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In vivo regulation of interleukin-2 receptor α gene transcription by the coordinated binding of constitutive and inducible factors in human primary T cells

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IL-2Rα transcription is developmentally restricted to T cells and physiologically dependent on specific stimuli such as antigen recognition. To analyse the mechanisms used to activate IL-2Rα transcription as well as those used to block it in non-expressing cells, we determined the protein–DNA interactions at the IL-2Rα locus in three different cell types using the DMS/LMPCR genomic footprinting method. CD25/IL-2Rα can be efficiently induced in primary human T cells since ~100% express this gene when receiving an appropriate combination of mitogenic stimuli. To understand why IL-2Rα is not expressed in other haematopoietic cell types, we analysed BJAB B lymphoma cells which do not express the IL-2Rα gene and contain constitutively active nuclear NF-κB. Primary fibroblasts from embryo and adult skin were selected to examine the mechanisms that may be used to keep the IL-2Rα gene inactive in non-haematopoietic cells. The three main results are: (i) the stable *in vivo* occupancy of IL-2Rα κB element in resting T cells, most probably by constitutive NF-κB p50 homodimer that could impair SRF binding to the flanking SRE/CArG box; (ii) its inducible occupancy by NF-κB p50–p65 associated with the binding of an SRE/CArG box DNA-binding factor upon mitogenic stimulation; and (iii) a correlation between the precommitment of T cells to activation and the presence of stable preassembled protein–DNA complexes in contrast with the bare IL-2Rα locus in non-T cells.

Keywords: IL-2Rα/NF-κB/primary T cells/SRF/transcription

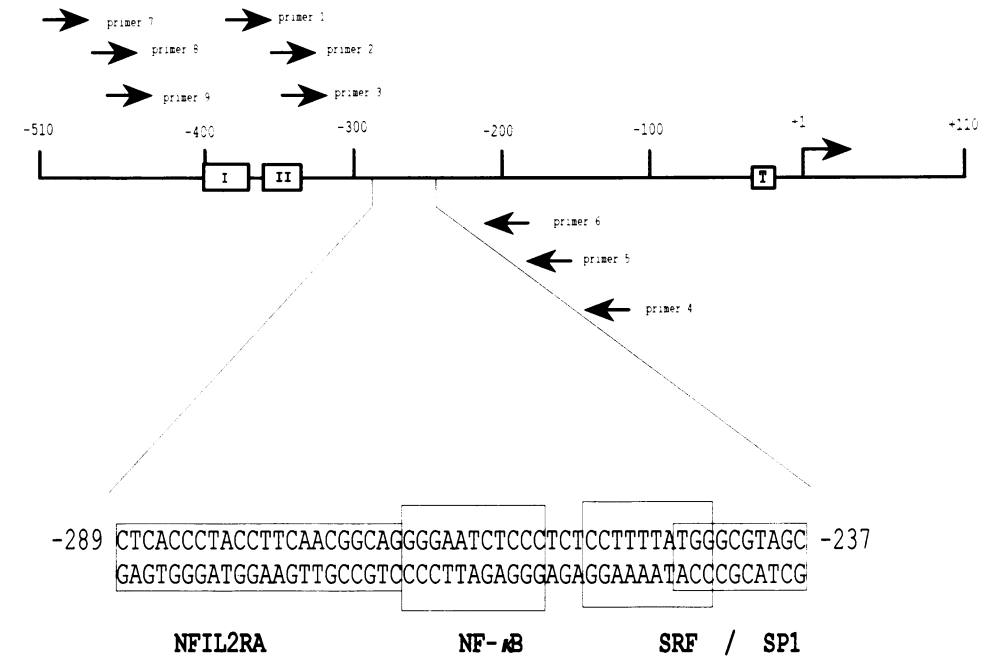
Introduction

The interaction between interleukin 2 (IL-2) and its receptor (IL-2R) plays a major role in peripheral blood T lymphocyte activation (reviewed in Taniguchi and Minami, 1993). Upon mitogenic stimulation, the expression of the IL-2Rα gene encoding the alpha chain of IL-2 receptor (CD25/IL-2Rα) allows the formation of high-affinity receptors for IL-2 through the association of the α chain with at least two distinct polypeptide chains constitutively expressed at the surface of resting T cells, respectively designated β and γ (reviewed in Minami *et al.*, 1993). In addition to its signal-dependent transcriptional control, the IL-2Rα gene is under a strict cell type-specific regulation (reviewed in Leonard, 1992). Thus, expression of IL-2Rα is mainly restricted to the T-cell lineage and its commitment is likely to emerge during T-cell ontogeny (reviewed in Leonard, 1992; Rothenberg, 1992).

Previous studies have shown that IL-2Rα expression is at least in part regulated by an enhancer region located between nucleotide positions –289 and –237 relative to the major transcription initiation site which can drive expression in a stimulation dependent, T cell-restricted fashion (reviewed in Muegge and Durum, 1989; Leonard, 1992). In contrast to the IL-2 minimal enhancer/promoter composed of multiple regulatory elements (Ullman *et al.*, 1990), the IL-2Rα promoter contains only four positive regulatory elements among which, in T cells, a κB motif is the only binding site characterized to date for inducible nuclear factors related to the Rel/NF-κB family (Figure 1A). The κB and SRE/CArG box regulatory elements are apparently required for activation by both TAX (transcription activator protein of HTLV-1) and mitogenic stimulation (Bohnlein *et al.*, 1988; Leung and Nabel, 1988; Ruben *et al.*, 1988; Ballard *et al.*, 1989; Cross *et al.*, 1989; Lowenthal *et al.*, 1989b; Toledano *et al.*, 1990). We have previously shown that the inhibition of both NF-κB nuclear activation (Costello *et al.*, 1993b) and NFKB1 gene induction (Costello *et al.*, 1993a) was directly correlated with a block of the proliferative response of primary T cells stimulated via CD2+CD28. However, because of its ubiquitous expression, NF-κB cannot account alone for the strict IL-2Rα gene transcription regulation (Baeuerle and Henkel, 1994). Cells that are not transcribing IL-2Rα gene might have steadily bound repressors at the IL-2Rα locus or partially assembled enhancer complexes. Alternatively, developmentally committed resting naive T cells might display a pattern of protein–DNA interactions different from that of cells incompetent for IL-2Rα expression. Hence, the study of IL-2Rα transcriptional control might permit discrimination between regulatory factors involved in the developmental restriction that blocks its expression and those responsible for its induction. In this respect, IL-2Rα differs from developmentally restrained genes which are temporally and physiologically coupled to cell differentiation. The regulation of IL-2Rα raises two main questions: (i) is the developmental commitment of T cells reflected in molecular terms by stable interactions between available factors and the IL-2Rα enhancer putative regulatory elements? and (ii) what molecular mechanism supports the functional cooperation between IL-2Rα regulatory factors (DNA-binding, factor activity or a combination of the two)?

Most studies of nuclear *trans*-acting factors involved in the regulation of IL-2Rα gene transcription have used tumour T-cell models such as the human leukaemia T-cell line Jurkat or the chemically-induced murine thymoma

A



B

Primers		Sequences	Tm
1	-370	TGACTCCTGAGGACGTTA -353	60°C
2	-343	GGGGAGGACTCAGCTTATGAAGTGC -319	63°C
3	-333	CAGCTTATGAAGTGCTGGGTGAGACCACT -314	66°C
4	-118	GATGACATATAGTTTGCGG -136	56°C
5	-164	CAAGTTCAATTGCTGGAGGAGGTGTGGG -188	63°C
6	-179	GAGGTGTGGGCTGGGGTTGATGAG -203	68°C
7	-511	CAGGTTATAAGCCTTGGGG -493	60°C
8	-480	ATTCTCAGGATCCTTCAGTTCGCCG -456	63°C
9	-473	GGATCCTTCAGTTCGCCGCATCCTTCTCC -445	68°C
Common linker		5′-GCGGTGACCCGGGAGATCTGAATTC-3′ 3′-CTAGACTTAAG-5′	

Fig. 1. (**A**) Schematic representation of IL-2Rα promoter organization and partial nucleotide sequence. The positive regulatory elements previously characterized are indicated below the sequence. Boxes represent the location of NRE I site (I), NRE II site (II) and TATA box (T). The arrows indicate the approximate position of the primers used for ligation-mediated PCR genomic DNA amplification. (**B**) Nucleotide sequences and location of the primers used for ligation-mediated PCR amplification.

EL-4. Because the regulation of IL-2Rα promoter activity is uncoupled from cellular proliferation, the physiological significance of the observations is questionable. We focused on the analysis of the mechanism that controls cell type restriction and signal dependence of IL-2Rα transcription in primary human T cells purified from peripheral blood. Primary T-cell culture is a good model of an IL-2Rα-expressing cell type since ~100% can be induced to express CD25/IL-2Rα when receiving an appropriate combination of mitogenic stimuli such as CD28-specific monoclonal antibodies (mAbs) in association with either CD2 or CD3 mAbs (Cerdan *et al.*, 1992) that mimic several of the physiological aspects of T lymphocyte activation (reviewed in Olive *et al.*, 1994). High-level expression of IL-2Rα at the cell surface induced by CD2+CD28 co-stimulation is sustained by a potent stimulation of IL-2Rα gene transcription and mRNA stabilization (Cerdan *et al.*, 1992). In order to understand why IL-2Rα is not expressed in other haematopoietic cell types, we analysed BJAB human B lymphoma cells which do not express IL-2Rα gene (Harada and Yanagi, 1992) and, as mature B cells, express active NF-κB constitutively (Johansson *et al.*, 1990; Grilli *et al.*, 1993). Human primary fibroblasts from embryo and from adult skin were also selected to examine the mechanisms that may be used to keep IL-2Rα gene inactive in non-haematopoietic cells.

Using a high-resolution dimethylsulfate/ligation-mediated PCR (DMS/LMPCR) genomic footprinting method we have demonstrated the constitutive occupancy of the NF-κB element of IL-2Rα in unstimulated cells and a dramatic change within both κB and SRE sites upon stimulation of T cells to proliferate. Furthermore, the strong inducible modification of the NF-κB element as well as the flanking SRE was completely inhibited upon treatment by pyrrolidine dithiocarbamate (PDTC), the most specific NF-κB inhibitor available. Taken together, our results support the key role played by NF-κB in the activation of CD25/IL-2Rα gene in primary human T lymphocytes stimulated to proliferate. They provide evidence for the stable *in vivo* occupancy of a genomic κB element, most probably by constitutive NF-κB p50 homodimer, that could impair SRF binding to the flanking SRE/CArG box. Our data therefore strongly reinforce the hypothesis of transcription silencing by constitutive p50 homodimers replaced by activated nuclear NF-κB complexes upon stimulation (Kang *et al.*, 1992). Moreover, we observed a correlation between precommitment to activation and stable preassembled protein–DNA complexes in T cells in contrast with the bare IL-2Rα locus detected by *in vivo* footprinting in non-T cells.

Results

Identification of nuclear factors that bind sites of IL-2Rα promoter/enhancer in vitro in primary resting and stimulated T cells

The proximal IL-2Rα promoter/enhancer region contains four putative binding sites for nuclear factors that apparently regulate positively its transcription (NFIL2RA, NF-κB, SRF and SP1) as well as two negative regulatory elements (NRE I and II) (Figure 1A). The two negative regulatory elements, NRE I and NRE II, are located between nucleotides –400 and –368 (Smith and Greene, 1989) and between nucleotides –317 and –342 (Lowenthal *et al.*, 1989b), respectively. NRE I contains a 11 bp core element (TTCATCCCAGG) identical to a binding site present in the LTR of the human type-1 immunodeficiency virus (HIV-1) that binds *in vitro* to a 50 kDa protein (SP50) (Smith and Greene, 1989). Apparently these elements act as transcriptional silencers since their presence decreases significantly both basal and inducible IL-2Rα promoter activity. To determine the availability of the positively acting factors implicated in the control of IL-2Rα expression, nuclear extracts were prepared from the same primary T-cell populations used for LMPCR *in vivo* footprint assays and phenotypic controls. These extracts were tested to identify the factors able to bind specifically *in vitro* to the IL-2Rα regulatory elements by electrophoretic mobility shift assays (EMSAs) (Figure 2). Upstream element 1 [UE1 (–289,–269)] is the binding site for a poorly defined 56 kDa nuclear DNA-binding factor, NFIL2RA, apparently involved in basal transcription activity (Ballard *et al.*, 1989; Lowenthal *et al.*, 1989b; Toledano *et al.*, 1990). UE1 oligonucleotide probe was constitutively bound by factors present in almost constant amounts both in resting and in CD2+CD28-activated T cells (Figure 2A). As previously reported (Costello *et al.*, 1993b), the κB oligonucleotide probe was specifically bound by NF-κB p50 homodimers (Figure 2B, C complex) constitutively present

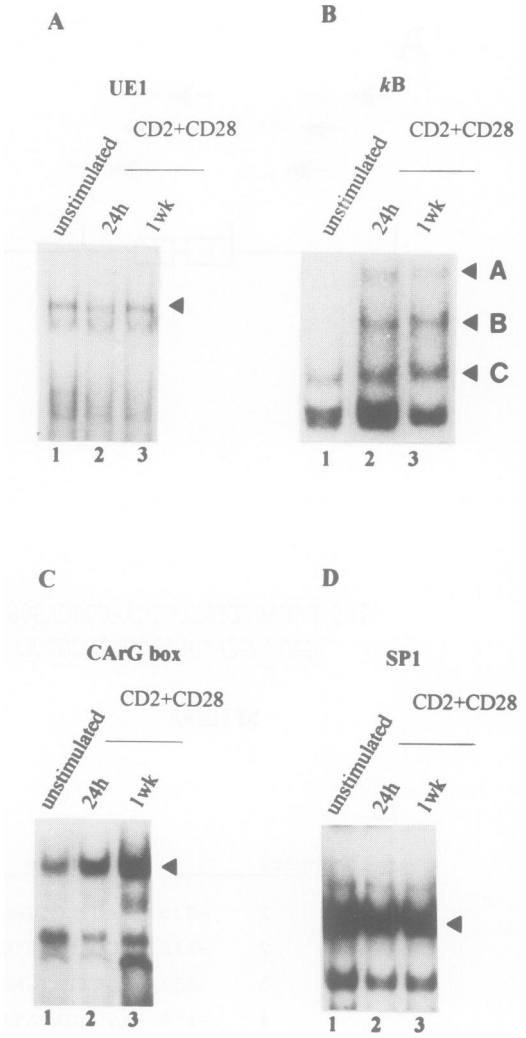


Fig. 2. *In vitro* binding activities on the individual positive regulatory elements of IL-2Rα promoter detected by electrophoretic mobility shift assays. Purified human T lymphocytes were stimulated by saturating concentrations of CD2 and CD28 mAbs. Cells were harvested at different times indicated at the top of the lanes. Unstimulated cells (lanes 1) were harvested at 24 h after cell seeding. Nuclear extracts were prepared as described in Materials and methods. (**A**) $UE1_{IL\text{-}2R\alpha}$, (**B**) $\kappa B_{IL\text{-}2R\alpha}$, (**C**) $SRE_{IL\text{-}2R\alpha}$ probe, (**D**) $SP1_{SV40}$. Arrowheads indicate the major and specific binding complexes for each probe. For specificity control, reaction mix incubations were performed in presence of 200-fold excess of their respective unlabelled oligonucleotide DNA (data not shown). The unmarked, constitutive complexes were identified as non-specific binding proteins since they were abolished by competition with non-specific oligonucleotides (data not shown).

in the nuclei of resting T cells and, after CD2+CD28 stimulation, by NF-κB p50–p65 heterodimers (Figure 2B, B complex) and probably c-Rel homodimers (Figure 2B, complex A). SRE/CArG box oligonucleotide probe was bound by a specific complex tentatively identified as SRF, which increased significantly when T cells are actively proliferating (Figure 2C). One striking feature of the IL-2Rα minimal enhancer/promoter is that the SRE/CArG (–253,–244) and SP1/GC (–246,–237) boxes overlap by three nucleotides (Figure 1A). An SV40-derived GC box was chosen as an SP1-specific oligonucleotide probe to avoid the formation of poorly analysable protein–DNA

complex patterns (Roman *et al.*, 1990). The SV40-derived SP1 oligonucleotide probe was specifically bound by an upper complex identified as SP1 (Figure 2D) since it co-migrated with the complex formed with recombinant SP1 and was displaced as a slower migrating complex by anti-SP1 antibody (data not shown). The amount of SP1 complex was apparently unaffected during CD2+CD28 stimulation. In parallel, specificity of the SRF complex was determined by competition with a molar excess of unlabelled oligonucleotides including a c-fos dyad symmetry element (c-*fos* SRE) which contains a CArG box and binds SRF with high affinity (Treisman, 1987) and an SV40 early promoter double SP1 site that avidly binds SP1 (data not shown). Taken together, these data show that among the four candidates for the binding to IL-2Rα promoter/enhancer: (i) only NF-κB was fully inducible; and (ii) SRF increased significantly in proliferating T cells whereas NFIL2RA and SP1 were constitutively present in the nuclei of human primary T cells stimulated via CD2+CD28.

IL-2Rα proximal promoter/enhancer in T cells is bound by several trans-acting factors before mitogenic stimulation

To analyse *in vivo* the putative regulatory protein–DNA interactions at the human IL-2Rα locus, live primary T cells were treated with DMS, which methylates G residues in the major groove of DNA and to a lesser extent the A residues in the minor groove (Maxam and Gilbert, 1980). Close protein contacts will protect them from, or make them hypersensitive to, methylation. The subsequent piperidine cleavage of the extracted DNA followed by LMPCR amplification generates a G-specific sequence ladder. This technique has revealed specific major groove contacts at several sites known to be required for IL-2 expression (Brunvand *et al.*, 1993; Chen and Rothenberg, 1994; Garrity *et al.*, 1994). As illustrated by the representative experiments presented in Figure 3, multiple significant differences were observed between the G ladder pattern obtained from both strands between DNA from unstimulated resting T cells and naked DNA controls (compare lanes 1 and 2 in panels A and B). Among the putative regulatory elements previously shown to be functionally important by site-directed mutagenesis (Ballard *et al.*, 1989; Cross *et al.*, 1989; Lowenthal *et al.*, 1989b; Roman *et al.*, 1990; Toledano *et al.*, 1990), we observed a hyperreactivity of G residue –268 and a protection of the three downstream Gs within κB. Two Gs are significantly protected on a non-coding strand within the same site, hence demonstrating strong specific major groove contacts within the κB site of IL-2Rα in resting T cells (Figure 3B, lane 2). Several Gs of the non-coding strand within the UE1 element were also protected to a lesser extent, but no differences were observed in the coding strand. In contrast, no significant differences were detected within both SRE/CArG and SP1/GC boxes. Upstream of the minimal enhancer, several protected Gs were observed within both NREs on both strands. Moreover, several protections or hypersensitivities were detected that do not correlate with any functionally active site previously characterized. For example, in the vicinity of NRE, at least four Gs appear to be protected on the non-coding strand. Similarly, downstream of the SP1/GC box, the G nucleotide –232 on the non-coding strand is almost completely protected (Figure 3A) and the two A nucleotides –224 and –223 appear hypersensitive to methylation on the coding strand (Figure 3B). These results confirmed the stable occupancy of several sites of the IL-2Rα locus in primary resting T cells in the absence of any detectable IL-2Rα gene transcription by run-on analysis (data not shown; see also Cerdan *et al.*, 1992).

Mitogenic stimulation of primary T cells results in the coordinated binding of at least two proteins to the IL-2Rα promoter/enhancer

Changes in the pattern of *in vivo* protein–DNA interactions at the active IL-2Rα locus were analysed using a LMPCR footprint assay in primary human T cells stimulated to proliferate. Purified resting T cells were stimulated either by a combination of anti-CD2 and -CD28 mAbs or of PMA and PHA. Both combinations of stimuli activate resting T cells to proliferate and to express the IL-2Rα gene. DMS-treated cells were harvested after 24 h when IL-2Rα gene transcription is switched on, as determined by run-on analysis (Cerdan *et al.*, 1992 and data not shown). First, note that T-cell activation resulted in only two major modifications of site occupancy in the IL-2Rα promoter/enhancer. The main change was detected within the κB site on both strands (Figure 3A and B, lanes 3 and 4) and correlated with NF-κB nuclear translocation detected by EMSA. The second affected two Gs within the CArG/GC box overlap at position (–244,–245) on the coding strand, strongly suggesting inducible binding of SRF and/or SP1 (Figure 3A, lanes 3 and 4). Since no significant protection was observed on the non-coding strand within the SP1/GC box, we propose that an SRF-like factor could occupy the SRE/CArG box *in vivo*. Alternatively, since the SRF footprint does not strongly protect the two κB-proximal Gs, as would be expected from published data on SRF–DNA interactions, the SRE/CArG box (Herrera *et al.*, 1989) could be occupied by another protein such as YY1 which has been shown *in vitro* to occupy the downstream part of the c-*fos* CArG box (Natesan and Gilman, 1993). No other significant changes were detectable in multiple independent experiments analysed by densitometry scanning using the Millipore BioImage analyser. Interestingly enough, site occupancies within both NRE were remarkably stable (Figure 3C). LMPCR genomic footprint experiments on each strand of the IL-2Rα locus were also performed using primary T cells harvested at 6, 24, 48, 72 and 96 h after either CD2+CD28 or PMA+PHA' indicating the stable occupancy of both constitutive and inducible sites during the time-course analysed (data not shown).

PDTC treatment blocks NF-κB nuclear translocation and inducible protein–DNA interactions at κB and SRE/CArG box sites in CD2+CD28-stimulated T cells

PDTC, a free radical scavenger, is the most potent inhibitor available of the nuclear activation of NF-κB (Molitor *et al.*, 1991; Schreck *et al.*, 1991, 1992). We have previously shown that the drug almost completely blocks NF-κB nuclear activation in primary human T cells stimulated via CD2 and CD28 as detected by EMSA

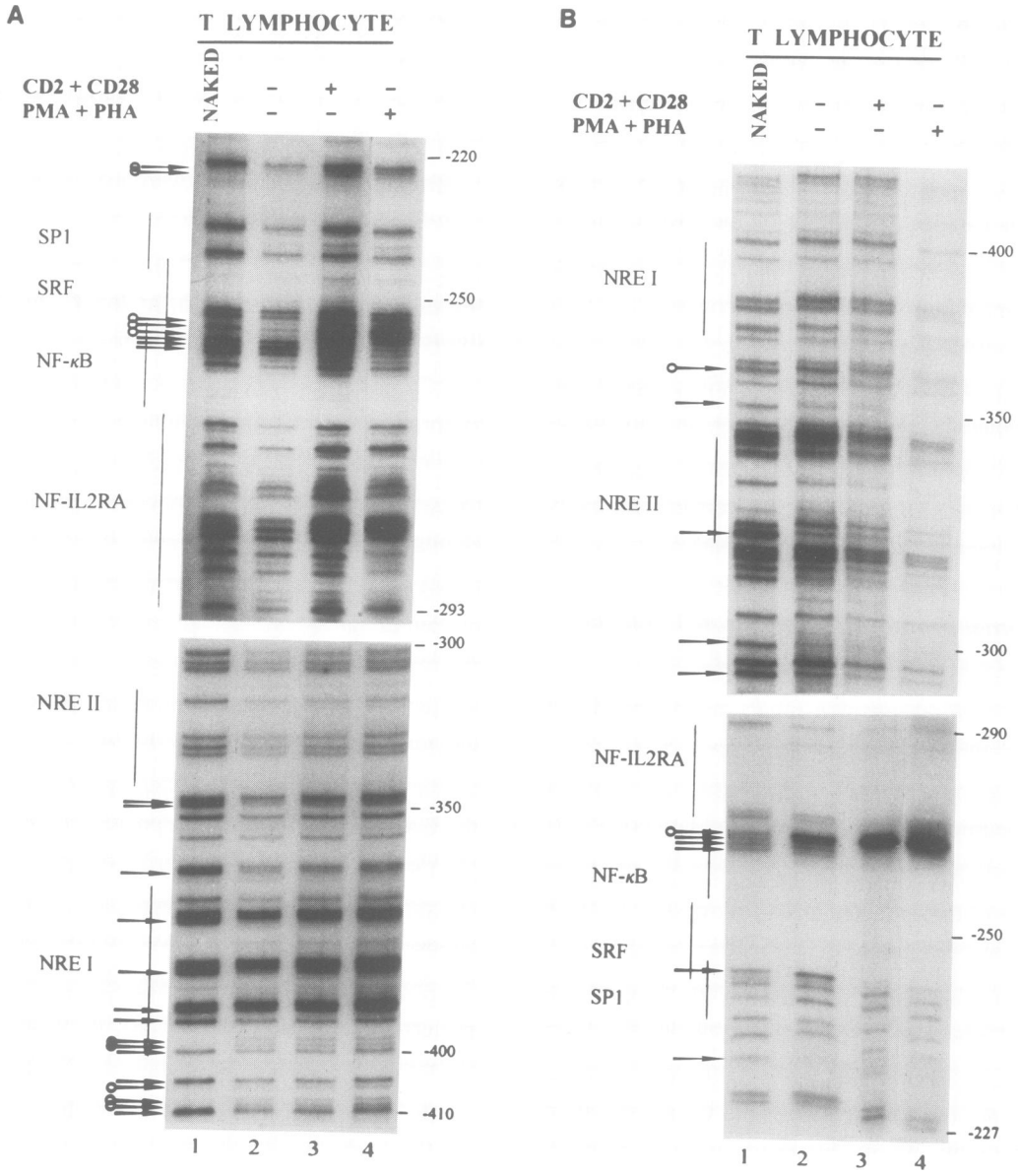


Fig. 3. *In vivo* occupancy of the IL-2Rα promoter was determined by DMS genomic footprinting on highly purified T cell before or after activation. (**A**) Non-coding strand. (**B**) Coding strand. *In vitro* methylated DNA G ladder (lanes 1), unstimulated cells (lanes 2), CD2+CD28 24 h stimulation (lanes 3), PMA+PHA 24 h stimulation (lanes 4). All DNA samples were amplified by LMPCR with the set of appropriate primers for the coding and non-coding strands. Radiolabelled amplified DNA was then loaded into a sequencing gel. Numbers on the right side of each panel correspond to the location of G residues within the IL-2Rα promoter with respect to the major transcription initiation site. Plain arrows represent protected nucleotides; arrows with open circles indicate hypersensitive sites.

(Costello *et al.*, 1993b). As illustrated in Figure 4, proliferation of T cells triggered by CD2+CD28 was dramatically inhibited in presence of 1 μM PDTC (panel A). Furthermore, CD25/IL-Rα cell surface expression was efficiently reduced by 10 μM PDTC (Figure 4B), although individual IL-2Rα regulatory elements showed diverse sensitivities to the free radical scavenger (panel C). A 1 h pretreatment with 1 μM PDTC caused a dramatic inhibition of NF-κB nuclear translocation, whereas none of the constitutive DNA-binding activities to the individual IL-2Rα regulatory elements was affected. Hence, PDTC provides a powerful tool to address the question of the nuclear factors which occupy the IL-2Rα promoter/enhancer region after T-cell stimulation. LMPCR genomic footprinting was performed using *in vivo* methylated genomic DNA prepared from batches of primary T cells used simultaneously for phenotypic and EMSA controls. As illustrated in Figure 5, 1 μM PDTC completely reverted the inducible occupancies observed in stimulated cells on both coding and non-coding strands. In sharp contrast, 1 mM pyrrolidine—an end-product of PDTC oxidation which lacks a dithiocarboxy group—had no detectable effect. Since both inducible protein interactions were affected whereas only NF-κB nuclear translocation was altered, we conclude that the inducible occupancy within the SRF/CArG box is dependent on the stable occupancy by active NF-κB in primary T cells stimulated *via* CD2+CD28.

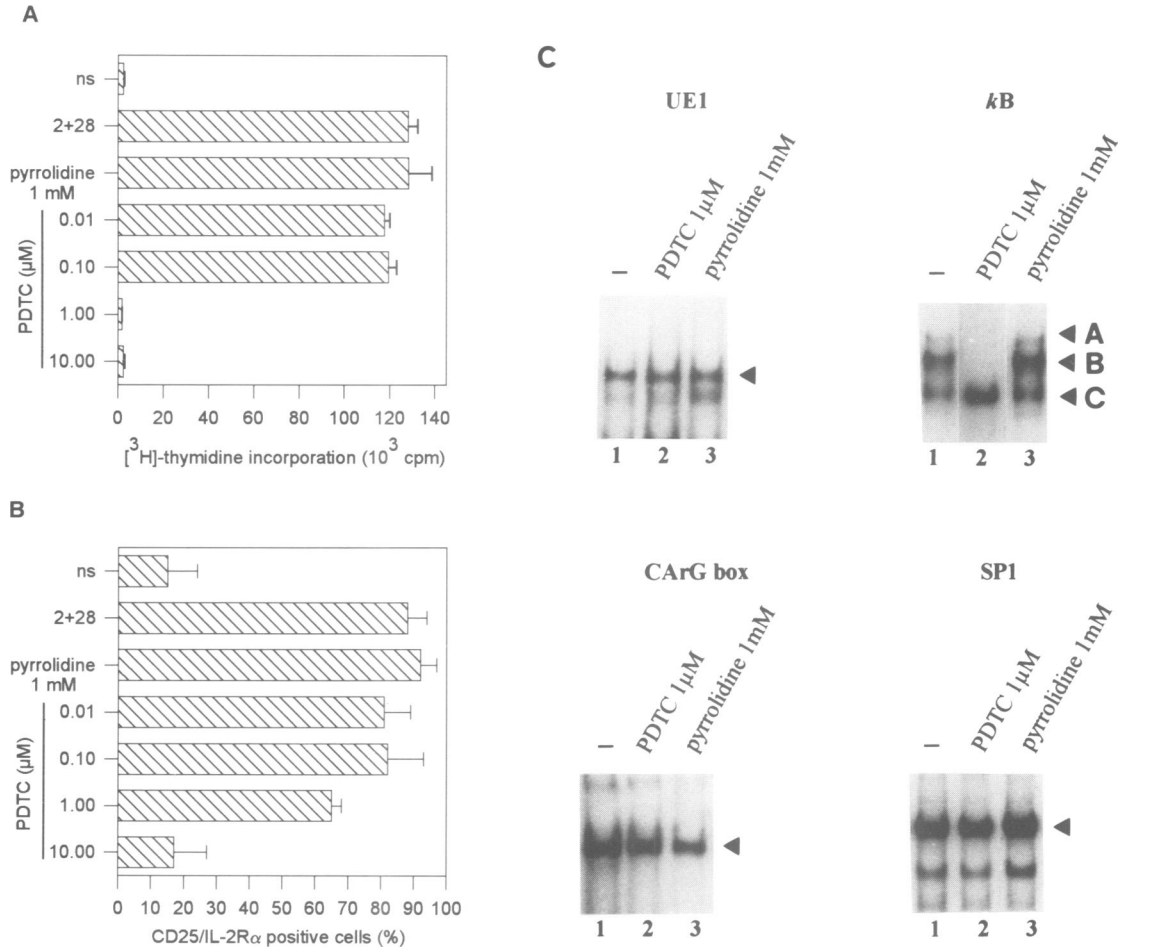


Fig. 4. Effects of NF-κB inhibitor on T-cell proliferation and CD25 expression. (**A**) [^{3}H]thymidine incorporation. Cells were pretreated with PDTC 30 min before stimulation by saturating concentrations of CD2 and CD28 monoclonal antibodies. The different concentrations of PDTC are indicated. Pyrrolidine was used at 1 mM 30 min before stimulation by CD2 and CD28. The incorporated radioactivity was counted after 3 days of [^{3}H]thymidine incorporation. (**B**) CD25/IL-2Rα cell surface expression was determined at day 3 by flow cytometry analysis. Non-toxicity of PDTC was assessed by trypan blue staining. (**C**) Effects of PDTC on *in vitro* nuclear DNA-binding activities. Electrophoretic mobility shift assays were performed with nuclear extracts of highly purified T lymphocytes stimulated by CD2+CD28 (lanes 1). At 30 min before stimulation, cells were treated with 1 μM PDTC (lanes 2) or 1 mM pyrrolidine (lanes 3). The individual regulatory elements present in the ^{32}P-labelled oligonucleotide probes are indicated at the top of each panel. Arrowheads indicate the specific binding activities.

Occupancy of the κB$_{IL\text{-}2Rα}$ element by NF-κB p50 homodimer impairs the in vitro binding of an SRF-like factor to the flanking SRE/CArG box

Our and other data from the fibroblast system (Treisman, 1992) suggest that it cannot be the DNA-binding of SRF itself that changes upon T-cell activation. The apparent inability of SRF constitutively present in the nuclei of resting T cells to bind *in vivo* the SRE/CArG box of IL-2Rα raises the question whether SRF binding could be impaired by the presence of stably bound p50 homodimer. We tested this hypothesis by EMSAs performed using an oligonucleotide probe containing both NF-κB and SRF binding sites (nucleotides –274 to –240 of the 5′ untranslated region of the human IL-2Rα gene). In accordance with previously published data (Pimentel-Muinos *et al.*, 1994a,b), assays performed with an excess of labelled probe showed two specific DNA–protein complexes present in resting T cells (Figure 6A, lane 1) and three complexes in CD2+CD28-stimulated T cells (Figure 6A, lane 2). Their specificity was characterized by competition with unlabelled –276 to –240 oligonucleotide probe (data not shown) and cross-competition with smaller internal probes, which included κB, SP1/GC and SRE/CArG boxes. In stimulated cells, we detected two bands specifically competed by a 200-fold molar excess of the κB element present in IL-2Rα promoter (Figure 6A, lane 3). The lower complex was identified as NF-κB p50 homodimers since it disappeared in presence of serum anti-p50 n3 (Figure 6A, lane 7) that recognizes only p50 homodimers (Kieran *et al.*, 1990). The intermediate complex was mainly composed of NF-κB p50–p65 heterodimers since it disappeared in presence of anti-p105 n2 (Figure 6A, lane 6) that recognizes both p50–p50 and p50–p65 dimers. The upper complex was identified as an SRF-like factor by competition with a molar excess of unlabelled SRE/CArG box oligonucleotide (Figure 6A, lane 5). EMSAs performed with either a mutated κB site or a mutated SRE probe further confirmed the composition of each complex (data not shown). No DNA–protein complex could be competed by a 200-fold molar excess of SP1 oligonucleotide (Figure 6A, lane 4) although an SP1 binding site consensus is present between nucleotides –245 and –240 of IL-2Rα promoter in accordance with previous results (Pimentel-Muinos *et al.*, 1994a,b).

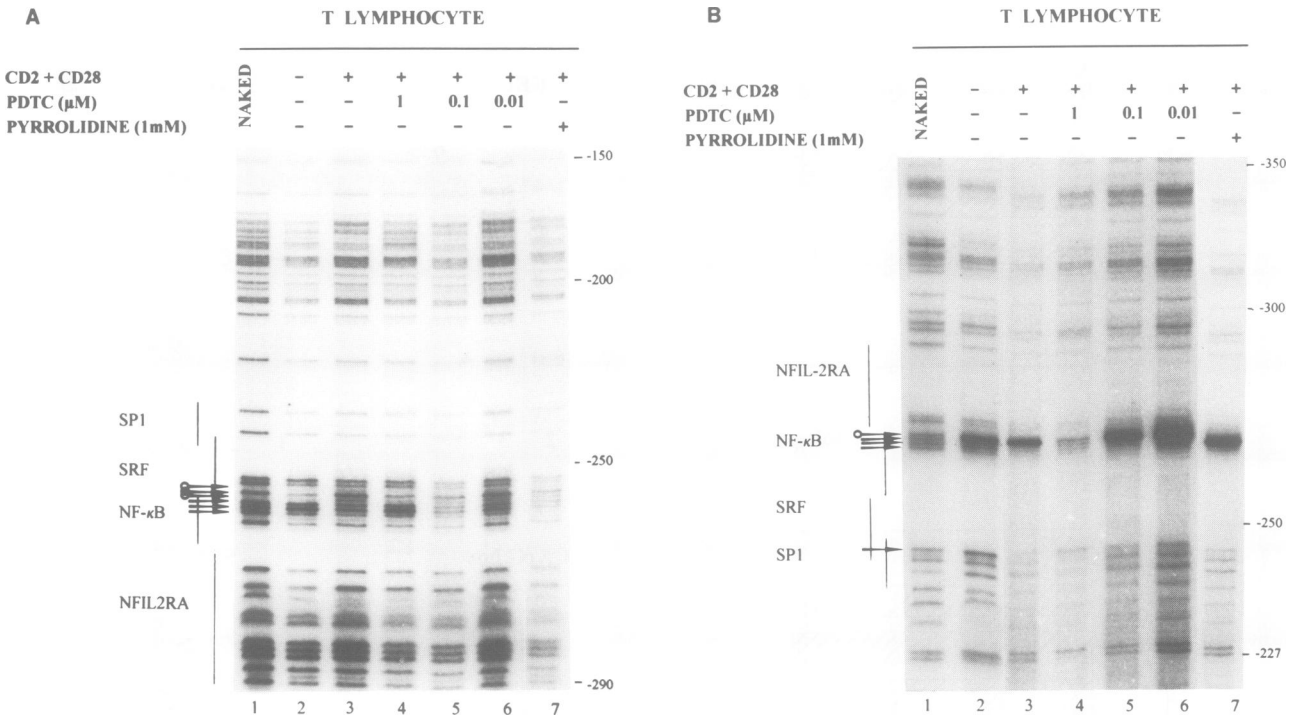


Fig. 5. Effects of PDTC on *in vivo* DNA-binding activities. DMS *in vivo* footprints were performed with DNA of T cells pretreated with different concentrations of PDTC or pyrrolidine 30 min before stimulation with a saturating concentration of CD2 and CD28 during 24 h. (**A**) Non-coding strand. (**B**) Coding strand. Naked DNA (lanes 1); DNA from unstimulated cells (lanes 2); T cells stimulated by CD2+CD28 (lanes 3); T cells treated with respectively 1, 0.1, 0.01 μM of PDTC 30 min before CD2+CD28 stimulation (lanes 4–6); cells treated with 1 mM pyrrolidine 30 min before stimulation by CD2+CD28 (lanes 7). In panel B (lanes 1 and 2), representatives of G-ladders obtained with *in vitro* methylated genomic DNA and from unstimulated primary T cells, are from lanes 1 and 2 in Figure 3B.

EMSAs performed under conditions of limited probe amount revealed that only the p50 homodimer complexes are detectable in resting T-cell nuclear extract (Figure 6B, lane 1). p50 homodimer inhibition by serum n3 allowed the appearance of the two new complexes identified as p50–p65 heterodimer and an SRF-like complex (Figure 6B, lane 2). In CD2+CD28-stimulated T cells, the simultaneous detection of p50 homodimer and p50–p65 heterodimer DNA-binding activity is explained by the partially limiting amount of probe used in this assay required for unambiguous identification of the retarded DNA–protein complexes (Figure 6B, lane 3). In sharp contrast, selective inhibition of the p50-homodimer–DNA complex by anti-p50 n3 allowed the detection of an SRF-like DNA-binding activity (Figure 6B, lane 5). In agreement with *in vivo* footprint observations, these data, when taken together, clearly demonstrated that occupancy of the $\kappa B_{IL\text{-}2\alpha}$ element by NF-κB p50 homodimer impairs the binding of an SRF-like factor to the flanking SRE/CArG box *in vitro*.

Absence of detectable in vivo protein–DNA interactions at the IL-2Rα locus in cells that are not committed for T-cell differentiation

Since the IL-2Rα promoter/enhancer is bound by multiple *trans*-acting factors before mitogen stimulation, LMPCR *in vivo* footprint assays were performed on several cell types of non-T origin that do not express IL-2Rα (data not shown) to establish the relationship between site occupancies and commitment to T cell-specific differentiation. As illustrated in Figure 7, we did not detect *in vivo* protein–DNA interactions in any of the non-expressing cells examined, including G_0-arrested and actively growing primary fibroblasts and the B-cell lymphoma BJAB. We propose that primary human T cells, as yet uninduced for IL-2Rα expression, are developmentally committed by means of preassembled protein–DNA complexes within IL-2Rα promoter/enhancer region, in sharp contrast to the bare IL-2Rα locus in non-T cells.

Discussion

IL-2Rα transcription is under a strict developmental regulation and depends physiologically on specific stimuli such as antigen recognition. Band shift and DNase I footprint assays have defined *in vitro* DNA–protein interactions at the IL-2Rα promoter/enhancer *cis*-elements, indicating the apparent major role played by the NF-κB transcription factor. However, if NF-κB is a key element in the rapid response to environmental signals, this ubiquitous transcription factor lacks the specificity characteristics required to explain the strict IL-2Rα gene transcription regulation. Furthermore, most published data were obtained using actively growing tumoral T-cell models which are not necessary representative of resting T lymphocytes present in peripheral blood. In order to address the role of NF-κB and its interplay with the other *trans*-acting factors involved in IL-2Rα promoter/enhancer in almost physiological conditions, we have analysed the nature of *in vivo* protein–DNA complexes in human primary resting T cells and compared them with the sets of nuclear factors present in CD2+CD28-activated T cells and in a selection of cell types of non-T origin.

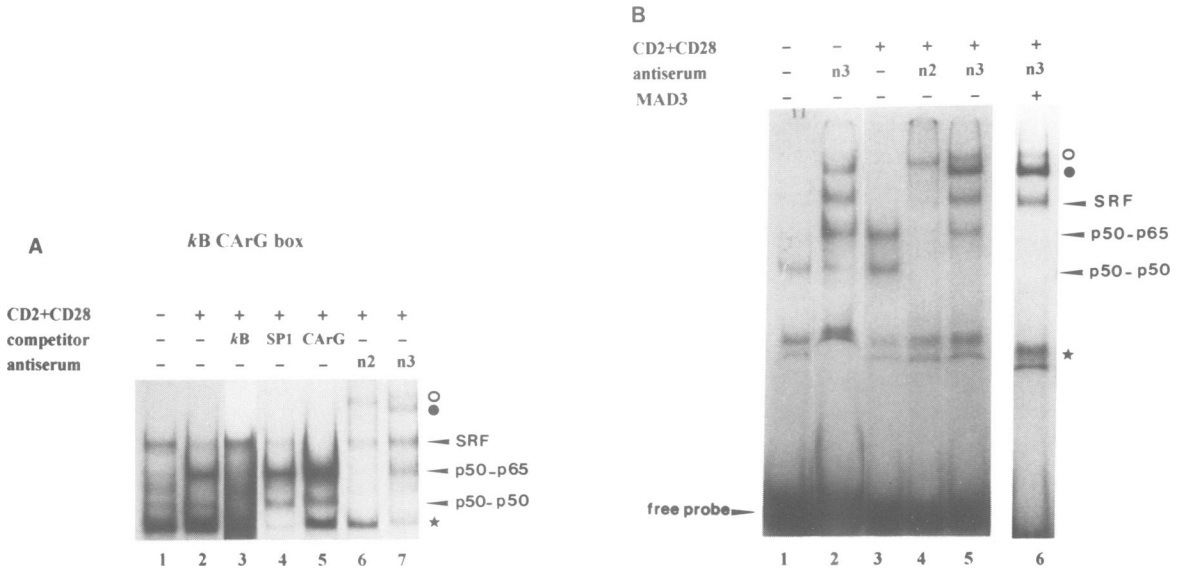


Fig. 6. DNA–protein complexes from unstimulated and CD2+CD28-stimulated T cells obtained with the –276 to –240 fragment of IL-2Rα promoter. Purified human T lymphocytes were harvested at 24 h after cell seeding and nuclear extracts were prepared as described in Materials and methods. As indicated above the lanes, either a 200-fold molar excess of different double-stranded unlabelled oligonucleotides or 1 μl of two NF-κB-specific antisera (n2 or n3) were added to the reaction mixture just before the radiolabelled probe containing nucleotides –276 to –240 of human IL-2Rα promoter. Serum anti-p105 n2 recognizes both KBF1/p50 homodimers and NF-κB p50–p65 heterodimers and anti-p50 n3 recognizes only KBF1/p50 homodimers (Kieran *et al.*, 1990). ○, DNA-binding activity contributed by rabbit antisera; *, non-specific DNA-binding complexes; ●, unidentified protein–DNA complex. (**A**) EMSAs performed in conditions of probe excess (14 h exposure). Lane 1, unstimulated T cells; lanes 2–7, T cells stimulated by saturating concentration of CD2 and CD28 mAbs. (**B**) EMSAs performed in conditions of probe limitation (6 day exposure). 5×10^3 c.p.m. of radiolabelled probe were used on the basis of probe titration performed in presence of 4 μg nuclear extract as the minimal amount required for unambiguous identification of the retarded DNA–protein complexes (data not shown). The faster migrating, non-specific complex observed in panel A is absent in these limiting conditions. Lanes 1–2, unstimulated T cells; lanes 3–6, CD2+CD28-stimulated T cells. In lane 6, 1 μl of recombinant IκBα protein MAD-3, which inhibits DNA binding of both NF-κB and c-Rel (Zabel *et al.*, 1993), was added to the reaction mixture in addition to 1 μl of anti-p50 n3. The specificity of the complexes was determined by competition using unlabelled double-stranded oligonucleotides and was identical to the results presented in Figure 6A. A 200-fold molar excess of $\kappa B_{IL\text{-}2R\alpha}$ oligonucleotide competed for the two bands corresponding respectively to NF-κB p50–p50 and p50–p65, but not the complex corresponding to SRF. The upper binding complex detected in the presence of anti-p50 n3 was competed by a 200-fold molar excess of SRE/CArG box unlabelled oligonucleotide without effect on both NF-κB p50–p50 and p50–p65 complexes (data not shown).

In vivo role of NF-κB

EMSAs with the $\kappa B_{IL\text{-}2R\alpha}$ element demonstrate that nuclear NF-κB p50 homodimers are readily detectable in unstimulated primary T cells (Costello *et al.*, 1993b). In response to CD2+CD28 or CD3+CD28 co-stimulation, two new nuclear NF-κB complexes are activated. We have previously shown that inhibition of both NF-κB nuclear activation (Costello *et al.*, 1993b) and NFKB1 gene overexpression (Costello *et al.*, 1993a) directly correlated with a blocked primary T-cell proliferative response to CD2+CD28. Transient transfection assays in Jurkat T cells have shown that overexpression of the NF-κB p50 subunit, which lacks a strong transactivation domain, could repress IL-2 promoter activity (Kang *et al.*, 1992). *In vivo* footprints of IL-2Rα locus in primary T cells and EMSAs performed with limiting amounts of an oligonucleotide probe containing both κB and SRE/CArG box elements, are consistent with this model where p50 homodimers could play the role of repressor, displaced by activated NF-κB complexes (Figure 8). Genetically engineered mice lacking NF-κB p50 subunit present no developmental abnormalities but rather a defect in the proliferative response of purified T cells stimulated by CD3+CD28 (Sha *et al.*, 1995). Taken together, these data strongly suggest that beside its ubiquitous role of general mediator of the inflammatory response, the NFKB1 gene encoding the p50 subunit plays a critical role in co-stimulation of T-cell proliferation.

Role of the cooperative binding between constitutive and inducible factors at the IL-2Rα locus in primary T cells

Evidence consistent with cooperativity as a substantial component of IL-2Rα transcriptional control comes from *cis*-element mutational analysis, in which the individual alteration of any of a number of different sites strongly decreases overall promoter/enhancer activity (Cross *et al.*, 1987; Ruben *et al.*, 1988; Ballard *et al.*, 1989; Lowenthal *et al.*, 1989a,b; Pomerantz *et al.*, 1989; Smith and Greene, 1989; Lin *et al.*, 1990). An additional feature of the IL-2Rα regulatory region that favours dependence on cooperative interactions is that its κB site deviates significantly from the NF-κB consensus sequence and acts as a weak *cis*-element (Perkins *et al.*, 1992). Occupancy modification of IL-2Rα *cis*-acting elements upon mitogenic stimulation apparently occurs in the presence of the pre-existing bound factors. This observation therefore suggests that interactions between preassembled constitutive factors and inducible factors are required for the specific induction of IL-2Rα transcription in primary cells. Beside the κB element, G residues within the flanking SRE/CArG box were dramatically modified, thus indicating the binding of at least two inducible factors. The proximity of κB and SRE/CArG box raises the possibility that both elements act in cooperation, as suggested by previous regulatory element mutagenesis and EMSA studies (Kuang *et al.*, 1993). This hypothesis is reinforced by the correla-

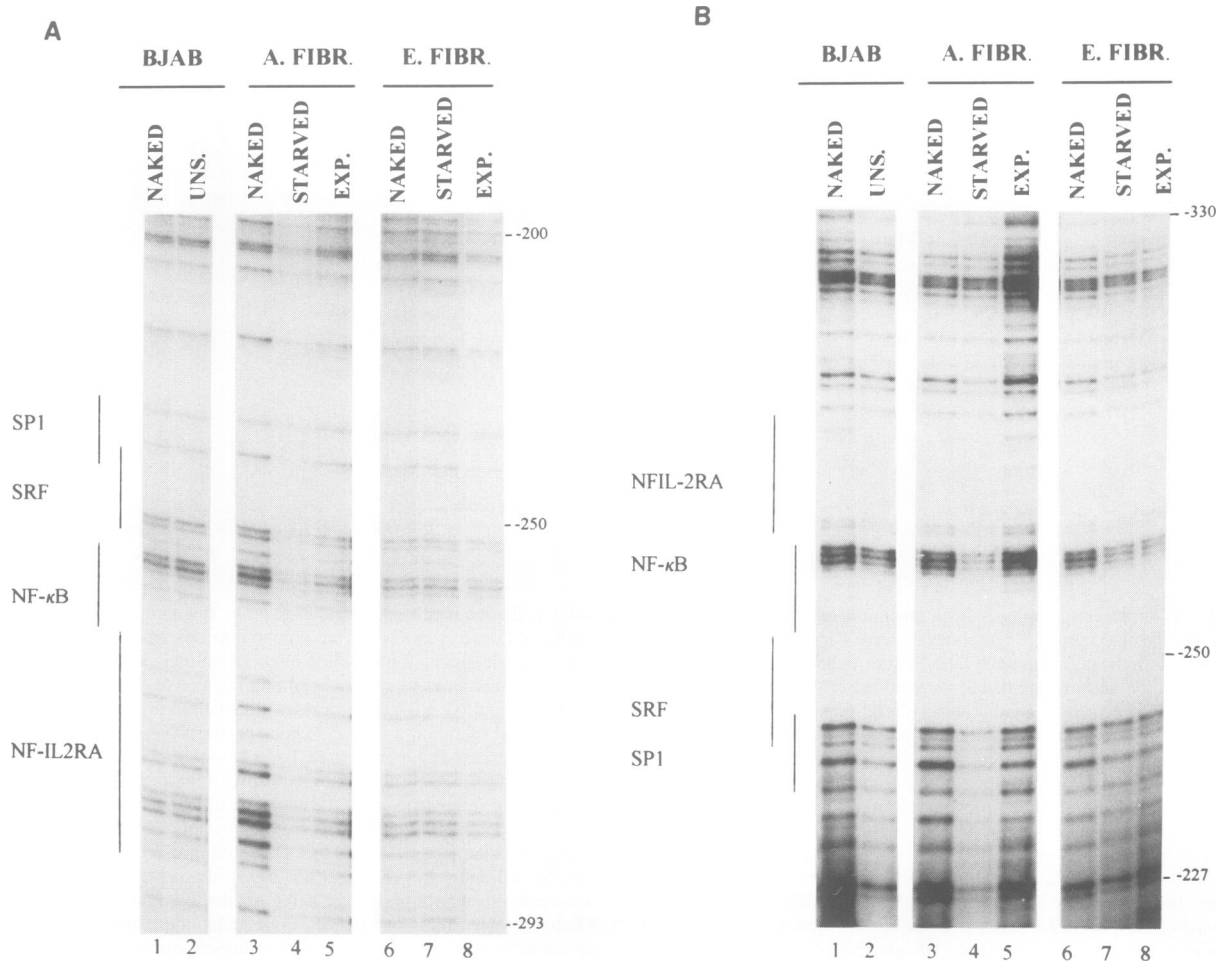


Fig. 7. *In vivo* genomic footprinting of IL-2Rα locus in non-expressing cells. (**A**) Non-coding strand. (**B**) Coding strand. As indicated at the top of the lanes, experiments were performed using DMS methylated genomic DNA from human BJAB cells (BJAB) or from adult skin fibroblasts (A.FIBR) and embryo primary fibroblasts (E.FIBR). Naked DNA (lanes 1, 3 and 6); unstimulated BJAB (lane 2); fibroblasts arrested in G_0 by serum starvation (lanes 4 and 5); serum-stimulated exponentially growing fibroblasts (lanes 5 and 8).

tion between *in vivo* occupancy of SRE and nuclear translocation of active NF-κB demonstrated by PDTC treatment. One possibility is that engagement of an SRF-like factor on IL-2Rα promoter requires NF-κB binding. At the c-*fos* SRE, a ternary complex forms between the ubiquitous factor SRF and members of the Ets domain protein family, the ternary complex factors (TCFs) that bind an Ets binding site, 2 bp upstream of SRE. Recent findings indicate that regulation of TCF activity by phosphorylation is an important mechanism by which the SRE responds to growth factor signals (reviewed in Treisman, 1994). Our results suggest that similar contacts could occur *in vivo* between the κB and SRE binding factors. Nonetheless, the molecular mechanisms at play must certainly be different since the same specific DNA–protein architecture is apparently maintained at the c-*fos* SRE in human A431 cells (Herrera *et al.*, 1989), regardless of changes in the transcription state of the gene, whereas occupancy of κB/SRE at the IL-2Rα locus appears inducible. We propose that, *in vivo*, the binding of SRF, readily present in the nuclei of resting T cells, to the SRE/CArG box of IL-2Rα is impaired by stably bound p50 homodimer to the flanking κB element. Moreover, the levels of SRF constitutively expressed in T cells are consistently higher than in other cell types and overexpression of SRF in B cells causes the IL-2Rα enhancer to function as well as it does in T cells (Kuang *et al.*, 1993). In our T-cell model, the increased band shifting activities seen with NF-κB and SRF probes appear sustained by an increase in the amounts of the corresponding proteins upon stimulation, as detected by Western blotting (2- to 4-fold increase for NF-κB p50, no significant variation for NF-κB p65 and 2- to 3-fold increase for SRF, Costello *et al.*, 1993a, and our unpublished data). Altogether, these data suggest that the high level of SRF binding in T cells is functionally important. However, we cannot conclude definitively at this stage that SRF is involved in IL-2Rα gene control, since the *in vivo* footprint differs from the protection pattern expected from published data concerning SRF–DNA interactions (Herrera *et al.*, 1989). Alternatively, the SRE/CArG box could be occupied by another CArG box-binding protein such as YY1, which recognizes the downstream part of the CArG box (Natesan and Gilman, 1993). YY1 is a ubiquitously expressed zinc finger protein with relatively degenerate DNA-binding specificity (Hahn, 1992) that apparently positively or negatively influences

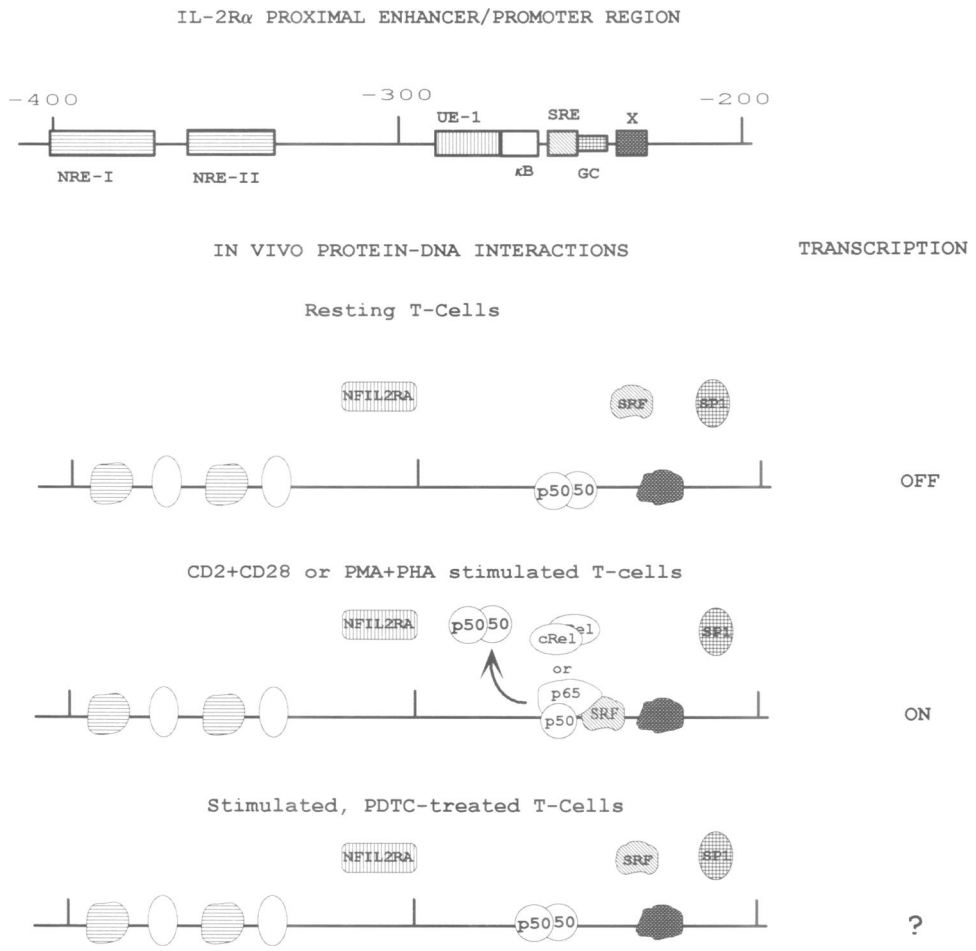


Fig. 8. Summary of the protein–DNA interactions detected in human primary T cells and non-T cells by *in vivo* footprint analysis and the presence of nuclear factors in these cells determined by *in vitro* DNA-binding assay. The X box designates a homologous nucleotide sequence found in the 5′ flanking region of IL-2 and some other T cell-specific genes (Fujita *et al.*, 1986). Unidentified factors correspond to constitutive occupancies detected by *in vivo* footprinting.

promoter activity by affecting promoter structure rather than by directly contacting the transcriptional machinery (Natesan and Gilman, 1993). Although most of the previously reported data are in favour of a direct involvement of SRF in human IL-2Rα gene regulation (Lin *et al.*, 1990; Roman *et al.*, 1990; Toledano *et al.*, 1990; Kuang *et al.*, 1993; Pimentel-Muinos *et al.*, 1994a,b), further investigations are required to address its interplay with active NF-κB p50–p65 complex and the apparent discrepancy between *in vivo* and *in vitro* data. However, if cooperative interaction between NF-κB and SRF plays a role in activation of IL-Rα transcription in human, the fact that the mouse IL-2Rα proximal enhancer contains only two identified κB sites and is devoid of any flanking SRE or GC box consensus argues against its general involvement in the T cell-specific regulation of IL-2Rα gene (Sperisen *et al.*, 1995).

Despite the constitutive binding and IL-2Rα promoter activities of NFIL2RA and SP1 detected *in vitro* by EMSA and CAT assay (reviewed in Leonard, 1992), their binding sites are not steadily occupied at the IL-2Rα locus *in vivo*. One of the striking features of IL-2Rα enhancer/promoter is that CArG and GC boxes overlap by three nucleotides (Figure 1). Site-directed mutagenesis and transient transfection assays in Jurkat T cells suggest that SP1 acts as a repressor of IL-2Rα expression, most likely by competition for binding of SRF-like nuclear factor(s) to the overlapping SRE/CArG box (Roman *et al.*, 1990). Our results argue against a repressor role for SP1 by steric hindrance. Furthermore, our and other data using extended oligonucleotide probes containing all four IL-2Rα regulatory elements evidenced that only three specific complexes, respectively identified as SRF and NF-κB p50–p50 and p50–p65 dimers, can be detected *in vitro* by EMSAs but failed to reveal NFIL2RA or SP1 complexes (Pimentel-Muinos *et al.*, 1994a,b). This suggests that neither of the two factors play a major role in IL-2Rα transcription.

The two negative regulatory elements NRE I and NRE II (Cross *et al.*, 1987; Smith and Greene, 1989) are apparently steadily occupied in *in vivo* unstimulated T cells. The lack of detectable modifications after activation strongly suggest that IL-2Rα transcription does not rely upon the release of repressors from the NRE binding sites. We have identified several new putative binding sites in the vicinity of both NREs. These constitutive occupancies coincide with two previously unidentified elements which contain the consensus binding site [C(A/T)(T/G)(T/G) ANN(C/T)] of a poorly characterized regulatory element, originally described as a gamma interferon response

element, γ-IRE (Yang *et al.*, 1990). Similarly, the footprint observed downstream of IL-2Rα GC box, correlates with a conserved sequence found in the 5′ flanking region of IL-2 and other T cell-specific genes (Fujita *et al.*, 1986). Further experiments are required to characterize the proteins bound to these sites and to determine their role in the regulation of IL-2Rα transcription.

Precommitment of T cells results in preassembled protein–promoter/enhancer at the IL-2Rα locus

Because the differentiation of helper T cells is a separate event from their activation to express the IL-2 Rα chain, stable protein–DNA interactions at the IL-2Rα locus might occur in T cells before activation. A partially assembled protein–enhancer complex would distinguish them from non-T cells, thereby setting the stage for an induction event. Alternatively, such developmental marking might take the form of specific repressive interactions in non-T lineages selectively removed in T cells. We tested these possibilities by comparing *in vivo* protein–DNA interactions in T cells with those in other haematopoietic and non-haematopoietic cells before induction. We found evidence for a stable, lineage-dependent molecular commitment at the IL-2Rα locus, in contrast with the bare IL-2Rα locus in non-T cells (summarized in Figure 8). Hence, the cell-specific competence of the IL-2Rα locus to be induced could be detected at the level of protein–DNA contacts in the major groove. These observations do not rule out the possibility of repressive or permissive interactions to which DMS had low sensitivity, such as binding in the minor groove or to recognition sites without G residues. The possible involvement of minor groove-binding factors belonging to the High Mobility Group (HMG) protein family (reviewed in Clevers *et al.*, 1993), such as DSP1 and HMG I(Y) which could determine whether NF-κB acts as an activator or a repressor of transcription (Thanos and Maniatis, 1992; Lehming *et al.*, 1994), remains to be investigated. Nevertheless, there is clearly no evidence, in any of the non-expressing cells examined, of an *in vivo* occupancy of the sites that become occupied in peripheral blood T cells. This observation argues against a straightforward competition between activator and repressor proteins for binding site contacts in the major groove as a way of enforcing cell-type- and signal-dependent restriction of IL-2Rα in primary T cells.

In the absence of any detectable difference in binding affinity *in vitro*, it has been proposed that changes in chromatin structure and/or DNA modifications determine the binding of factors to their DNA targets. This level of regulation could involve the packaging of DNA into higher-order chromatin structures, or the presence or absence of a nucleosome at a protein binding site (Becker *et al.*, 1987). Stable modification of DNA in protein-binding sites may also prevent the interaction of ubiquitous factors in non-expressing cells. The lack of an *in vivo* footprint, despite the presence of factors, has been noted for other genes (Becker *et al.*, 1987; Kara and Glimcher, 1991). Activation of the IL-2Rα gene may require a higher-order chromatin configuration controlling the accessibility of the inducible factors to specific DNA elements, whereas basal level promoter activity and T-cell specificity can be achieved by preassembling steadily bound factors which pre-exist in resting T cells. In agreement with our observations at the IL-2Rα locus, several regulatory elements are apparently steadily occupied at the IL-2 locus in human Jurkat T cells before induction (Brunvand *et al.*, 1993). After stimulation, three new occupancies were mapped within the AP-1, NFAT and OAP^{40} proximal binding sites. In sharp contrast, in unstimulated EL4 cells, a murine tumoral T-cell model, no sites appear occupied within the IL-2 enhancer/promoter region (Chen and Rothenberg, 1994; Garrity *et al.*, 1994). This discrepancy could result in either an unexpected species specificity of IL-2 gene precommitment during T-cell development, or in a difference in the stage of differentiation of the two different T-cell models used in both studies. We favour the second proposition, since EL4 is derived from a chemically-induced thymoma, whereas Jurkat is derived from a more mature T cell leukaemia. The recent characterization of a second positive regulatory region (PRRII) located between nucleotides −137 and −67 (John *et al.*, 1995) reinforces the hypothesis that T-cell specificity of IL-2Rα expression could be achieved through the interaction between cell type-specific factors such as Elf-1 and T cell-specific preassembled protein–DNA complexes, whereas factors like NF-κB and SRF more potently contribute to the inducibility of this gene. Another non-exclusive hypothesis is that unidentified *cis*-acting T cell-specific elements, localized either downstream or upstream of the previously characterized sequence, could also participate in the cell type-specific control of IL-2Rα transcription. In agreement with our hypothesis of a precommitment to express IL-2Rα through a partial preassembling of its promoter/enhancer region during T-cell differentiation, three T cell-specific DNase I hypersensitive sites within the murine IL-2Rα locus have been reported after completion of this work (Soldaini *et al.*, 1995).

Materials and methods

Cells

BJAB, an EBV genome-negative Burkitt lymphoma cell line, was grown as described (Menezes *et al.*, 1975). Human adult primary fibroblasts were derived from normal skin biopsies. Human embryo fibroblasts were prepared from whole embryonic limbs. Peripheral lymphocytes were isolated from voluntary, healthy blood donations and purified as previously described (Cerdan *et al.*, 1991). Purity of T-cell preparations (>99%) was checked by immunofluorescence labelling with various mAbs specific for monocytic, B and NK cells. Contamination by monocytes was also controlled by the absence of T-cell proliferation upon optimal stimulation with PHA. Primary T cells were maintained in RPMI 10% FCS. Stimulations were done with the following mAbs, used alone or in combination, at saturating concentrations. Anti-CD2 mAb 39C1.5 (rat IgG2a) and 6F10.3 (mouse IgG1) were used as purified mAbs at 10 μg/ml each (Olive *et al.*, 1986a). Anti-CD28 248 ascitic fluid (mouse IgM), obtained from Dr A.Moretta (Cancer Institute, Genoa, Italy), was used at a 1/400 dilution. T-cell activation was controlled by proliferation assays (Costello *et al.*, 1993b) and CD25/IL-2Rα expression at the cell membrane. CD25/IL-2Rα expression was detected according to standard procedures with fluorescein isothiocyanate-labelled antibody 33B3.11 (Olive *et al.*, 1986b) using a FACSCAN (Becton Dickinson). *In vivo* footprint, band-shift, CD25/IL-2Rα expression and proliferation assays were performed in parallel using the same batches of activated T cells. All experiments were performed at least three times using the T lymphocytes of three volunteer donors.

Electrophoretic mobility shift assays

Nuclear extracts of purified T lymphocytes and electrophoretic mobility shift assays (EMSAs) were performed as previously described (Costello *et al.*, 1993b). Briefly, 1 μg of nuclear extract protein and 1 μg of poly

Table I. Synthetic double-stranded oligonucleotide probes used in EMSAs

Name	Sequence	Origin
UE1	GATCGCTCACCCTACCTTCAACGGCA CGAGTGGGATGGAAGTTGCCGTCTAG	IL-2Rα (–290,–269)
κB	CAACGGCAGGGGAATCTCCCTCTCCTT GTTGCCGTCCCCTTAGAGGGAGAGGAA	IL-2Rα (–276,–250)
SRE/CArG	GATCCTCTCCTTTTATGGGCGT GAGAGGAAAATACCCGCACTAG	IL-2Rα (–257,–240)
κB/SRE	CAACGGCAGGGGAATCTCCCTCTCCTTTTATGGGCGT GTTGCCGTCCCCTTAGAGGGAGAGGAAAATACCCGCA	IL-2Rα (–276,–240)
muκB/SRE	CAACGGCACTCGAATCTCCCTCTCCTTTTATGGGCGT GTTGCCGTGAGCTTAGAGGGAGAGGAAAATACCCGCA	IL-2Rα (–276,–240)
κB/muSRE	CAACGGCAGGGGAATCTCCCTCTCCTTCGTTGGGCGT GTTGCCGTCCCCTTAGAGGGAGAGGAAGCAACCCGCA	IL-2Rα (–276,–240)
SP1/GC-box	CGATGGGCGGAGTTAGGGACGGGA TACCCGCCTCAATCCCTGCCCTGC	SV40

Sequences are in 5′ to 3′ orientation. Nucleotides substituted to disrupt κB and SRE/CArG boxes respectively, are underlined.

[(dI-dC)×(dI-dC)] were incubated with ~5×10^4 c.p.m. of ^{32}P-labelled oligonucleotide probe in the presence of 10 μg of BSA. After 30 min incubation at room temperature samples were loaded in a 5% polyacrylamide non-denaturing gel. The different oligonucleotide probes used in EMSAs are presented in Table I.

In vivo footprint analysis of protein–DNA interactions within the IL-2Rα minimal enhancer/promoter region

The genomic footprint analysis was performed by the DMS/ligation-mediated PCR method (Mueller and Wold, 1989; Garrity and Wold, 1992). Briefly, 10^7 purified T cells (with or without stimulation) were concentrated in 1 ml of RPMI 10% FCS containing 40 μl 0.5 M HEPES, pH 7.3, and treated for 1 min with 5 μl of DMS (Aldrich Chemicals). The reaction was stopped by centrifugation and the cell pellet washed twice in 1 ml ice-cold PBS containing 2% mercaptoethanol. Cells were lysed in 2 ml Tris buffer (20 mM Tris, pH 7.5, 10 mM NaCl, 10 mM EDTA) supplemented with 100 μl 10% SDS, 100 μl 10% NP40, 200 μl proteinase K at 10 mg/ml and incubated overnight at 50°C on a rotating device. Proteins were removed by centrifugation for 40 min at 8000 r.p.m. after addition of 1.2 ml 5 M NaCl. After ethanol precipitation, nucleic acids were cleaved by a 30 min incubation at 90°C in 200 μl TE containing 20 μl piperidine. Genomic DNA concentration was determined spectrophotometrically at 260 nm. For naked DNA control, DNA was extracted from cells and then treated with 1 μl 98% DMS. PCR amplification was performed using VENT DNA polymerase (New England Biolabs). Synthetic oligonucleotides primers used for IL-2Rα promoter *in vivo* footprint are indicated in Figure 1. The final labelled PCR products were separated on a 5% Hydrolink™ Long Ranger sequencing gel (Bioprobe Systems). Quantification of band intensity was performed using a BioImage Analyser (Millipore). To determine the pattern of naked DNA, genomic DNA was treated with DMS *in vitro* and analysed in parallel. To encompass the IL-2Rα proximal enhancer/promoter (Figure 1A), two sets of specific primers (primers 1–6) were used to analyse methylation patterns between positions –227 to –350 on the coding strand and positions –293 to –200 on the non-coding strand (Figure 1B). A third set of primers (primers 7–9) was used to further characterize region (–425,–271) containing two putative negative regulatory elements (NRE I and II). Eight independent experiments were performed to analyse the coding strand and seven for the non-coding strand at the IL-2Rα locus in primary human T cells. Finally, the human B lymphoma BJAB cell line was used as a non-T cell haematopoietic counterpart and primary embryonic and adult skin fibroblasts as non-haematopoietic controls.

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