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Li, Mian, Ph.D.

City University of New York, 1993

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CONTROL OF THE INTERLEUKIN-2 PROMOTER BY THE HTLV-I TRANSACTIVATOR

by

MIAN LI

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1993

1993

MIAN LI

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Abstract

CONTROL OF THE INTERLEUKIN-2 PROMOTER BY THE HTLV-I TRANSACTIVATOR

by

Mian Li

Advisor: Miriam Siekevitz, Ph.D.

The 40-kDa nuclear protein *tax* encoded by human T cell leukemia virus type I (HTLV-I) can transcriptionally activate the interleukin-2 (IL-2) enhancer and prevent inhibition of IL-2 gene expression by the immunosuppressant Cyclosporin A (CsA). We have identified a *tax* responsive element (TxRE) from -164 to -145 bp in the IL-2 enhancer which is sufficient to confer *tax* responsiveness. A 45-kDa nuclear protein (TxREF), which is expressed in Jurkat-*tax* cell lines but not in Jurkat cells without *tax*, specifically interacts with 5'TxRE sequences from -164 to -154. Deletion or mutation of 5'TxRE removes the binding of TxREF *in vitro* and dramatically reduces *tax* activity *in vivo*. Although the TxREF binding site contains an NF-kB like motif, TxREF is distinct from NF-kB. While the TxRE and NF-kB sites contribute to *tax* plus PMA inducibility of the IL-2 enhancer, the TxRE and NFAT sites are the important sites contributing to the synergistic effect of *tax* plus PHA inducibility of the IL-2 enhancer. These results demonstrate that TxREF is a novel *tax* inducible DNA binding protein and that TxRE plays a crucial role in

mediating *tax* induced IL-2 gene expression.

ACKNOWLEDGEMENTS

I am extremely grateful to my parents and my wife for their continual help and encouragement.

I would like to thank Dr. Miriam Siekevitz for guidance in my scientific research.

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INTRODUCTION

1. Transforming properties of *tax*

HTLV-I is the etiologic agent of human Adult T cell Leukemia/Lymphoma (ATL) which is endemic in certain parts of Japan and the West Indies and is found sporadically elsewhere in the world. The transforming properties of HTLV-I have been the focus of intensive study. Unlike acute transforming retroviruses, HTLV-I does not contain a cellular oncogene counterpart which can induce malignancy quickly; unlike most slow transforming retroviruses, HTLV-I lacks a selected proviral integration site which induces tumors after a long latent period through the aberrant activation of adjacent cellular genes. Hence the mechanism of transformation by HTLV-I may be unique among retroviruses (75).

Sequence analysis of HTLV-I has revealed that its genome is composed of the stuctural genes *gag, pol*, and *env* as well as two nonstructural viral genes, *tax* and *rex* (81). *Tax*, a 40kd protein, has been shown to increase viral gene transcription by activating its own long terminal repeat (LTR). Its activity is crucial for virus replication since *tax*-defective HTLV-I mutants are not infectious (82). In HTLV-I infected cells, *tax* is approximately 0.15% of the total protein estimated by immunoprecipitation and has a half life of 120 minutes. Most of the *tax* protein in HTLV-I infected cells is found in the nucleus. *Tax* is not detectable in the virion, suggesting that *tax* is not packaged in significant amounts in the virus (84).

The discovery that *tax* can activate the transcription of cellular genes,

including IL-2Ra, IL-2 (80), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) (60), and c-*fos* (18), led to a model for HTLV-I transformation: After HTLV-I infects cells, cellular transcription factors help the provirus to make small amounts of the viral regulatory proteins *tax* and *rex*. *Tax* and *rex* then function on the viral genome to increase transcription establishing the infectious stage (97). Once a cell is productively infected, *tax* induces the expression of a set of cellular genes that are required for T cell growth, in particular IL-2 and IL-2Ra. This deregulation of normal IL-2 and IL-2Ra gene expression may result in autocrine or paracrine T cell growth even in the absence of antigen. Unregulated T cell growth may both facilitate the spread of viral infection and initiate the process of T cell transformation. Eventurally IL-2 is no longer needed for continued cell growth and *tax* is undetectable in freshly isolated leukemic cells. Thus, *tax* may be responsible for initiating the transformation process, but it may not be required for maintaining this process. Furthermore, it has been shown that HTLV-I particles have a mitogenic effect on T cells, suggesting that a combination of various abnormal signals and multiple steps (for example, chromosomal breakage) are required to establish transformation (100).

HTLV-I may also cause HTLV-I associated myelopathy or tropical spastic paraparesis (HAM/TSP). In contrast to late-stage acute ATL, HAM/TSP may represent an early autocrine phase of HTLV-I infection in which *tax* induces the production of IL-2 and its receptor, leading to polyclonal T cell activation with subsequent inflammatory nervous system damage (87,98).

Direct evidence of the transforming properties of *tax* come from several observations. Mitogen-activated cord blood lymphocytes and thymocytes transfected by a *tax*-expressing Herpesvirus Saimiri virus vector can be immortalized *in vitro* and have the same phenotype (CD4+, CD5+, HLA ClassII+, IL-2Ra+) as lymphocytes transformed by cocultivation with HTLV-I (29). In addtion, mice expressing *tax* as a transgene developed neurofibromas (30). It is unclear, however, why the tumors found are mesenchymal rather than lymphoid in origin. *Tax* can also cooperate with the *ras* oncogene to induce neoplastic transformation of rat embryo fibroblasts (68,86).

2. Targets of *tax* activation

A. *Tax* activation of the HTLV-I LTR

The earliest experimental evidence implicating *tax* as a viral gene transactivator was from Haseltine *et al* (84). *Tax* expression vectors were made and cotransfected into various cell lines with constructs containing the chloramphenicol acetyltransferase (CAT) gene under the control of the HTLV-I LTR. Cotransfection with *tax* led to a marked increase in the level of HTLV-I LTR directed CAT gene expression. Shimotohno *et al.* (79) further defined the target for tax activation in the HTLV-I genome as the conserved 21 base pair (bp) repeats within a pair of 51bp repeats in the U3 region of the LTR. Two copies of the 21-bp sequence are sufficient to confer *tax* responsiveness. Although the orientation of the 21-bp

sequence in the LTR did not affect CAT activity, the space between each 21-bp repeat may affect CAT activity. Tandem repeats of a 27-bp segment, which includes the 21-bp sequence, have slightly less CAT activity than corresponding constructs with an equal copy number and the same orientation of the 21bp sequence.

The HTLV-1 LTR contains several sequences which share homology with the cyclic AMP responsive element (CRE) and with an AP-1-like element. Both cAMP and PMA have been shown to activate the HTLV-I LTR. The sequence requirements for the activation by *tax*, cAMP and PMA were examined to elucidate the mechanism of *tax* activity (67,20,70). Although some of the sequences required for cAMP induction of the HTLV-I LTR are within the 21bp repeat and are also part of the sequence responsive to *tax*, these two inducers function through different pathways. For example, *tax* does not require elevated levels of cAMP for activity and cAMP can augment saturating levels of *tax* activity. An element containing the 8-bp dyad-symmetric consensus CRE, TGACGTCA, is not *tax* responsive. Moreover, increases in LTR activity in response to cAMP range between 3 to 7 fold, whereas a 100 fold increase was found in response to *tax*. It also appears that there are separate pathways for PMA and *tax* activation. The sequences required for the response to PMA have been shown to be within a 60bp region which spans 42nts from one copy of the HTLV-I 51bp motif and 18nts from the adjoining 51bp element. This region contains one copy of the 21bp motif which includes the AP-1-like element. One copy of the 60bp sequence was not

responsive to *tax* but was responsive to PMA. Tandem repeats of the 21bp motif placed upstream of a SV-40 promoter-CAT construct were responsive to *tax* but not to PMA. The nuclear proteins which bind the 60bp PMA responsive element are different before and after TPA treatment as shown by gel retardation analysis and DNase I footprinting. Although there is a putative AP-1 sequence, purified AP-1 cannot footprint this site. These results indicate that the sequences required for activation by cAMP, PMA and *tax* overlap each other, but are not identical. Mutational analysis of the 21bp sequences indicated that the bases TGACG are crucial for *tax* activity and the putative consensus sequence of the *tax*-responsive elements in the LTR is (A/T)(G/C)(G/C)CNNTGACGC(T/A) (85).

Tax does not appear to bind DNA directly. Rather, it may modify the activity of DNA binding proteins. A number of proteins from Hela cells or lymphoid cells that bind the HTLV-I 21-bp-repeat have been identified using DNA affinity chromatography or UV crosslinking of nuclear proteins to the DNA. Although the TGACG sequence is crucial for the binding activity of these cellular proteins (67,85), it is not clear whether these proteins are directly involved in *tax* mediated transactivation. Purified recombinant *tax*, when added to a reaction mixture containing radiolabeled HTLV-I 21-bp repeat and purified Hela proteins that can bind the 21-bp repeat, increases the binding of one of the protein-DNA complexes to the core sequence TGACG as detected by gel shift mobility assay (50). In addtion, purified *tax* protein, when added into a reaction mixture containing radiolabeled HTLV-I LTR 21-bp repeat and nuclear proteins from Jurkat T cells,

can interact with the proteins bound to the 21-bp repeat to form a protein-DNA complex of apparent higher molecular weight (101). Hence, *tax* may activate transcription of the HTLV-I LTR may through direct interaction with constitutively expressed cellular factors that bind to the 21-bp repeat (50,63,101).

B. Tax activation of cellular gene promoters

IL-2Rα

It would be of interest to find the cellular genes regulated by *tax* since these genes may be directly involved in the HTLV-I transformation of normal cells. *Tax* transactivation of the IL-2Ra gene has been studied in detail. (*Tax* transactivation of the IL-2 gene will be discussed under section 3. IL-2 gene regulation). Transient transfection assays were performed using the Jurkat $CD4^+$ T lymphocytic leukemia cell line, which shares with normal T cells a similar pattern of gene activation following mitogenic stimulation. Either PHA or PMA alone is sufficient to induce IL-2Ra gene expression. Siekevitz *et al* (80) showed that *tax* can stimulate a 3- to 6- fold increase in IL-2Ra promoter activity in Jurkat T cells cotransfected by *tax*-expression vectors and CAT reporter constructs. Stable expression of *tax* in permanently transfected Jurkat T cells also leads to the activation of the IL-2 and IL-2Ra genes (95). *Tax* activates IL-2Ra gene expression, in part, by increasing the binding of a cellular protein, HIVEN86, to the nuclear factor kappa B (NFkB) motif (GGGGAATCTCCC) in the promoter region (3) (See section 3 for discussion of NF-kB). Deletion of this region abolishes CAT

activity induced by *tax*. A 18-bp IL-2Ra promoter segment spanning the NF-kB motif is sufficient to confer *tax* inducibility (7 to 10 fold) to the Herpes Simplex Virus (HSV) thymidine kinase (TK) promoter in Jurkat T cells stably expressing tax. The stimulatory effects were independent of insert orientation and were amplified when the segment was reiterated. Two dimensional gel analysis and UV-cross linking revealed that, in addition to HIVEN86, there are six proteins interacting with the NF-kB motif. HIVEN86 is only present in *tax*-expressing T cell lines but not in antisense-*tax* T cell lines. The interaction between HIVEN86 and the other peptides bound to the NF-kB motif may be required for the *tax*-mediated activation of the IL-2Ra gene. HIVEN86 is also involved in the mitogen-mediated activation of the IL-2Ra gene through the NF-kB motif, but additional sequences located upsteam of the NF-kB element are required for maximal mitogen activation, although not for maximal *tax* activation (3).

The importance of *tax* activation of NF-kB to cellular transformation can be demonstrated by inhibiting NF-kB expression with anti-sense oligodeoxynucleotides. The growth of culture-adapted *tax*-transformed mice fibroblasts and an HTLV-I transformed human lymphocyte line were both inhibited by this treatment (41). Mice treated with antisense oligodeoxynucleotides to NF-kB experience rapid regression of transplanted fibrosarcomas, suggesting that NF-kB activity is necessary for maintenance of the malignant phenotype (41).

GM-CSF is a hematopoetic growth factor that stimulates the proliferation of myeloid progenitor cells and enhances the functional capacity of mature myeloid

effector cells. Expression of GM-CSF by HTLV-I infected lymphocytes may contribute to the granulocytosis and eosinophilia frequently seen in patients with HTLV-I induced ATL (60). Like IL-2 and IL-2Ra, GM-CSF is expressed by activated T lymphocytes. HTLV-I infected T cell lines constitutively produce GM-CSF, in contrast to uninfected T cells lines which only express GM-CSF when stimulated by mitogens. Using transient co-transfection assays, Nimer. *et al* identified a 90-bp region (-53 to +37) in the human GM-CSF promoter, which itself is sufficient to confer *tax* responsiveness. A 22-bp sequence within this region is required for *tax* responsiveness as well as for mitogen induction. CATT(A/T) repeats within this 22-bp-region are critical in the response to *tax* (59). Unlike the IL-2Ra promoter, the NF-kB domain in the GM-CSF promoter region can be deleted without loss of *tax* transactivation. In the mouse GM-CSF promoter, NF-kB like sequences and the cytokine-1 (CK-1, GGAGATTCCA) and cytokine-2 (CK-2, TCAGGTA) sequences are required for *tax* inducibility (54,73,77). However, when they were examined in the context of the human GM-CSF promoter, they did not have *tax* responsive regulatory activity .

c-*fos* gene transcription is also stimulated by *tax* in a variety of cell types including Hela carcinoma cells and HepG2 hepatoma cells (18). The sequences responsible are not well defined, but upstream regions (-362 to -276) in the promoter are responsive to *tax* activation. This DNA fragment contains multiple elements including a dyad symmetry element that encompasses the serum responsive element, an AP-1 like element, the v-*sis* conditioned-medium

responsive element and an E1a-inducible element. Fujii *et al* (19) demonstrated that the important sequence in this enhancer for *tax* activation is "GC(AT-rich)GG" (CArG Box), which forms part of the dyad symmetry element and is also required for induction of the c-*fos* gene with serum and other growth factors. $p67^{SRF}$, a CArG-binding factor which is constitutively localized in the nucleus, was fused with a DNA binding domain from GAL4. The carboxy-terminal portion of $p67^{SRF}$, which is required for protein-protein interaction, is sufficient to direct *tax* mediated activation as measured by cotransfection assays (19). The DNA binding domain and dimerization domain of $p67^{SRF}$ were dispensable, suggesting that $p67^{SRF}$ mediates transcriptional activation through interaction with *tax* (19).

The critical region in the TGF-ß promoter which is required for *tax* induction contains sequences which are similar to an AP-1 binding site (40).

Comparison of the sequences required for *tax* induction in the HTLV-I LTR and the GM-CSF and IL-2Ra promoters show no consensus sequences, implying that *tax* acts in an indirect fashion by interacting with or modifing cellular transcriptional factors rather than by direct binding to DNA. Smith *et al* reported that *tax* transactivation of an NF-kB site containing promoter and the HTLV-I LTR appears to be mediated through different functional domains of *tax*, suggesting that *tax* transactivates viral and cellular promoters through at least two distinct mechanisms of host transcription factor activation (83).

3. IL-2 gene regulation

IL-2 gene expression is restricted to activated T lymphocytes. Two signals are required for optimal IL-2 production. One signal is through T cell receptor (TCR)-antigen interaction and can be substituted by PHA, Concanavalin A (ConA) or anti-CD3 monoclonal antibody. The second signal is through accessory cells or mitogens that activate protein kinase C (PKC). Human IL-2 gene expression is regulated at the level of transcription initiation (43). It is under the control of a promoter region between -319 and -52 upstream of the transcription initiation site (13). A number of sites in this promoter region were initially characterized as nuclear protein binding sites (12,9). They are from -89 to -73 (A), -158 to -135 (B), -208 to -188 (C), -255 to -217 (D), and -279 to -263 (E) (Fig. 1). Deletion mutants which encompass the A, B, D, or E sites had approximately 5%, 11%, 41%, or 31% of wild type activity when tested by transfection into Jurkat T cells (12). Deletion of the C site and the surrounding sequences showed no diminished CAT activity relative to control wild type, but site-directed mutagenesis in this site impairs mitogen-mediated activation by 80% (32).

The sequences responsible for the signal initiated from the TCR are around the A and E sites, also called antigen receptor responsive element-2 (ARRE-2) and antigen receptor responsive element-1 (ARRE-1), respectively. "Nuclear factor of activated T cells", NFAT, which is only present in activated T cells, binds to ARRE-2 which is located between -285 and -255 (78). NFAT is composed of two subunits (61). NFAT-C is T cell-specific and confined to the cytoplasm in unactivated cells. The other subunit, localized in the nucleus by activation of PKC,

is ubiquitous. Both jun/fos and junB/fra-1 have both been reported to be the nuclear component of NFAT (93,7). Tandem repeats of the NFAT-binding sequence are sufficient to confer inducibility from signals initiated at the TCR and to make a promoter T cell specific (78). ARRE-1, located between -93 to -63, shares sequence homology with the octamer motif and binds to the constitutively expressed Oct-1 transcription factor and an inducible protein OAP[40]. Tandem repeats of this sequence also lead to increased activities in response to signals initiated at the TCR (12).

The sequences which respond to the activation of PKC by PMA are less precisely defined, since several sites may independently contribute to the PKC response. The NF-kB motif is crucial for IL-2Ra gene activation by mitogens or by the HTLV-I *tax* protein. It is also required for the maximal induction of the IL-2 gene by mitogens (31). One of the sequences in the C site (-206 to -195, AGGGATTTCACC) has an NF-kB motif and binds at least two NF-kB-specific proteins induced by either PHA or PMA, a 51kDa protein and HIVEN86, as shown by Hoyos *et al.* (31). Tandem repeats of the 12bp NF-kB motif are not sufficient to confer PMA-induced CAT activity, although a 25 bp element surrounding this motif is induced by PMA plus *tax* (32). NF-kB was originally discovered as a B cell specific transcription factor regulating immunoglobulin kappa gene expression via the NF-kB sequence (GGGACTTTCC) in the k gene enhancer. It has since been found in many other cell types. NF-kB activity resides in a heterotetrameric protein complex including p50 plus p85 or p55 plus p65-75. Both forms of NF-kB bind to

DNA and are NF-kB-sequence specific (56). These four proteins are immunologically related to the *v-rel* oncoprotein. p85 appeares to be identical to the *c-rel* protein. All of these proteins are found in the cytoplasm of resting T cells, where they are in an inactive form which is bound to an inhibitor protein IkB. Upon induction by various agents including PMA, IkB becomes phosphorylated . As a result, NF-kB dissociates from IkB and migrates to the nucleus to bind DNA. PMA induction of NF-kB is biphasic, with the 55-kDa and 75-kDa species appearing in the nucleus within minutes, whereas the 50-kDa and 85-kDa species appear only several hours after cellular stimulation (55).

There is an AP-1 like sequence -AGAGTCA- in the B site. It is not clear what protein(s) interacts with this AP-1 like sequence since the core AP-1 like sequence "AGAGTCA" does not bind purified c-Jun/Fos heterodimer (71). However, a nuclear factor from an untransformed murine T cell clone Ar-5 binds to this site in the mouse IL-2 promoter (36). This binding disappears after prolonged PMA treatment of Ar-5. It is difficult to correlate this observation to the potential binding activity in the human IL-2 enhancer. There is also an NF-kB like sequence in the B site upstream of this AP-1 like sequence. The role of this site regarding PKC responsiveness awaits further study.

The activation of the IL-2 gene can be inhibited by Cyclosporin A (CsA). Cyclosporin A, a cyclic fungal undecapeptide produced by *Tolypocladium inflatum*, is an immunosuppressive drug used widely in preventing rejection of transplanted organs. The immunosuppressive activity of CsA may be due to the inhibition of

lymphokine production by activated T cells. IL-2, IL-4 and gamma-interferon production are specifically inhibited by CsA (14,76). CsA does not inhibit the immediate membrane events associated with triggering the antigen receptor such as IP3 generation or calcium mobilization but does inhibit their downstream events. Kronke *et al* showed that IL-2 mRNA accumulation in induced Jurkat cells is diminished by CsA in a dose dependent manner and CsA acts by blocking IL-2 mRNA transcription initiation (43). The mechanism of CsA inhibition of IL-2 gene expression is due partly to the impaired binding of NFAT to the E site and the impaired activity of Octamer-binding proteins at the A site (74). Both OAP^{40} and the nuclear component of NFAT are members of the jun family (93). Crabtree *et al.* (93) proposed that the activity of jun proteins may be regulated by two different pathways which respond differently to PMA. The CsA sensitive pathway, which regulates both OAP^{40} and NFAT activity, is triggered by the influx of calcium. The CsA resistent pathway activates PKC to increase AP-1 activity (93).

Intracellular immunophilines, which are identical to the rotamases which catalyze the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate slow rate-limiting steps in the folding of several proteins (91), can bind CsA as well as the other immunosuppressants FK506 and rapamycin. However, the isomerase activity is not correlated to immunosuppresive activity. The Immunophiline-CsA complex can also bind calcineurin A which is a Ca^{++}- and calmodulin-dependent protein phosphotase. The relevence of the interaction between calcineurin and immunophilines to immunosuppression is not clear. It has

been proposed that the activity of calcineurin A may control NFAT-C translocation into the nucleus (72). Erlanger proposed that the important receptor of CsA is a lymphocyte cell-surface receptor but not a cytosolic immunophiline (15).

Nuclear factors similar to those studied in Jurkat T cells which interact with the A, B, C, and E sites were identified in primary T cells from human periphral blood by Granelli-Piperno *et al* (25,26). In addtion to NFAT binding of site E, the binding activity of a nuclear factor which bound site B after PHA plus PMA treatment was also inhibited by CsA (27). The identification of this factor is unclear.

CD28, a 44kd homodimeric glycoprotein expressed on 80% of human peripheral blood T cells, is involved with other signal transduction pathways triggered from the TCR during T cell activation. Costimulation of resting T cells with either anti-CD3 plus anti-CD28 or PMA plus anti-CD28 can increase the levels of IL-2, gamma-IFN, and GM-CSF in culture supernatants by inhibiting the degradation of lymphokine mRNAs (39). This stimulation occurs even in the presence of CsA. Increased production of these cytokines may also result from increased transcription, since Fraser *et al.* (17) showed that stimulation of Jurkat T cells with anti-CD28 combined with ionomycin plus PMA can induce IL-2 promoter (-164 to -51) activity fivefold. They propose that a protein complex induced by this combination of stimuli binds to the promoter at -164 to -154 and may be involved in the anti-CD28 activation process. GM-CSF, IL-3, and gamma-IFN promoter activities were also increased 3-6 fold by stimulation of CD28 in the presence of TcR-like signals (16). The IL-2 CD28RE (-160 to -152)-homologous

sequences contained within these promoters are required for CD28-induced activity. Verweij *et al.* showed that anti-CD28 plus anti-CD3 can also increase IL-2 enhancer activity 6 fold. They reported that an NF-kB like factor is induced by anti-CD28 plus anti-CD3 or anti-CD28 plus PMA and binds the IL-2 promoter from -162 to -150 (94).

The pronounced inhibitory effect of CsA on IL-2 gene expression can be circumvented by the HTLV-I *tax* protein. *Tax* alone has little or no effect on IL-2 promoter (-584 to +51) activity but markedly synergizes with other mitogenic stimuli (PHA, PMA or anti-CD3 monoclonal antibody), which alone are ineffective. Either *tax* plus PHA or *tax* plus PMA can activate IL-2 promoter expression in the presence of CsA at a concentration which totally inhibits IL-2 gene expression induced by PHA plus PMA. This circumvention effect displayed by *tax* is believed to occur at the transcriptional level (78). The mechanism of transcriptional regulation of the IL-2 enhancer by *tax* is unclear. Our previous study showed that an NF-kB site contributes to the *tax* inducibility of the IL-2 enhancer (31). The other cis-elements in the IL-2 enhancer mediating *tax* responsiveness are less well studied. We report here that we have identified another *tax* responsive element (TxRE) from -164 to -145 in the IL-2 enhancer. Mutation of constructs containing this sequence and internal deletions of the IL-2 enhancer further indicates that the 5'TxRE sequence from -164 to -154 is crucial for *tax* induction of IL-2 gene expression. A 45-kDa nuclear protein (TxREF), which is expressed in Jurkat-*tax* cell lines but not Jurkat cells without *tax*, specifically interacts with TxRE. Although

the binding site contains an NF-kB like motif, TxREF is a novel *tax* inducible DNA binding protein distinct from NF-kB.

MATERIALS AND METHODS

Cells and reagents

Jurkat, Jurkat-*tax* (J-*tax*-9 and J-*tax*-19), Jurkat-anti-*tax* (J-anti-*tax*-2 and J-anti-*tax*-10) human T cell lines (95), Hut 102 cells (Human cutaneous T cell lymphoma, HTLV-I infected, ATCC), Hut 78 cells (Human cutaneous T cell lymphoma, ATCC), and 5637 cells (Human bladder carcinoma cells, ATCC) were cultured in RPMI-1640 medium supplemented with 7% fetal calf serum, 2mM glutamine, 50U/ml penicillin, and 50mg/ml streptomycin. Hela, a human carcinoma cell line, was grown in minimum essential medium supplemented with 5% FCS, 2mM glutamine, 50U/ml penicillin, and 50mg/ml streptomycin. Phytohemagglutinin (PHA, Wellcome Diagnostics, England), phorbol 12 -myristate 13 -acetate (PMA, Sigma), Cyclosporin A (CsA, Sandoz Research Institute, NJ), ionomycin (CALBIOCHEM, CA), monoclonal anti-CD3 IgG2a (kindly provided by Dr. Tom Moran) and mouse anti-CD28 monoclonal antibody ascites 9.3 (9.3) (Bristol-Meyers) were used as described below.

DNA constructs

Oligonucletides were synthesized on an Applied Biosystems DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis as described (6). The sequences of the oligonucleotides are: BL (AAAGAAATTCCAAAGAGT-CATCAGAAGAGGAAAAATGAA; -164 to -126 within the IL-2 promoter); m2/4BL (AAAGAAATTTTAAATTGACATCAGAAGAGGAAAAATGAA); m4/5BL

(AAAGAAATTCCAAATTGACATCAGATATTTAAAAATGAA); BT (AAAGAA-ATTCCAAAGAGTCA; -164 to -145); m2BT (AAAGAAATTTTAAAGAGTCA); BU (AAAGAAATTCCA), ILAP-1 (AAGAGTCAT), AP-1 (ATGAGTCAT). BO, BM, BR are depicted in Fig. 4. All of the oligonucleotides contain GATC overhanging ends. They were subsequently cloned into the BamHI site at position -105 on a plasmid with the thymidine kinase (TK) promoter linked to the chloramphenicol acetyl transferase (CAT) gene. The TK promoter has a "CCAAT" box at position -80, a "GC" box at -56 and a "TATAA" box at -24 from the transcription start site (53). TK-CAT shows basal activity and is only weakly inducible by mitogens in Jurkat cells. The positive clones were screened by hybridization with radiolabeled oligonucleotides and confirmed by DNA sequencing. The constructs we used are outlined in Figure 4. The *tax* expression plasmid (spFMTLTR/82-2C) and the antisense-*tax* expression plasmid (spFMTLTR/62-6) are as described (80). pIL-2CAT contains DNA sequences from -576 to +42 of the IL-2 promoter (13). pAA-CAT contains two copies of the IL-2 promoter sequences from -326 to -164. pBB-CAT contains two copies of the IL-2 promoter sequences from -164 to -51. pNFAT-1 contains 3 copies of DNA sequence from -257 to -286. pOCT contains 4 copies of sequences from -94 to -65. pAA-CAT, pBB-CAT, pNFAT-1 and pOCT contain the gamma-fibrinogen promoter (12).

DNA transfection

The plasmid DNAs were prepared by Qiagen Max Kid (Qiagen Inc) based on instruction of the manufacturer or by the alkaline lysis method (48). Briefly, the

DNA was precipated by isopropanol, treated with RNase and phenol:chloroform, followed by precipitation by PEG. Supercoiled plasmid DNAs were transfected into cells by the DEAE-dextran method (69). Five micrograms of each DNA construct were transfected into 10^7 Jurkat T cells and cotransfected with either 5 micrograms of *tax* expression plasmid (*tax*) or antisense *tax* expression plasmid (anti-*tax*). Twenty four hours after transfection, PHA (1ug/ml), ionomycin (1uM), PMA (50ng/ml), CsA (1 ug/ml), anti-CD3 IgG2a (500ng/ml), anti-CD28 (9.3, 1:1000) or a combination of these reagents were added into the culture medium as described in the figure legends. Cells were collected after an additional 18 hours of incubation. CAT assays were performed as described (24). Protein concentration was determined by Bio-Rad protein assay. Results were normalized for total protein content from $2x10^6$ cells.

Nuclear extracts and gel retardation analysis

Cells were typically collected after 4 hrs induction with PHA (1ug/ml), PMA (50ng/ml), Ionomycin (1uM), anti-CD28 (9.3, 1:1000), CsA (1ug/ml) or a combination of these stimuli as described in the figure legends and treated with hypotonic buffer plus 0.2% NP-40. Nuclear extracts were made as described (14). Briefly, nuclei were pelleted by centrifugation. After consecutive .3M and 1.5M $(NH_4)_2SO_4$ precipitations, nuclear proteins were obtained. The protein concentration was determined by Bio-Rad protein assay. Alternatively, nuclear extracts were also made as described (11) with minor modifications. Briefly, 1 x 10^9 cells were incubated in buffer A (10mM Hepes, PH 7.9; 1.5mM $MgCL_2$, 10mM

KCL, and 0.5mM DTT) on ice for 10 minutes. Cells were then disrupted in a dounce homogenizer (type B). The nuclei were recovered by centrifugation and disrupted in buffer C (20mM Hepes, PH 7.9; 25% glycerol, 0.42 M NaCL, 1.5mM $MgCL_2$, 0.2mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) in a homogenizer. After 30 minutes incubation at 4^0C with gentle shaking, cell debris was removed by centrifugation. The supernatants were dialysed with at least 100 volumes of Buffer D (20mM Hepes, PH 7.9; 20% glycerol, 0,1M KCL, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT) for 2-3 hours.

The following double-stranded oligonucleotides containing overhanging GATC ends were labelled with P^{32}-dATP by fill-in with the Klenow fragment of DNA polymerase I: BT m2BT, ILAP-1 and AP-1 were described above. m1BT (AAGAGGATTCCAAAGAGTCA); m3BT (AAAGAAATTCCGGGAAGTCA); m4BT (AAAGAAATTCCAAATTGACA); HIV-kB (AGGGACTTTCC); IL2R-kB (GGCAGGGGAATCTCCCT); IL2-kB (AAAGAGGGATTTCACCT); IL6-kB (TGGGATTTTCCCA) (57); HT-21 (AAGGCTCTGACGTCTCCCCCC), NFAT (-286 to -257 of the IL-2 enhancer, GGAGGAAAAACTGTTTCATACAGAAGGCGT); Oct-1 (TTTGAAAATATGTGTAATATGTAAAACATTTTG); CK-1 (-103 to -79 of the GM-CSF promoter, AAGGGCCAGGAGATTCCACAGTT). Fifteen micrograms of nuclear extract were preincubated with 2 ug PolydI-dC on ice for 10 mins in the presence or absence of non-radioactive competitor oligonucleotides followed by incubation with 150pg (80,000cpm) of radiolabeled probe on ice for 30 mins. The binding reaction was carried out in a solution containing 50mM KCL, 10mM $MgCL_2$,

50mM Hepes (pH 7.9), 1mM EDTA, 1mM DTT, 1mg/ml BSA, 30% glycerol (V/V). The final volume was 20 microliters which was loaded onto a 5% non-denaturing acrylamide gel in 0.25xTBE to separate the bound complex from the free probe. 5' end-labeled HinfI PBR 322 and 1 kb ladder fragments were coelectrophoresed as markers.

UV Cross-linking

The gel retardation analysis was done as described above except that the radiolabeled probes contain bromodeoxyuridine incorporated into the antisense strand as described (2). Following electrophoresis, the gel was subjected to UV irradiation (254 nm, 250 mJ, 30 minutes) using a Stratalinker model 1800 apparatus (Stratagene, La Jolla, Calif.). After UV irradiation, the gel was exposed to X-ray film for 3-4 hrs and the area of the gel containing the band of interest was excised and incubated in sodium dodecyl sulfate (SDS) running buffer for 30-60 minutes on ice. The gel slice was placed directly in the well of a 10% SDS-polyacrylamide gel and electrophoresed at 15 mA overnight.

Gel filtration chromatography

Protein purification were performed by the Protein Chemistry Core Facility (Mount Sinai Medical Center). 1 ml of nuclear extract (24mg) of J-*tax*-19 cells was passed through Superdex 75 (Phamacia) based on instructions of the manufacturer. One hundred and forty fractions of 1.0 ml each were collected at 0.7 ml/min. The molecular weight of proteins eluted from the colum was determined by comparison to molecular weight standards (BSA, 80 Kd; Lysozeme,

18.5 Kd) which had been prerunned on the colum.

FACS Analysis

5 x 10^5 cells were incubated with 200ul of PBS containing antibodies on ice for 1 hour. anti-CD28 9.3 ascites was used at 1:200 dilution. Anti-CD3-containing supernatant from cultured hybridoma cells (kindly provided by Rouqing Huang) was used undiluted. After incubation with primary antibody, cells were washed twice with PBS, and then incubated with 200ul of $F(ab)'_2$ goat anti-mouse IgG-FITC conjugate (10ug/ml). 1% paraformadehyde was then added. The flow cytometric analysis and sorting were performed by Flow Cytometry Core Facility (Mount Sinai Medical Center).

CHAPTER I

Characterization of the *Cis* element mediating *tax* inducibility of the IL-2 enhancer

Characterization of the *Cis* element mediating *tax* inducibility of the IL-2 enhancer

RESULTS

Two regions in the IL-2 enhancer can be activated by *tax*

We have studied the effect of the HTLV-I *tax* protein on IL-2 promoter activity by cotransfecting a *tax* expression vector or an anti-sense-*tax* (anti-*tax*) expression vector with a plasmid containing the IL-2 promoter (-576 to +42) linked to the CAT gene into Jurkat T cells. As shown in Fig. 2A, although *tax* alone has little effect on the IL-2 promoter, it is able to synergize with either PHA or PMA to dramatically increase CAT activity, 32 fold in the presence of *tax* plus PHA and 21 fold in the presence of *tax* plus PMA. In addition, *tax* can augment the response induced by optimal doses of PHA plus PMA. In the absence of *tax*, PHA plus PMA increases pIL-2 CAT activity 31 fold; in the presence of *tax*, PHA plus PMA increases pIL-2 CAT activity 53 fold. These results suggest that *tax* may function in part through a different activation process from that of PHA or PMA. These data are consistant with our previous report (80).

To define the cis-elements that *tax* requires for its function, we examined the *tax* inducibility of a series of IL-2 enhancer internal deletion mutants with CAT as the reporter gene. These deletions encompass most of the IL-2 enhancer (12). No single deletion can abolish *tax* induced activity (discussed below). To determine whether two independent *tax* responsive elements exist in the IL-2

enhancer, we examined the *tax* inducibility of two fragments (A and B) which together comprise the IL-2 enhancer (-326 to -52). Both *tax* plus PHA and *tax* plus PMA produce significant increases in CAT activity when plasmids containing either two copies of the B fragment (-164 to -52) or two copies of the A fragment (-326 to-164) linked to the gammma-fibrinogen promoter and the CAT gene were cotransfected into the Jurkat T cell line with a *tax* expression vector. As shown in Fig. 2A, *tax* plus PHA induces a 30 fold increase in pBB-CAT activity and a 12 fold increase in pAA-CAT activity. *Tax* plus PMA induces a 25 fold increase in pBB-CAT activity and a 14 fold increase in pAA-CAT activity. Hence, at least two independent cis-elements in the IL-2 enhancer contribute to *tax* inducibility.

CsA has been shown to inhibit IL-2 promoter activity induced by PHA plus PMA, while *tax* circumvents this inhibition (80). As shown in Fig. 2C, CsA inhibits the induction of the IL-2 promoter by PHA plus PMA. In the presence of *tax*, CAT activity is restored. CsA also has an inhibitory effect on both the A and B fragments (Fig. 2C). While these inhibitory effects of CsA on the induction of pAA-CAT are partially reversed by *tax* (an increase from 3% to 14% relative transacetylation as shown in Fig. 2C.), CsA has no inhibitory effect on either the PHA or the PHA plus PMA induced expression of pBB-CAT when *tax* is present (Fig. 2B, Fig. 2C). These observations indicate that there is more than one site mediating *tax* inducibility of the IL-2 promoter and that *tax* has a stronger effect on the site in the B fragment than the one in the A fragment.

Sequences from -164 to -145 are sufficient to confer *tax* responsiveness

We have demonstrated that two distinct regions of the IL-2 enhancer are independently activated by *tax.* To further explore the mechanism of *tax* inducibility of the IL-2 enhancer and its circumventory effect on CsA inhibition, we examined the cis-elements within the A and the B fragments. Within the A fragment, there are three potentially functionally important sites known which interact with proteins induced by PHA or PMA (see Figure 1). One is the NFAT binding site from -285 to -255 (12). The others are the NF-kB motif from -206 to -195 (31) and a potential Oct-1 binding site from -259 to -234 (12). A plasmid containing 3 copies of the 31bp NFAT binding sequence linked to the gamma-fibrinogen promoter and the CAT gene was tested by cotransfection into the Jurkat T cells. As shown in Fig. 3A, *tax* has no direct effect on NFAT-directed CAT gene expression and CsA can completely inhibit the PHA induced CAT activity of this plasmid even in the presence of *tax.* The NFAT-1 construct was also transfected into permanently transfected Jurkat-anti-*tax* cells which contain an anti-sense *tax* expression vector, and Jurkat-*tax* cells which express *tax* protein (Fig. 3B). In these cells, *tax* has no effect on the expression of pNFAT-1. In addition, CsA inhibited PHA induced activity in both Jurkat-anti-*tax* and Jurkat-anti-*tax* cells. The 25bp sequence surrounding the NF-kB motif is also responsive to *tax* plus PMA (32). Site directed mutagenesis of the NF-kB site reduced CAT gene expression to less than 50% of pIL-2CAT activity induced by *tax* plus PMA (31). We did not examine the *tax* inducibility of the potential Oct-1 site in the A fragment. However, the Oct-1 site in the B fragment was examined and *tax* inducibility was not

observed (Fig. 3A). In addition, CsA inhibition was not reversed by *tax*. These preliminary results suggest that the NF-kB sequence may be responsible for the *tax* inducibility of pAA-CAT.

Since pBB-CAT shows a higher *tax* inducibility than pAA-CAT, and activation of pBB-CAT by *tax* plus PHA was completely resistant to CsA inhibition, we investigated the B fragment in detail. The sequences (-164 to -65) nearly spanning the B region (-164 to -52) were dissected into several pieces (BL, BT, BU, ILAP-1, BU, BO, BM, and BR) and cloned into TK-CAT constructs (Fig. 4A). The Oct-1 region of the promoter has been placed in front of the gamma-fibrinogen promoter and the CAT gene (12). The only plasmid which responds to PHA is pOCT (Fig. 3A, discussed above). None of the other constructs respond to PHA alone, PMA alone, PHA plus PMA or to PHA plus *tax* (data not shown). As shown in Fig. 4B, only the BL (-164 to -126, pBL-5 and pBL-4) and the BT (-164 to-145, pBT-7, pBT17, and pBT-5) regions, which contain putative NF-kB and AP-1 sites, show a response to *tax* plus PMA above the activity of TK-CAT. Two copies of the BL region (pBL-5) produce a 15 fold increase above the level of expression found in the absence of *tax* or PMA. Two copies of the BT region (pBT-7) produce a 25 fold induction. Similar results are found when the effect of *tax* by itself is examined. There is a 4 fold stimulatory effect of *tax* on pBT-7 and on pBL-4 which contains 3 copies of the BL region. Increases in the number of copies of BT results in a larger response to *tax*: 8 to 9 fold induction by *tax* on pBT-5 which contains four copies of the BT sequence. *Tax* induction of the pBL and pBT

constructs is not inhibitable by CsA (Fig.5). The expression of pBL-4 was also examined in Jurkat-anti-*tax* cells and Jurkat-*tax* cells (Fig.6). In contrast to the low activity in the J-anti-*tax*-10 cells, pBL-4 expresses a 27 fold higher constitutive level of CAT activity in J-*tax*-19 cells.

Analysis of the BL and BT regions

The BT region from -164 to -145 contains all the information necessary to respond to *tax* and to *tax* plus PMA. The contribution of sequences within this *tax* responsive element (TxRE) was examined. The BT region was further subdivided (Fig. 4A) into BU (5'BT containing the NF-kB motif) and ILAP-1 (3'BT containing a putative AP-1 site). Threee copies of BU are less responsive to *tax* and to *tax* plus PMA than three copies of BT (Fig. 4B). pBU-5 has a minimal response to *tax* compared to 8 to 9 fold induction in pBT-17. It responds to *tax* plus PMA with a 7 fold induction compared to 32 fold in pBT-17. pILAP-1 is non-responsive to *tax* or to *tax* plus PMA and PHA. As shown in Fig. 7, there is no difference between the expression of pILAP-1, which contains 6 copies of ILAP-1, when it is cotransfected with either the *tax* or anti-*tax* expression vector or when PHA plus PMA is added. ILAP-1 differs from the consensus AP-1 sequence by a single nucletide (See legend to Fig. 7). We also examined the response of pCOLLAP-1, which contains 5 copies of the consensus AP-1 sequence, to *tax* and *tax* plus PMA. Like pILAP-1, no change in expression was detected when *tax* was added, although a small increase in expression was observed when PHA and PMA were added (3 to 4 fold in the presence of either anti-*tax* or *tax* (Fig. 7). In addition, the

constitutive basal level of expression of pCOLLAP-1 was higher than pILAP-1. The question of whether the AP-1 like motif functions as an AP-1 binding site is discussed in detail in Chapter II.

We examined the contribution of the 5'BT and 3'BT sequences further by introducing mutations into BT. Since the 5'BT sequence (from -164 to -154, AAAGAAATTCC) resembles both an NF-kB like motif in the antisense strand (94) and a CK-1 like motif in the sense strand (16,77), and the 3'BT sequence from -153 to -145 (AAAGAGTCA) resembles an AP-1 like motif, we decided to change these sequence motifs by mutation and then examine their function. Since the -CC- bases in either the NF-kB like motif or the CK-1 like motif are crucial for their activity (1,17,31), we changed those bases to -TT- in pm2BT-20 (Fig. 4). These two changes destroyed the response of the BT region to *tax*. As shown in Fig. 4B, pmBT-20, which contains 6 copies of the mutated BT sequence, has no activity above that found with TK-CAT. Therefore the 5'BT sequence appears critical to the function of the TxRE. Mutation of GAGT to TTGA in the AP-1 like motif that should destroy the AP-1 binding site was made in pm4BT-8 (Fig. 4A). pm4BT-8, which contains 7 copies of the mutated sequences, responds to *tax* and *tax* plus PMA, although dramatically less than pBT-7, which contains only 2 copies of the wild type sequence. From these results and the absence of a response in pILAP-1, we can conclude that the 3'BT sequence apparently contributes to the activity of the TxRE, although it is not in itself sufficient.

To further define the role of the NF-kB and AP-1 like sequence motifs in the

tax mediated activation, we examined the effects of mutations in BL. pm4BL-2, which contains mutations only at the AP-1 like motif retained full responsiveness to *tax* and to *tax* plus PMA (Fig. 4B) as did pm4/5BL, which contains additional mutations in the BO region downstream of ILAP-1 (Fig. 4A). In contrast, pm2/4BL with mutations in both the 5'BT and the 3'BT, was totally unresponsive to *tax* or to *tax* plus PMA. It therefore appears that the -CC- bases in the 5'BT are particularly critical for *tax* activity in both BL and BT, while the contribution of 3'BT is only apparent in the BT constructs.

Sequences from -164 to -154 are necessary for *tax* inducibility of the IL-2 enhancer

To determine the role of the TxRE in the intact enhancer, we examined the *tax* inducibility of a series of IL-2 enhancer internal deletion mutants with CAT as the reporter gene (12). No single deletion can abolish *tax* plus PMA induced CAT activity (Fig. 8A). pID159/151, which contains a partial deletion of 5'TxRE, shows the most prominent negative effect. pID208/174, which contains a deletion of the NF-kB site, shows 51% of pIL-2CAT activity. Deletion of 3'BT (3'TxRE) has reduced CAT activity (pID152/121) but not as prominently as pID159/151 or pID 208/174. This result is reminiscent of pm4BT-8 with mutations in the AP-1 like motif, which did not respond to *tax* as strongly as the wild type constuct. The data indicate that no single deletion can abolish *tax* activity in the IL-2 enhancer. Both the TxRE and the NF-kB site appear to be important in mediating the *tax* plus PMA effect on the IL-2 enhancer. These mutants were also tested for their *tax* plus

PHA inducibility (Fig. 8B). pID159/151 shows the same dramatic negative effect. In addition, deletion of the NFAT site (pID279/263) as well as its adjacent sites (pID317/286, pID255/217) resulted in reduced CAT activity. Interestingly, deletion of the Oct-1 site in pID98/73 had no apparent effect on the synergistic effect of *tax* and PHA, in contrast to the marked loss of PHA plus PMA inducibility (12). These results demonstrate that the synergistic effect of *tax* plus PHA on the IL-2 enhancer is mostly contributed by TxRE and the NFAT site.

The effect of PHA, PMA and CsA on these deletion mutants has been previously reported (12,14). Many of these mutations reduce the effect of PHA plus PMA. In all cases, however, CsA reduces the induction still further, in most cases down to the basal level (14 and Fig. 8C, closed bars). As previously discussed (Fig. 2C), the presence of *tax* can rescue wild type CAT expression even when CsA is added. The CAT expression of all but one of the deletion mutants, pID151/159, is still rescuable by *tax* (Fig. 8C, compare open to closed bars). However, since deletion of the 5'TxRE in pID151/159 interferes with both *tax* inducibility and PHA plus PMA inducibility, the precise interpretation of the data from the deletion analysis is feasible only when the data from the oligonucletide analysis are also considered. Taken together, these results indicate that the sequence from -164 to -154 (5'TxRE) is necessary for maximal *tax* inducibility of the IL-2 enhancer. This site is also necessary for *tax* to abrogate the CsA inhibition of the IL-2 enhancer activity by *tax*.

It has been reported that sequences from -160 to -150 are important for

activation of IL-2 gene transcription by anti-CD28 (17,93). We have also examined the effect of monoclonal anti-CD28 antibody 9.3 on IL-2 enhancer activity in Jurkat T cells. As shown in Fig. 9, we have found that 9.3 has a small effect on the expression of the pIL2-CAT in anti-CD3 plus PMA treated cells (Fig. 9). When pIL2-CAT was cotransfected into these cells with the *tax* expression vector, 9.3 increased the CAT activity still further. We also tested the response of pBT-5 to stimulation with anti-CD28. 9.3 had no effect on the expression of pBT-5 in anti-CD3 plus PMA treated cells, either in the presence or in the absence of *tax*. We conclude that the BT sequence, which is sufficient to confer *tax* inducibility, is not sufficient to confer anti-CD28 mediated activation. However, the BT sequence may contain a site which is necessary for the anti-CD28 mediated increase in IL-2 enhancer activity.

Discussion

The IL-2 enhancer in Jurkat T cells can be activated by various combinations of stimuli, including PHA plus PMA, *tax* plus PMA and *tax* plus PHA. No single mutation within the IL-2 enhancer specifically destroys *tax*-activation while leaving PHA plus PMA activation intact. It is therefore difficult to isolate the specific effects of *tax*. Nevertherless, preliminary data suggested that more than one site is involved in mediating *tax* activity in the IL-2 enhancer. Both pAA-CAT and pBB-CAT responded to *tax* and confirmed the hypothesis that at least two cis-elements mediate *tax* inducibility of the IL-2 enhancer (Fig. 2). The B fragment contains a stronger *tax* responsive site than the one in the A fragment. Using oligonucletides from the B fragment linked to the TK promoter, we defined the BT sequence from -164 to -145 as a *tax* responsive element (TxRE) in the IL-2 enhancer (Fig. 4). These pBT-CAT constructs do not respond to PHA, PMA, and PHA plus PMA but do respond to *tax* alone and to *tax* plus PMA.

We also examined the contribution of particular sequences in this TxRE to the expression of the pBT and pBL plasmids. Mutation of two bases within the NF-kB motif in 5'BT destroys the ability of pBT-CAT and pBL-CAT to respond to *tax* or *tax* plus PMA. The inducibility of BT is reduced substantially, but not completely, when the 3'BT is either mutated (pm4BT-8, Fig. 4B) or deleted (pBU-5, Fig. 4B). These mutations in the 3'BT have no effect on pBL-CAT activity. One explanation for this apparent disparity is that a factor could bind in the intact enhancer at the border of BT and BO (see Fig. 4A). Sufficient binding for *tax* activation may occur

in wild type BT and mutated BL, but not in mutated BT. Alternatively, sequences in BL downstream of BT, may bind a factor which can substitute for binding at 3'BT.

The 3'BT sequence (AAAGAGTCA) contains an AP-1 like motif which had been suggested to be a target of protein kinase C (36). Since the *c-fos* gene is inducible by *tax* (19), and PMA can activate *AP-1*, it appeared possible that *tax* plus PMA induces *AP-1*, which subsequently activates the TxRE. Neverthless, a functional AP-1 binding site is not required for PMA-enhanced *tax* activity since pBL-CAT constructs containing mutations at the AP-1 like motif that should abolish *AP-1* binding retained the full response to *tax* plus PMA (Fig. 4). In addition, pILAP-1 which contains tandem copies of the core AP-1 like sequence in the IL-2 enhancer did not respond to either *tax* or *tax* plus PMA. pCOLLAP-1, which contains tandem copies of the consensus AP-1 sequence, expresses a higher basal CAT activity than pILAP-1 in Jurkat cells. As we show in Chapter II, AP-1 is constitutive in these cells, but it cannot recognize the ILAP-1 sequence.

Analysis of the *tax* inducibility of internal deletion mutants in the IL-2 enhancer further indicates that 5'TxRE is also necessary for *tax* inducibility of the intact IL-2 enhancer (Fig. 8). pID159/151 (with a deletion in TxRE) responded the least to stimulation by *tax* plus PMA or *tax* plus PHA compared to all the other pID mutants. Although this reduced *tax* plus PHA or *tax* plus PMA activity could be due entirely to the contribution of this site to PHA and PMA induction, we believe this is unlikely. As shown in Fig. 8, the induction of all of the pID plasmids by PHA

plus PMA is completely inhibited by CsA. However, cotransfection by *tax* can dramatically reverse this effect for all of the plasmids except pID159/151. These results indicate that the *tax* activation of 5'TxRE is required for the abrogation of the CsA inhibitory effect on the IL-2 enhancer. It is possible that another site in combination with the TxRE may also be required to overcome CsA inhibition since we did not examine all the sequences in the enhancer. However, combined data obtained from the oligonucleotide analysis and deletion analysis show that TxRE is a major *tax* responsive element within the IL-2 enhancer.

Tax increases pBB-CAT expression by synergizing with either PHA or PMA. Added singly, these fail to stimulate CAT gene expression. The activation of pBB-CAT activity by *tax* plus PMA may be due to the activation of TxRE alone, since pBL and pBT-CAT constructs respond strongly to *tax* plus PMA. The activation of pBB-CAT activity by *tax* plus PHA is less clear. Although the BL region also contains a potential NFAT site (74,90), pBL-CAT constructs do not respond to PHA, either alone or in combination with *tax* or PMA. However, we can not rule out the possibility that this potential NFAT site is only active when it is present on the large B fragment in pBB-CAT. *Tax* plus PHA induced pBB-CAT activity may also be explained by *tax* activation of the TxRE and PHA activation of the Oct-1 site downstream of BL. Although deletion of this Oct-1 site has no negative effect on the *tax* plus PHA inducibility of the intact enhancer (Fig. 8), it is possible that the distal NFAT site on the A fragment is sufficient for expression. In fact, deletion of either the distal NFAT site or 5'TxRE diminished PHA plus *tax* induced IL-2

enhancer CAT activity drastically (Fig. 8), suggesting that these two sites are the two most important contributors to the PHA plus *tax* induced activity of the intact enhancer.

It is unclear how *tax* reverses the CsA inhibition of PHA-induced IL-2 gene expression. NFAT and Octamer-binding proteins appear to be the functional targets of CsA inhibition (74). We have found that *tax* has no effect on NFAT or Oct-1 directed CAT gene expression, either directly or in the presence of CsA (Fig. 3, summarized in Fig. 10). *Tax* plus PHA induced pIL-2CAT activity is partially resistant to CsA and *tax* plus PHA induced pBB-CAT activity is totally resistant to CsA inhibition. However, pBL-CAT, which also contains the TxRE, does not respond to *tax* plus PHA. It is only when the *tax* and PHA responsive sites are found on the same piece of DNA that resistance to CsA is found. Therefore, in addition to activating TxRE, *tax* must induce other nuclear events in synergy with the PHA induced signal to restore activation at the NFAT or the Oct-1 sites which are normally inhibitable by CsA. Both the NFAT site and the TxRE must be active in order to obtain a *tax* plus PHA effect on the IL-2 enhancer (Fig. 8C). It is possible that these two sites together are also sufficient to confer such activity. To test this hypothesis we are currently examining the *tax* plus PHA plus CsA responses of CAT constructs which contain only the NFAT and TxRE sequences.

It has been shown that the NF-kB site in the A fragment contributes to the *tax* inducibility of IL-2 gene expression (31). Both site-directed mutagenesis (31) and deletion of the NF-kB site (pID208/174, Fig. 8A) reduces *tax* plus PMA induced

activity to 50% of that produced by wild type. It is not clear whether the NF-kB site contributes to the *tax* plus PHA inducibility of the IL-2 enhancer since the data obtained through deletion analysis (12; Fig. 8B) differ from that obtained by site-directed mutagenesis (31). Within the A fragment, in addition to the NF-kB site, there is an inverted copy of a possible TxREF binding site at -295 to -285 upstream of the NFAT site (17). It is possible that these two sites are both involved in *tax* mediated pAA-CAT activity.

We have mapped the TxRE in the same area as the CD28RE, which raises the possibility that the two activation signals may share a common pathway. It has been reported that anti-CD28 activation of IL-2 gene expression in a Jurkat T cell clone is sensitive to CsA inhibition (96). In contrast, IL-2 gene expression in peripheral blood T lymphocytes is either completely resistant (anti-CD28 plus PMA) or partially resistant (anti-CD28 plus PHA) to CsA inhibition (37,38,89). The discrepancy between Jurkat T cells and PBLs in the responses to anti-CD28 and CsA can be explained if Jurkat T cells are missing cellular components present in PBLs which are required for circumvention of CsA inhibition. For example, transmission of the CD-28 signal from the membrane may be inhibited by CsA in Jurkat T cells but not in PBLs. The pattern of activation of IL-2 gene expression in PBLs in the presence of CsA is similar to that seen with PHA, PMA, *tax,* and CsA in Jurkat T cells (for example Fig. 2). This similarity can be explained if *tax* can substitute in the transfected Jurkat T cells for components missing in the CD28 pathway . For example, *tax*, which is resident in the nuclei of the transfected

Jurkat cells, may replace the CsA-sensitive downstream component of the CD-28 triggered activation pathway. Since it has not been demonstrated that activation of transcription initiation by anti-CD28 is resistant to CsA in PBL, it is also possible that *tax* and CD-28 utilize distinct activation pathways but share part of the *cis* element in the IL-2 enhancer.

CHAPTER II

Identification of the transacting factors mediating *tax* activation of the IL-2 enhancer

Identification of the transacting factors mediating *tax* activation of the IL-2 enhancer

Introduction

We have demonstrated that the TxRE is both sufficient to confer *tax* responsiveness and necessary for *tax* inducibility of the IL-2 enhancer. Constructs containing tandem copies of TxRE (BT) transfected into Jurkat T cells respond to either *tax* or *tax* plus PMA but not to PHA plus *tax* or PHA plus PMA. pBT-CAT constructs have higher activity in Jurkat-*tax* cells than in Jurkat anti-*tax* cells. The 5'TxRE is particular crucial for *tax* mediated IL-2 enhancer activity. The -CC- bases in the 5'TxRE, which resembles an NF-kB motif, are also required for *tax* mediated pBT-CAT activity.

A region from -162 to -150 within the TxRE, was also reported to be important for the activity induced in Jurkat cells by anti-CD28 antibody in the presence of ionomycin plus PMA. A protein complex (CD28RC) induced by these reagents can interact with the 5'TxRE (17). CD28RC can also bind to CK-1 sites, which are homologous to the 5'TxRE, in the enhancers of the GM-CSF, gamma-IFN, and IL-3 genes (16). These CK-1 sites are important for the anti-CD28 activation of the GM-CSF, gamma-IFN and IL-3 enhancers in Jurkat T cells. CD28RC was defined as a CK-1 binding complex which contains 35, 36, and 44kDa proteins as determined by UV cross-linking. NF-kB can also bind the CK-1 sequence from the

GM-CSF enhancer (77). It was also reported that a NF-kB like factor induced by anti-CD28 plus either anti-CD3 or PMA can also bind to the CD28RC binding site in the IL-2 enhancer and may play an important role in mediating anti-CD28 activation of the IL-2 gene (94). NF-GMa, a nuclear protein present in a number of cell types including K5637 cells (a human bladder carcinoma cell line), also interacts with the CK-1 sequence (77). It is unclear whether NF-GMa is related to CD28RC.

Since *tax* induction of NF-kB is critical for *tax* mediated IL-2Rα gene expression, we wish to determine the relationship between NF-kB and the factors which bind to the 5'TxRE. The 3'TxRE, which contains an AP-1 motif, is less critical for activation by *tax*. The question of the involvement of AP-1 in the activation of the TxRE will also be considered in this chapter.

We have shown that *tax* plus PHA can activate the IL-2 enhancer in the presence of CsA. Deletion analysis of the IL-2 enhancer revealed that both the TxRE and NFAT sites are required for *tax* plus PHA induced IL-2 enhancer activity. However, *tax* plus PHA can not induce the expression of pBT-CAT. In addition, pNFAT-1 (with tandem copies of NFAT binding sites linked to the gammma fibrinogen promoter) does not respond to *tax*, and CsA inhibits PHA induced pNFAT-1 expression even in the presence of *tax*. Therefore, in addition to activating TxRE, *tax* must induce other nuclear events to restore activity at the NFAT site. *Tax* may also have effects on the Oct-1 site since *tax* plus PHA can activate pBB-CAT in the presence of CsA. The effect of *tax* on the binding of

NFAT and Oct-1 is explored in this chapter.

Results

A DNA binding protein induced by *tax* in Jurkat T cell line specifically interacts with TxRE

To determine which nuclear factors interact with the TxRE, gel retardation analysis was performed using radiolabeled BT (-164 to -145). We examined nuclear extracts from several cell lines including Jurkat-anti-*tax* cells, which contain an anti-sense *tax* expression vector, and Jurkat-*tax* cells, which express *tax* protein. As shown in Fig. 11, a retarded band with fast mobility was only observed using nuclear extracts from the *tax* expressing cells (arrow, lanes 2,3) but not Jurkat-anti-*tax* cells (lane 1), Jurkat cells stimulated by PHA plus PMA (lane 4), or Hela cells stimulated by PMA (lane 5). We tentatively refer to this protein as *tax* responsive element binding factor (TxREF). As shown in Fig. 12A, TxREF can not be detected by radiolabeled m2BT (lane 9) and HIV-kB (lane 12). Radiolabeled BU (5'BT which contains the NF-kB motif alone) does not bind TxREF (Fig. 12A lane 11 and Fig. 12B lane 2, a longer exposure). TxREF binding to BT can be competed by BT itself (lanes 3,4) but not by HIV-kB (lanes 5,6) or BU (lanes 7,8).

TxREF is a 45kDa protein whose *in vitro* binding activity is correlated with the *in vivo* activation of TxRE by *tax*.

TxREF is the major retarded band detected by radiolabeled BT in extracts from Jurkat-*tax* cells, but not Jurkat-anti-*tax* cells or Jurkat cells stimulated with PHA plus PMA. Competition analysis was performed to determine the sequence

requirements for the binding of TxREF. As shown in Fig.13, wild type BT (lanes 14-16) and oligonucleotides M3 and M4 (lanes 8-13), which contain mutations in 3'BT, are efficient competitors for the binding of wild type BT to TxREF. Mutations in 5'BT prevent M2 from competing for binding to TxREF (lanes 5-7) and decrease the ability of M1 to compete (lanes 2-4). In addition, the binding activity of TxREF is not influenced by CsA treatment of Jurkat-*tax* cells (Fig. 17A, lanes 1 and 2). The results of these *in vitro* protein binding assays correlate with the *in vivo* transfection analysis (Fig. 4B). The binding site of TxREF appears to be mainly within the 5'TxRE (-164 to 154).

UV cross-linking experiments were performed to determine the molecular weight of TxREF. As shown in Fig.14, we found that TxREF is a 45-kDa protein. The size of TxREF was also estimated using gel filtration chromatography (Fig. 15). TxREF binding activity was recovered in fractions 58,59 and 60 (lanes 7,6 and 5), which contain proteins from 48kDa to 40kDa molecular weight.

TxREF is distinct from NF-kB

To determine whether TxREF is an NF-kB like factor, we examined the binding of TxREF to the TxRE and to previously characterized NF-kB binding sites. As shown in Fig.16A, binding of TxREF to the BT probe (lane 1) can be competed by itself (lanes 2,3) but not by oligonucleotides containing NF-kB binding sites: IL2-kB (lane 4), IL6-kB (lane 5) and HIV-kB (lanes 6,7). It is of interest that the HTLV-I LTR 21bp repeat does not compete for TxREF binding either (lane 8). The IL2R-kB probe recognizes a nuclear protein complex in Jurkat-*tax* cells, which migrates

more slowly than TxREF (Fig. 16B, lane 8) and has been identified as NF-kB(3,46,55). Binding of NF-kB to the IL2R-kB probe is efficiently competed by IL-2RkB (lanes 9-12), while BT is an inefficient competitor (lanes 13-16). TxREF can recognize the BT probe (lane 1) but not the IL2R-kB probe, and can only be competed by itself (lanes 5-7) but not IL2R-kB (lanes 2-4). Therefore, the DNA sequence specificity of TxREF is different from NF-kB.

TxREF is distinct from CK-1 binding proteins

To determine whether TxREF was related to CD28RC or NF-GMa, radiolabeled CK-1 from the GM-CSF promoter was used to detect DNA binding proteins from Jurkat-*tax* cells (Fig. 17A). TxREF, which interacts with the BT probe (lower band, lanes 1,2), does not interact with the CK-1 probe (lanes 5,6), indicating that TxREF is not identical to either previously described CD28RC or NF-GMa. We tested nuclear extracts from K5637 cells, which contain NF-GMa, using radiolabled BT or CK-1 as probes. The protein-DNA complexes formed by nuclear extracts from K5637 cells, untreated or treated with PMA, with either the BT probe (lanes 3,4) or the CK-1 probe (lanes 7,8) have different mobilities from TxREF (lanes 1,2). BT and CK-1 may recognize similar nuclear factors from K5637 cells (lane 3,7) and K5637 cells treated with PMA (lanes 4,8). TxREF binding to BT can be competed by BT itself (Fig. 17B, lanes 2 and 3) but not by CK-1 (lanes 4,5). These data indicate that TxREF is distinct from the previously described CK-1 binding proteins.

The IL-2 AP-1 motif does not bind AP-1

We have shown that the AP-1 motif is not required for *tax* induced IL-2 enhancer activity. Transfection assays show that the sequences from IL-AP1 and consensus AP-1, which differ in a single nucleotide, result in a dramatic difference in CAT gene expression (Fig. 7). It was of interest to test nuclear extracts from Jurkat T cells using ILAP-1 (-152 to -145 GATCCAAGAGTCAT, the 3' half of the TxRE), and consensus AP-1 (GATCCATGAGTCAT) as probes. As shown in Fig. 18, nuclear extract from Hela cells, as a control for the presence of AP-1, bound strongly to AP-1 (lane 16), while no or very low levls of binding were seen when radiolabeled ILAP-1 was used (lane 11). Radiolabeled AP-1 also bound AP-1 from Jurkat T cells (lane 6). There is apparently no increase in the amount of AP-1 detected when PMA is added to either the Jurkat cells (lane 7) or the Hela cells (lane 13). This binding to AP-1 can only be competed by AP-1 (lane 9) but not by ILAP-1 (lane 8). The complex detected by ILAP-1 (lanes 1-5) has a faster mobility than that detected by AP-1. Detection of the ILAP-1-protein complex is not reproducible. The data indicate that ILAP-1 motif does not bind or binds very weakly to AP-1.

The Influence of *tax* on NFAT and Oct-I DNA binding activity in Jurkat T cells

To understand the mechanism of *tax* plus PHA activation of the IL-2 enhancer, we investigated the influence of *tax* on NFAT and Oct-I binding activity in Jurkat T cells. As shown in Fig. 19, NFAT binding activity is induced in Jurkat-*tax*, Jurkat-anti-tax, and Jurkat cells treated with ionomycin plus PMA (lanes 3,5,9) and in Jurkat cells treated with PHA plus PMA (lane 8). CsA inhibits the formation of the

NFAT-DNA complex in extracts from Jurkat-anti-*tax* or Jurkat cells treated with ionomycin plus PMA plus CsA (lanes 6, 10). In contrast, CsA treatment of ionomycin plus PMA stimulated Jurkat-*tax* cells does not prevent formation of the NFAT-protein complex (lane 4). Apparently, the presence of *tax* can partially circumvent the CsA inhibition of NFAT binding to DNA.

The interaction of *tax* with CsA was also examined using radiolabeled Oct-1 (Fig. 20). The Octamer-binding proteins are constitutively expressed in Jurkat T cells (lane 7), Jurkat-anti-*tax* cells (lane 4) and Jurkat-*tax* cells (data not shown). CsA does not affect the Oct-1 binding in these cells (data not shown and lane 1). After a nine hour incubation of Jurkat (lane 9) and Jurkat-anti-*tax* cells (lane 5) with ionomycin plus PMA, the Octamer-binding complexes observed have a faster mobility than the constitutive complex. CsA has no effect on the binding activity of these complexes (lanes 6,10). In contrast to this slow form of the complexes found in Jurkat-anti-*tax* cells, a nine hour stimulation of Jurkat-*tax* cells does not change the mobility of the Octamer binding complex (lane 2,3). The significance of this effect of *tax* on the induced Octamer-binding complex formed in mitogen stimulated cells is unclear.

Discussion

We have identified a DNA binding protein, TxREF, in nuclear extracts from Jurkat-*tax* cell lines which binds to radiolabeled BT. TxREF is only detected in nuclear extracts from Jurkat-*tax* cells but not from Jurkat-anti-*tax* cells even when they had been stimulated by PHA plus PMA. *In vitro* binding of TxREF is correlated with *in vivo tax* activity. The upstream portion of the TxRE (5'TxRE) is most critical to TxREF binding and to *tax*-mediated activation of the BT- and BL-CAT constructs. Mutation of CC to TT in the 5'TxRE (M2), which abolishes *tax* responsiveness (pm2BT-20 and pm2/4BL, Fig. 4B) also prevents TxREF complex formation (Fig. 12A, lane 9 and Fig. 14). Mutation of GAGT to TTGA in the AP-1 like motif (M4), which did not interfere with *tax* responsiveness (pm4/5BL and pm4BL-2) did not interfere with the binding of TxREF to the TxRE. In addtion, deletion of the 5'TxRE also destroys *tax* inducibility of the IL-2 enhancer (Fig. 8). However, the 5'TxRE (BU) is not as efficient as BT: pBU-5 responds to *tax* weakly (Fig. 4) and TxREF is not detected by radiolabeled BU (Fig. 12B, lane 2). The TxREF binding site may therefore extend to the 3'TxRE, or the 3'TxRE sequence may influence the stability of TxREF-BT interaction. The 3'TxRE contains a potential AP-1 binding site. However, the one base pair difference between ILAP-1 and the consensus AP-1 sequence has a dramatic effect on AP-1 binding activity. We have shown that the AP-1 found in either Hela or Jurkat nuclear extracts does not bind this sequence *in vitro* (Fig. 18), and the activity of pCOLLAP-1 transfected into Jurkat cells is much greater than that of pILAP-1 (Fig. 7). Although TxREF

binding to BT is dependent on part of the NF-kB motif, TxREF is distinct from the members of NF-kB/rel family reported so far. For example, TxREF can not be induced by PHA plus PMA treatment of Jurkat T cells and pBT-CAT and pBL-CAT constructs do not respond to PHA plus PMA (Fig. 11, data not shown). In contrast, PHA or PMA treatment of Jurkat T cells induces NF-kB activity and increases CAT gene expression directed by the HIV-kB motif, the IL2Rα-kB motif, and the IL2-kB motif (4,32, data not shown). In addition, TxREF has a size of 45-kDa (Fig. 14), which is smaller than the NFkB-specific proteins found in activated Jurkat T cells and in Jurkat-*tax* cell lines (3,21,55). TxREF is therefore the second nuclear protein induced by *tax* to be identified (after NF-kB) and represents a novel DNA binding protein playing an important role in *tax* mediated IL-2 gene activation.

TxREF and CD28RC both recognize DNA sequences contained within -164 to -154 and TxREF has a size of 45kDa which is close to the size of the 44kDa subunit of CD28RC (35,36, and 44kDa). It is clear that TxREF is distinct from the CD28RC described by Fraser *et al.* (17) since the CK-1 sequence from the GM-CSF promoter, which has been shown to bind CD28RC, did not compete for TxREF binding and TxREF did not recognize CK-1 (Fig. 17A and Fig. 17B). We have not been able to reproduce the experimental results reported by Fraser *et al* who described CD28RC as a protein complex bound to 5'BT induced in Jurkat T cells by anti-CD28 plus ionomycin plus PMA. At this point we cannot explain this discrepancy. The precise relationship between TxREF and CD28RC requires further studies. The Jurkat T cells, Jurkat-anti-*tax* cell lines, and Jurkat-*tax* cell

lines we used all express CD28 molecules on the cell surfaces as determined by FACS analysis (data not shown) and we have been able to identify NF-kB activity (CI) detected by BT in Jurkat T cells treated with either anti-CD28 plus anti-CD3 or anti-CD28 plus PMA (data not shown), in accord with the observations reported by Verweij *et al.* (94). We have also found that anti-CD28 can enhance anti-CD3 plus PMA induced pIL-2CAT activity (Fig. 9). Therefore, NF-kB may be partially responsible for increased pIL-2CAT activity induced by anti-CD28.

We have shown that inducible NFAT binding activity was only marginally inhibitable by CsA in Jurkat cells which express the *tax* protein (Fig. 20). One explanation is that *tax* can circumvent the inhibitory effect of CsA on NFAT-C translocation into the nucleus. However, *tax* cannot rescue the activity of pNFAT-1 in the presence of CsA (Fig. 3A), indicating that CsA can inhibit the transcriptional activity of NFAT even when NFAT is apparently bound to the DNA. It has been shown that the nuclear components of NFAT contain members of the jun family (7.93). OAP^{40} belongs to the jun family as well, and its transcriptional activity but not its binding activity, is also diminished by CsA (93). The jun related components of NFAT may therefore be the direct targets of CsA. CsA may also inhibit the activity of a unidentified coactivator which is required for NFAT site activation. The following model may explain how *tax* synergizes with PHA to circumvent CsA inhibition of IL-2 enhancer activity. *Tax* may facilitate the translocation of NFAT-C into the nucleus, induced by an increase in intracellular Ca++, even when CsA is present. In addition, the binding of TxREF to TxRE may

activate bound NFAT, for example by direct interaction or through the interaction of another, as yet undefined, site on the enhancer. These effects of *tax* on both cytosolic and nuclear components would restore TcR triggered activation of the NFAT site and allow expression of the IL-2 enhancer in the presence of CsA.

Tax also has an effect on the Octamer-binding complex. After a 9 hour stimulation by mitogens, the Octamer-binding complexes in Jurkat cells without *tax* have a different mobility from those in Jurkat-*tax* cells. The significance of this effect of *tax* on the induced Octamer-binding complex is unclear. Since Oct-1 transcriptional activity can be regulated by phosphorylation/dephosphorylation, its tempting to speculate that the different mobility of the octamer binding proteins are due to the phosphorylation status of these proteins. Treating the nuclear extracts by phosphotase and then examining the mobility of the Oct-1-protein binding complex may test this hypothesis.

By studying the nuclear factors interacting with the elements that are required for *tax* activation of the IL-2 enhancer we have gained insight into the mechanism of *tax* deregulation of IL-2 gene expression. IL-2 is the major growth factor for T cell proliferation. Its secretion is tightly regulated by various stimuli. The utilization of TxREF by HTLV-*tax* results in uncontrolled IL-2 gene activation and hence the begining of T cell transformation. Characterization of TxREF will be helpful not only in understanding the molecular basis of HTLV-I *tax* induced T cell transformation but also in understanding the requirements for IL-2 gene regulation in maturing T cells: At a certain point in the activation of peripheral blood T

lymphocytes, IL-2 gene expression becomes NFAT-independent and resistant to CsA inhibition (88), suggesting that a changing constellation of DNA-binding proteins may participate in the activation of the IL-2 enhancer. TxREF activity is also NFAT-independent (Fig. 8C) and CsA resistant, and may be an important determinant of the CsA insensitive mode of IL-2 gene activation.

FIGURES AND FIGURE LEGENDS

Fig. 1. A, B, C, D and E represent the sequences which were initially characterized as nuclear protein binding sites in the IL-2 enhancer (12,9).

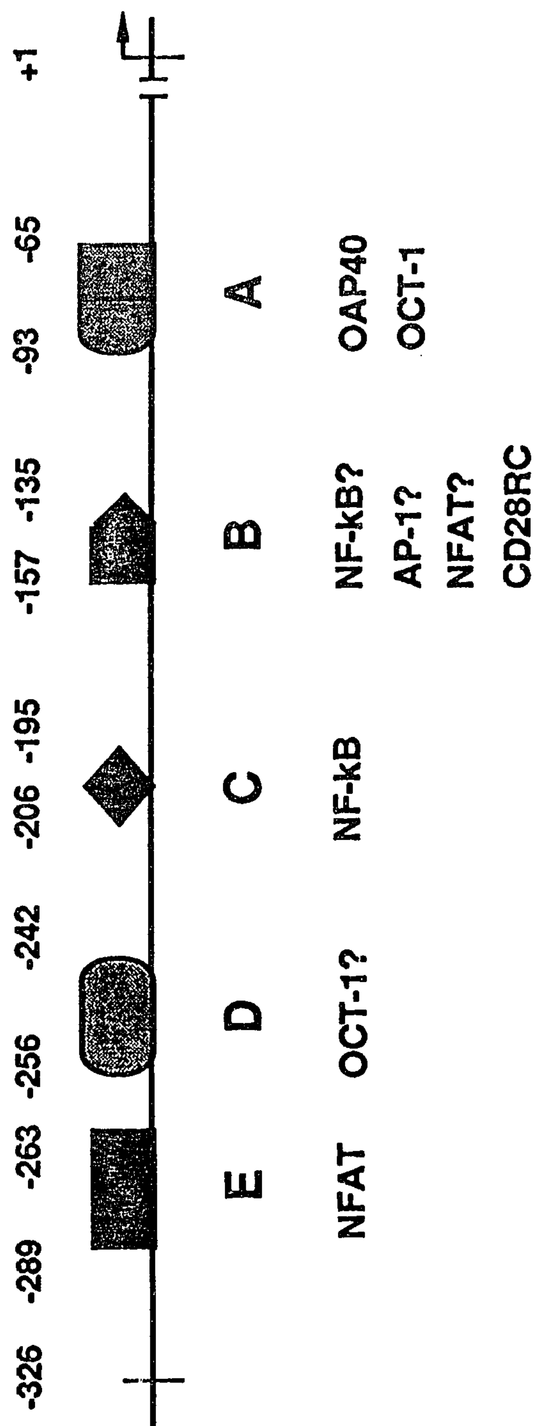


Fig. 2 (A) Two distinct regions of the IL-2 enhancer are independently activated by *tax.*

The CAT-expression vectors, pIL2-CAT (containing the IL-2 promoter from -576 to +42), pAA-CAT (containing 2 copies of the A fragment from -326 to -164), and pBB-CAT (containing 2 copies of the B fragment from -164 to -52) were cotransfected into Jurkat T cell lines with either a *tax* expression vector (+) or an antisense-*tax* expression vector (-). CAT assays were performed as described in "Materials and Methods". The results reflect data from 3 to 4 experiments with at least 2 independent plasmid preparations for each construct. The data are presented as % transacetylation (mean±SD) normalized to 1% transacetylation without inducer (medium alone). The % transacetylation before normalization (mean ± SD) of each plasmid without inducers are as follows: in the presence of anti-*tax*, pIL2-CAT 1.1 ± 0.5, pAA-CAT 1.0 ± 0.7, pBB-CAT 1.5 ± 1.2; in the presence of *tax*, pIL2-CAT 0.9 ± 0.4, pAA-CAT 2.6 ± 1.7, pBB-CAT 3.6 ± 3.4. * For these conditions, the transfections were performed twice.

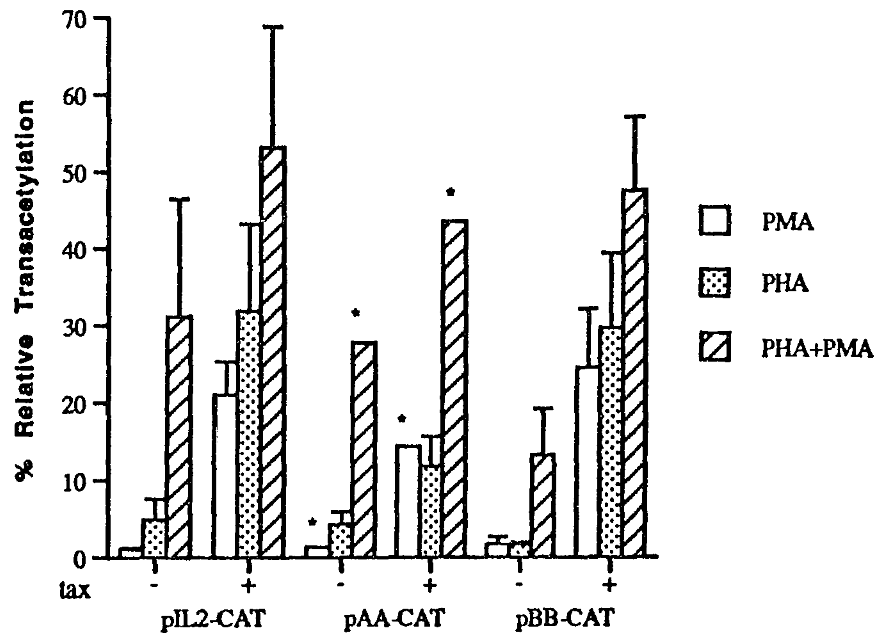

A
% Relative Transacetylation
70
60
50
40
30
20
10
0
tax
-
+
-
+
-
+
pIL2-CAT
pAA-CAT
pBB-CAT
PMA
PHA
PHA+PMA

Fig. 2 (B) The effects of CsA on PHA induced-IL-2 enhancer activity.

The experiments were performed as described in Fig. 2A. The values of basal activity (% transacetylation) are the same as in Fig. 1A.

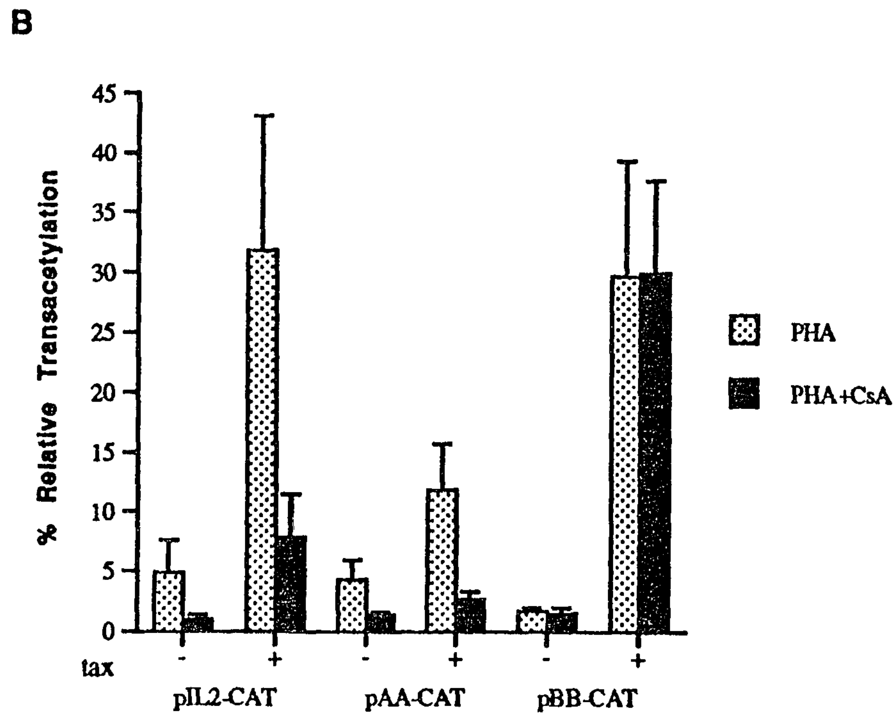


Fig. 2(C) The effects of CsA on PHA plus PMA-induced IL-2 enhancer activity. The experiments were performed as described in Fig. 2A. The values of basal activity are the same as in Fig. 1A.

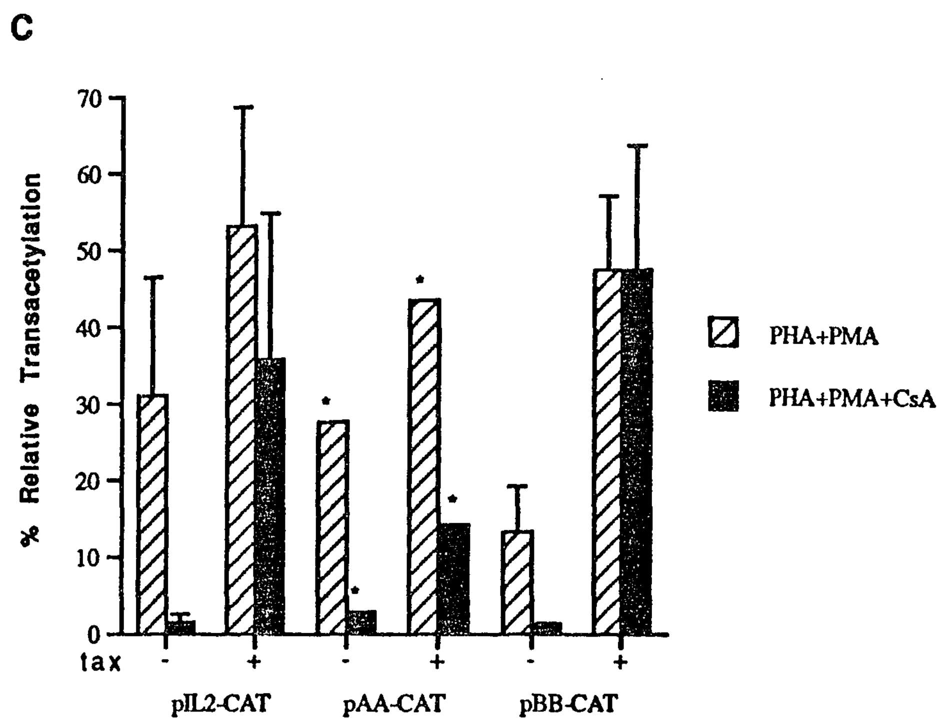


Fig. 3(A). NFAT and Oct-1 are not responsive to *tax.*

pNFAT-1, containing 3 copies of NFAT linked to the gamma-fibrinogen promoter, and pOCT-1, containing 4 copies of Oct-1 linked to the gamma-fibrinogen promoter, were transfected into Jurkat T cells with either an anti-*tax* or a *tax* expression vector. CAT assays were performed as described in "Materials and Methods". The fold increase in CAT activity for pNFAT-1 was as follows: in the presence of anti-*tax*, PHA plus CsA 1.3 and PHA alone 31.2; in the presence of *tax*, PHA plus CsA 1.3 and PHA 30.0. The fold increase in CAT activity for pOCT was as follows: in the presence of anti-*tax*, PHA plus CsA 1.2 and PHA 7.2; in the presence of *tax*, PHA plus CsA 1.4 and PHA alone 7.3.

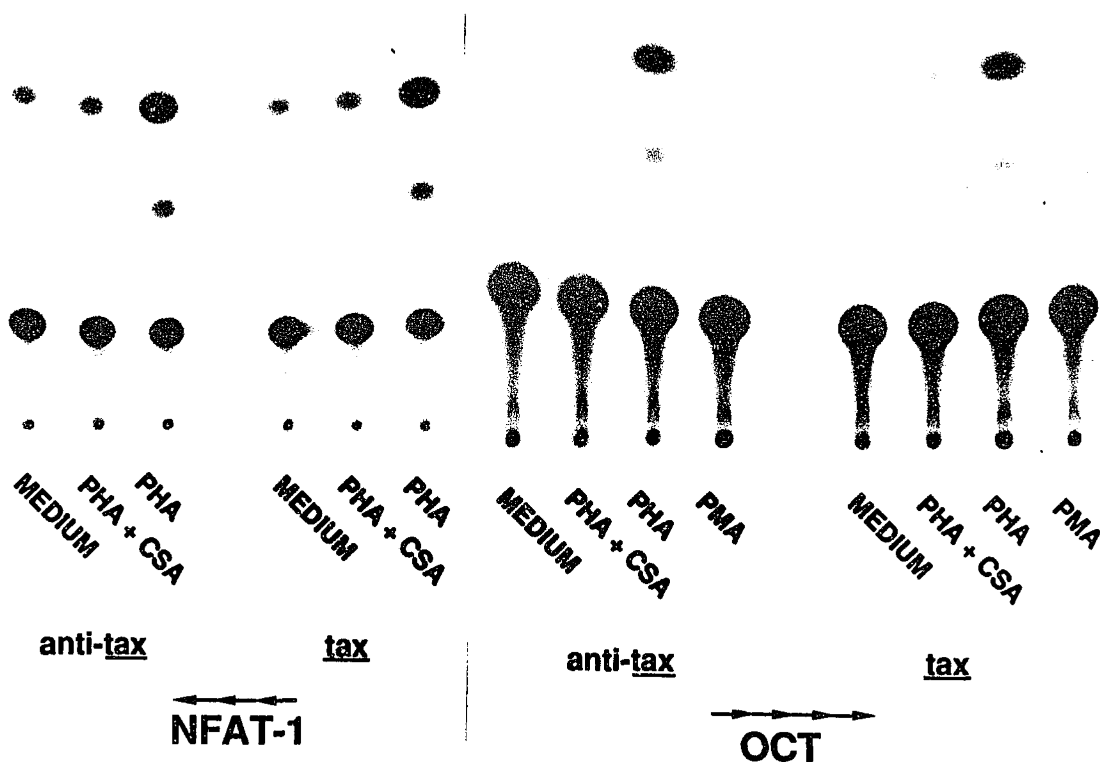


Fig. 3(B) pNFAT-1 was transfected into J-anti-*tax*-10 cells and J-*tax*-19 cells. CAT assays were performed as described in "Materials and Methods". The % transacetylation of pNFAT-1: Jurkat-anti-*tax*, medium alone 1.5, PHA plus CsA 4.1, PHA 46.4; Jurkat-*tax*, medium alone 1.0, PHA plus CsA 0.9, PHA 50.9.

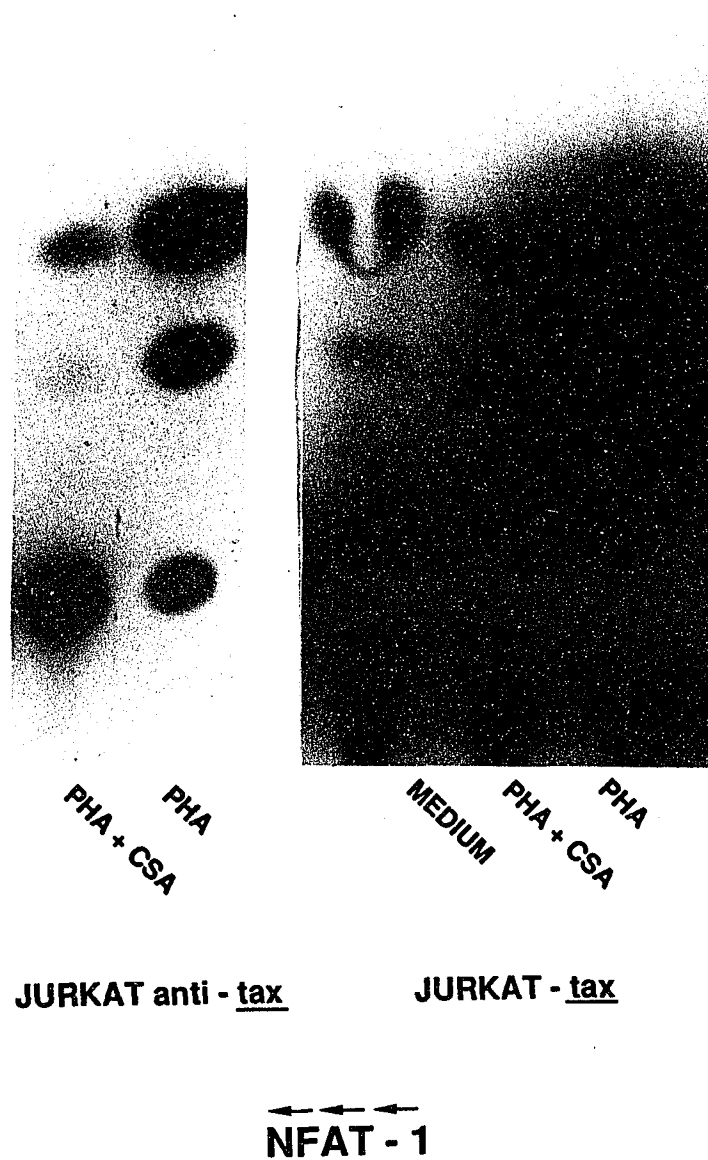


Fig. 4 *Tax* activation of DNA regions within the B fragment.

(A) The sequences spaning the B region (-164 to -52) were dissected into several pieces and cloned into TK-CAT. The Oct-1 region of the enhancer has been placed in front of the gamma-fibrinogen promoter and the CAT gene (12).

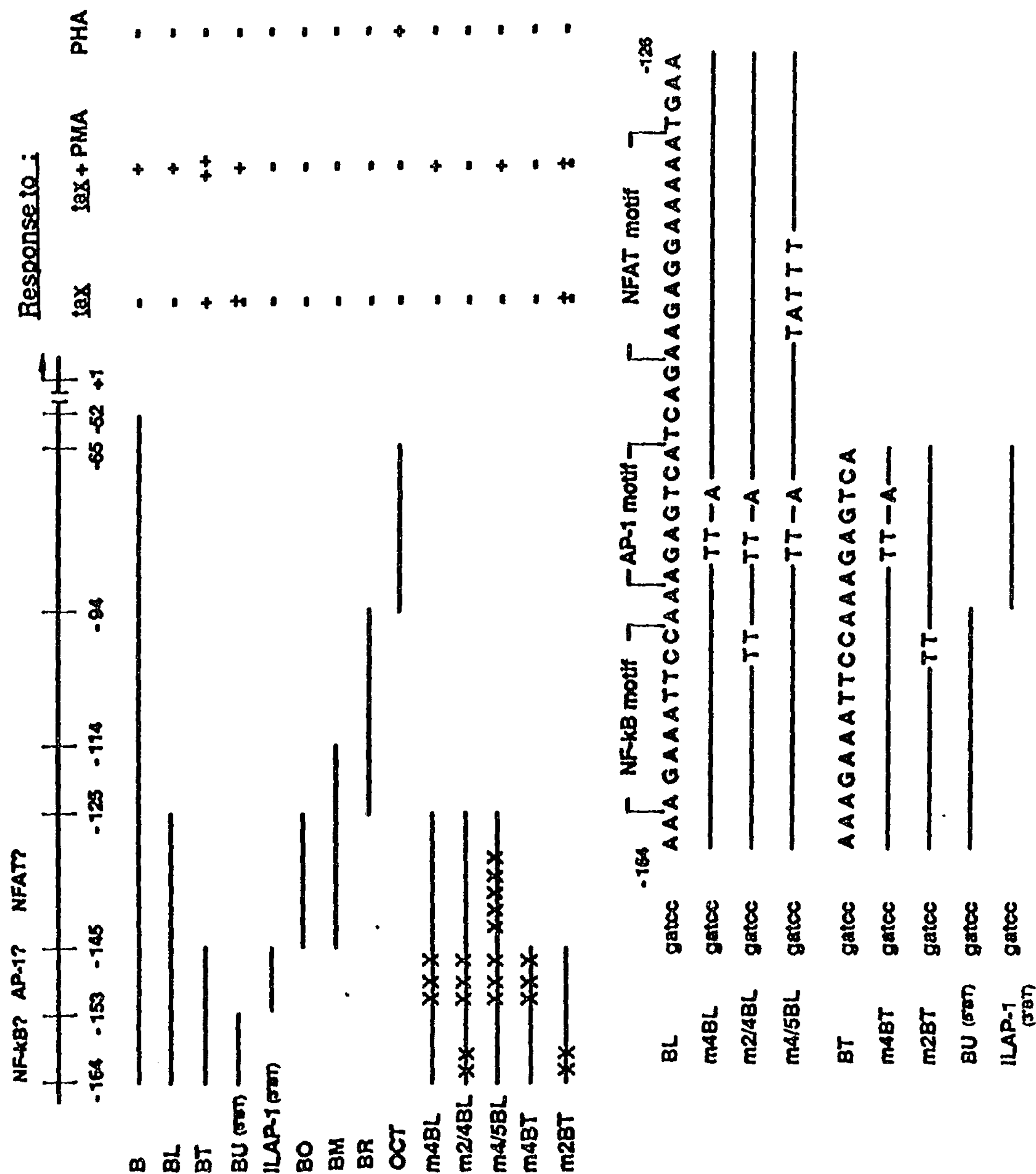


Fig. 4(B) The oligonucletides in Fig. 4A were cloned into CAT expression plasmids. The arrows represent the number of copies of each insert in the TK-CAT plasmid. The direction of the arrowhead indicates the orientation of the insert in the TK-CAT plasmid. These constructs were cotransfected into Jurkat T cells with either a *tax* expression vector or an antisense-*tax* expression vector. CAT assays were performed as described in "Materials and Methods". The results are based on 2 to 6 experiments with at least 2 independent plasmid preparations for each construct except that pBL-4, pBO-4, pBU-5 and pOCT were tested only once. Data are presented as described in Fig. 1. The % transacetylation before normalization (mean±SD) of each plasmid without inducers in the absence of *tax* are as follows: pBL-5 1.4 ± 0.5, pBL-4 0.9 ± 0.4, pm4/5BL 1.6 ± 0.4, pm2/4BL 1.6 ± 0.9, pBT-7 0.6 ± 0.2, pBT-17 0.98 ± 0.23, pBT-5 0.61 ± 0.31, pm2BT-20 1.3 ± 0.3, pm4BT-8 0.9 ± 0.3, pBU-5 1.2, pBO-4 0.72, pBM-7 1.4 ± 0.8, pBR-18 0.7 ± 0.4, pOCT 1.0, TK-CAT 3.1 ± 1.2.

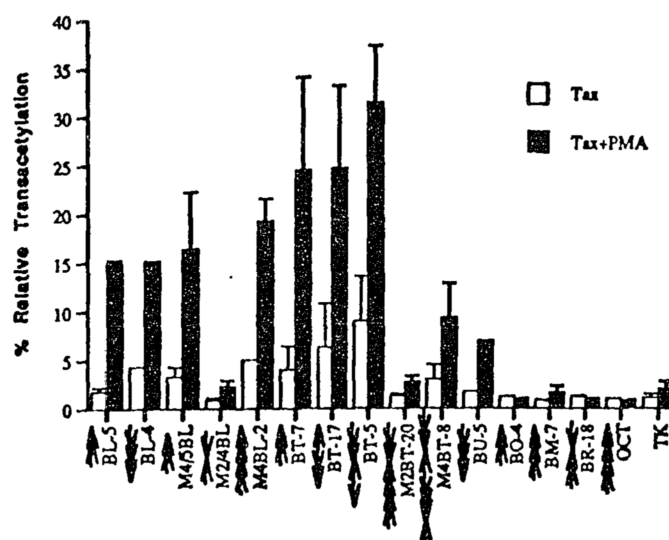


Fig. 5. pBL-5 and pBT-17 were transfected into Jurkat T cells with either an anti-*tax* or a *tax* expression vector. CAT assays were performed as described in "Materials and Methods". The % transacetylation of pBL-5: in the presence of anti-*tax*, 3.4 (medium alone), 4.2 (PMA); in the presence of *tax*, 12 (medium alone), 48 (PMA plus CsA), 69 (PMA). The % transacetylation of pBT-17: in the presence of anti-*tax*, 3 (medium alone), 2.7 (PMA); in the presence of *tax*, 33 (medium alone), 84 (PMA plus CsA), 85 (PMA).

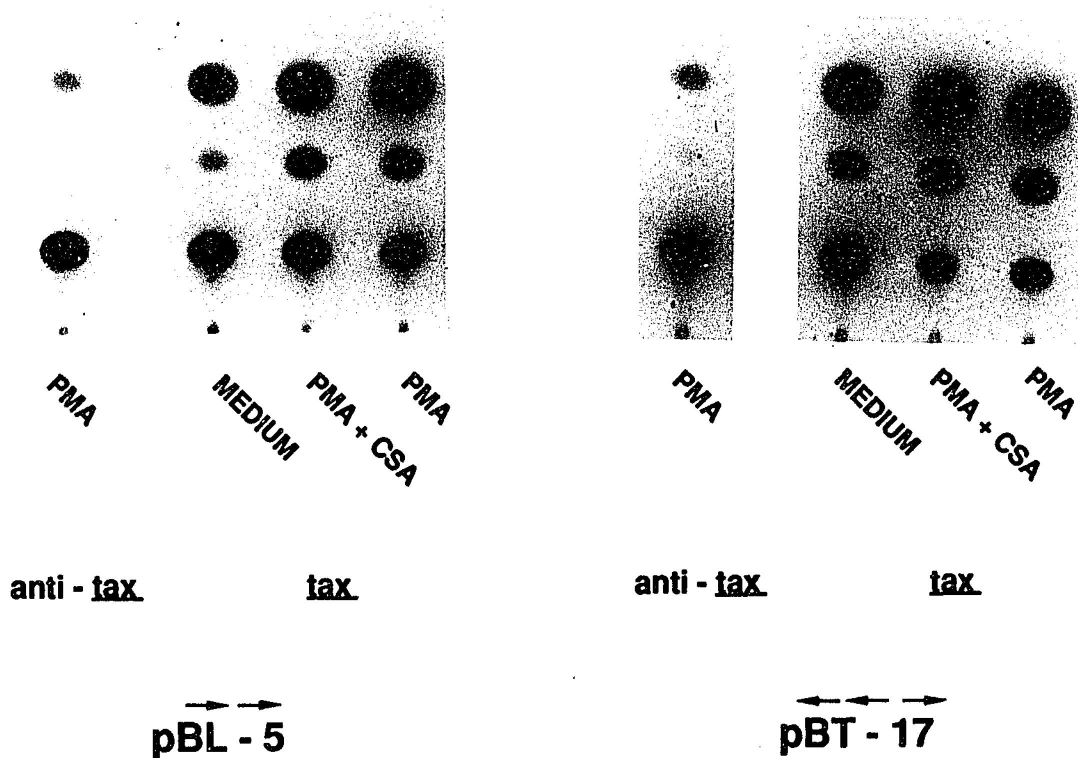


Fig. 6. pBL-4 was transfected into J-anti-*tax*-10 and J-*tax*-19 cells. CAT assays were performed as described in "Materials and Methods". The % transacetylation of pBL-4: J-anti-*tax*-10, 0.6 (medium alone), 0.6 (PHA plus CsA), 0.7 (PHA); J-*tax*-19, 16.3 (medium alone), 25.9 (PHA plus CsA), 29.7 (PHA).

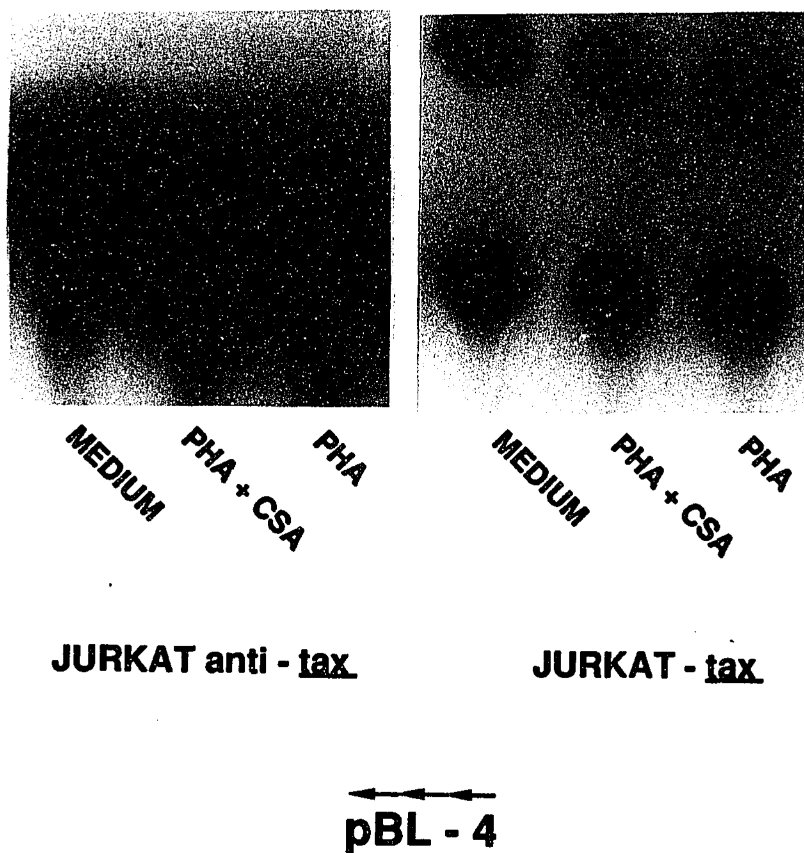


Fig. 7. Activity of pILAP-1 and pCOLLAP-1.

pILAP-1, containing 6 copies of ILAP-1 linked to the TK-CAT construct, or pCOLLAP-1, containing 5 copies of consensus AP1 linked to TK-CAT, were transfected into Jurkat T cells with either an anti-*tax* or *tax* expression vector. CAT assays were performed as described in "Materials and Methods". The % transacetylation of each plasmid are as follows: pILAP-1, anti-*tax* 2.8, anti-*tax* plus PHA plus PMA 3.4, *tax* 2.2, *tax* plus PHA plus PMA 6.0; pCOLLAP-1, anti-*tax* 15.3, anti-*tax* plus PHA plus PMA 45, *tax* 9.0, *tax* plus PHA plus PMA 39.3, control plasmid TK-CAT, anti-*tax* 3.9, anti-*tax* plus PHA plus PMA 9.0, *tax* 3.9, *tax* plus PHA plus PMA 9.0.

ILAP-1: GATCCA<u>A</u>GAGTCA. COLLAP-1: GATCCA<u>T</u>GAGTCA.

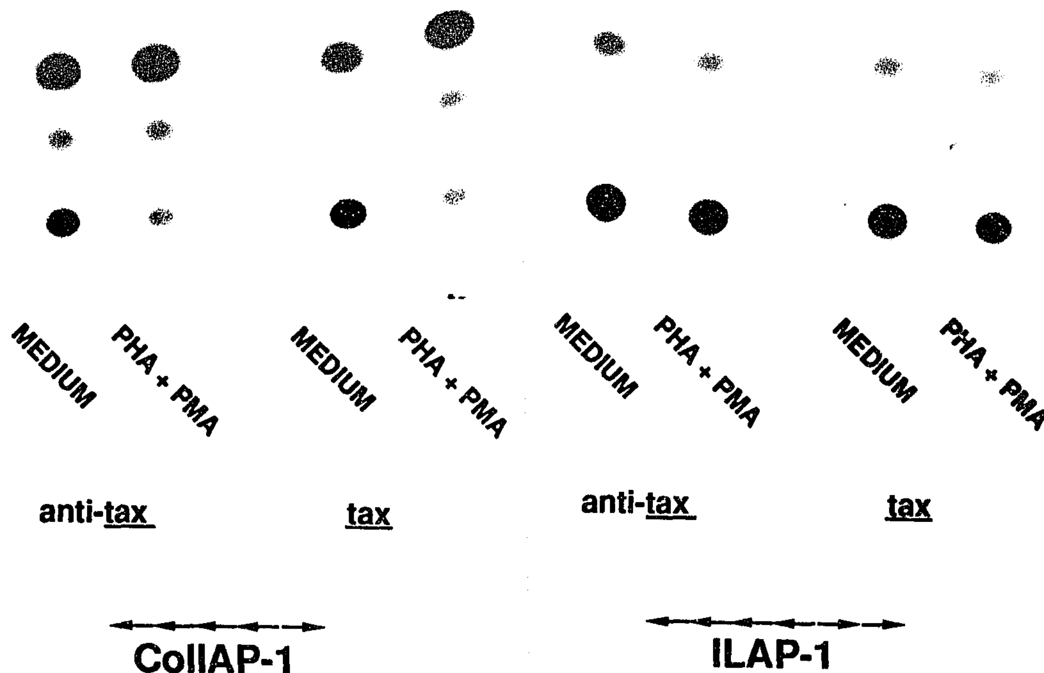


Fig. 8. *Tax* inducibility of the IL-2 enhancer internal deletion mutants.

(A) Transfections were performed as described in Fig. 2. The results represent 2-5 experiments with 2 independent plasmid preparations for each construct. The % transacetylation of each plasmid in the presence of PMA is normalized to 1% transacetylation in the absence of PMA. The open bars represent the activity of deletion mutants relative to the wild type IL-2 enhancer (-576 to +42) which is set at 100 in PMA treated cells cotransfected with *tax*. The numbers following ID represent 5' to 3' deleted bases of each mutant. For comparison, the numbers at the bottom of the figure represent the activity of plasmids cotransfected with anti-*tax* in PHA plus PMA treated cells relative to 100, the activity of wild type in PMA treated cells cotransfected with *tax*. The % transacetylation of the wild type IL-2 enhancer (pIL-2CAT) in PMA treated cells cotransfected with *tax* is 8.3±1.1. The %transacetylation (mean±SD) of each plasmid in the presence of either anti-*tax* or *tax* (in parentheses) without inducers are as follows: pIL2-CAT 0.24±0.14 (0.37±0.35), ID317/286 0.72±0.27 (1.20±0.50), ID279/263 0.69±0.10 (0.50±0.10), ID255/217 0.80±0.36 (0.55±0.26), ID208/174 0.86±0.64 (1.5±0.67), ID159/151 0.80±0.61 (0.94±0.37), ID152/121 0.24±0.10 (0.78±0.50), ID89/73 1.9±1.8 (0.58±0.42).

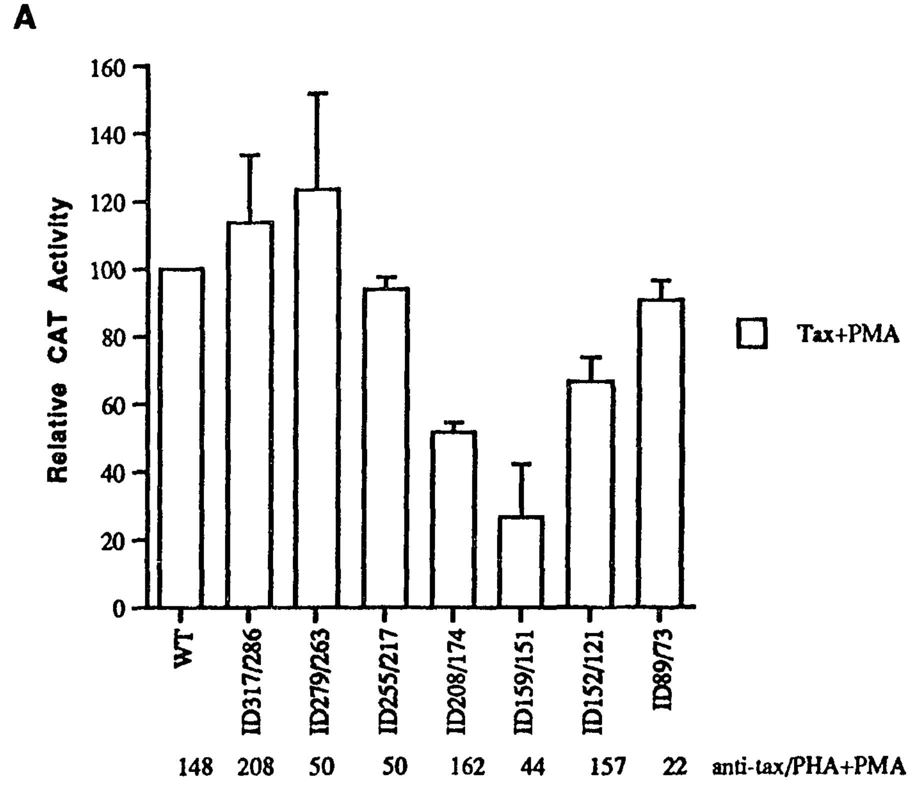

A
Relative CAT Activity
0
20
40
60
80
100
120
140
160
WT
ID317/286
ID279/263
ID255/217
ID208/174
ID159/151
ID152/121
ID89/73
148
208
50
50
162
44
157
22
anti-tax/PHA+PMA
Tax+PMA

Fig. 8 (B) Transfections were performed as described in Fig.8A except that cells were treated with PHA. The numbers at the bottom of the figure represent the activity of plasmids cotransfected with anti-*tax* in PHA plus PMA treated cells relative to 100, the activity of wild type cotransfected with *tax* in PHA treated cells. The % transacetylation of pIL-2CAT in PHA treated cells cotransfected with *tax* is 11.8±4.2. The values of basal activity for the plasmids are the same as in Fig.8A.

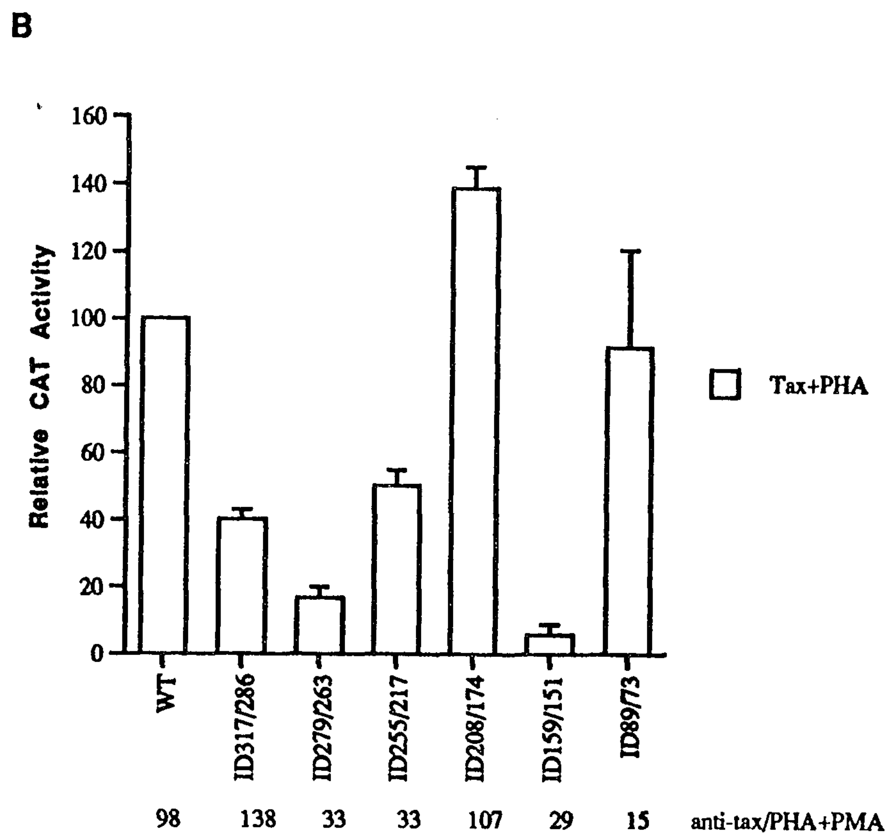


Fig. 8 (C) Transfections were performed as described in Fig.8A except that cells were treated with PHA plus PMA with CsA. The closed bars represent the activity of each mutant cotransfected with anti-*tax* relative to that cotransfected with *tax*. The numbers at the bottom of the figure represent the activity of plasmids cotransfected with either anti-*tax* or *tax* in PHA plus PMA treated cells relative to 100, the activity of wild type cotransfected with *tax* in PHA plus PMA plus CsA treated cells. The % transacetylation of pIL-2CAT in PHA plus PMA plus CsA treated cells cotransfected with *tax* is 12.2$\pm$6.0.

The values of basal activity for the plasmids are the same as in Fig.8A. ID59/45 was only tested once and its value of basal activity is 1.49 (1.46).

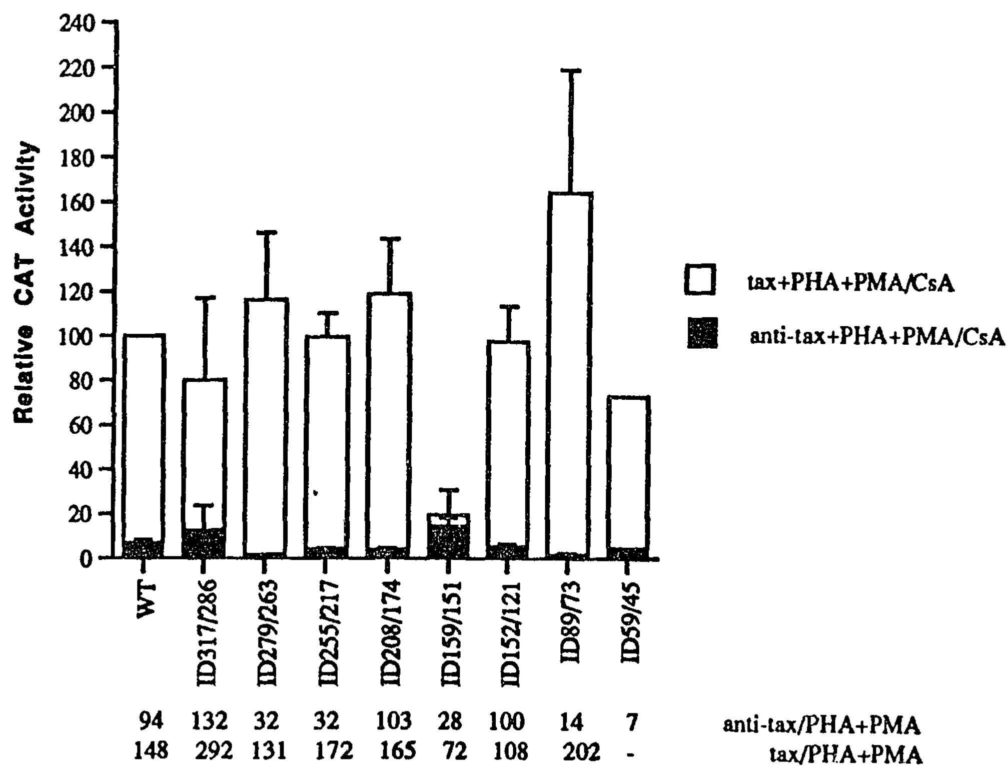


Fig. 9. pIL-2CAT and pBT-5 were cotransfected into Jurkat cells with either an *anti-tax* expression vector (anti-*tax*) or a *tax* expression vector. CAT assays were performed as described in "Materials and Methods". The data are presented as % transacetylation normalized to 1% transacetylation without inducer (medium alone) in the presence of anti-*tax*. The % transacetylation of each plasmid without inducers are as follows: pIL-2CAT 0.22 $\pm$ 0.02; pBT-5 0.61.

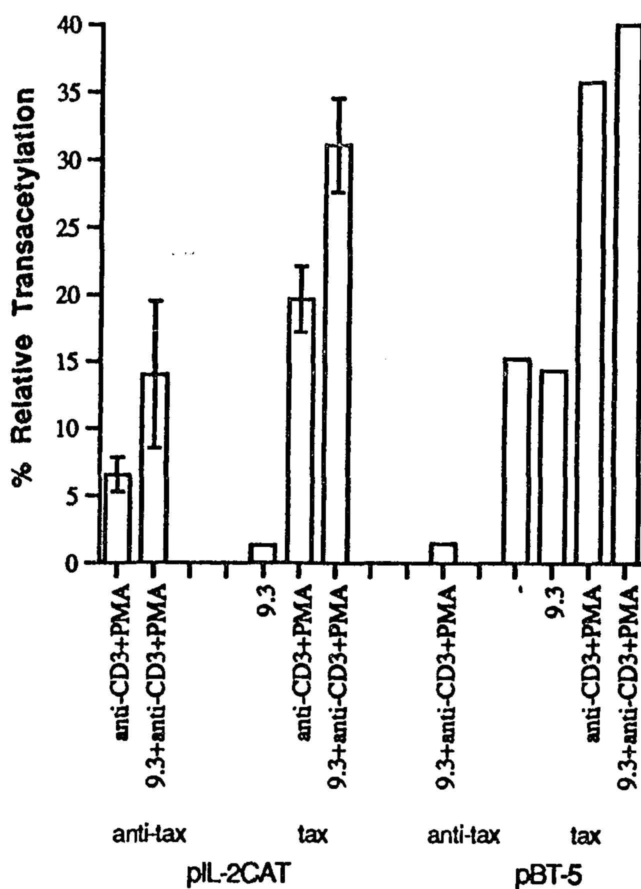


Fig. 10. The response to PHA, PHA plus CsA, and PHA plus CsA in the presence of *tax* were examined of the IL-2 enhancer, the A fragment, the B fragment, NFAT binding site, NF-kB binding site, BL region, TxRE (BT), and Oct-1 binding site (OCT) are shown. (-) repesents "No-response". (+) represents "Response". (+++) represents "very strong response" (See results for details).

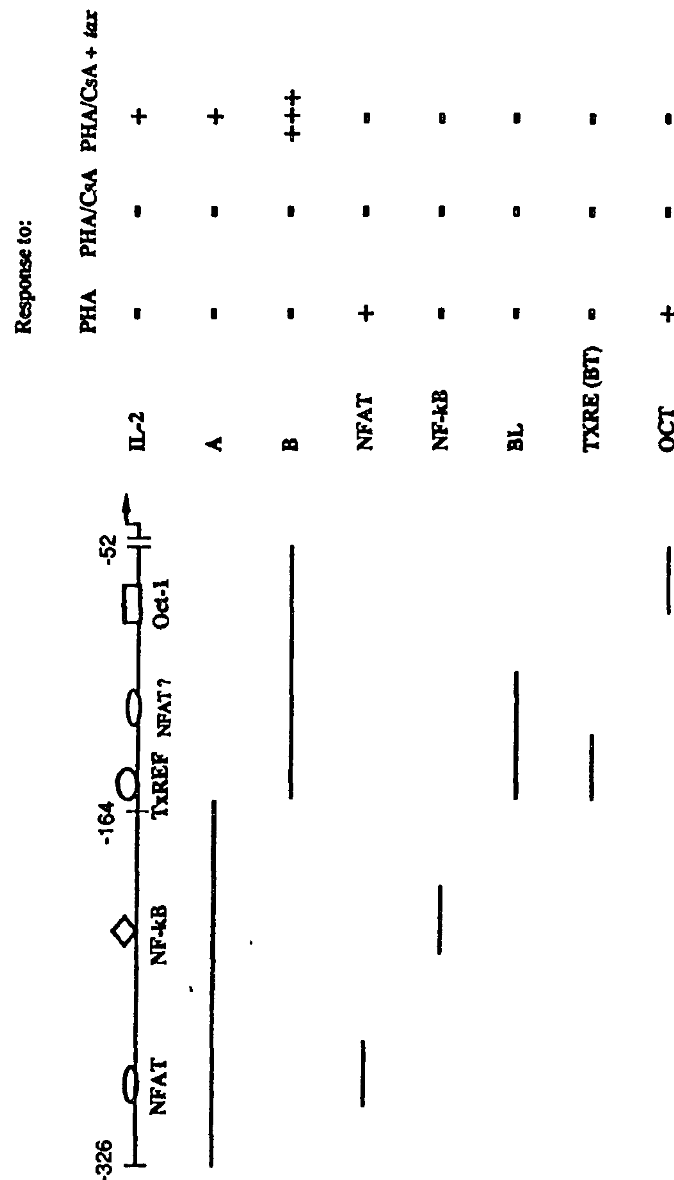


Fig.11. TxREF is only present in Jurkat-*tax* cells.

Radiolabeled BT (-164 to -145 bp in the IL-2 enhancer) was incubated with nuclear extract from J-anti-*tax*-2 cells stimulated with PMA (lane 1), J-*tax*-9 cells in medium alone (lane 2) or stimulated with PMA (lane 3), Jurkat cells stimulated with PHA plus PMA (lane 4) and Hela cells stimulated with PMA (lane 5). DNA binding, competition and gel retardation analysis was performed as described in "Materials and Methods". TxREF is denoted by the arrow.

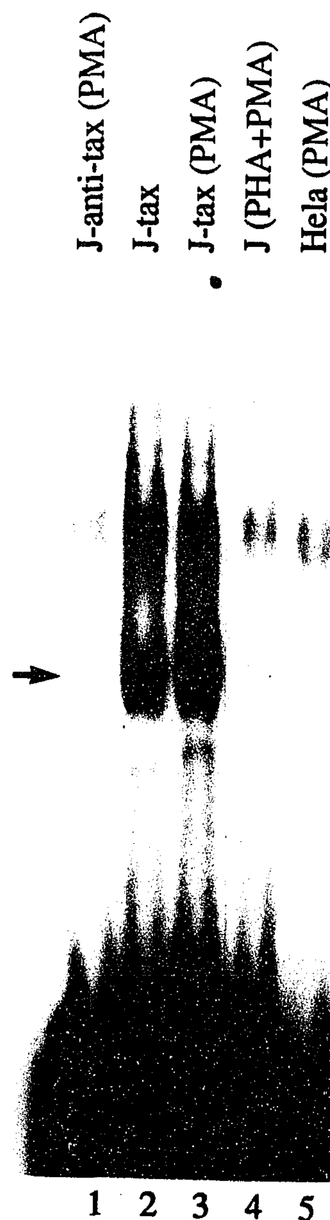
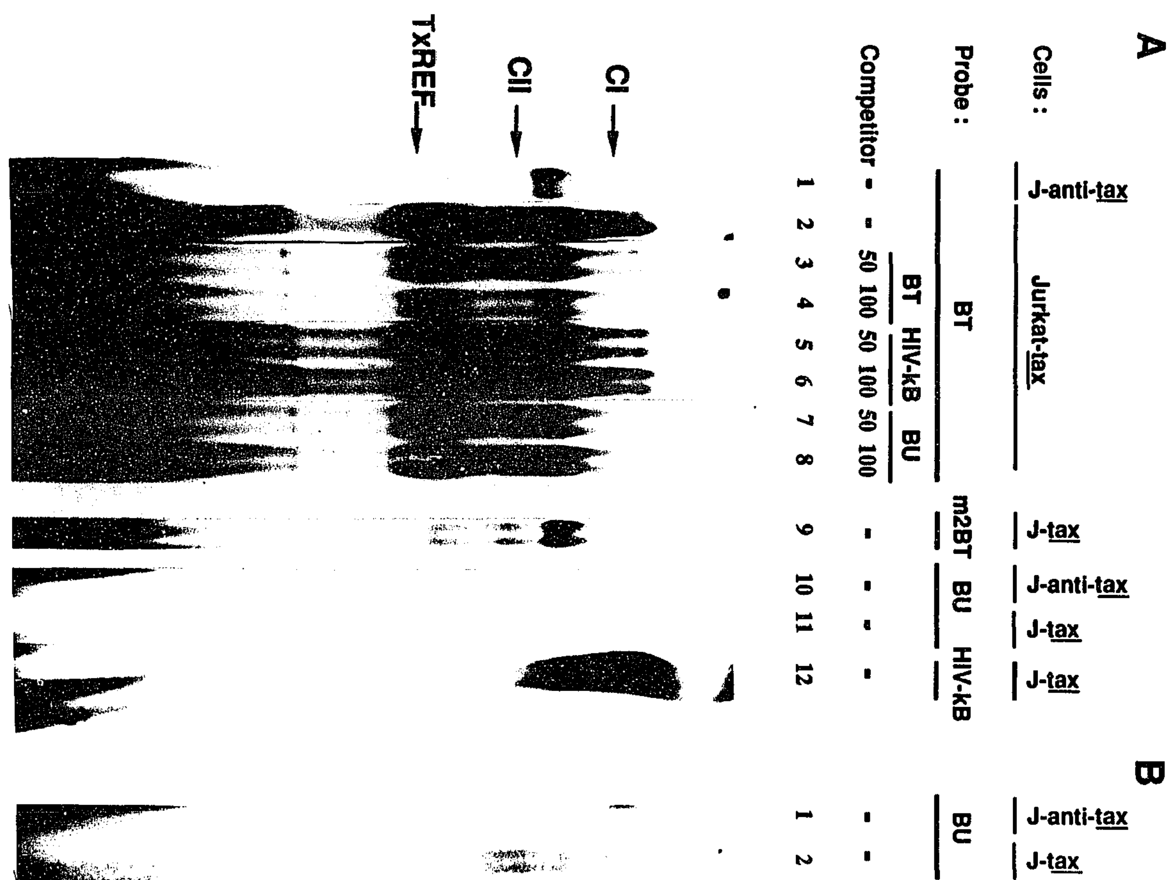


Fig. 12 (A) Radiolabeled BT was incubated with nuclear extract from J-anti-*tax*-2 cells (lane 1) or J-*tax*-9 cells in the presence of unlabeled BT (lanes 2-4), HIV-kB (lanes 5,6), or BU (lanes 7,8). The relative molar ratio of unlabeled oligonucletides versus labeled probe in the reaction mixture are indicated at the top of each lane. Nuclear extract from J-*tax*-9 cells was also incubated with radiolabeled m2BT (lane 9), BU (lane 11), or HIV-kB (lane 12). Complexes CI, CII and TxREF are indicated by arrows.

(B) Longer exposure of Figure 15A; lanes 10 (lane 1) and 11 (lane 2).



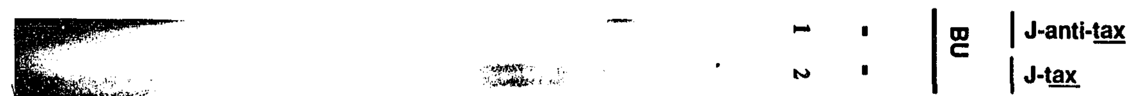
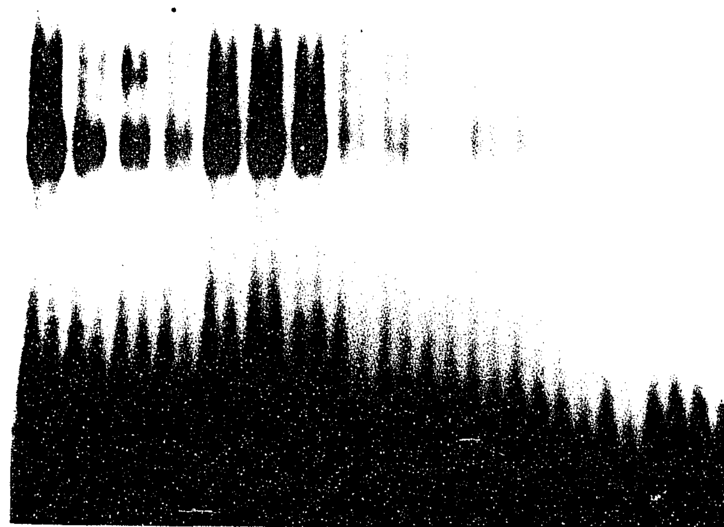


Fig. 13 TxREF specifically interacts with -164 to -154 bp.

Radiolabeled BT was incubated with nuclear extract from J-*tax*-9 cells. A 50, 100 or 200 fold molar excess of unlabeled competitors BT (lanes 14-16) or its mutated versions M1 (lanes 2-4), M2 (lanes 5-7), M3 (lanes 8-10), and M4 (lanes 11-13) were added to the binding reactions. BT: AAAGAAATTCCAAAGAGTCA; M1BT: AAGAGGATTCCAAAGAGTCA; M2BT: AAAGAAATTTTAAAGAGTCA; M3BT: AAAGAAATTCCGGGAAGTCA; M4BT: AAAGAAATTCCAAATTGACA.

Competitor: — M1 M2 M3 M4 WT

50 100 200 50 100 200 50 100 200 50 100 200 50 100 200



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 14 TxREF is a 45-kDa protein.

Radiolabeled BT with BrdU incorporated into the antisense strand was incubated with nuclear extract from J-*tax*-9 cells. The resulting complex was separated by electrophoresis in a 5% nondenaturing gel. After UV irradiation, the TxREF band was excised and subjected to 10% SDS-PAGE. The 45-kDa band is denoted by the arrow. The molecular weight of protein standards in kilodaltons are shown.

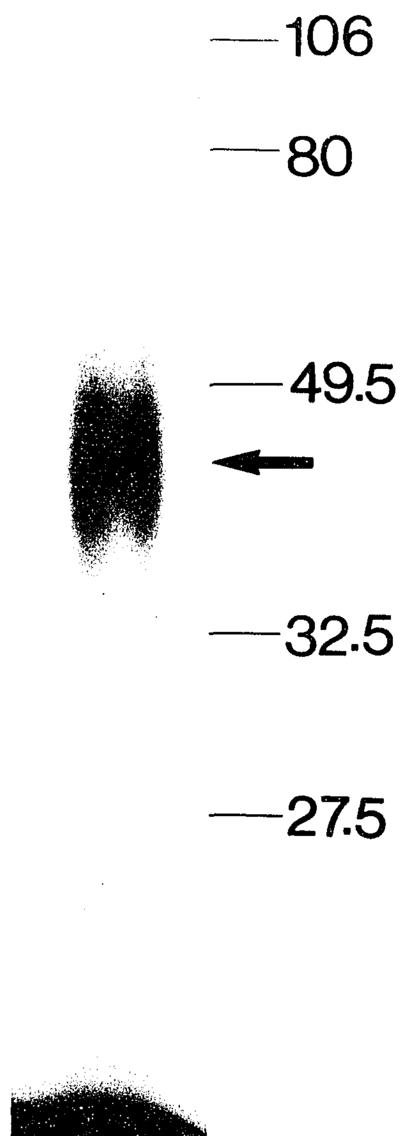


Fig. 15 Radiolabeled BT was incubated with fractions eluted from a Superdex 75 column: Fractions 70 (lane 1), 66 (lane 2), 64 (lane 3), 62 (lane 4), 60-50 (lane 5-15), 47 (lane 16) and 45 (lane 17). Radiolabeled 1kB DNA marker was coelectrophoresed (lane M).

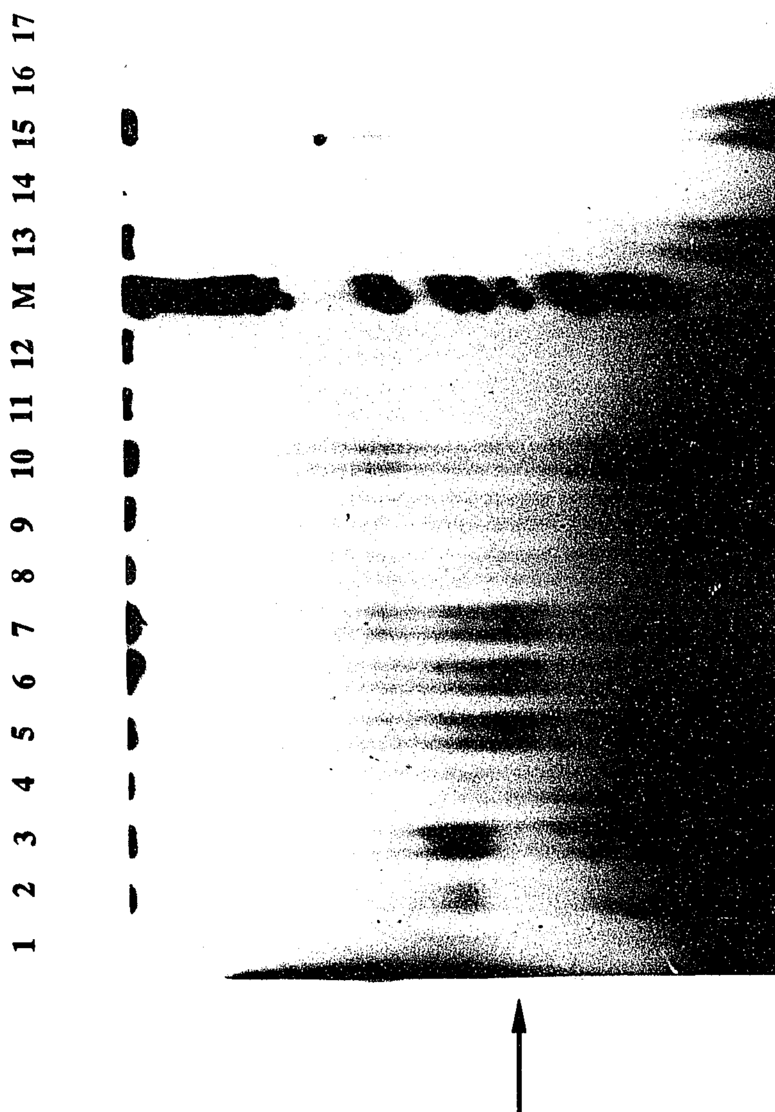


Fig. 16 TxREF is distinct from NF-kB.

(A) Radiolabeled BT was incubated with nuclear extract from J-*tax*-19 cells in the presence of 50 or 200 fold molar excess of unlabeled competitors BT (lane 2,3), 200 fold excess of IL2-kB (lane 4), 200 fold excess of IL6-kB (lane 5), 200 or 500 fold excess of HIV-kB (lane 6,7), or 500 fold excess of HTLV-I LTR 21bp repeat (lane 8).

Competitor: — BT IL2-kB IL6-kB HIV-kB HT-21

50 200 200 200 200 500 500

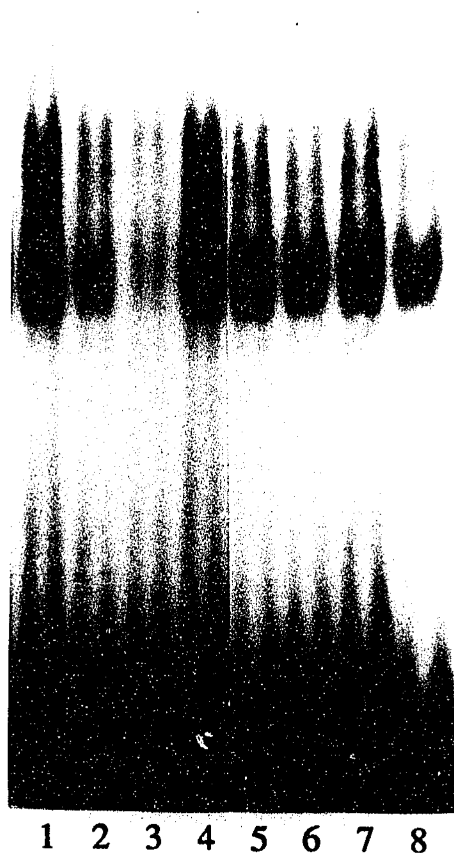


Fig. 16(B) Radiolabeled BT (lanes 1-7) or radiolabeled IL2R-kB (lanes 8-16) was incubated with nuclear extract from J-*tax*-19 cells in the presence of unlabeled competitors IL2R-kB (lanes 2-4 and 9-12) or BT (lanes 5-7 and 13-16).

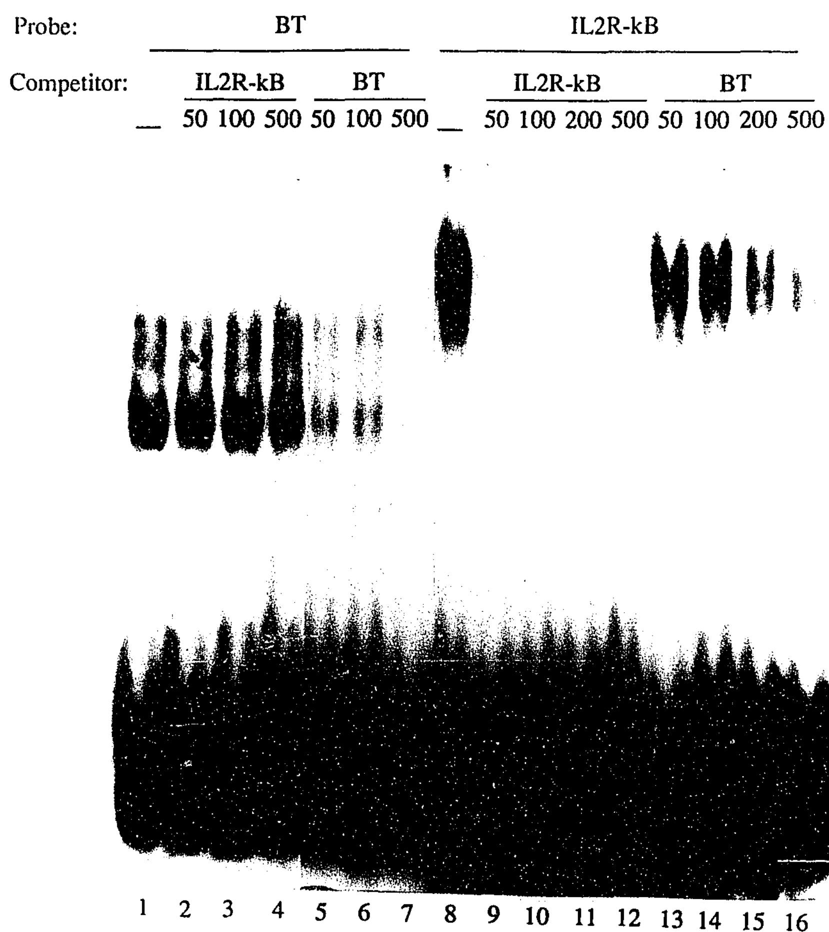


Fig. 17(A) Radiolabeled BT was incubated with nuclear extracts from J-*tax*-19 cells (lane 1), J-*tax*-19 cells treated with CsA (lane 2), K5637 cells (lane 3), or K5637 cells stimulated with PMA (lane 4). Radiolabeled CK-1 probe was incubated with nuclear extracts from J-anti-*tax*-2 cells (lane 5), J-*tax*-19 cells (lane 6), K5637 cells (lane 7) or K5637 cells stimulated with PMA.

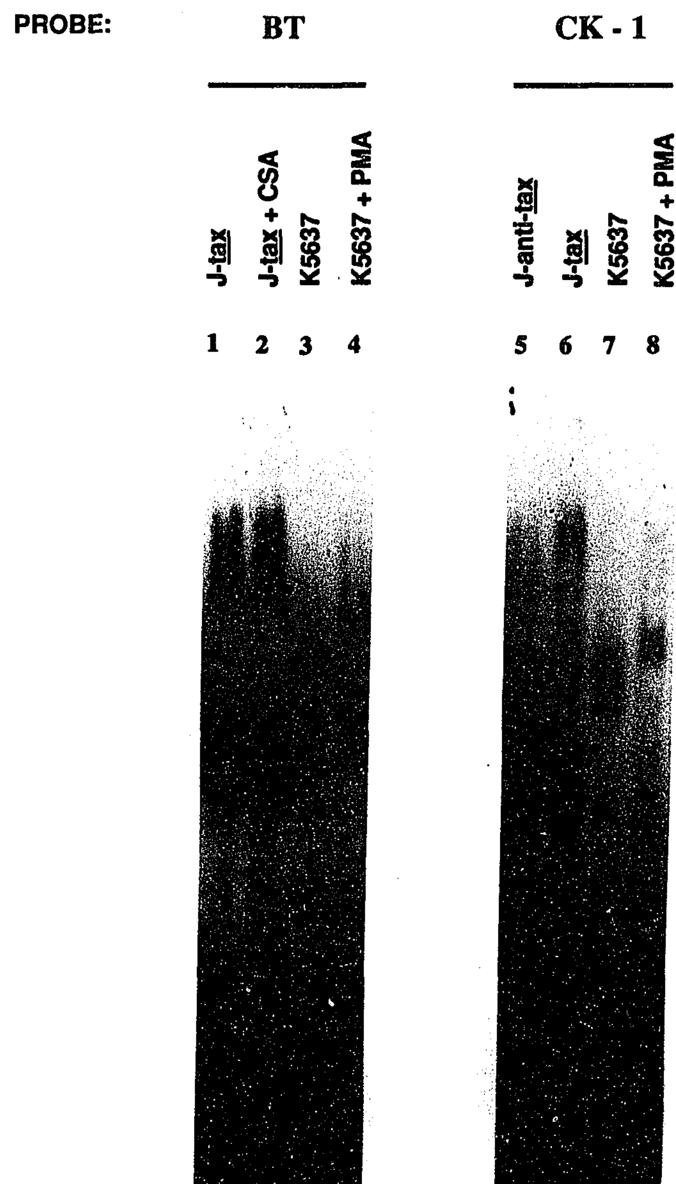


Fig. 17(B) Radiolabeled BT was incubated with nuclear extracts from J-*tax*-19 cells stimulated by PMA in the presence of 100 fold (lane 2) or 200 fold (lane 3) unlabeled BT or 100 fold (lane 4) or 200 fold (lane 5) unlabeled CK-1.

JURKAT-tax + PMA

PROBE : BT

COMPETITOR : BT CK - 1

- 100 200 100 200

1 2 3 4 5

Fig. 18. ILAP-1 does not bind AP-1

Radiolabeled ILAP-1 (-152 to -145 gatccAAGAGTCA) was incubated with nuclear extracts from Jurkat cells either unstimulated (lane 1) or stimulated with PMA (lanes 2-5), Hela cells either unstimulated (lane 11) or stimulated with PMA (lane 12). Radiolabeled consensus AP-1 (gatccATGAGTCA) was incubated with nuclear extracts from either unstimulated (lane 6) or stimulated by PMA (lanes 7-10), Hela cells either unstimulated (lane 16) or stimulated with PMA (lanes 13-15). Cold competitors ILAP-1 (lanes 3,8,15), AP-1 (lanes 4,9,14), BO (lane 5) or BH8 (lane 10) are present in a 100 fold molar excess of radiolabeled probes. BH8 was used as nonspecific competitor.

BH8: AGCTGGTGTAACAAGCTGGTGTTCTCTCCTTTATTG.

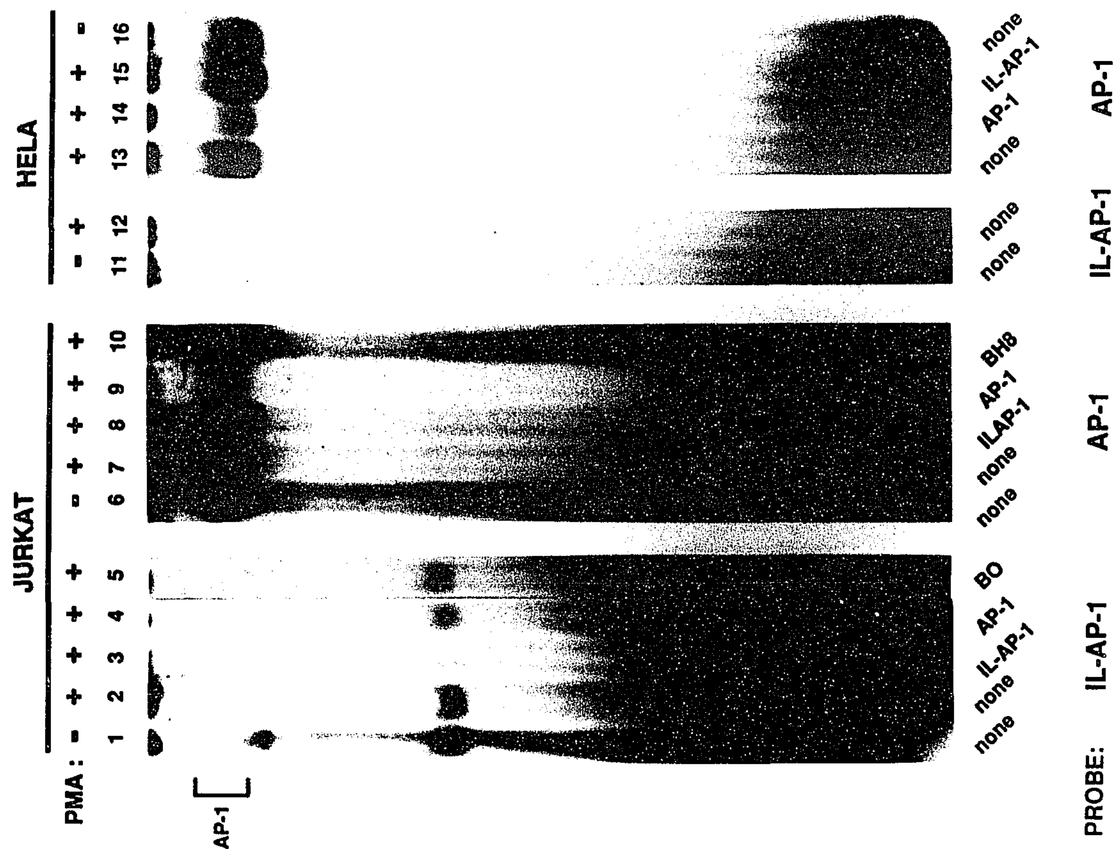


Fig. 19 Radiolabeled NFAT was incubated with nuclear extracts from Jurkat-*tax* cells in medium alone (lane 1), treated by CsA (lane 2), ionomycin plus PMA (lane 3), or ionomycin plus PMA in the presence of CsA (lane 4); Jurkat-anti-*tax* cells stimulated by ionomycin plus PMA (lane 5) or ionomycin plus PMA in the presence of CsA (lane 6); Jurkat cells (lane 7), stimulated by PHA plus PMA (lane 8), ionomycin plus PMA (lane 9) or ionomycin plus PMA in the presence of CsA (lane 10).

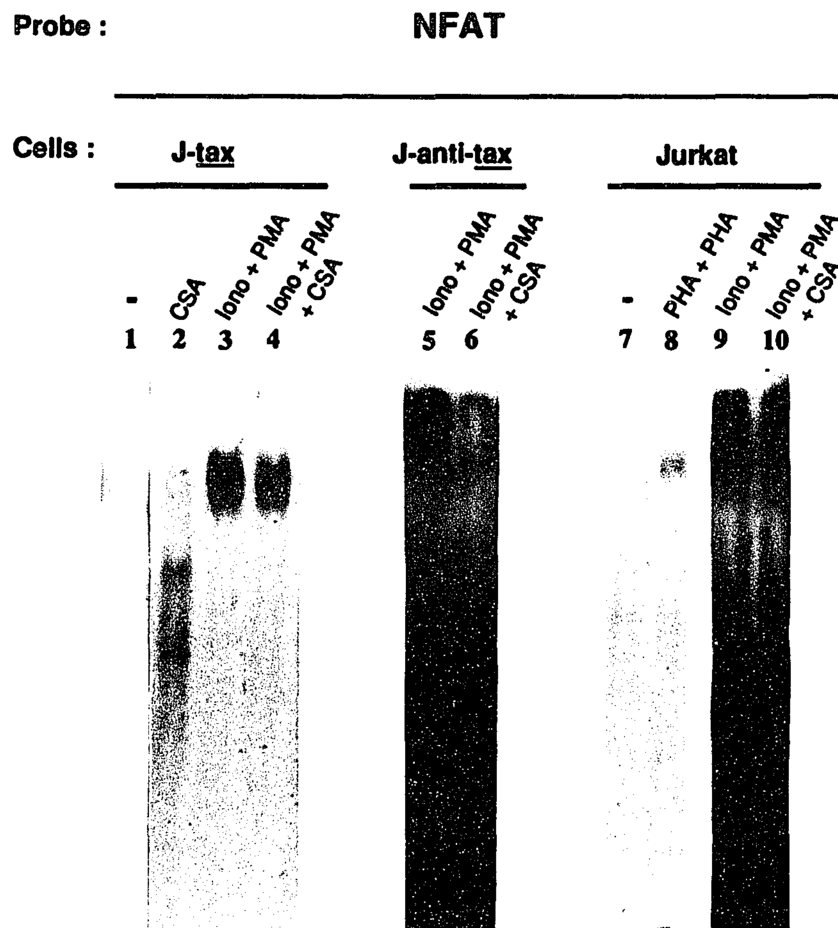
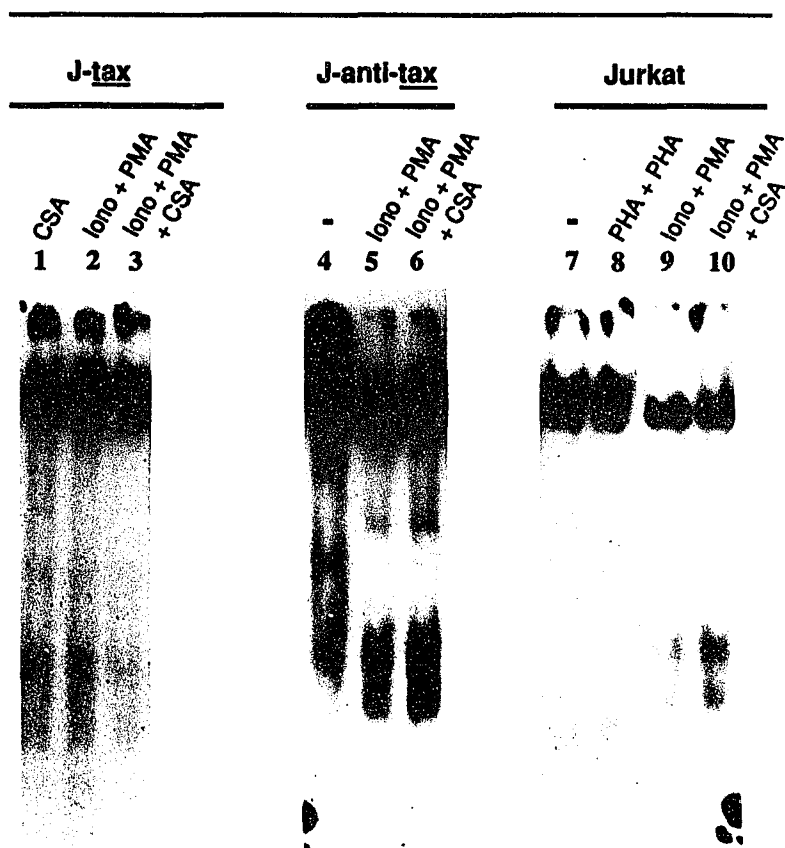


Fig. 20 Radiolabeled Oct-1 was incubated with nuclear extracts from J-*tax*-19 cells treated by CsA (lane 1), stimulated by ionomycin plus PMA (lane 2) or ionomycin plus PMA in the presence of CsA (lane 3); J-anti-*tax*-2 cells (lane 4), stimulated by ionomycin plus PMA (lane 5) or ionomycin plus PMA in the presence of CsA (lane 6); Jurkat cells (lane 7), stimulated by PHA plus PMA (lane 8), ionomycin plus PMA (lane 9) or ionomycin plus PMA in the presence of CsA (lane 10).



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