A Novel Nuclear Factor Binds to the NF-κB Motif in the Interleukin-2 Enhancer

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Introduction

An important aspect of T-lymphocyte physiology is the necessity for exquisitely tight control of interleukin-2 (IL-2) gene expression. IL-2 is produced only by T cells with extremely transient kinetics [1] (S. Kang, M. Lenardo, unpublished results). Moreover, in nontransformed T cells, significant transcription of the IL-2 gene appears to occur only in the presence of T-cell receptor signaling coupled with an as yet ill-defined "costimulatory signal" present on the surface of competent antigen presenting cells [2, 3]. Further evidence of fine control in IL-2 production is found in the TH_1/TH_2 subdivision of T cells in the murine system: after signaling through apparently the same T cell receptor apparatus, the TH_1 phenotype produces a lymphokine profile which includes IL-2 while the TH_2 phenotype does not [4].

Transcription of the IL-2 gene appears to be controlled in a T cell-specific manner by multiple *cis*-acting elements lying within 300 nucleotides upstream of the transcriptional start site [5]. Although the elements controlling the IL-2 gene have been vigorously investigated, the basis of the cell specificity remains unclear. Most of the activating nuclear factors which bind to these control elements, NF-κB, AP-1, and the octamer-binding proteins, are either not T-cell restricted or are ubiquitous. Only NF-AT appears to be T-cell specific; however, deletion of the NF-AT binding site does not eliminate the inducibility of the IL-2 promoter activity in T cells [6, 7]. We are studying these various enhancer elements to define the molecular basis of the tight regulation of IL-2 gene transcription.

Nearly all detailed studies of gene regulation have utilized transformed cell lines as their model system. Although transformed cells have clearly been informative, they have important limitations. Most significantly, they divide indefinitely without a stimulus. In addition, oncogene products in these cells may by themselves, or by affecting various cellular pathways, perturb normal gene regulation. Perhaps for these reasons, many interesting phenomena of normal cells cannot be reproduced with transformed cells. We have therefore undertaken to study IL-2 gene regulation in murine T-helper lymphocyte clones.

Using murine $CD4^+$ TH_1 cells, we have identified a novel nuclear factor which binds to a site in the IL-2 enhancer shown previously to interact with the well-characterized nuclear factor NF-κB [8, 9]. This factor also appears to bind with lower affinity to other NF-κB sites. Interestingly, the apparent regulation of this factor in normal T cells would suggest that it acts to repress IL-2 gene transcription. In addition, this factor appears to be regulated aberrantly in transformed cell lines.

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

A Novel Factor Binds to the κB Motif

The IL-2 enhancer contains a site at −207 to −196 which has been shown by Lenardo et al. to bind purified NF-κB [9]. However, the site is unusual compared to other known NF-κB binding sites in that it contains a purine at the end of 3′ half-site. The IL-2 site has been shown by Serfling et al. [10] to be a "T-cell element" because it is a functionally active enhancer element only in T cells. The IL-2 site also resembles the "cytokine consensus" sequences defined by Shibuya et al. [11]. Cytokine consensus sites are found in numerous lymphokine genes, but appear to bind a variety of nuclear factors depending on fine sequence differences. For example, the "cytokine consensus site" in granulocyte macrophage–colony stimulating factor (GM-CSF) binds and is regulated by a factor NF-GMa [12].

NF-κB sites	κ-light chain:	GGGACTTTCC
	β-interferon:	GGGAAATTCC
IL-2 site		GGGATTTCACC
"Cytokine consensus"	GM-CSF:	GAGATTCCAC
	IL-3:	GAGATTCCAC

Nuclear proteins from the T-cell clone A.E7 [2] were assayed using a electrophoretic mobility shift assay (EMSA) [7]. Using an oligo containing the NF-κB site in the κ-light chain enhancer, we detected a small amount of constitutive NF-κB in these T cells, with marked induction of binding activity upon antigenic stimulation (Fig. 1). Unexpectedly, we also detected a faster migrating band in the unstimulated extracts which seemed to disappear in the stimulated extracts. We designate this factor as NF-CYT1. Thus, two deoxyribonucleic acid (DNA) binding complexes of apparently different molecular size and distinct responses to antigenic stimulation were detectable with the κB site from the κ-light chain enhancer.

NF-CYT1 Displays Distinct Binding Affinities from that of NF-κB

To determine the relative apparent affinities of the two factors for the motifs in the IL-2 gene and the κ-light chain enhancer, cross competition studies were carried out with resting A.E7 extract. Figure 2 shows that NF-CYT1 has an approximately 6-fold higher affinity for the site in the IL-2 gene than for the site in the κ-light chain enhancer (lanes 1–9). Reciprocally, NF-κB appears to have an approximately 6-fold higher affinity for the site in the κ-light chain enhancer than for the site in the IL-2 enhancer (lanes 10–18). The HIV LTR κB site and the IL2R κB site appear to bind NF-CYT1 and NF-κB with apparent affinities similar to the κ-light chain enhancer (data not shown). The apparent affinity differences between NF-κB and NF-CYT1 make it unlikely that the NF-CYT1 complex is a degradation product of NF-κB.

NF-CYT1 is Distinct from NF-GMa

NF-GMa is a tumor necrosis factor (TNF)-inducible nuclear factor thought to contribute to the transcriptional regulation of both IL-3 and GM-CSF [12]. NF-GMa binds to sites in the IL-3 and GM-CSF enhancers which bear resemblance to the IL-2 site (see above). Therefore, we tested the possibility that this factor also binds to the IL-2 site. Figure 3 shows that the complex bound by the IL-2 oligo can be competed with the IL-2 oligo but not with either the IL-3 or the GM-CSF oligos. The complex bound by the GM-CSF oligo is slower migrating

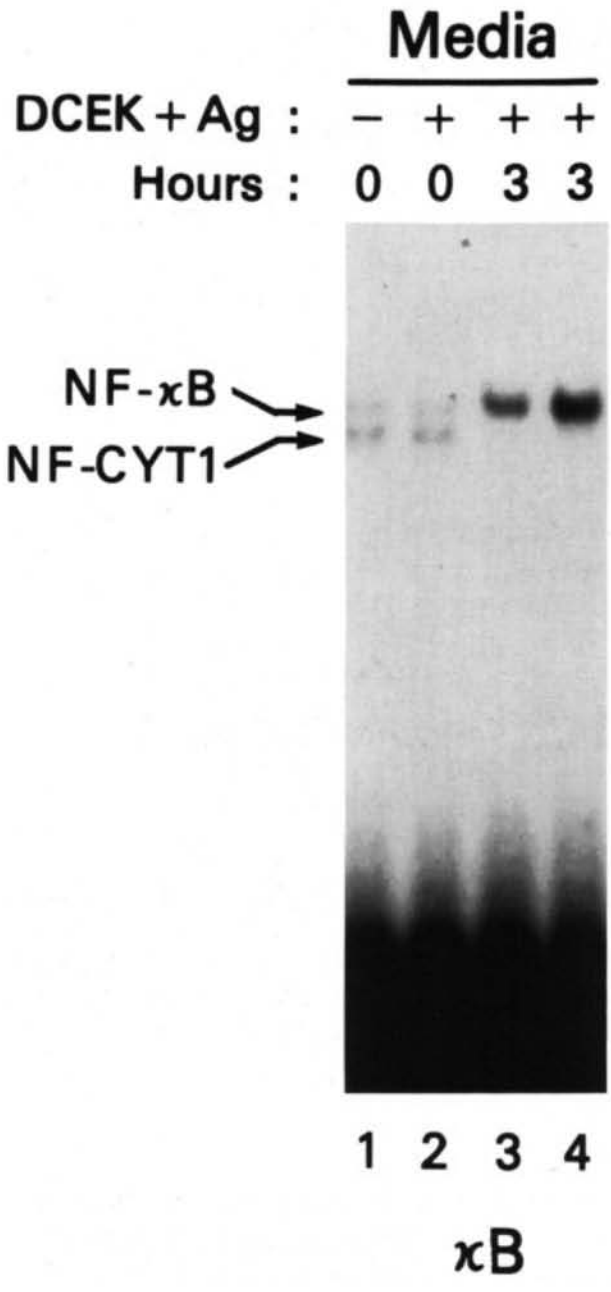

Fig. 1. EMSA using the κB site from the κ-light chain enhancer used as radiolabelled probe. *Lanes 1*, and *2*, unstimulated A.E7 extract (lane has antigen presenting cells but no antigen (denoted DCEK); *lanes 3* and *4*, extracts from A.E7 stimulated for 3 h with 10 μ*M* pigeon cytochrome C (frag. 81–104) and antigen presenting cells. Gel conditions essentially as described in Staudt et al. [13]

than that bound by the IL-2 oligo; further, it is competed for by the GM-CSF and IL-3 oligos (lanes 11–14), but not by the IL-2 oligo (lanes 15–17). Thus, NF-CYT1 has different binding characteristics from NF-GMa.

Loss of NF-CYT1 Binding Activity is Correlated with IL-2 Production

To explore the regulation of NF-CYT1 more fully, we analyzed nuclear extracts from cells which do and do not make IL-2 (Fig. 4). In the A.E7 clone which is an IL-2 producer, a dramatic decrease in NF-CYT1 activity (the lower band in lanes 1–4) can be seen with antigenic stimulation. The decrease is seen as early as 1.5 h after antigenic stimulation, and appears to plateau at 3 h. Note that the reference lane (lane 1) is not a 0-h time point, but a 4.5-h time point after incubation with antigen presenting cells, without antigen. We have reproducibly observed that binding activity of NF-CYT1 diminishes only in the presence of antigen (S. Kang and M. Lenardo, unpublished results). Also note that the level of NF-κB (the light upper complex in lanes 1–4) in the 4.5-h/no antigen lane appears to be at least as high as at 4.5 h with antigen. In contrast to NF-CYT1, which requires antigen for modulation, we have found that NF-κB can be induced in the presence of antigen presenting cells alone (S. Kang and M. Lenardo, unpublished results). This finding underscores the differential regulation of NF-CYT1 and NF-κB.

The D10 T-cell clone is a murine $CD4^+$ lymphocyte of the Th2 phenotype: it produces IL-4, IL-5, and IL-6, but not IL-2 or gamma-interferon [4]. We stimulated the D10 clone with concanavalin A (sufficient for full induction of lymphokine activity in these cells) and prepared nuclear extracts at the times indicated (lanes 12–15). NF-CYT1 activity in these cells does not decrease with stimulation but rather appears to increase.

Other non-IL-2 producing cells were tested for NF-CYT1 binding activity. Figure 4 shows that significant NF-CYT1 binding activity is found in BJAB cells (a human B-cell lymphoma), phorbol ester (PMA) stimulated HELA cells (a human cervical carcinoma), and DCEK cells (a mouse fibroblast line). The bands of higher mobility than NF-

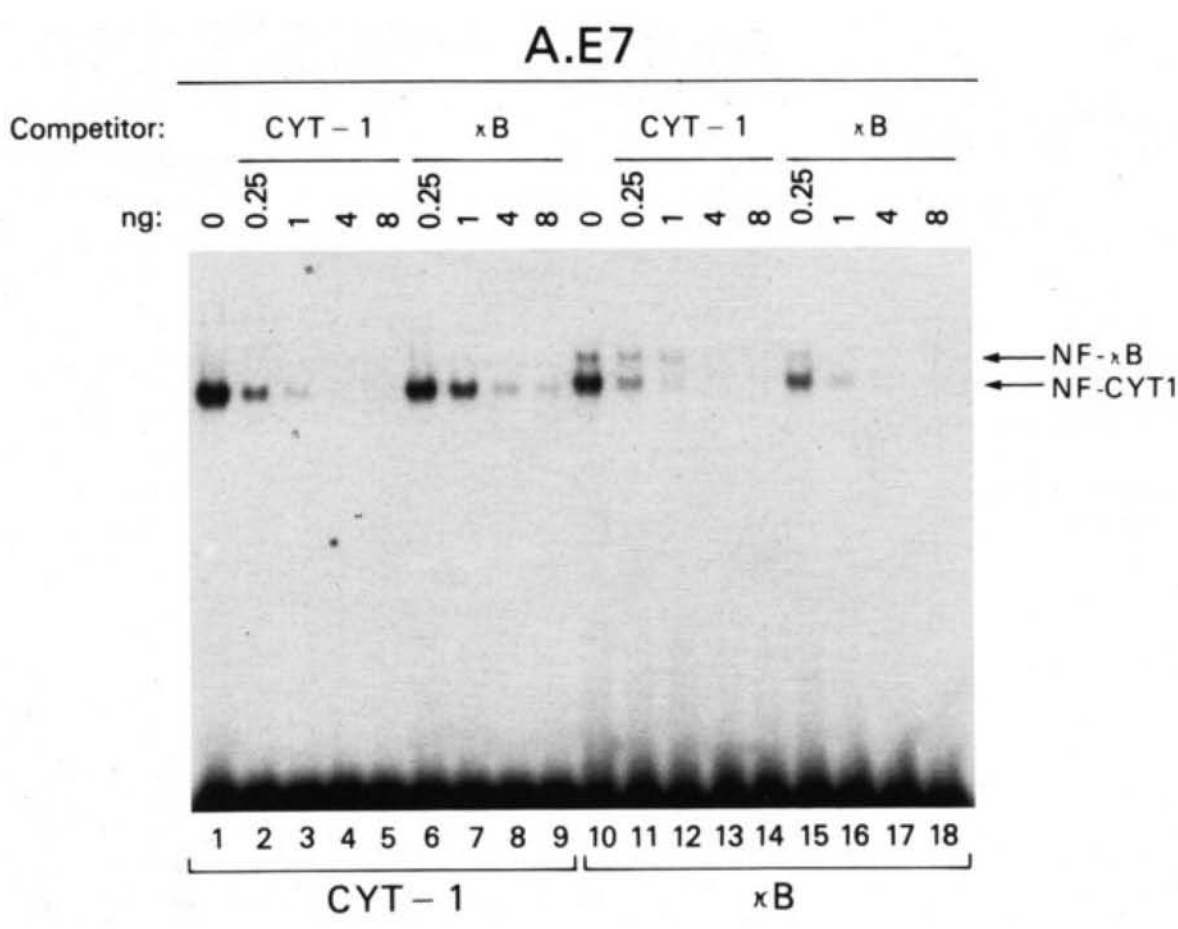

Fig. 2. EMSA: extract from resting A.E7 T-cell clones. *Lanes 1–9*, IL-2 site used as labelled probe; *lanes 10–18*, κB site from the κ-light chain enhancer used as labelled probe. Competitions as noted above the lanes, representing approximately 2.5-, 10-, 40-, and 80-fold molar excess. Sequences of synthetic oligos (with Sal 5′ overhangs): IL-2 site TCGACCAAGAGGGATTTCACCTAAAT CC and κ-light chain site TCGACAGAG GGGACTTTCCGAGAGGG

CYT1 are non-specific, as assayed by competition studies (not shown).

In summary, we have found the decrease in NF-CYT1 activity only in a T-cell clone which produces IL-2. We have also found significant binding activity in non-T cells.

NF-CYT1 is Regulated Differently in Transformed T cells

We examined the regulation of NF-CYT1 activity in the well-studied T-cell tumor lines EL-4 (a mouse thymoma line), and Jurkat (a human T-cell line) (Fig. 4). Both lines produce IL-2 upon stimulation with PMA, and phytohemagglutinin (PHA) + PMA, respectively. In EL-4 cells, a significant amount of binding activity was seen in unstimulated extracts (lane 8). In contrast to A.E7, however, the binding activity was increased with stimulation at 3 h (lane 9). A similar result was obtained in the Jurkat line (lanes 10, 11). Thus, the regulation of NF-CYT1 in the transformed T cells appears to differ from that found in normal T-cell clones. The answer may lie in the observation that these tumor lines have different kinetics of IL-2 induction than in the T-cell clone A.E7: for example, IL-2 message peaks at 4 h in A.E7 and at 8 h in EL-4. Thus, transcriptional events may be taking place at a later time point. Studies are underway to determine whether the difference in regulation of NF-CYT1 between normal and transformed T cells is simply a kinetic one.

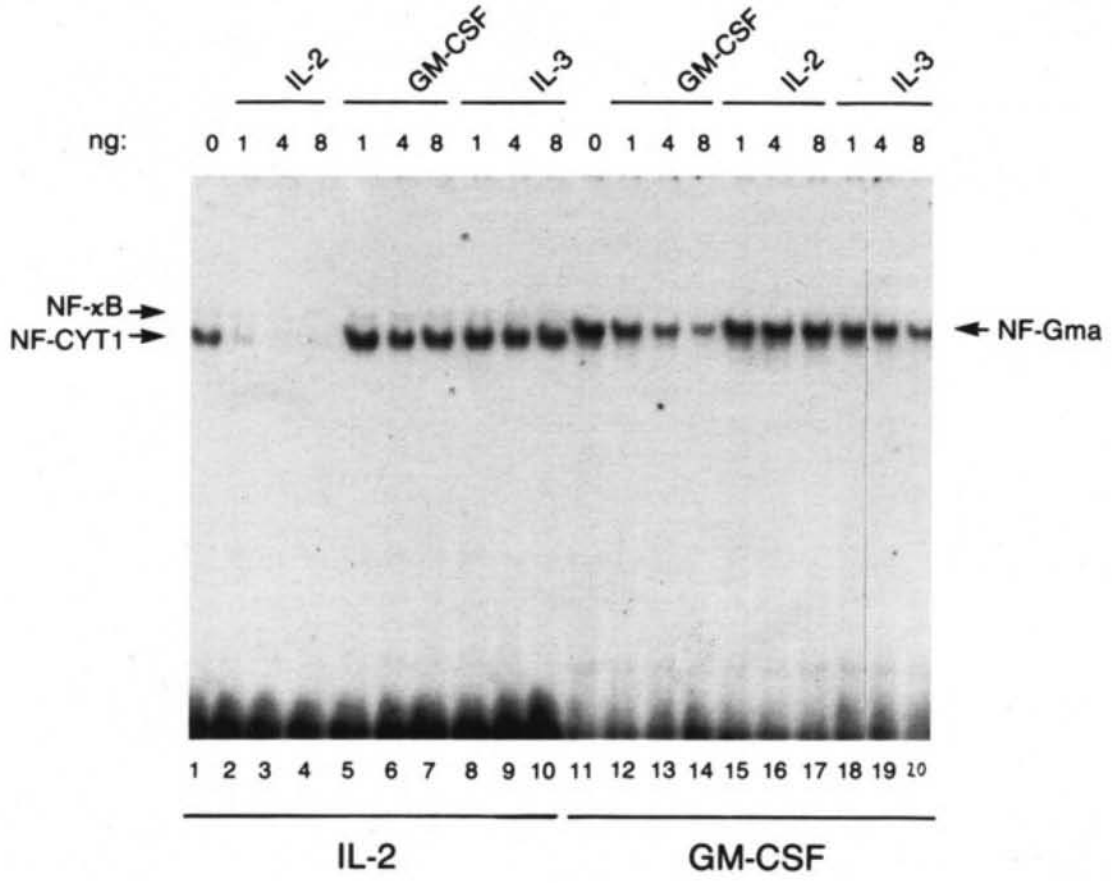

Fig. 3. EMSA: PMA stimulated EL4 extract used in all lanes. *Lanes 1–10*, IL-2 site used as labelled probe; *lanes 11–20*, GM-CSF site used as labelled probe. Competitors are noted above the lanes, representing approximately 10-, 40-, and 80-fold molar excess. GM-CSF oligo: TCGAAGGGCCAGGAGATTCCA CAACT. IL-3 oligo: TCGAAGGATGAGA TTCCACTGCATA

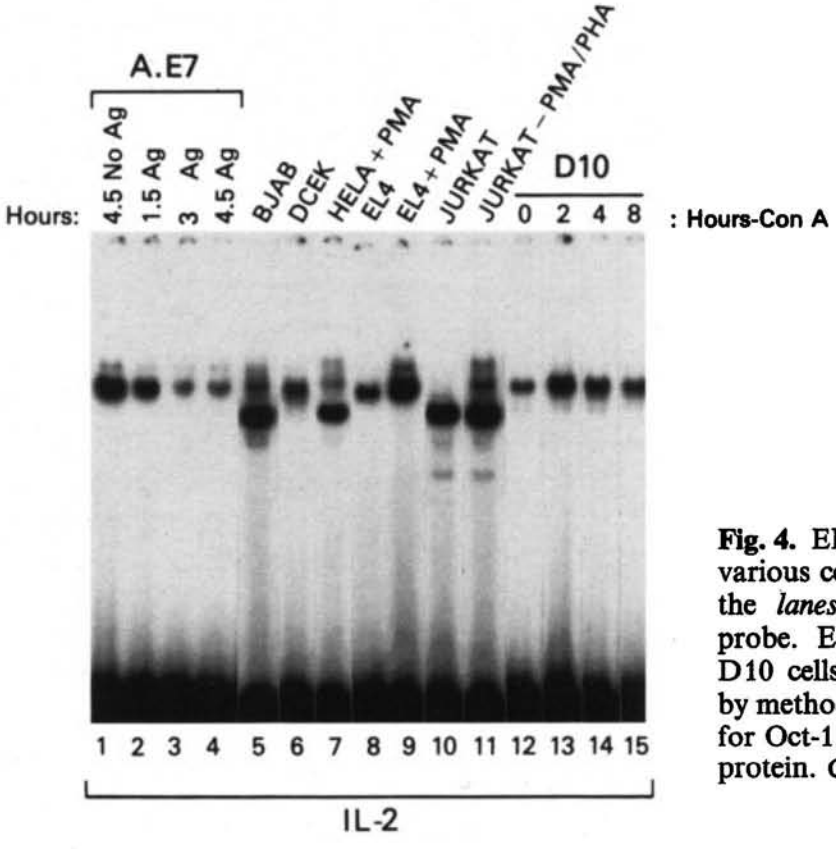

Fig. 4. EMSA: comparison of CYT1 in various cell types. Extracts noted above the *lanes*.. IL-2 site used as labelled probe. Extracts from A.E7 cells and D10 cells were normalized for protein by method of Bradford, and by assaying for Oct-1, a ubiquitous and constitutive protein. *Con A:* concanavalin A

Table 1. DNA binding proteins of the κB site family

Factor	Site	Genes regulated	Distribution	Inducers	Mode
NF-κB	GGGACTTTCC (most frequent)	Igκ light chain β-interferon, TNF-α IL-2 receptor-α, *HIV* IL-6, lymphotoxin serum amyloid A, angiotensinogen	Precursor is ubiquitous; constitutive – B and some T cells	Phorbols TNF-α viruses ds RNA IL-1, $p40^{tax}$	Post-translational
H2- TF1	GGGGATTCCCC	MHC H2-K^b	ubiquitous	LPS	N.D.
KBF-1	GGGACTTTCC	β_2-microglobulin MHC H2-K^b	?ubiquitous	N.D.	N.D.
HIVEN86A	GGGACTTTCC	IL-2 receptor-α, HIV	?T lymphocytes	PMA/PHA	N.D.
EBP-1	GGGACTTTCC	HIV, SV-40	?ubiquitous	N.D.	
PRDII-BF1 MBP-1	GGGAAATTCC	?β-interferon	almost ubiquitous	serum viruses	transcriptional
NF-CYT1	GGGATTTCACC	IL-2	almost ubiquitous	Ag + APC (decrease levels)	N.D.

Summary

We have identified a novel nuclear factor, NF-CYT1, which is present in both T and non-T cells and binds with highest affinity to the κB motif in the IL-2 gene. NF-CYT1 appears to bind with approximately 6-fold lower apparent affinity to the NF-κB sites in the κ-light chain enhancer, the HIV LTR, and the IL-2R alpha chain enhancer. Preliminary studies appear to implicate the purine residue in the second half-site as the major contributor to the binding specificity (Kang et al., unpublished results).

We believe that NF-CYT1 is a new member of the "κB site binding" family (Table 1). We show here that NF-CYT1 is distinct from NF-κB and NF-GMA, based on affinity and regulatory differences. We believe that NF-CYT1 is not related to H2-TF1, KBF-1 or EBP-1 because these have been found to be constitutive factors. The relationship to HIVEN86A and PRDII-BF-1 is unclear because these factors have not been demonstrated in mobility shift assays. It will be interesting to compare the structural differences between NF-CYT1 and NF-κB once they are cloned, to help understand how two factors can bind to similar sequences, but with reciprocal affinities.

The mechanism of the decrease in binding activity of NF-CYT1 and how it is restricted to activated TH1 cells is unknown. The rapid kinetics of the decrease make a posttranslational mechanism more likely. Studies may provide insights into the signal transduction pathways involved in T-cell activation.

NF-CYT1 is the first κB-motif binding factor shown to decrease in binding activity with cellular activation. In fact, it may be the first transcription factor that apparently decreases during lymphocyte activation. The functional implications of this decrease are unclear at present. However, the regulation of NF-CYT1 in various cell types is consistent with that of a repressor molecule. The observation that NF-CYT1 binds with highest affinity to the IL-2 site and only decreases with activation in T cells which make IL-2 suggests that NF-CYT1 may be important in keeping IL-2 gene transcription off in situations inappropriate for IL-2 production. However, NF-CYT1 may also play a modulatory role in the transcription of genes to which it can bind with lower affinity. Functional studies will serve to define the interplay of NF-CYT1 and NF-κB in the transcriptional regulation of both T- and non-T-cell restricted genes.

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