open arrow indicates the supershifted complex, which has a similar mobility to a non-specific complex detected when serum alone is incubated with oligonucleotide probe (lanes 1,2).

(Figure 6b): again p50 mRNA was induced by anti-CD3ε (compare lanes 1 and 3) but not by IL2 (lane 2). Induction in response to anti-CD3ε was not inhibited by anti-IL2R antibodies (lane 4) but was significantly inhibited by CsA (lane 5; ~75% decrease).

These results suggest that the cyclosporin sensitivity of the NF-κB site might be due to the ability of cyclosporin to inhibit induction of p105 gene expression and ultimately inhibit production of p50 protein. To determine whether p50 was present in the IL-2Ra κB-binding complex, we tested the ability of a p50 antiserum to react with the IL-2Ra κB-binding proteins in the gel shift assay. When anti-p50 antiserum (38) was included in the gel shift assay, the complex binding to the murine IL-2Rα κB oligonucleotide was diminished in intensity and a new complex of lower mobility appeared (Figure 7, lane 2). Preimmune serum had no effect on the specific NF-κB complex (lane 1); the lower mobility complex seen with preimmune serum results from non-specific DNA-binding proteins present in serum (lanes 3,4). The antisera were specific for the p50 protein since they had no effect on complexes formed using the same extracts and an NFAT oligonucleotide (lanes 5 and 6). The ability of anti-p50 antisera to supershift the specific protein-DNA complex completely suggests that the majority of the IL-2Rα kB-binding complexes contain p50. Thus, it is likely that some or all of the reduction in binding activity and transcriptional activity of the NF-κB site caused by cyclosporin is due to the inhibition of *de novo* p50 production.

DISCUSSION

We have shown that CsA inhibits induction of IL2Rα mRNA in untransformed murine T cells stimulated through the TCR/CD3 complex. Moreover we have confirmed that the NF-κB site of the murine IL2Rα promoter is required for IL2Rα gene induction in these cells. Induction of a reporter plasmid containing a multimer of the IL2Rα NF-κB site is also sensitive to CsA,

implicating the NF-κB site of the IL2Rα promoter as a target for CsA action.

The T cells we have used are maintained in IL-2 and thus express low resting levels of IL2Rα mRNA and protein. Gel retardation assays using an oligonucleotide for the IL2Rα κB site indicate that these cells also express low resting levels of nuclear NF-κB, which are rapidly increased (15 min) following TCR/CD3 stimulation. In gel shift assays, p50 NF-κB can be detected as a major component of the protein complex that binds to the IL-2Rα NF-κB site. The early phase of induction of the p50-containing NF-κB complex is insensitive to CsA and protein synthesis inhibitors and is likely to represent activation of preexisting NF-κB. At later times (2−6 h), mRNA for the p105 precursor of the p50 subunit of NF-κB is induced in stimulated T cells, suggesting the existence of a second phase of NF-κB induction involving *de novo* synthesis of p105 mRNA and protein. Since CsA inhibits the induction of p105 mRNA, this second phase of NF-κB induction would be expected to be inhibited by protein synthesis inhibitors and by CsA. Indeed, gel retardation assays show partial inhibition of NF-κB induction by protein synthesis inhibitors and by CsA at later times after activation (1−4 h). A comparable two-phase induction of NF-κB after TNFα stimulation of HL-60 cells has been reported [51]. We propose that while the first phase of NF-κB activation may initiate the increase in IL2Rα gene transcription in activated T cells, the second phase may be required for sustained expression of IL2Rα mRNA and protein. Thus the sensitivity of IL2Rα mRNA induction to CsA and to protein synthesis inhibitors may reflect, at least in part, the CsA sensitivity of p105 mRNA induction and the requirement for *de novo* synthesis of p105 protein respectively.

We have observed a difference in the CsA sensitivity of IL2Rα mRNA induction between human and murine cells: while induction of IL2Rα mRNA in murine Ar-5 cells is highly CsA-sensitive, transcription of the IL2Rα gene and induction of IL2Rα mRNA in PHA/PMA-stimulated human PBL is completely insensitive to CsA or FK506 [4], as is induction of the human 5′IL2Rα-CAT plasmid in PMA/ionomycin-stimulated JURKAT cells [52]. In contrast, induction of NF-κB p105 mRNA is inhibited by CsA in both murine and human T cells [this report and ref. 53], and transcriptional activation of some NF-κB sites has been shown to be sensitive to CsA in Jurkat cells [54,55]. It is possible that the inability of CsA to inhibit IL2Rα induction in human cells results from the participation of additional transcription factors other than NF-κB in promoter induction. At least four elements in the promoter are required for IL2Rα gene induction in JURKAT cells [20,44−46]. These are an upstream element (UE-1 or NF-IL2RA) located just 5′ of the NF-κB site [20,44], the NF-κB site itself, a serum response element or CArG site [56] located just 3′ of the NF-κB site, and an Sp1 site partially overlapping the 3′ end of the CArG site [45,46]. When murine Ar-5 T cells were transfected with −317 human IL2Rα-CAT plasmids containing site-specific mutations in each of the above elements, only mutations in the NF-κB and the Sp1 sites showed diminished induction (P.G. McCaffrey, unpublished data). The murine promoter contains several sequence changes compared to the human promoter in the region around the NF-κB site, and the loss of the UE-1 and CArG sites in the murine promoter may be associated with a concomitant loss of factors capable of binding to these sites in murine cells. The cyclosporin sensitivity of both the human and murine promoters in murine cells may therefore indicate that the NF-κB site plays a relatively more important role in promoter induction in murine T cells than in human T cells.

We have previously shown that the CsA-sensitive transcription factor NFAT can bind to certain NF-κB sites [57]. However, the CsA sensitivity of IL2Rα mRNA induction does not appear to be due to interaction of the IL2Rα κB site with a CsA-sensitive factor related to NFAT. In repeated experiments performed under a variety of conditions, we have been unable to demonstrate binding of NFAT or related factors to either the murine or human IL2 or IL2Rα κB sites. Rather the complexes binding to both sites appear similar to p50/p65 NFκB, as judged by their mobility and, in the case of the IL-2Rα κB site, reactivity with antisera to p50. We propose that the CsA sensitivity of both the 3×IL2Rακ B and the 5×TCEd plasmids can be explained by the CsA sensitivity of p50 mRNA induction. At present we have no information on whether related p50-containing complexes, such as p50/c-rel, that bind to the IL2Rα κB sites [18] contain other rel family members that are induced in a cyclosporin-sensitive manner.

The mechanism by which CsA inhibits p105 mRNA induction remains to be determined. Since only steady-state levels of p105 mRNA were measured in this work, CsA could either inhibit transcription of the p105 gene or potentiate the degradation of p105 mRNA. The former possibility appears more likely given that CsA is known to inhibit lymphokine gene transcription [4,7]. By comparison with the NFAT sites in the IL-2 promoter [32], the regulatory region of the p105 gene [58] does not contain any obvious binding sites for the cyclosporin-sensitive transcription factor NFAT. The elucidation of the elements in the p105 promoter that are involved in regulation of gene expression in activated T cells will be necessary to determine the basis for the cyclosporin sensitivity of p105 mRNA induction.

ACKNOWLEDGEMENTS

We thank Dr. Warner Greene for promoter deletion mutants and site-specific mutants of human 5′IL2Rα-CAT plasmids, Dr. E. Serfling for the 5×TCEd plasmid, Dr. S. Ghosh for cDNA probes for the p50 and p65 subunits of NF-κB, Dr. Michael J. Leonardo for anti-p50 antisera, and Dr. Anne Goldfeld for confirming the biological activity of our commercial preparation of TNFα. This work was supported by NIH grant CA42471 (to A.R.). and an institutional Biomedical Research Support Grant (to P.G.M.). P.G.M. is the recipient of a Leukemia Society Special Fellow Award. V.V.-A. was supported by a USDA training grant in Biotechnology (84-GRAD-9-0018).

REFERENCES

1. Ullman, K.S., Northrop, J.P., Verweij, C.L., Crabtree, G.R. (1990) *Ann. Rev. Immunol.*, 8, 421−452.
2. Smith, K.A. (1988) *Science*, 240, 1169−1176.
3. Kronke, M., Leonard, W.J., Depper, J.M., and Greene, W.C. (1985) *J. Exp. Med.*, 161, 1593−1598.
4. Tocci, M.J., Matkovich, D.A., Collier, K.A., Kwok, P., Dumont, F., Lin, S., Degudicibus, S., Siekierka, J.J., Chin, J., and Hutchinson, N.I. (1989) *J. Immunol.*, 143, 718−726.
5. Shaw, J.-P., Utz, P.J., Durand, D.B., Toole, J.J., Emmel, E.A., and Crabtree, G.R. (1988) *Science*, 241, 202−205.
6. Hoyos, B., Ballard, D.W., Bohnlein, E., Siekevitz, M., and Greene, W.C. (1989) *Science*, 244, 457−460.
7. Kronke, M., Leonard, W.J., Depper, J.M., Arya, S.K., Wong-Staal, F., Gallo, R.C., Waldmann, T.A., and Greene, W.C. (1984) *Proc.Natl.Acad.Sci. USA*, 81, 5214−5218.

8. Mattila, P.S., Ullman, K.S., Fiering, S., Emmel, E.A., McCutcheon, M., Crabtree, G.R., and Herzenberg, L.A. (1990) *EMBO J.*, 9, 4425–4433.
9. Bohnlein, E., Ballard, D.W., Bogerd, H., Peffer, N.J., Lowenthal, J.W., and Greene, W.C. (1989) *J. Biol. Chem.*, 264, 8475–8478.
10. Ruben, S., Poteat, H., Tan, T.-H., Kawakami, K., Roeder, R., Haseltine W., and Rosen, C.A. (1988) *Science*, 241, 89–92.
11. Ballard, D.W., Bohnlein, E., Lowenthal, J.W., Wano, Y., Franza, B.R., and Greene, W.C. (1988) *Science*, 241, 1652–1655.
12. Lowenthal, J.W., Ballard, D.W., Bohnlein, E., and Greene, W.C. (1989) *Proc. Natl. Acad. Sci. USA*, 86, 2331–2335.
13. Lowenthal, J.W., Ballard, D.W., Bogerd, H., Bohnlein, E., and Greene, W.C. (1989) *J. Immunol.*, 142, 3121–3128.
14. Freimuth, W.W., Depper, J.M., and Nabel, G.J. (1989) *J. Immunol.*, 143, 3064–3068.
15. Liou, H.-C. and Baltimore, D. (1993) *Current Opinion in Cell Biology*, 5, 477–487.
16. Ghosh, S., and Baltimore, D. (1990) *Nature*, 344, 678–682.
17. Baeuerle, P.A., and Baltimore, D. (1988) *Science*, 242, 540–546.
18. Molitor, J.A., Walker, W.H., Doerre, S., Ballard, D.W., Greene, W.C. (1990) *Proc. Natl. Acad. Sci. USA*, 87, 10028–10032.
19. Cross, S.L., Halden, N.F., Lenardo, M.J., and Leonard, W.J. (1989) *Science*, 244, 466–469.
20. Toledano, M.B., Roman, D.G., Halden, N.F., Lin, B.B., and Leonard, W.J. (1990) *Proc. Natl. Acad. Sci. USA*, 87, 1830–1834.
21. Rao, A., Faas, S.J., and Cantor, H. (1984) *J. Exp. Med.*, 159, 479–494.
22. Valge, V.E., Wong, J.G.P., Datlof, B.M., Sinskey, A.J., and Rao, A. (1988) *Cell*, 55, 101–112.
23. Wong, J.G.P., and Rao, A. (1990) *J. Biol. Chem.*, 265, 4685–4693.
24. Jamieson, C., McCaffrey, P.G., Rao, A., and Sen, R. (1991) *J. Immunol.*, 147, 416–420.
25. Glimcher, L.H., Hamano,T., Asofsky, R., Sachs, D.H., Pierres, M., Samelson, L.E., Sharrow, S.O., and Paul, W.E. (1983) *J. Immunol.*, 130, 2287–2294.
26. Leo, O., Foo, M., Sachs, D.H., Samelson, L.E., and Bluestone, J.A. (1987) *Proc. Natl. Acad. Sci. USA*, 84, 1374–1378.
27. Malek, T.R., Robb, R.J., and Shevach, E.M. (1983) *Proc. Natl. Acad. Sci. USA*, 80, 5694–5698.
28. Ceredig, R., Lowenthal, J.W., Nabholz, M., and MacDonald, H.R. (1985) *Nature*, 314, 98–100.
29. Valge-Archer, V.E., de Villiers, J., Sinskey, A.J., and Rao, A. (1990) *J. Immunol.*, 145, 4355–4364.
30. Valge-Archer, V.E. (1991) *Gene regulation in T cell activation*. Ph.D. thesis, Massachusetts Institute of Technology.
31. Luckow, B., and Schutz, G. (1987) *Nucl. Acids Res.*, 15, 5490–5490.
32. Serfling, E., Barthelmas, R., Pfeuffer, I., Schenk, B., Zarius, S., Swoboda, R., Mercurio, F., and Karin, M. (1989) *EMBO J.*, 8, 465–473.
33. Kanamori, H., Ishida, N., Shimizu, A., Yaoita, Y., Nikaido, T. and Honjo. T. (1987) *J. Biol. Chem.*, 262, 5079–5086.
34. Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I. and Howard, B. (1982) *Proc. Natl. Acad. Sci. USA*, 79, 6777–6781.
35. Kim, P.K. (1991) *Positive regulator sites in the promoter for the murine Interleukin-2 receptor a gene*. Undergraduate senior thesis, Harvard University.
36. Jain, J., Valge-Archer, V.E., Sinskey, A.J. and Rao, A. (1992) *J. Exp. Med.*, 175, 853–862.
37. Bradford, M. (1976) *Anal. Biochem.*, 72, 248–254.
38. Kang, S.-M., Tran, A.-C., Grilli, M. and Lenardo, M.J. (1992) *Science*, 256, 1452–1456.
39. Sen, R., and Baltimore, D. (1986). *Cell*, 46, 705–716.
40. Depper, J.M., Leonard, W.J., Drogula, C., Kronke, M., Waldmann, T.A., and Greene, W.C. (1985) *Proc. Natl. Acad. Sci. USA*, 82, 4230–4234.
41. Goldfeld, A.E., McCaffrey, P.G., Strominger, J.L., and Rao, A. (1993) *J. Exp. Med.*, 178, 1365–1379.
42. Lowenthal, J.W., Bohnlein, E., Ballard, D.W., and Greene, W.C. (1988) *Proc. Natl. Acad. Sci. USA*, 85, 4468–4472.
43. Smith, M.R., and Greene, W.C. (1989) *Proc. Natl. Acad. Sci. USA*, 86, 8526–8530.
44. Ballard, D.W., Bohnlein, E., Hoffman, J.A., Bogerd, H.P., Dixon, E.P., Franza, B.R., and Greene, W.C. (1989) *New Biol.* 1, 83–92.
45. Pomerantz, J.L., Mauxion, F., Yoshida, M., Greene, W.C., and Sen, R. (1989) *J. Immunol.*, 143, 4275–4281.
46. Roman, D.G., Toledano, M.B. and Leonard, W.J. (1990) *New Biol.*, 2, 642–647.
47. Randak, C., Brabletz, T., Hergenrother, H., Sobotta, I., and Serfling, E. (1990) *EMBO J.*, 9, 2529–2536.
48. Brabletz, T., Pietrowski, I., and Serfling, E. (1991) *Nucl. Acids Res.*, 19, 61–67.
49. Nolan, G.P., Ghosh, S., Liou, H.-C., Tempst, P., and Baltimore, D. (1991) *Cell*, 64, 961–969.
50. Ghosh, S., Gifford, A.M., Riviere, L.R., Tempst, P., Nolan, G.P., and Baltimore, D. (1990) *Cell*, 62, 1019–1029.
51. Hohmann, H.-P., Remy, R., Scheidereit, C., and van Loon, A.P.G.M. (1991) *Mol. Cell. Biol.*, 11, 259–266.
52. Banerji, S.S., Parsons, J.N., and Tocci, M.J. (1991) *Mol. Cell. Biol.*, 11, 4074–4087.
53. Bours, V., Villalobos, J., Burd, P.R., Kelly, K., and Siebenlist, U. (1990) *Nature*, 348, 76–80.
54. Emmel, E.A., Verweij, C.L., Durand, D.B., Higgins, K.M., Lacy, E., and Crabtree, G.R. (1989) *Science*, 246, 1617–1620.
55. Schmidt, A., Hennighausen, L., and Siebenlist, U. (1990) *J. Virol.*, 64, 4037–4041.
56. Kuang, A.A., Novak, K.D., Kang, S.-M., Bruhn, K., and Lenardo, M.J. (1993) *Mol. Cell. Biol.*, 13, 2536–2545.
57. McCaffrey, P.G., Jain, J., Jamieson, C., Sen, R., and Rao, A. (1992) *J. Biol. Chem.*, 267, 1864–1871.
58. Ten, R.M., Paya, C.V., Israel, N., LeBail, O., Mattei, M.-G., Virelizier, J.-L., Kourilsky, P., and Israel, A. (1992) *EMBO J.*, 11, 195–203.