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**Transcriptional regulation of interleukin-2 gene expression
through NF-kB and RW-BF**

Hoyos, Beatrice, Ph.D.

City University of New York, 1993

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TRANSCRIPTIONAL REGULATION OF INTERLEUKIN-2 GENE

EXPRESSION THROUGH NF-kB AND RW-BF

by

Beatrice Hoyos

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

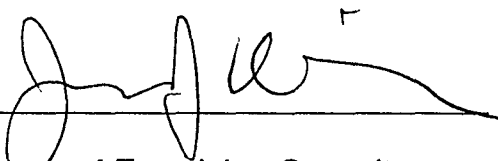
Beatrice Hoyos

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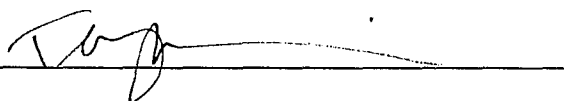
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THE CITY UNIVERSITY OF NEW YORK

ABSTRACT**TRANSCRIPTIONAL REGULATION OF INTERLEUKIN-2 GENE
EXPRESSION THROUGH NR-kB AND RW-BF**

by

Beatrice Hoyos

Advisor: Miriam Siekevitz, Ph.D.

Interleukin-2 (IL-2) is a T cell growth factor required for the clonal expansion of activated T cells. This lymphokine is regulated at the level of transcription, and several cis-elements and transacting factors that regulate its level of expression have been identified. This thesis work has concentrated on the identification and characterization of DNA binding proteins interacting with the sequences -206 to -195 and -197 to -183 upstream of the transcription initiation site in the IL-2 promoter.

Inspection of the IL-2 promoter led to the recognition of the sequence -206 to -195 as an element that shares 7 out of 10 bases with the NF-kB binding motif.

Mutagenesis of this sequence resulted in 30- to 75% down regulation of the promoter function. The factor binding this motif was identified as Nuclear Factor-kappa B (NF-kB) based on several criteria.

Mutagenesis of the sequence (termed RW) situated immediately downstream of NF-kB at position -197 to -183 was conducted to determine its contribution to the regulation of IL-2 gene expression. The results showed a 25- to 50% decrease in promoter function. The binding of the complex RW-binding factor (RW-BF) was characterized and differentiated from NF-kB binding.

To identify the protein binding to this consensus sequence a HUT78 cDNA expression library was screened using as probe the sequence -197 to -183. Two identical clones were isolated. Northern blot analysis of Jurkat cell indicated the presence of a single band of approximately 2500 bases. The nucleotide sequence of the clones share a 99.5% similarity with a previously identified protein, namely DNA binding protein B (Dbpb).

Altogether, we have identified two transcription factors important in the regulation of IL-2 gene expression, namely NF-kB and RW-BF. The cDNA encoded protein has not yet been proven to be the protein that interacts with the -197 to -183 sequence in the Jurkat cells nuclear extracts, namely RW-BF.

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Finally, thanks to my family and friends for their support and encouragement. These years would have been very difficult without you.

Format of Thesis

This thesis was prepared in accordance with guidelines of the City University of New York. Chapter II contains results published as Beatrice Hoyos, Dean W. Ballard, Ernst Bohnlein, Miriam Siekevitz, and Warner C. Greene. *Science* 244: 254-60 (1989). The data for figures 3 and 4 were contributed by the collaborators (this work is used with the publisher's permission). Chapter IIIA, IIIB, and IIIC contain unpublished results. Each section has an Abstract, Introduction, Materials and Methods, Results, and Discussion. There is a general introduction and general discussion at the beginning and end of the thesis. To avoid redundancy the references for all the chapters have been consolidated.

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INTRODUCTION

T cells play a central role in the immune system. Their quiescent state is terminated by their interaction with antigen and accessory cells. This activation results in blast transformation, proliferation, differentiation, and immune function. Interleukin-2 (IL-2), a T cell growth factor, is essential for the clonal expansion of activated cells. The expression of IL-2 as well as that of IL-2 receptor alpha chain (IL-2R alpha) are indicative of the activated state of the T cells (for review see Crabtree, 1989).

There is a two signal requirement for the optimal initiation of IL-2 gene expression in normal peripheral blood T cells as well as in Jurkat T cells. One signal is mediated by antigen interacting with the T cell receptor (TCR), which results in cytoplasmic influx of calcium. This signal can be substituted by phytohemagglutinin (PHA), concanavalin A (Con A), calcium ionophores (Ionomycin and A23187), and anti-CD3 antibodies (Weiss et. al., 1984; Larsson et. al., 1980; Smith, 1980; Truneh et. al., 1985). The second signal is mediated through interaction with antigen presenting cells (APC), which release monokines such as IL-6 and IL-1 that activate protein kinase C (PKC). This second signal may be replaced by phorbol esters such as phorbol 12-myristic 13-acetate, PMA (Larsson et. al., 1980; Weiss et. al., 1984). Transcription factors respond to the transduced activation signals and activate the IL-2 gene.

The human T cell leukemia virus type 1 (HTLV-1) transcriptional transactivator tax can induce IL-2 gene expression. Tax alone results in low levels of IL-2 expression; however, it acts in synergy with PHA and PMA to induce high levels of IL-2 promoter function (Maruyama et. al., 1987; Siekevitz et. al., 1987).

Cyclosporin A (CsA) is an immunosuppressant that has been shown to inhibit IL-2 transcription (Kronke et. al., 1984; Elliott et. al., 1984; Bickel et. al., 1987). However, its mechanism of inhibition is not yet understood. There are two hypotheses: One, the binding of transcription factors important for the regulation of IL-2 expression is weakened or prevented by CsA (Emmel et. al., 1989; Granelli-Piperno et. al., 1990; Randok et. al., 1990; Baumann et. al., 1991). Second, CsA interacts with its intracellular receptor cyclophilin (Takahashi et. al., 1989) forming an inhibitory complex which then binds to calcineurin. Calcineurin is a calcium/calmodulin-dependent phosphatase (Liu et. al., 1991; Liu et. al., 1992; Clifton and Crabtree, 1992) that activates the IL-2 promoter (O'Keefe et. al., 1992). Binding of the inhibitory complex to calcineurin results in the inhibition of the nuclear translocation of the cytosolic subunit of nuclear factor of activated T cells (NFAT-1), a factor essential for IL-2 expression. Consequently, IL-2 transcription initiation is inhibited (Flanagan et. al., 1991; Jain et. al., 1992; Schreiber and Crabtree, 1992).

IL-2 is regulated at the level of transcription initiation and post-transcriptionally at

the level of mRNA stability. Early studies demonstrated the presence of IL-2 mRNA only in activated cells (Efrat et. al., 1984; Elliot et. al., 1984). Furthermore, nuclear run-on assays suggested the regulation to be at the level of transcription initiation (Kronke et. al., 1984). Evidence for the posttranslational regulation come from the study by Efrat and colleagues (1984) that showed the level of IL-2 mRNA to be susceptible to a repressor protein that could be inhibited by cycloheximide treatment only after IL-2 mRNA transcription had initiated. Recent work (Lindsten et. al., 1989), showed enhanced IL-2 expression as a result of augmented stability of the IL-2 mRNA in cells that had been stimulated by mitogen plus anti-CD28. How anti-CD28 activation leads to IL-2 mRNA stability is not yet known.

Detailed analyses of the IL-2 promoter and enhancer element have been conducted. The minimum IL-2 promoter region consists of 380 nucleotides upstream from the transcription initiation site. This region is sufficient to confer T cell specificity, the two signal requirement, sensitivity to CsA, and responsiveness to tax activation, all of which are characteristic of IL-2 regulation, upon a heterologous promoter (Fujita et. al., 1986; Durand et. al., 1987; Williams et. al., 1987). Mutational analyses of the IL-2 enhancer element as well as DNase protection studies identified several cis-elements essential for optimal enhancer activity (Durand et. al., 1988; Brunvand et. al., 1988; Shibuya et. al., 1989).

NFAT-1 is a T-cell-specific factor having a major regulatory function in the IL-2

promoter. It is a PHA-induced protein with kinetics of appearance (Shaw et. al., 1988) and sensitivities to immunosuppressants paralleling those of IL-2. Mutagenesis or deletion of the NFAT motif at position -285 to -255 results in greater than 50% promoter inhibition (Durand et. al., 1988). Furthermore, transgenic mice studies show that the NFAT binding element is able to regulate the transcription of a reporter gene (Verweij et. al., 1990). As mentioned above, NFAT is sensitive to CsA. This has been demonstrated by inhibition of NFAT binding in the presence of CsA, and by the inhibition on NFAT directed transcription (Emmel et. al., 1989; Schmidt et. al., 1990; Mattila et. al., 1990). Tax, however, has no effect on NFAT binding activity (Miam Li, personal communication). Within the promoter there is a sequence -145 to -158 which share homology with the NFAT element and has been shown to be sensitive to CsA and is known as NFIL2B (Mattila et. al., 1990). This sequence may represent a second binding site for NFAT.

NFAT consists of two subunits: one is cytosolic and constitutively expressed, the other is an inducible nuclear protein, possibly AP1 (Jain et. al., 1992). It is suggested that CsA inhibits the nuclear translocation of the cytosolic subunit preventing NFAT formation (Flanagen et. al., 1991).

Oct-1 is a ubiquitous, PHA-induced transcriptional regulatory factor demonstrated to have two binding sites within the IL-2 promoter at positions -242 to -255 and -63 to -93. Point mutations within these elements, or deletions result in 30 to 50%

inhibition of promoter function respectively (Durand et. al., 1988; Shibuya et. al., 1989; Kamps et. al., 1991). The proximal element has been implicated in the negative regulation of promoter (Nabel et. al., 1988). This implication in negative regulation may have resulted from its proximity to the binding site (-110 to -101) of NIL-2-a which negatively regulated IL-2 expression (Williams et. al., 1991). Oct-1 and an inducible protein, octamer associated protein (OAP40), function to regulate the IL-2 promoter (Ullman et. al., 1991). The element -63 to -93 has been shown to be sensitive to CsA inhibition by DNase protection assays (Siebenlist et. al., 1986) and by inhibition of Oct-1 directed transcription (Mattila et. al. 1990). The activity of Oct-1 is not affected by tax activation (Mian Li, personal communication).

AP1 is a PMA inducible transcription factor (Lee and Tjian, 1987; Chiu et. al., 1987) for which two binding sites are found within the enhancer element at positions -185 to -174 and -151 to -140. The proximal site has been shown by mutagenesis to play an important role in the IL-2 gene regulation (Serfling et. al., 1989). AP1 consists of two subunits c-fos and c-jun (Neuber et. al., 1989). The c-fos expression is induced by mitogen signals and tax (Muegge et. al., 1989, Nagata et. al., 1989) while c-jun is activated by IL-1 and PMA (Muegge et. al., 1989). Although AP1 is able to respond to tax activation through its c-fos subunit (Nagata et. a., 1989), it does not mediate tax inducibility to the IL-2 promoter (Mian Li, personal communication). In addition, CsA treatment does not interfere with the binding activity of AP1 (Emmel et. al., 1989), the activity of the AP1 element, or

c-fos mRNA synthesis (Mattila et. al., 1990).

CD28-RC is the recently identified CD28-responsive complex which upregulates IL-2 gene expression through interaction with the sequence -164 to -154 (Fraser et. al., 1991; Verweij et. al., 1991). This element has been demonstrated to be essential for tax activation of the promoter (Mian Li, personal communication). In addition, point mutations or deletion of this element results in impaired promoter function (Durand et. al., 1988), or lack of responsiveness to anti-CD28 (Fraser et. al. 1991). Furthermore, anti-CD28 antibody, 9.3 (Hasen et. al., 1980) leads to CsA resistant IL-2 transcription (Thompson et. al., 1989; June et. al., 1989).

NF-kB plays an important role in the regulation of IL-2 gene expression. Two elements are present in the promoter at positions -206 to -195 and -163 to -153. This laboratory characterized the binding and function of the -206 to -195 sequence (Hoyos et. al., 1989). This factor is PMA inducible and responds to tax activation. In contrast to AP1, NF-kB is able to mediate tax activation of the IL-2 enhancer. In addition, the binding to NF-kB is weakly or not affected by CsA.

Together, PHA induction of IL-2 is mediated through NFAT and Oct-1; PMA induction activates NF-kB and AP1; and anti-CD28 induces CD28-RC which augments the effects of PHA plus PMA. The effect of tax transactivation is mediated by NF-kB and a factor that interacts with the sequence -164 to -154

which is recognized also by NF- κ B and CD28-RC. The CsA inhibitory effect on IL-2 transcription is mainly mediated through NFAT and Oct-1. In addition, the only human T cell specific factor so far identified is NFAT. While these are factors that activate IL-2 transcription, the factor Nil-2-a is a negative regulator of IL-2 gene expression.

In the mouse IL-2 promoter a T cell specific factor has been demonstrated by DNase protection assays of the enhancer element using semipurified extracts from PMA induced and uninduced Hela and EL-4 T-cells over the sequence:

-212 G A C C A A G A G G G A T T T C A C C T A A A T C -188

The complex interacting with this sequence was termed TCF-1 for T cell factor-1 (Serfling et. al., 1989). However, gel retardation assays using extracts from PMA induced and uninduced Hela and EL-4 cells or Nawalka, a B cell line, show the presence of TCF-1 in all of cell lines including uninduced Hela cells. Furthermore, the binding of TCF-1 requires an intact IL2- κ B-like motif and is competed by the κ B motif (Padler-Pohl et. al. 1990). The T-cell restricted activity of the sequence was demonstrated by transient transfection of a construct containing 5 copies of the sequence upstream of the TKCAT vector, 5x-TCE (Serfling et. al. 1989). However, in-vitro transcription assays using 5x-TCE and the IL-2CAT construct for control failed to demonstrate the inducibility of the activity. In addition, in this assay activity was detected in uninduced Hela cells for both constructs (Padler-Pohl et. al., 1990). The investigators find that TCF-1 as well as NF- κ B are able to bind to

the kB-like element within this sequence. They believe that it is TCF-1 which directs the T-cell restricted activity of the element (Briegel et. al., 1991), since the transactivating activity of NF-kB is not T-cell restricted (Pierce et. al., 1988). In addition, they find that TCF-1 consists of two polypeptides of approximately 50 and 105 kD (Briegel et. al. 1991).

The investigation presented in this thesis analyzes the contribution of the kB-like element at position -206 to -195 and the sequence immediately downstream from it (-197 to -183), termed RW, to the T cell specific regulated expression of human Interleukin-2. This investigation demonstrates that the transcription factor nuclear factor-kappa B (NF-kB) interacts with the kB-like motif, and that a novel complex RW-binding factor (RW-BF) binds to the RW sequence. The data present evidence for the binding of NF-kB and RW-BF to the sequence -211 to -187 that may result in a lymphoid specific regulation of IL-2. Ultimately, the findings contribute to the understanding of the transcriptional regulation of IL-2 gene expression.

Kappa B-Specific DNA Binding Proteins: Role in the Regulation of Human Interleukin-2 Gene Expression

Abstract

Transcriptional activation of the human interleukin-2 (IL-2) gene, like induction of the IL-2 receptor alpha ((L-2R alpha) gene and the type 1 human immunodeficiency virus (HIV-1), is shown to be modulated by a kB-like enhancer element. Mutation of a kB core sequence identified in the IL-2 promoter (-206 to -195) partially inhibits both mitogen and HTLV-I Tax-mediated activation of this transcription unit and blocks the specific binding of two inducible cellular factors. These kB-specific proteins (80 to 90 and 50 to 55 kilodaltons) similarly interact with the functional kB enhancer present in the IL-2R alpha promoter. These data suggest that these kB-specific proteins have a role in the coordinate regulation of this growth factor-growth factor receptor gene system that controls T cell proliferation.

Results and Discussion

The growth of human lymphocytes is regulated in part by antigen or mitogen induced expression of the cellular genes encoding IL-2 and the alpha subunit of the high-affinity IL-2 receptor (IL-2R alpha, Tac, and p55) (Greene et. al., 1986; Taniguchi et. al. 1986). Similarly, the long terminal repeat (LTR) of the type 1 human immunodeficiency virus (HIV-1) is stimulated by T cell mitogens (Nabel et. al. 1987; Siekevitz et. al. 1987; Kaufman et. al. 1987 Tong-Starksen et. al. 1987), which contributes to the heightened state of HIVp1 replication that occurs in activated CD4+ T lymphocytes but not in resting cells (Salahuddin et. al. 1985; Zagury et. al. 1986; Mc Dougal et. al. 1985; Folks et. al. 1986). The induced expression of both the HIV-1 LTR (Nabel et. al. 1987; Siekevitz et. al. 1987; Kaufman et. al. 1987 Tong-Starksen et. al. 1987) and the IL-2R alpha promoter (Bohnlein et. al. 1988; Ballard et. al. 1988; Leung et. al. 1988; Ruben et. al 1988) in Jurkat T cells by mitogens such as phytohemagglutinin (PHA), phorbol 12-myristic 13-acetate (PMA), or the human T cell lymphotropic virus type 1 (HTLV-1)-derived Tax protein may involve the action of related kB enhancer elements. These enhancer motifs directly interact with at least two inducible cellular proteins, NF-kB (51 kD) (Kawakami et. al. 1988) and HIVEN86A (86 kD) (Bohnlein et. al. 1988; Ballard et. al. 1988; Franza et. al. 1987).

We now describe our studies on the role of identical or related factors in the coordinated activation of the human IL-2 gene. Inspection of the 5' flanking region of the IL-2 gene (Fujita et. al. 1983; Diegenlist et. al. 1986) revealed a 12-bp promoter sequence located between nucleotides -206 and -195 (AGGGATTTCACC) that resembled the kB enhancer elements present in the IL-2R alpha promoter (GGGAAATCTCCC) and the HIV-1 LTR(A/GGGGACTTTCC). Although several functional domains have been defined in the IL-2 promoter (Durand et. al. 1988; Nabel et. al. 1988; Fujita et. al. 1986; Williams et. al. 1988; Brunvand et. al. 1988; Shaw et al 1988), regulatory effects mediated through this putative kB site have not been described.

To examine whether this IL-2 kB-like element function in IL-2 promoter induction, we used oligonucleotide-directed mutagenesis to alter selectively various nucleotides (see legend to figure 1) within the putative kB site of the wild-type IL-2 promoter (Durand et. al. 1988; Nabel et. al. 1988; Fujita et. al. 1986; Williams et. al. 1988; Brunvand et. al. 1988). One clustered mutation, designated M1 (GGG to CTC at -205 to -203), was specifically selected because identical base substitutions in the kB elements of the IL-2R alpha promoter and HIV-1 enhancer reduced mitogen or tax induced promoter activation as well as kB-specific protein binding activity (Nabel et. al. 1987; Bohnlein et. al. 1988; Ballard et. al. 1988; Leung et. al. 1988; Ruben et. al 1988). The additional M4 and M5 mutations altered four and eight bases within this site, respectively, while the M6 mutation

changed three bases located immediately 5' of the kB-like element. The IL-2 M1, M4, M5, and M6 mutant promoters and wild-type counterpart (Shaw et. al.1988) were subsequently linked to the chloramphenicol acetyltransferase (CAT) gene for comparative transient expression studies. These recombinant reporter plasmids, either alone or in combination with sense or antisense tax expression vectors (spRMT2LTR 82-2C and 82-4, respectively Siekevitz et. al. 1987), were introduced into Jurkat T cells that were then stimulated with PHA or PMA (Fig. 1). In accord with our earlier studies (Siekevitz et. al., 1987), combinations of PHA plus PMA, Tax plus PHA, and Tax plus PMA increased CAT activity in cells transfected with pIL-2-CAT, whereas single agents were largely ineffective. In contrast, introduction of the M1, M4, and M5 mutation in IL-2 promoter inhibited the response obtained with each of these combinations of stimuli. The M6 mutation located outside of the kB element, however, did not alter activation induced by Tax and PHA or PMA and only modestly affected the response induced by PHA and PMA combined. Together, these findings suggest that the IL-2 kB-like motif is needed for optimal mitogen-induced activation of the IL-2 promoter in these Jurkat cells.

To ascertain whether inducible cellular proteins specifically interact with this IL-2 kB-like site, we performed gel retardation assays with 22-bp oligonucleotides spanning this region. Electrophoretically retarded DNA-protein complexes were detected with nuclear extracts isolated from Jurkat T cells stimulated for 8 hours with PHA, PMA, or PHA plus PMA but not with media alone (Fig. 2A). These

complexes of ^{32}P -labeled DNA and protein resulted from sequence specific binding in that their formation was blocked by an excess (500 times, molar) of unlabeled wild-type IL-2 kB probe (Fig. 2B, lane 2). Consistent with the results of the functional assay (Fig. 1), size-matched IL-2 kB oligonucleotides that contained the M1 mutation failed to inhibit nucleoprotein complex formation (Fig. 2B, lane 3). Protein binding was also blocked by the addition of unlabeled oligonucleotides corresponding to the 5' kB element of the HIV-1 enhancer (lane 4) or the kB element from the IL-2R alpha gene (lane 6), but not by complete HIV-1 enhancer oligonucleotides containing the M1 mutation in both kB motifs (lane 5). Together, these cross-competition studies suggest that proteins interacting with the IL-2 kB site also bind to the HIV-1 enhancer and IL-2R alpha kB elements but not to the M1 mutated version of these elements.

To compare quantitatively the binding of these inducible factors to the IL-2 kB element and to the HIV-1 enhancer (5' motif), we performed gel-retardation assays with ^{32}P -labeled IL-2 kB and HIV-1 as probes in the presence of graded amounts of unlabeled IL-2 and HIV-1 kB oligonucleotides (Fig. 2C). With IL-2 kB as the radioactive substrate (Fig. 2C, top), the IL-2 kB and the HIV-1 kB competitor DNAs produced an essentially identical degree of inhibition at each concentration tested. In contrast, when HIV-1 kB was used as the ^{32}P -labeled probe (Fig. 2C, bottom), the IL-2 kB oligonucleotide consistently proved less efficient as a competitor than the HIV-1 kB probe. The data suggest that these DNA binding proteins

preferentially interact with the HIV-1 LTR kB element relative to the IL-2 kB site.

To examine whether the same inducible proteins interact with the kB element of the IL-2 and IL-2R alpha genes, we used bromodeoxyuridine-substituted, ^{32}P -labeled oligonucleotides (27 bp) containing these related but nonidentical elements (Fig. 3) in ultraviolet light-induced cross-linking studies. Nuclear extracts from PMA-induced Jurkat T cells were incubated with these cross-linking probes in the presence or absence of unlabeled competitor oligonucleotides. After irradiation with ultraviolet light we used preparative gel retention to separate the nucleoprotein complexes from free DNA. Competition with the unlabeled wild-type IL-2R alpha and IL-2 kB oligonucleotides (Fig. 3A, lanes 2 and 5) or those from which the kB site had been deleted (lane 1 and 6) confirmed the kB-specific nature of the ^{32}P -labeled DNA-protein complex formed. Subsequent analysis of these complexes on denaturing SDS-8% polyacrylamide gels revealed that proteins of identical size were covalently captured by both IL-2 and IL-2R alpha kB probes. These protein-DNA adducts included two species migrating with an apparent molecular size of 80 to 90 kD and two additional species of 50 to 55 kD (Fig 3B). These findings suggest that the same set of inducible T cell nuclear proteins specifically bind to the IL-2 kB and IL-2R alpha kB elements. We obtained similar results with the HIV-1 kB elements except that lesser amounts of the 80 to 90 kD species were recovered.

Since the induction of KF-kB activity in B lymphocytes has been reported to be regulated at a posttranslational level (Sen and Baltimore 1986) and to involve the induced dissociation of this factor from a labile cytoplasmic inhibitor (Baeuerle and Baltimore 1988), we next investigated the effects of cycloheximide on the mitogen-inducible expression of these T cell DNA binding proteins. As was expected, nuclear extracts from unstimulated Jurkat cells lacked binding activity (Fig. 4A, lane 1), whereas extract from PHA and PMA induced cells contained proteins that formed complexes with the IL-2 kB probe (lane 2). Incubation of Jurkat T cells for 4.5 hours in 40 micromolar cycloheximide, a concentration sufficient to inhibit 97% of de novo protein synthesis (Ballard and Greene unpublished data), induced low amounts of this DNA binding activity (lane 3). The addition of cycloheximide 30 minutes before stimulation with PHA and PMA (4-hours) did not alter the induction of this DNA binding activity (lane 4), a result consistent with posttranslational activation. Competition experiment with unlabeled relatives of the IL-2 kB probe indicated that these mitogen-induced DNA binding proteins expressed in the cycloheximide treated cells specifically interacted with the kB site (Fig. 4B). An excess (200 times, molar) of unlabeled wild-type IL-2 kB oligonucleotide of identical probes containing three-base substitution (M3: CAA to GGG at -211 to -209) upstream of the kB element (-206 to -195) completely inhibited complex formation (Fig. 4B, lanes 2 and 6, respectively).

In contrast, unlabeled IL-2 kB probes containing either a 10-bp deletion in the kB

element (IL-2 dKB) or three base pair substitutions within the kB element at -205 to -203 (M1: GGG to CTC) or -201 to -199 (M2: TTT to CCC) failed to compete for protein binding (lanes 3, 4, and 5, respectively). Protein binding to the IL-2 kB probe was also blocked by unlabeled oligonucleotides corresponding to the kB element and flanking region of the IL-2R alpha gene (lane 7), the 5' and 3' reiterated element of the HIV-1 enhancer (lanes 9 and 10), the kB element from the k light chain immunoglobulin gene (lane 11) and the enhancer element from the murine class I major histocompatibility gene complex (H-2 K kB, lane 12). However, an IL-2R alpha oligonucleotide lacking the kB element did not compete (lane 8). Together, these results indicate that the kB elements from various viral and cellular genes are able to cross-compete for proteins binding to the IL-2 kB element and that these proteins remain inducible in Jurkat T cells despite the inhibition of de novo protein synthesis.

We next investigated the question of whether the kB-specific proteins alone were sufficient to support IL-2 gene activation in Jurkat T cells. IL-2R alpha and IL-2 mRNA expression were evaluated in Jurkat cells induced with PHA and PMA in the presence and absence of cycloheximide (Fig. 4C). Although IL-2R alpha mRNA expression was well preserved in the cycloheximide treated Jurkat T cells induced with PHA and PMA, IL-2 mRNA expression was undetectable. Similar results in Jurkat T cells have been reported by Shaw et. al. (1988) and Weiss et. al. (1987). Thus, although the kB-specific binding proteins are fully induced in the presence

of cycloheximide, IL-2 gene activation does not occur. These findings suggest that the de novo synthesis of an additional factor (or factors) is required for IL-2 gene expression in these Jurkat T cells. Recently, an inducible nuclear factor of activated T cells, NFAT-1, was shown to be involved in the control of IL-2 gene activation in Jurkat T cells (Shaw et. al. 1988). Since NFAT-1 expression is dependent on de novo protein synthesis in Jurkat cells, it is a candidate for being at least one of these additional factors. We (Kronke et. al. 1988) and others (Efrat et. al. 19984; Granelli-Piperino et. al. 1986) have found that IL-2 gene expression in mitogen-activated peripheral blood lymphocytes is independent of protein synthesis. Although the biochemical basis for this paradoxical result in Jurkat and primary T cells is unresolved, it may reflect biologically important differences in transcription factor production or action (or both) in these cell types.

Our findings support a role for kB-specific DNA binding proteins in the overall regulation of IL-2 gene activation in human Jurkat T cells. That these inducible factors also regulate IL-2R alpha gene activation suggests that these proteins contribute to the coordinated induction of these genes, both of which are critically involved in the control of human T cell growth.

Figures

Fig. 1 A kB-like element in the IL-2 gene is required for optimal promoter activation mediated by combination of PHA, PMA and Tax.

CAT expression vectors containing the wild-type IL-2 promoter (Durand et. al. 1988) and wild-type kB sequence (AGGGATTTCAT, -206 to -195), or various mutations of this element (M1: ACTCATTTCAC; M4: AGGGAGGACAG; and M5: ACTCCGAACAG) or a three-base alteration immediately 5' of the kB site (M6: AAG to TTC, -209 to -207) were cotransfected into Jurkat T cells in the presence or absence of antisense Tax expression plasmids (Siekevitz et. al. 1987). The cells were stimulated 24 hours later with PHA (1 ug/ml), PMA (50 ng/ml), or combinations of these agents. CAT activity and total protein recovery in the transfected cultures were measured after an additional 24 hours of culture (Siekevitz et. al. 1987). Data for the mutants are presented as the percentage of the induced response of the wild-type IL-2 promoter (shown as 100% for each set of stimuli). The results with the M1 mutant reflect cumulative data from five experiments with five independent plasmid preparations, whereas the M4, M5, and M6 results are cumulated from three experiments performed with two independent plasmid preparations. Error bars indicate standard errors of the mean.

Fig. 1

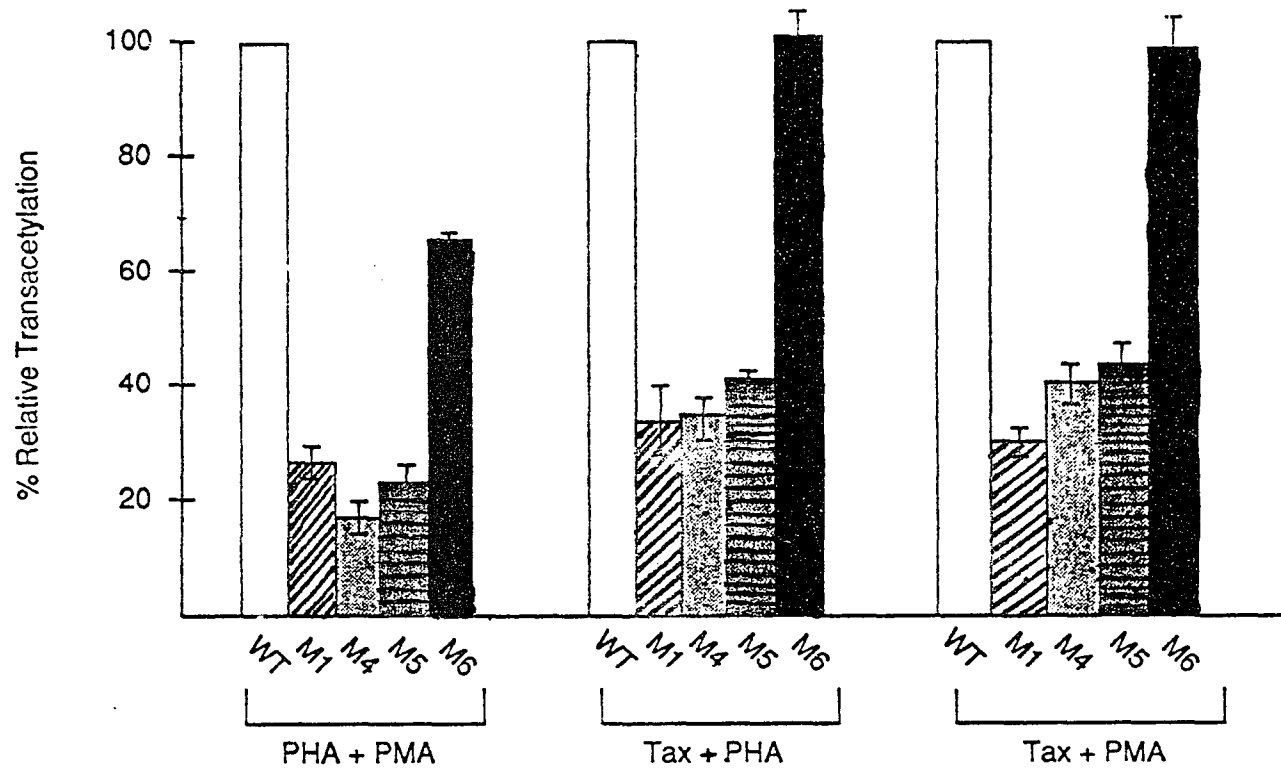


Fig. 2 Binding of inducible nuclear proteins to the IL-2 kB motif.

(A) Nuclear extracts (10 ug) from Jurkat t cells were prepared 8 hours after stimulation with PHA (2 ug/ml, lane 1), PMA (50 ng/ml, lane 2), or PHA plus PMA (lane 3), or in the absence of mitogen (lane 4), and mixed with ^{32} P-labeled IL-2 kB probe (Bohnlein et. al. 1988). Free DNA and protein-DNA complexes were separated by gel retardation (Bohnlein et. al. 1988) and detected by autoradiography. (B) Nuclear extracts from Jurkat T cells induced with PHA and PMA were prepared as in Fig. 2A and incubated with ^{32} P-labeled IL-2 kB oligonucleotides in the presence of an excess (500 times, molar) of the indicated unlabeled competitor oligonucleotides (IL-2 kB 5' gatccAAAGAGGGGATTTCACCT; IL-2 M1 5' gatccAAAGACTCATTTCACCT; HIV-R5'gatccAGGGACTTTCC; HIV-DR M1 5' ACACTCACTTTCCGCTGCTCACTTTCCAGGG; IL-2R alpha 5' gatccGGCAGGGAATCTCCCT; where gatcc represents nonspecific linker sequence added to facilitate cloning) as described (Bohnlein et. al. 1988). (C) Graded amounts of either unlabeled HIV-1 kB or IL-2 kB oligonucleotides were incubated with ^{32} P-labeled IL-2 kB (top) or HIV-1 kB (bottom) probes as described in (B). Only the retarded nucleoprotein complexes are shown.

Fig. 2

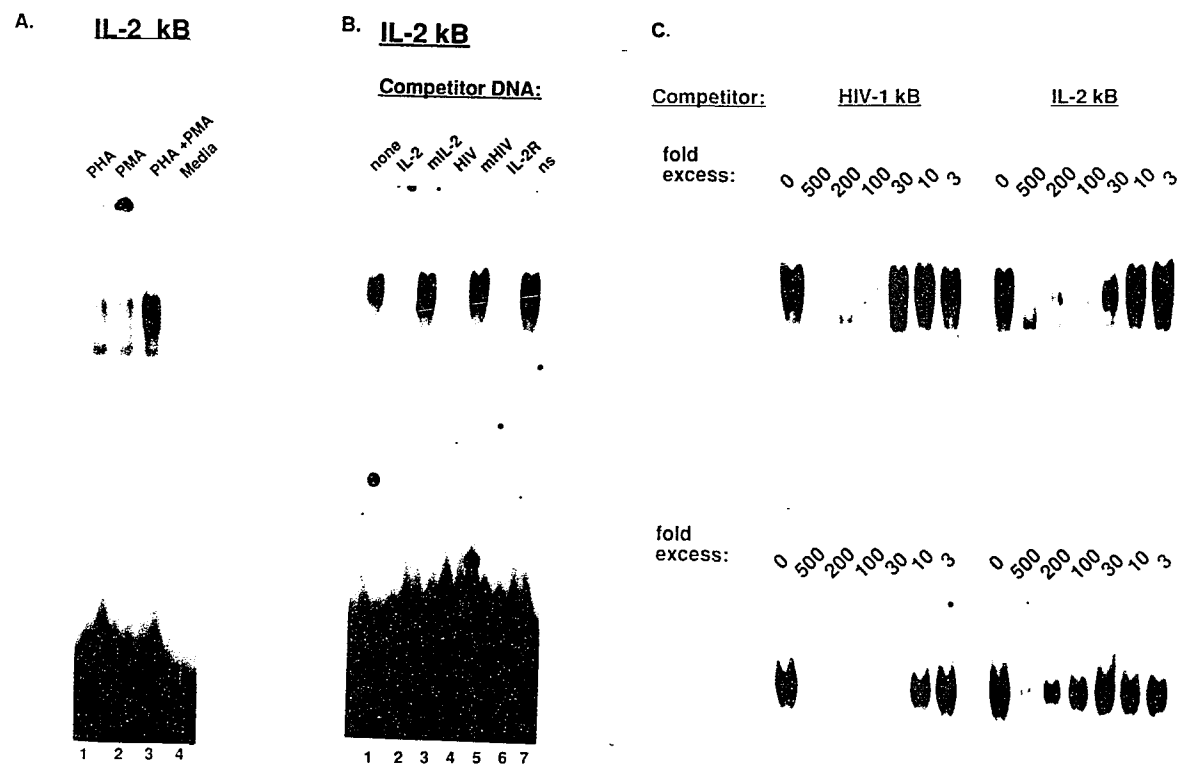


Fig. 3 The same nuclear factors directly bind to the kB elements present in the IL-2 and IL-2R alpha promoters.

(A) Nuclear extracts (20 ug) prepared from PMA stimulated Jurkat T cells (Bohnlein et. al. 1988) were incubated with size match, bromodeoxyuridine substituted, ^{32}P -labeled kB probes from either the IL-2R alpha (nucleotides -275 to -249) or IL-2 (nucleotides -212 to -186) promoters in the absence or presence of an excess (500 times, molar) of unlabeled competitor DNAs. Competitors included the homologous probe (WT) or a kB-deleted variant (dkB) with fused 5' and 3' sequence flanking the corresponding kB motif. The reaction mixtures were irradiated with ultraviolet (UV) light for 30 min, and free and protein-complex ^{32}P -labeled DNA species were separated by gel retardation (Bohnlein et. al. 1988). (B) Polyacrylamide slices containing adducts of the ^{32}P -labeled DNA and protein formed with ^{32}P -labeled IL-2R alpha kB or ^{32}P -labeled IL-2 kB were excised from the retention gels (A) and analyzed directly on denaturing SDS-8% polyacrylamide gels. Molecular size markers are indicated.

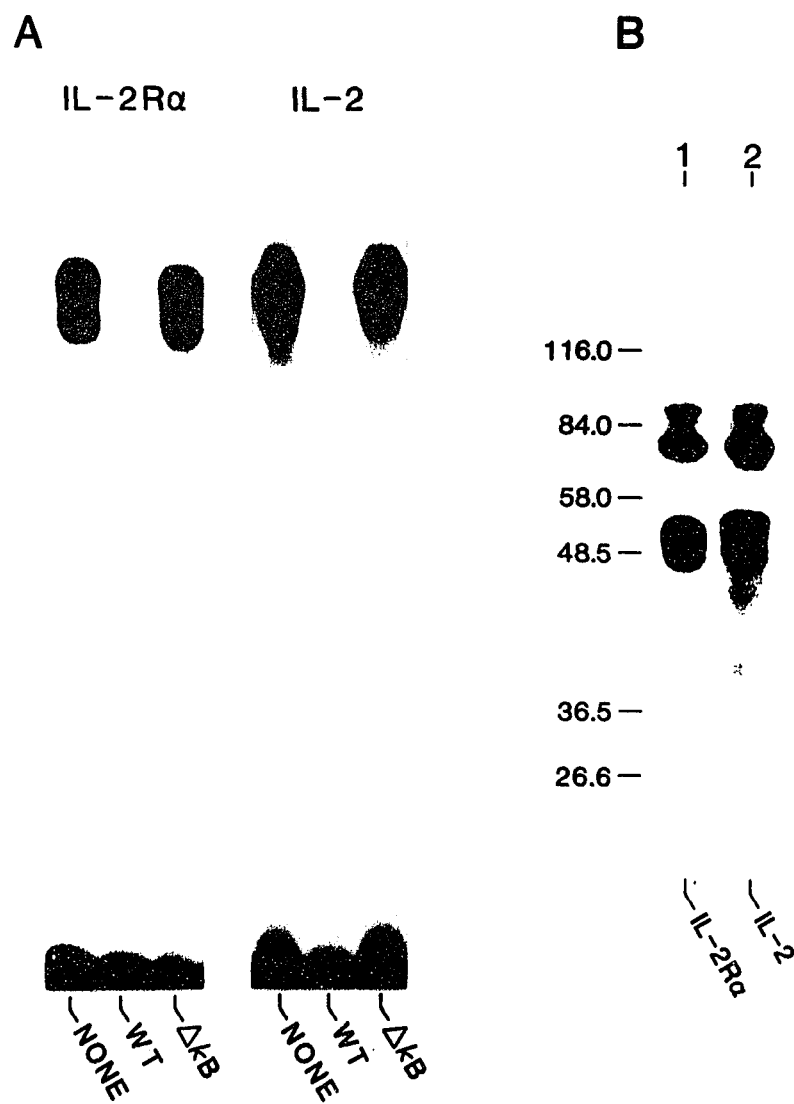


Fig. 3

Fig. 4 DNA binding properties of nuclear extract proteins from Jurkat T cells treated with cycloheximide.

(A) Effects of cycloheximide (CHX) on the induction of DNA binding proteins reactive with the IL-2 kB probe. Jurkat T cells were incubated with or without cycloheximide (40 μ M) for 30 min and then medium or combinations of PHA (1 μ g/ml), and PMA (50 ng/ml) were added. Nuclear extracts were prepared 4 hours later and gel retardation performed with the ^{32} P-labeled IL-2 kB probe. Culture conditions for each extract is indicated at the bottom of the gel. (B) Specificity of proteins that are induced by PHA and PMA in the presence of cycloheximide that bind to the ^{32} P-labeled IL-2 kB probe. Gel retardation assays were performed with and without an excess (200 times, molar) of the indicted unlabeled oligonucleotides. Only the electrophoretically retarded complexes are presented. (C) RNA blot analysis of IL-2R alpha and IL-2 mRNA expression in Jurkat T cell induced with PHA and PMA in the presence or absence of cycloheximide. The same Jurkat cell cultures described in (A) were used for the isolation of total cellular RNA. The upper and lower blots show expression of the 25S IL-2R alpha mRNA and 11S IL-2 mRNA under the indicated conditions of induction.

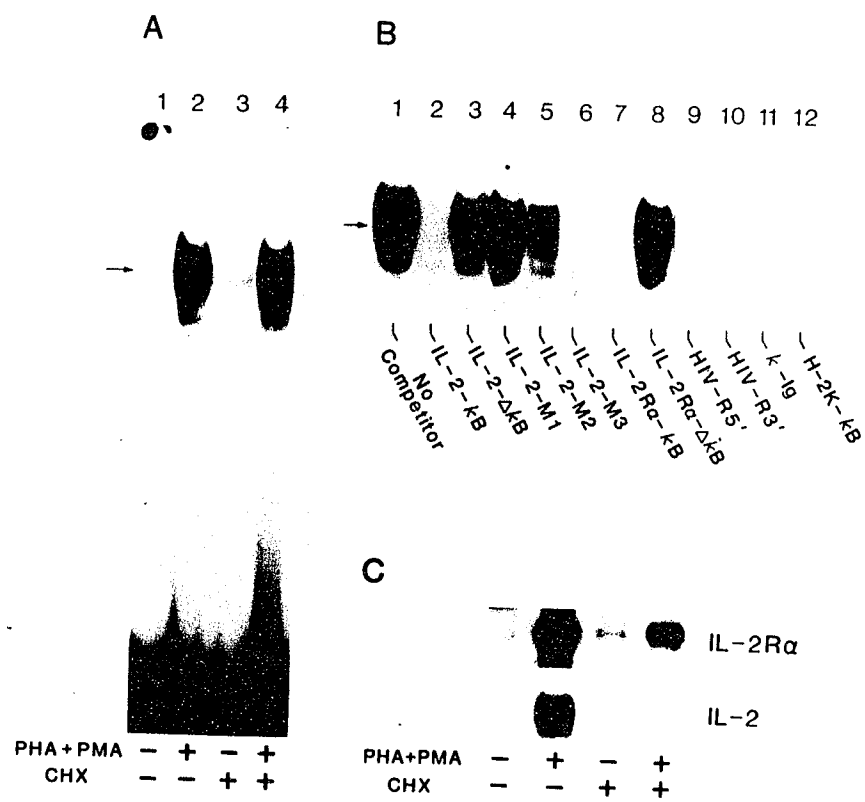


Fig. 4

Identification of RW-BF as a novel complex regulating IL-2 gene expression

Abstract

In the present study we demonstrate that the sequence -197 to -183 (termed RW) located immediately downstream of the IL2-kB-like element is important in regulating IL2 gene expression. This consensus sequence interacts specifically with a mitogen-induced nuclear factor that is sensitive to cycloheximide treatment and insensitive to tax activation and to cyclosporin A (CsA) an inhibitor of IL2 transcription. The complex, referred to as RW-BF, consists of two proteins of sizes 40 and 95 KDa. The binding of nuclear factor kappa B (NF-kB) and RW-BF do not show cell specificity; however, we present evidence suggesting the possible interaction of these two complexes that results in lymphocyte-specific transcriptional function.

Introduction

Nuclear factor kappa B (NF- κ B) is a transcription factor that is sequestered in the cytoplasm by the I κ B inhibitor. This inhibitor must be phosphorylated to release NF- κ B which then translocates to the nucleus (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1988; Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). NF- κ B binding and functional activity can be stimulated by LPS, PMA +/- PHA, and tax depending on the cell line used, B versus T or Hela cells (Sen and Baltimore, 1986; Nabel and Baltimore, 1987; Lenardo et. al., 1987; Pierce et. a., 1988; Bohnlein et. al. 1988; Cross et. al., 1989; Leung et. al., 1988; Nabel et. al., 1987). In addition, its binding can be superinduced by cycloheximide (Sen and Baltimore, 1986; Balwin et. al., 1988). However, in contrast to NFAT (Schreiber and Crabtree, 1992), NF- κ B is weakly or not affected at all by cyclosporin A treatment (Emmel et. al., 1989; Randok et. al., 1990; Baumann et. al. 1991).

NF- κ B consists of two subunits: p50 and p65 both of which have been cloned (Ghosh et. al., 1990; Kieran et. al., 1990; Nolan et. al. 1991, Ruben et. al. 1991). As a result, approximately 300 amino acids at the N-terminal of these proteins are shown to share homology with the rel proto-oncogene. This homology implies that a large family of rel-related proteins may interact with the κ B motif (Molitor et. al., 1990) since the DNA binding and dimerization domains of the proteins are located within the rel-homology region. Thus, NF- κ B previously thought to be a lymphoid

specific protein (Sen and Baltimore, 1986) is a ubiquitous factor regulating the function of several genes in different cell types (for review see Lenardo and Baltimore, 1989).

In the previous chapter the importance of NF-kB in the regulation of IL2 gene expression was demonstrated. However, the affinity of NF-kB for the IL2-kB-like element is lower (chapter II, fig. 2) than that for the kB motif. To determine the contribution of the 3' flanking sequence to both IL2 promoter function and NF-kB binding the present study was undertaken. The study shows that IL-2 promoter function is inhibited by mutagenesis of the -197 to -183 sequence (RW). Furthermore, a factor binds specifically to RW and its binding is differentiated from that of NF-kB based on several criteria. In addition, the IL2-2NF sequence, which encompasses both the IL2-kB-like and the RW elements is demonstrated to be required to confer transcriptional inducibility upon a heterologous promoter. Neither of these two elements is active on its own, suggesting the possible interaction of NF-KB and RW-BF.

Materials and Methods:

Cell lines:

Jurkat, a human leukemic T cell line, and Raji, a Burkitt's B cell lymphoma transformed by EBV were grown in RPMI supplemented with 10% fetal calf serum, 2mM glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin. Hela, a human carcinoma cell line, was grown in minimum essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin.

Oligonucleotides:

All oligonucleotides were synthesized on an Applied Biosystem DNA 380B synthesizer and purified by preparative denaturing acrylamide gel electrophoresis as described (Bohnlein et. al., 1988). Briefly, full length bands were visualized by UV long wave shadowing, excised, eluted by diffusion in TE pH 8 and precipitated with ethanol. Double stranded oligonucleotides were generated by heating equal molar amounts of the individual strands at 67°C for 5 minutes in annealing buffer (50 mM NaCl, 10 mM Tris pH 7.5, 0.2 mM EDTA) and allowing the strands to anneal during the cooling of the 67°C water bath to room temperature. Double-stranded oligonucleotides were radiolabeled using Klenow to fill-in the ends using ³²P alpha-dATP.

In-vitro mutagenesis of the IL-2 promoter:

The IL2-CAT construct (Siebenlist et. al., 1986) was mutagenized using an Amersham Kit following the protocols recommended by the manufacturer. Positive clones were screened by hybridization and mutations were confirmed by dideoxy sequencing.

Cloning of cis-elements in front of Herpes simplex virus thymidine kinase promoter:

We cloned preannealed oligonucleotides into the Bam HI site at position -105 on the TK-CAT plasmid (Mc Knight et. al., 1981). This vector shows basal activity and lacks inducibility by mitogens. The positive clones were screened by hybridization and the number and direction of the multimers was determined by restriction endonuclease digestion, and confirmed by sequencing.

DNA preparation:

All plasmid DNAs used were prepared by the alkaline lysis method (Maniatis 1989). The DNA was isopropanol precipitated, RNase treated and either passed through commercial Qiagen columns for further purification, or phenol:chloroform extracted, precipitated with ethanol followed by polyethylene glycol (PEG) precipitation.

Cell extracts:

Nuclear extracts were prepared as described (Dignam et. al., 1986) with minor modifications. Briefly, 1×10^9 cells were resuspended in 5X the cell pack volume (cpv) in buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT) incubated on ice for 10 minutes and centrifuged. The cells were then resuspended in 2X the cpv in buffer A and disrupted in a dounce homogenizer (type B). The nuclei were recovered by centrifugation and disrupted in 2.0 ml buffer B (20 mM Hepes pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) in a homogenizer. After 30 minutes incubation at 4°C with gentle shaking, cell debris was removed by centrifugation. The supernatant was applied to a PD-10 a Sephadex G-25 M desalting column (Pharmacia) that was equilibrated with buffer C (20 mM Hepes pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl [PMSF], 0.5 mM dithiothreitol [DTT]). Proteins were eluted in 3 ml buffer C and aliquots were frozen in liquid N_2 , and stored at -80°C. The yield was approximately 8 mg/ml of protein as determined by Bio-Rad protein assay.

Gel retardation assays:

The assay was performed as previously described (Bohnelein et. al., 1986, Scheidereit et. al., 1987) with the following modifications: Nuclear extracts (20-30 ug) were preincubated in binding buffer (4% Ficoll, 20 mM Hepes pH 7.9, 60 mM

KCl, 2 mM DTT, and 0.1 mg/ml BSA) for 10 minutes at room temperature in the presence or absence of competitors. This was followed by incubation for 20 minutes with 0.2-0.6 ng of double stranded oligonucleotide label with Klenow to fill-in the ends, in a final volume of 20 μ l. The complexes were separated from free probe in a 6% native polyacrylamide gel in 0.25X TBE. The gel electrophoresis was performed in 0.25X TBE.

UV-crosslinking:

Gel retardation assay was done using RW containing bromodeoxyuridine incorporated into the antisense strand and ^{32} PdATP in an agarose gel. Following electrophoresis the gel was UV-irradiated (312 nm for 15 minutes). After UV-irradiation the gel was exposed to X-ray film for 2 hours at 4°C. The pertinent bands were excised and boiled for 5 minutes in 2X protein denaturing buffer (3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 63 mM Tris pH 6.8) and loaded onto a 7.5 SDS/PAGE (Laemmli, 1970). Electrophoresis was done at 70 volts overnight. The gel was dried and exposed to X-ray film.

Transfection:

DNA was transfected into Jurkat cells by the DEAE-dextran method (Queen and Baltimore, 1983) with minor changes. Briefly, a total of 12 μ g/ml of DNA was

transfected into 1.5×10^7 cells in the presence of 500 ug/ml DEAE-dextran, after which the cells were aliquoted. 24 hrs posttransfection the cells were stimulated with medium alone, 1 ug/ml PHA, 50 ng/ml PMA, or PHA + PMA. Protein concentration was determined by Bio-Rad assay. CAT assay and thin-layer chromatography were performed as described (Gorman et. al., 1982). Spots were cut and counted with scintillation fluid. The percentage of acetylation of each sample was normalized for protein content of the cell lysate. The ratio of induction was calculated by dividing the percentage of acetylation of stimulated cells by the percentage of acetylation of uninduced cells.

Results

Mapping of the RW responsive element in the IL-2 promoter. Site-directed mutagenesis of the sequence -197 to -183 (termed RW) of the Interleukin-2 promoter was performed in order to analyze its functional role. This sequence is positioned immediately downstream from the NF-kB element that we have previously investigated (chapter II). As will be discussed below, the RW sequence with the IL-2 kB-like element are necessary to confer tax plus PMA inducibility upon a TKCAT promoter-reporter gene construct. Three mutants were made: M6B (TAC to AGG, -193 to -191), M8 (TAC to CTT, -193 to -191), and M9 (ATC to TAG, -190 to -188). As shown in fig. 5, cotransfection of mutants M6B and M8 with sense or antisense tax expression vectors into Jurkat cells resulted in 25-50% impairment of the promoter, whereas mutant M9 showed minimal effect. This suggested the possible regulatory function of the nucleotides at position -193 to -191 for promoter activity. To eliminate the possibility that this effect was due to interference with flanking sequences important for NF-kB binding, mobility gel shift assays were performed using a synthetic oligonucleotide corresponding to the sequence -197 to -183 (RW) and Jurkat nuclear extracts, a complex was observed to form with induced cell extracts (fig. 7A).

Sequence-specificity of the factor binding to RW. To differentiate the binding of RW-BF from that of other nuclear binding factors, specifically NF-kB, competition

experiments utilizing Jurkat cell extracts oligonucleotides representing the binding elements for NF- κ B, RW or modified RW sequences carrying base substitutions or 3' truncations were performed. The results (fig. 6A) indicate that the binding of this complex to RW is specific; high molar excess (200) fold of the nonspecific competitors could not prevent binding (lanes 2 to 4). In addition, 200-fold excess of specific competitors reduced binding. The 3' truncated RW oligonucleotide (ssRW) although able to bind RW-BF, was a poor competitor against RW (compare lane 6 and lane 8). Furthermore, no complex was detected with oligonucleotides containing base substitutions identical to M6B, M8 or with RWH, which has a nucleotide deletion at the most 5' end (fig. 6B).

To analyze the contribution of the Bam HI linker on RW to the binding of the factor, the linker was exchanged for that of Hind III (RWH). No RW-BF binding was detected in gel retardation assays to this new oligonucleotide (fig. 6B, lane 5). Surveying the sequence it was realized that the C at the 3' end of the linker (Bam HI) was common with the next upstream base of the gene. When the Hind III linker was added beyond this base, binding was then restored (fig. 6B, lane 7). Thus, RW-BF binds to the sequence CACCTACATCCATTC. Taken together, the mutagenesis and binding studies suggest a regulatory role for RW in IL-2 gene function. The sequence and binding specificities parallel the sequence requirement for optimal IL-2 gene expression.

Characterization of RW-BF binding. Nuclear extracts were prepared from cell that had been induced with PHA, PMA, PHA plus PMA, or with medium alone. As illustrated in fig. 7A, RW-BF is an inducible factor(s) and PMA is the most effective inducer. Furthermore, assays performed using extracts prepared from induced and uninduced Raji, Hela, and Jurkat cells indicated that the binding of this factor, although present in much lower levels, is not T-cell specific (fig. 7B). However, the binding is inducible in all the cell lines tested.

Cyclosporin A (CsA) is an immunosuppressive drug that blocks the transcription in IL-2. It has been suggested (Emmel et. al., 1989; Baumann et. al., 1991; Granelli-Piperno et al., 1990; Randak et. al., 1990) that this inhibitory effect is due to the ability of CsA to prevent the binding of regulatory factors to their elements in the promoter. To establish if this drug has any effect on the binding of RW-BF, Jurkat cells were treated with 1 ug/ml CsA for 30 minutes prior to and during activation with mitogen (Kronke et. al., 1984). Gel retardation assays were performed using nuclear extracts from these cells. The results (fig. 7C) show no difference in the binding whether CsA was present or absent. The same result is seen with the NF-kB binding as expected (Emmel et. al., 1989).

To further characterize RW-BF binding, the effect of cycloheximide on induction of complex formation was analyzed. This antibiotic is a protein synthesis inhibitor which blocks peptide transfer. Gel retardation assays were performed with nuclear

extracts prepared from Jurkat cell treated with 40 micromolar cycloheximide (Ballard and Greene unpublished results) for 30 minutes prior to and during mitogen induction. As control, binding to HIV-kB was included in this assay; it is known that binding to NF-kB is superinduced by cycloheximide treatment due most likely to inhibition of the repressor I κ B (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1988; Shirakawa, 1989; Ghosh and Baltimore, 1990). The findings shown in fig. 7D suggest that induction of RW-BF is affected by this treatment, indicating that it requires de-novo protein synthesis and therefore is not a member of a pre-existing pool of proteins. This question was addressed in more detail by treating both the cytoplasmic and the nuclear fractions of extracts from uninduced Jurkat cells with 0.2% sodium desoxycholate (DOC) and 27% formamide, conditions known to cause the dissociation of NF-kB/I κ B. These treatments had no effect (data not shown), indicating that RW-BF is not likely to be sequestered by a repressor (Baeuerle and Baltimore, 1988; Harel-Bellan et. al., 1989).

The effect of tax on the inducibility of RW-BF was also investigated. Tax is a transcriptional activator of HTLV-1, and has been shown (Siekevitz et. al., 1987; Maruyama et. al., 1987) to transactivate IL-2. A Jurkat cell line that constitutively expresses tax (19D) was induced with mitogens or medium alone and extracts were made (Siekevitz et. al. 1987). Gel retardation assays showed no effect of tax on the induction of RW-BF. These results are in contrast to the effect of tax on the binding of NF-kB (fig. 7E). Altogether, these results show that induction of RW-BF

binding by PMA/PHA requires de-novo protein synthesis and is insensitive to CsA inhibition and tax activation of IL-2 gene expression.

To determine the number and molecular size of the polypeptides forming the RW-BF complex, UV-crosslinking analysis was performed. The results show that RW-BF consists of two polypeptides of sizes corresponding to 40 and 95 kD (fig. 8)

RW alone is not able to mediate transcription. Many cis-elements not only show regulatory function within promoters but are themselves enhancer elements. To study the enhancer function of RW, 5 copies were cloned upstream of the minimal thymidine kinase (TK) promoter in a vector containing chloramphenicol acetyl transferase (CAT) as the reporter gene. These were transfected into Jurkat cells and induced with mitogens 24 hrs later. The results (fig. 9A) show that RW is not very active as an enhancer.

It has previously been demonstrated that as few as two copies of the kB motif are sufficient to regulate transcription of a heterologous promoter (Pierce et. al. 1988). Therefore, the enhancer ability of the IL2-kB-like element was tested. As many as 5 copies of this element could not direct transcription of the reporter gene. This could be explained by the low affinity of the NF-kB for the IL2-kB-like element. Thus, an additional oligonucleotide (LINF) was tested. This oligonucleotide consists of the IL2-kB-like element extended by three nucleotides at the 3' end; these three

nucleotides are important for IL2 promoter function (fig. 5). Seven copies of this oligonucleotide had poor transcriptional activity (fig. 9A).

To test whether NF-kB and RW-BF interact and together regulate IL-2 gene expression, an oligonucleotide encompassing the IL2-kB element plus the RW sequence -211 to -187 (termed 2NF) was synthesized and cloned as a concatamer into the TK-CAT vector. Transfection experiments using the resulting constructs show that as few as three copies of IL2-2NF are sufficient to confer PMA inducibility upon the heterologous promoter (fig. 9A). The interaction between NF-kB and RW-BF has been further analyzed by constructing clones containing concatamers of IL2-2NF mutated at either the NF-kB or the RW sequence. Transient transfection assays in Jurkat cells reveal these mutated constructs to be less active compared to that of the wild-type IL2-2NF (fig. 9B). The results suggest that binding of both the NF-kB and RW-BF factors to the IL2-2NF element are required for maximal transactivation.

The cell specificity of the effect was tested by performing transfections into Raji, and Hela cells. The results (fig 9C) show that IL2-2NF is lymphocyte specific. The 2NF construct is more active in Jurkat cells than in Raji cells, and not active at all in Hela cells.

Binding to the IL2-2NF oligonucleotide was tested in induced and uninduced Jurkat

cells. The results show an inducible factor which cannot interact with IL2-2NF if mutations within the kB-like element (M5), or the RW sequence (M6B) are present. Mutants M8 and M9 although within the RW sequence, show less effect on the binding (fig. 10A). This inducible binding is specific and can be abrogated by 100 fold molar excess of 2NF, L1NF, and HIV-kB. However, the RW sequence, RW2 or RW3 did not interfere in a detectable manner with this binding (fig. 10B).

Figures

Fig. 5 The IL2 promoter is regulated by the sequence CACCTACATCCATTC (-197 to -183) , termed RW.

Site-directed mutagenesis of RW. Wild-type IL2-CAT constructs and mutants M6B, M8, and M9 were cotransfected with sense and anti-sense tax-expression plasmids into Jurkat cells. The cells were stimulated 24 hrs. post transfection with 1 ug/ml PHA, 50 ng/ml PMA, PHA+PMA, and medium alone as described in materials and methods. Data for the mutants are presented as the percentage of the induced response of the wild-type IL-2 promoter. The error bars indicate standard error of the mean. The results are representative of 5 independent experiments. The sequence of the gene and the mutations introduced are:

Gene:	GTAACAAAGAGGGATTTCACCTACATCCATTCAGTCAGTC
IL2-kB	-206 AGGGATTTCAC -195
M6B:	GTAACAAAGAGGGATTTCACC <u>AGG</u> ATCCATTCAGTCAGTC
M8:	GTAACAAAGAGGGATTTCACC <u>CTT</u> ATCCATTCAGTCAGTC
M9:	GTAACAAAGAGGGATTTCACCTACT <u>AG</u> CATTCAGTCAGTC
RW:	-197 ACCTACATCCATTC -183

Fig. 5

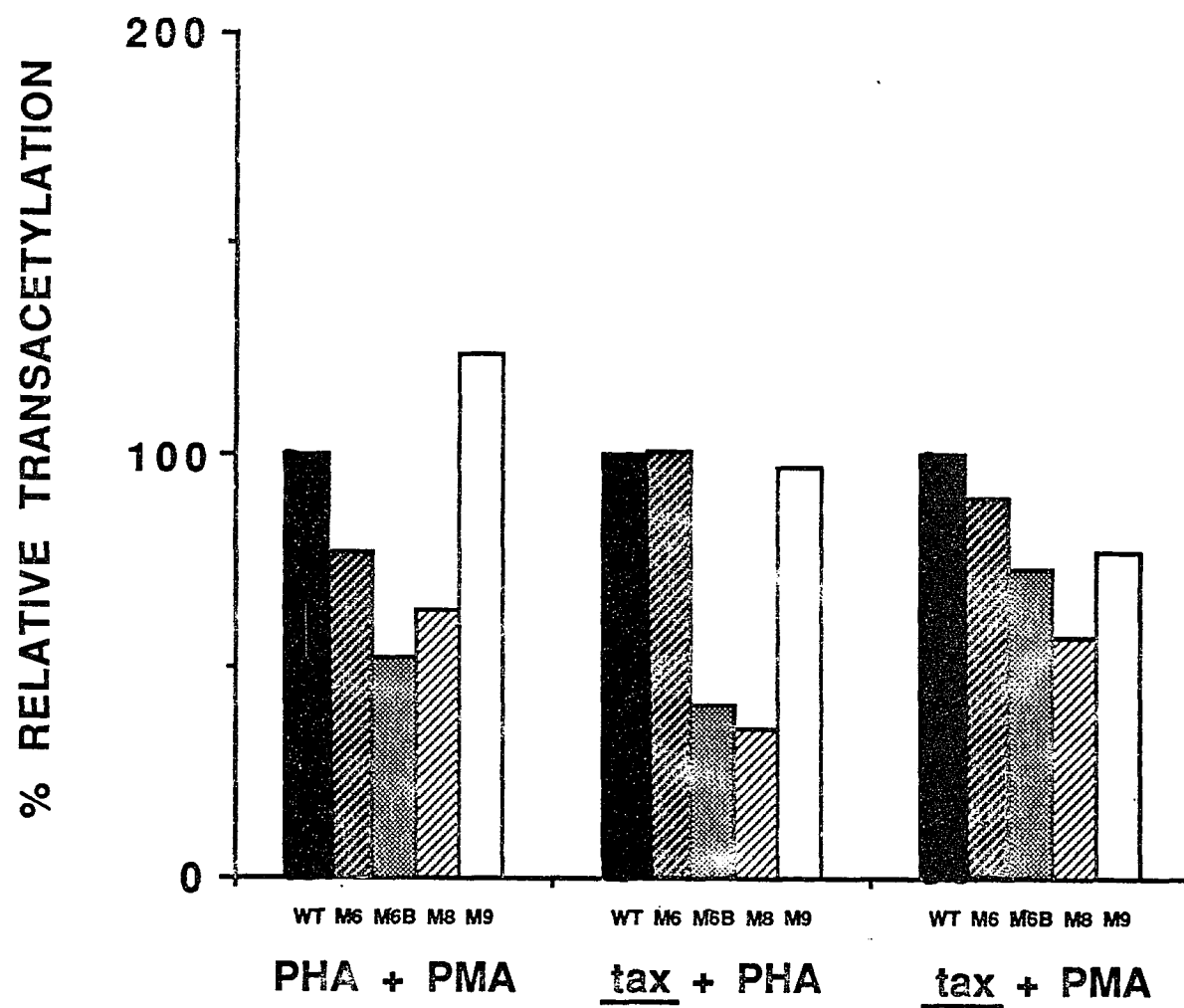
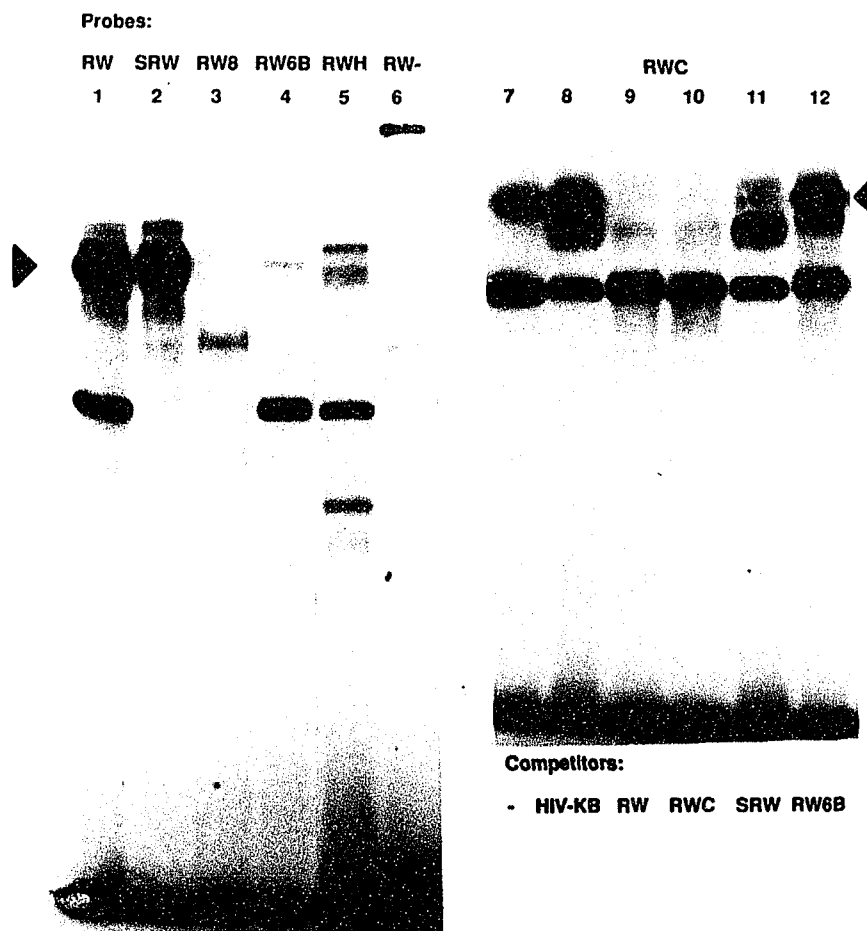


Fig. 6 Binding to RW is Sequence-specific.

(A) Competition for binding to RW in gel shift assays. The indicated oligonucleotides were used as competitors at a 200 molar excess. Nuclear extract from Jurkat cells stimulated with 50 ng/ml PMA is used.



(Fig. 6B) Binding to RW mutants. The indicated oligonucleotides were labeled to similar specific activities and used in gel shift assays using Jurkat cell extracts induced as indicated in panel A. The sequences of the oligonucleotides used in the gel shift assays as probes or competitors, where the small-case letter sequence represent nonspecific linker sequences added to facilitate cloning, are:

RW:	gatcc	ACCTACATCCATTC
sRW:	gatcc	ACCTACATCC
ssRW:	gatcc	ACCTAC
RW6B:	gatcc	ACC <u>AGG</u> ATCCATTC
RW9:	gatcc	ACCTACT <u>AG</u> CATTC
RWH:	<u>agctt</u>	ACCTACATCCATTC
RWC:	agctt	CACCTACATCCATTC
RW-:	-----	ACCTACATCCATTC
HIV-kB:	gatcc	AGGGACTTTCC
cAP1:		GGATGTTATAAAGCATGAGTCACTAGGGCGCA
IL2-AP1	gatcc	AAGAGTCAT
AP1		ATGAGTCAT

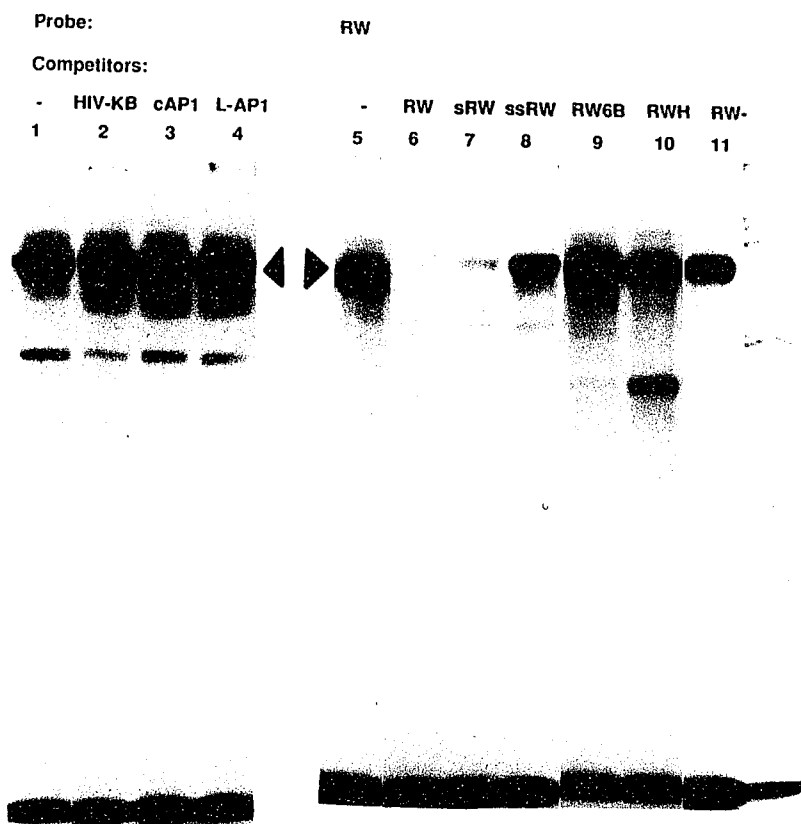


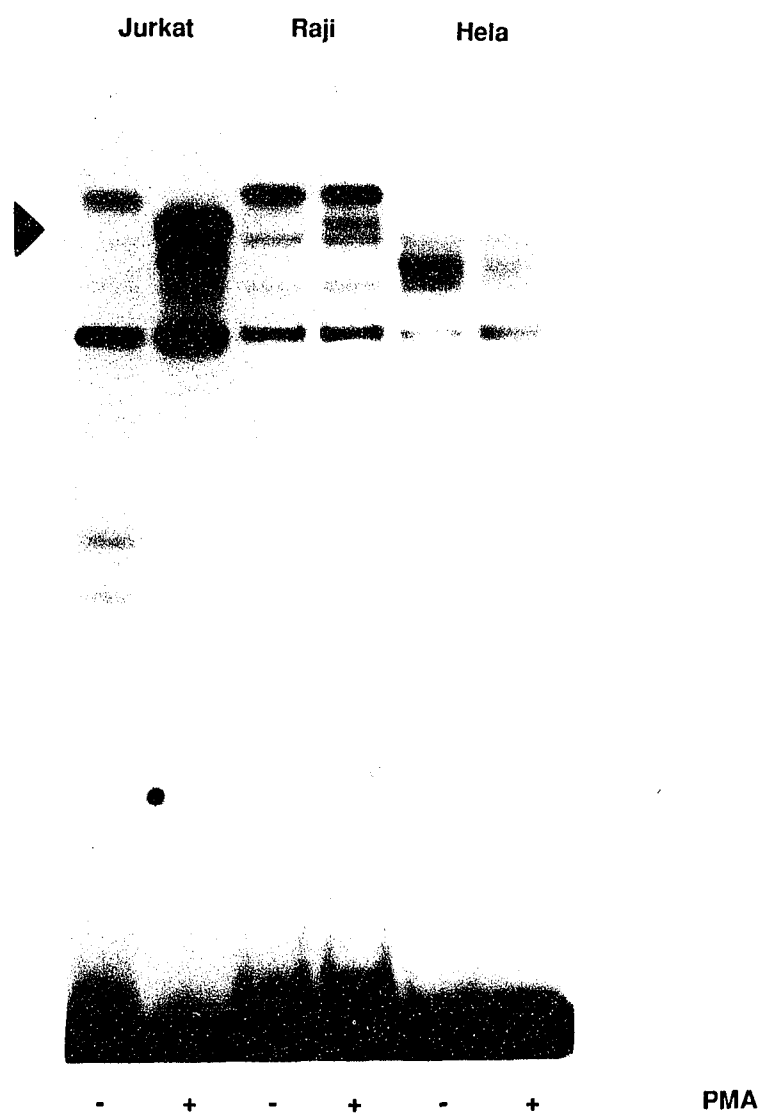
Fig. 6B

Fig. 7 Characterization of the complex binding to RW (RW-BF).

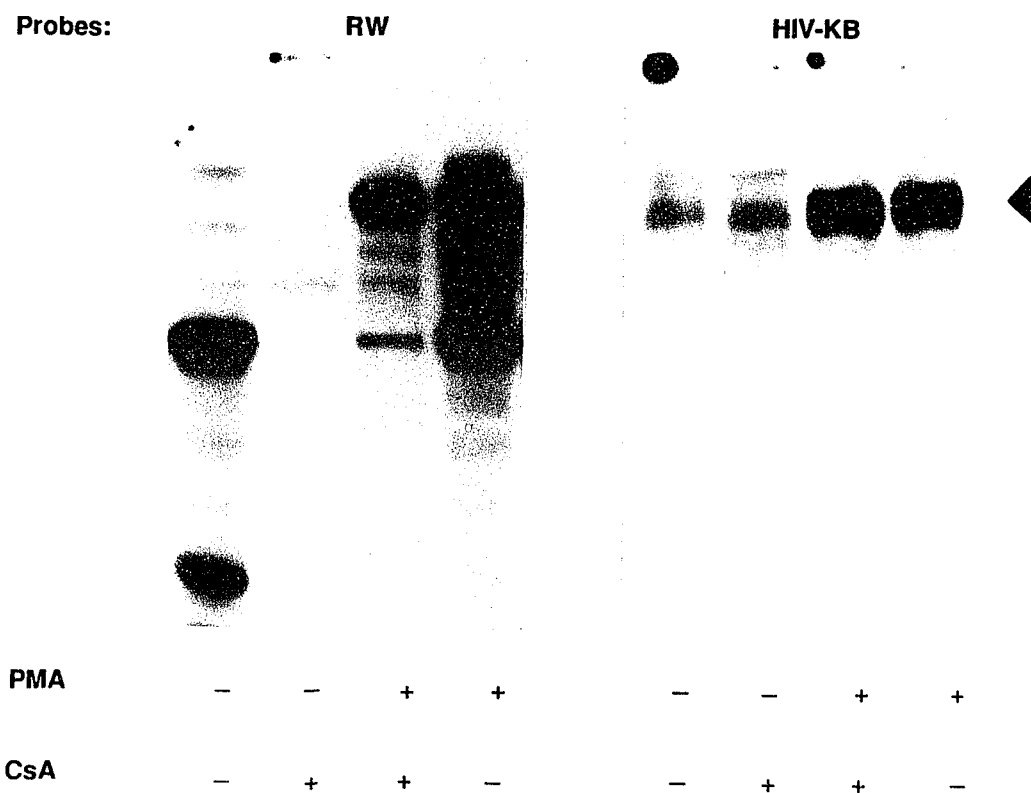
(A) Binding of RW-BF is induced by mitogen. Gel retardation assays were performed using RW as probe and nuclear extracts from Jurkat cells 4 hrs. stimulated with medium alone, 1 ug/ml PHA, 50 ng/ml PMA, and PHA+PMA.



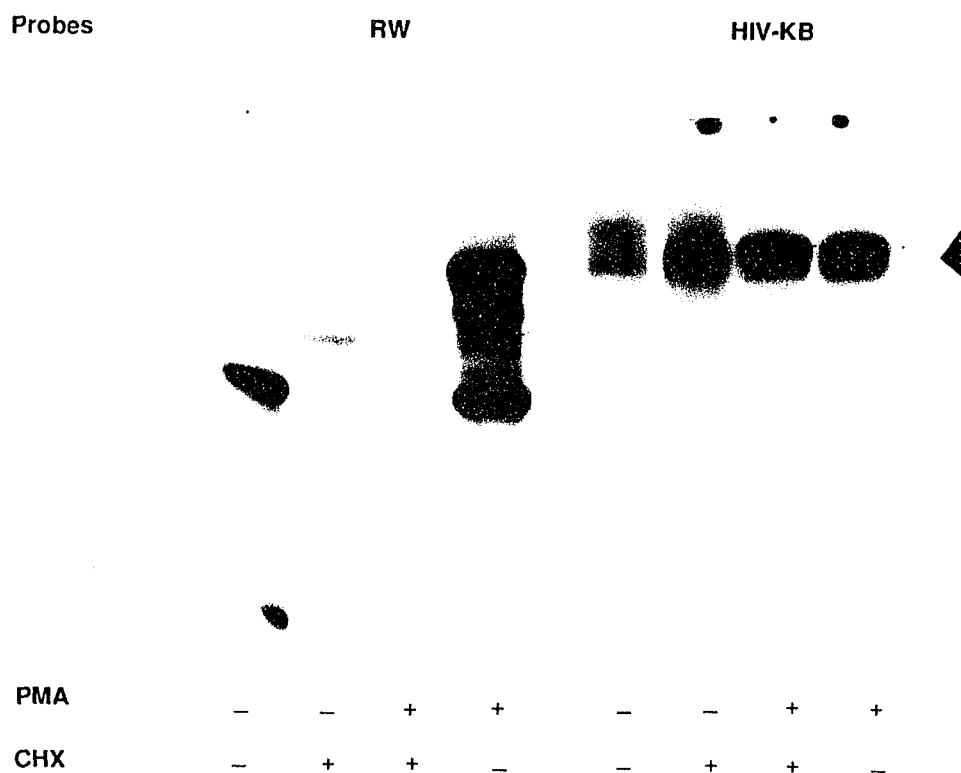
(Fig. 7B) RW-BF is not T-cell specific. Gel shift analysis of binding to RW using extracts from other cell lines. Labeled RW was incubated with nuclear extracts prepared from uninduced and PMA induced Jurkat cells, Raji cells, and Hela cells.



(Fig. 7C) RW-BF is not affected by CsA. Jurkat cells were treated with 1 mM CsA starting 30 min. before and during a 4 hours stimulation with 50 ng/ml PMA. Gel retardation assays were performed using these extracts. The probes used are indicated at the top of the figure, the stimulation conditions at the bottom. The appearance of the lower band showing sensitivity to CsA in this experiment is not a consistent result and has not been further investigated.



(Fig. 7D) Cycloheximide inhibits RW-BF formation. Jurkat cells were treated with 40 μ M Cycloheximide for 30 minutes prior to and during 4 hrs stimulation with 50 ng/ml PMA. Extracts were prepared and used in gel retardation assay. The probes are indicated at the top of the figure, the treatment conditions at the bottom.



(Fig. 7E) Tax does not induce RW-BF. 19D cell line is a Jurkat cell constitutively expressing tax. Gel retardation assays were performed using extracts prepared from cells as indicated at the bottom of the figure. Binding to HIV-kB is used as a control in these experiments.

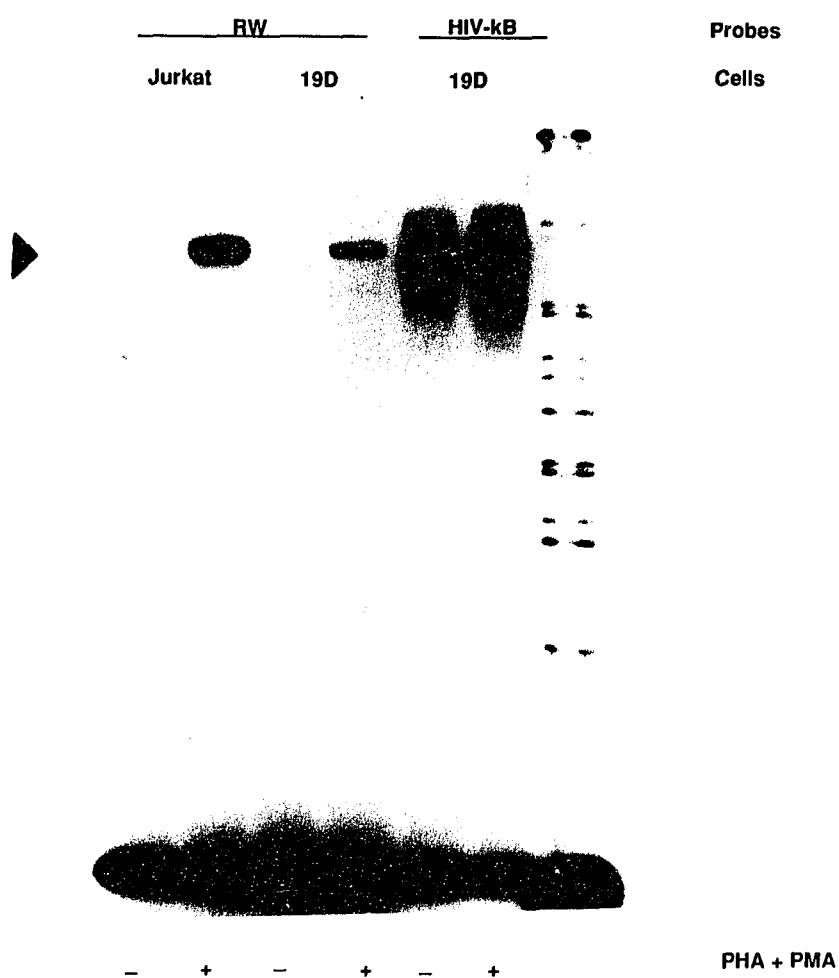


Fig. 8 UV-crosslinking of the complex binding to RW.

(A) Gel retardation assay control for the above UV-crosslinking study. Binding is specific as demonstrated by gel shift assays. RW was used as competitors at a 100 molar excess. Jurkat cells extracts from uninduced (lane 1) or PMA induced cell (lanes 2 and 3) were used in the assays. (B) Size determination of the polypeptides in the inducible binding complex formed with RW by UV-crosslinking analysis. Double stranded oligonucleotides were filled-in in the presence of BrdU and labeled with $^{32}\text{PdATP}$. DNA binding reaction were performed using these probes and nuclear extract from PMA induced Jurkat cells in the presence or absence of 100 molar excess of competitor. The complex that bound was exposed to UV irradiation and then analyzed under denaturing conditions on a 7.5% SDS-PAGE gel. Molecular size markers are shown in kilodaltons.

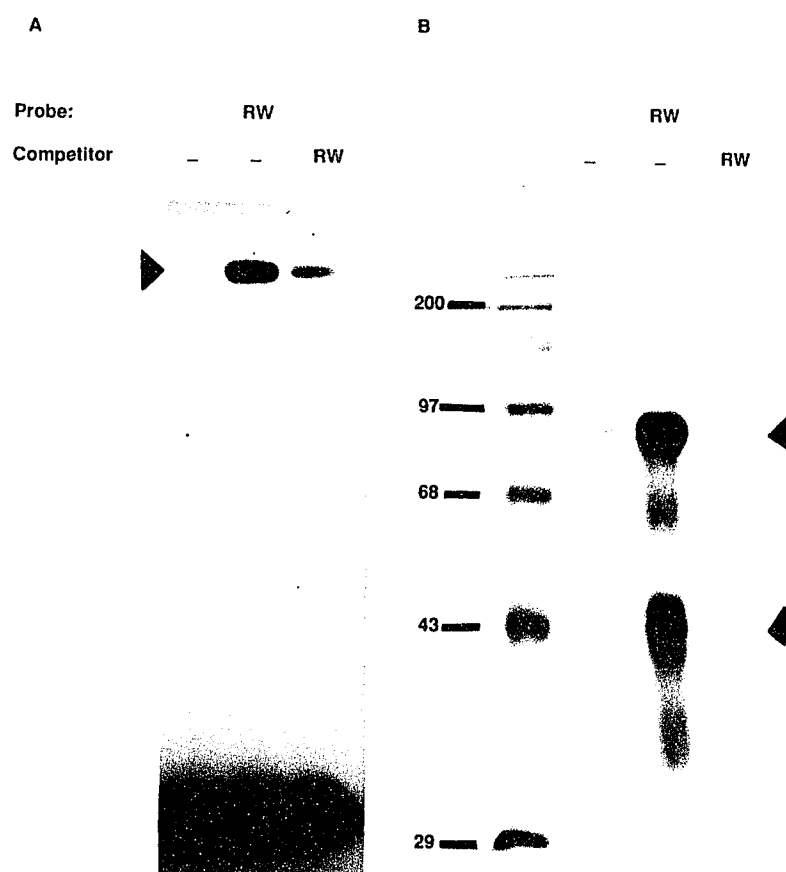


Fig. 8

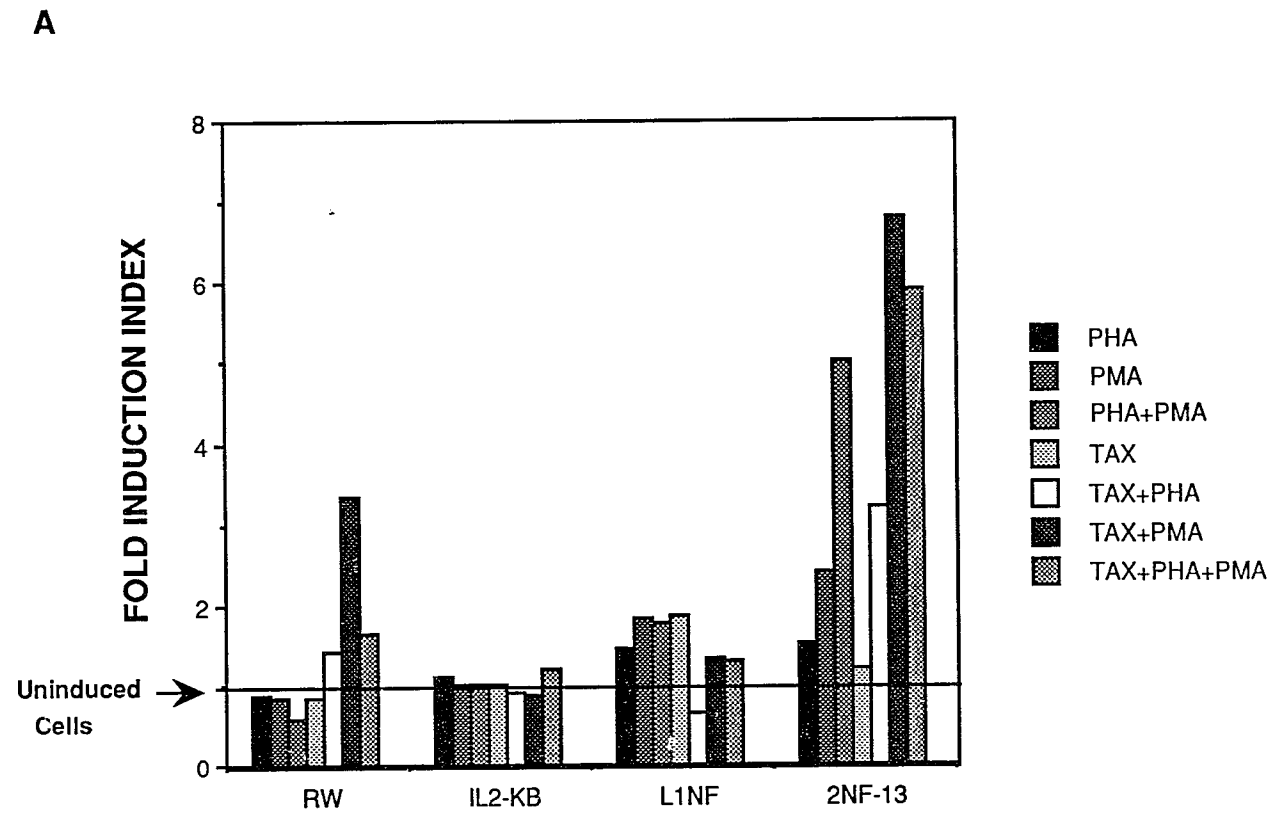
Fig. 9 The ability of RW-BF to cooperate with NF-kB to direct transcription of a reporter gene.

(A) The enhancer activity of 5 copies of RW, 5 copies of IL2-kB-like element, 7 copies of LINF, and 3 copies of 2NF upstream of a TKCAT vector were tested in Jurkat cells. The clones were cotransfected with sense or anti-sense tax expression constructs. 24 hours post-transfection the cells were stimulated with medium alone, 1 ug/ml PHA, 50 ng/ml PMA, and PHA+PMA. The results are representative of at least 3 independent experiments, and normalized for protein content. The data are presented as fold induction over the control TKCAT vector.

The sequence of elements are:

RW:	gatcc	ACCTACATCCATTC
IL2-kB:	gatcc	AAAGACCCATTTTCACC
L1NF:	gatcc	AAAGACCCATTTTCACCTAC
2NF:	gatcc	ACAAAGACCCATTTTCACCTACATCC

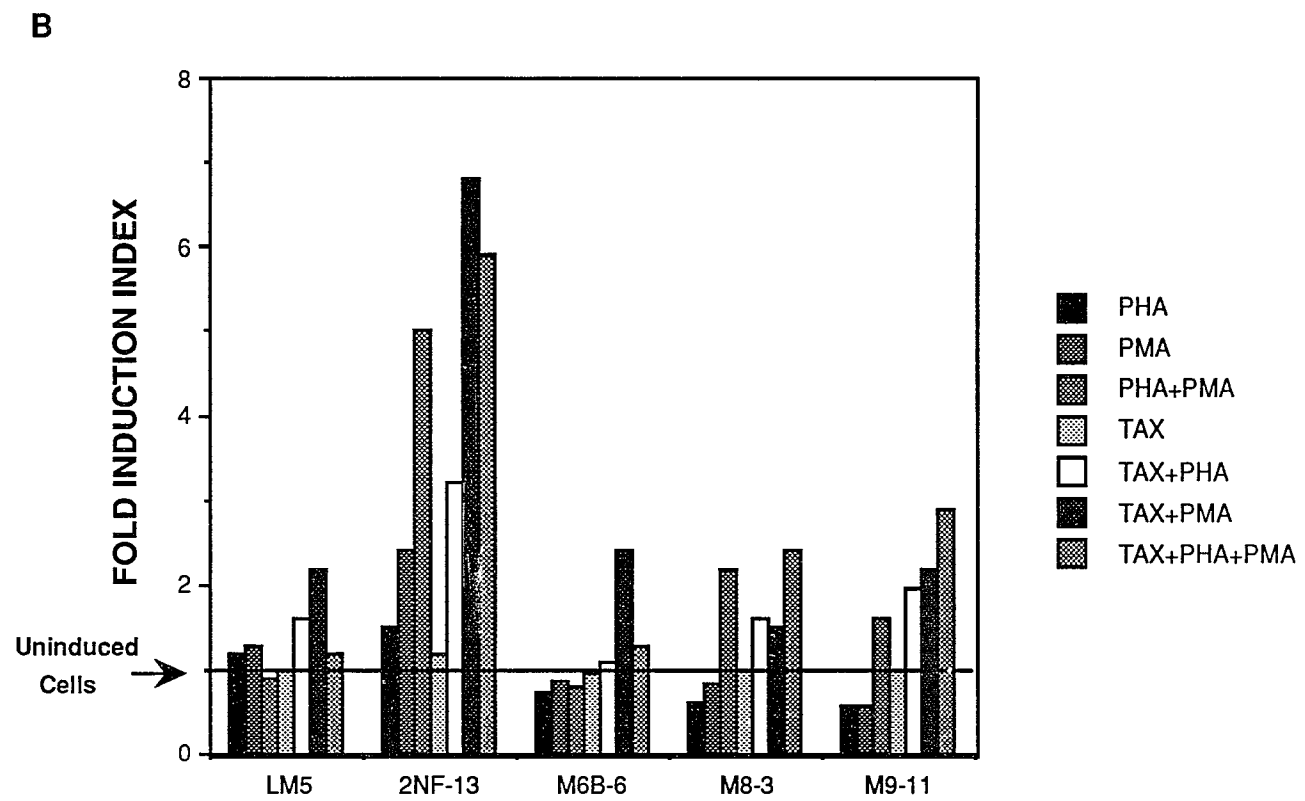
Fig. 9A



(Fig. 9B) Transient transfections using the IL2-2NF mutants were conducted only in Jurkat cells and the results are representative of two to three independent experiments. The mutants were cotransfected with sense or anti-sense tax constructs and stimulated 24 hrs post-transfection as indicated above for A. The sequence of the mutants are:

2NF-13:	gatcc	ACAAAGAGGGATTTACCTACATCC
LM5:	gatcc	ACAAAGACTCCGAACACGCTACTCC
M6B-6:	gatcc	ACAAAGAGGGATTTACCA <u>GG</u> ATCC
M8-3:	gatcc	ACAAAGAGGGATTTACCC <u>TT</u> ATCC
M9-11:	gatcc	ACAAAGAGGGATTTACCTACT <u>AGC</u>

Fig. 9B



(Fig. 9C) The ability of 2NF to direct transcription initiation in Jurkat, Raji, and Hela cells. 2NF-30 contains 7 copies of 2NF cloned upstream of the TKCAT vector. The cells were cotransfected and stimulated as indicated for A.

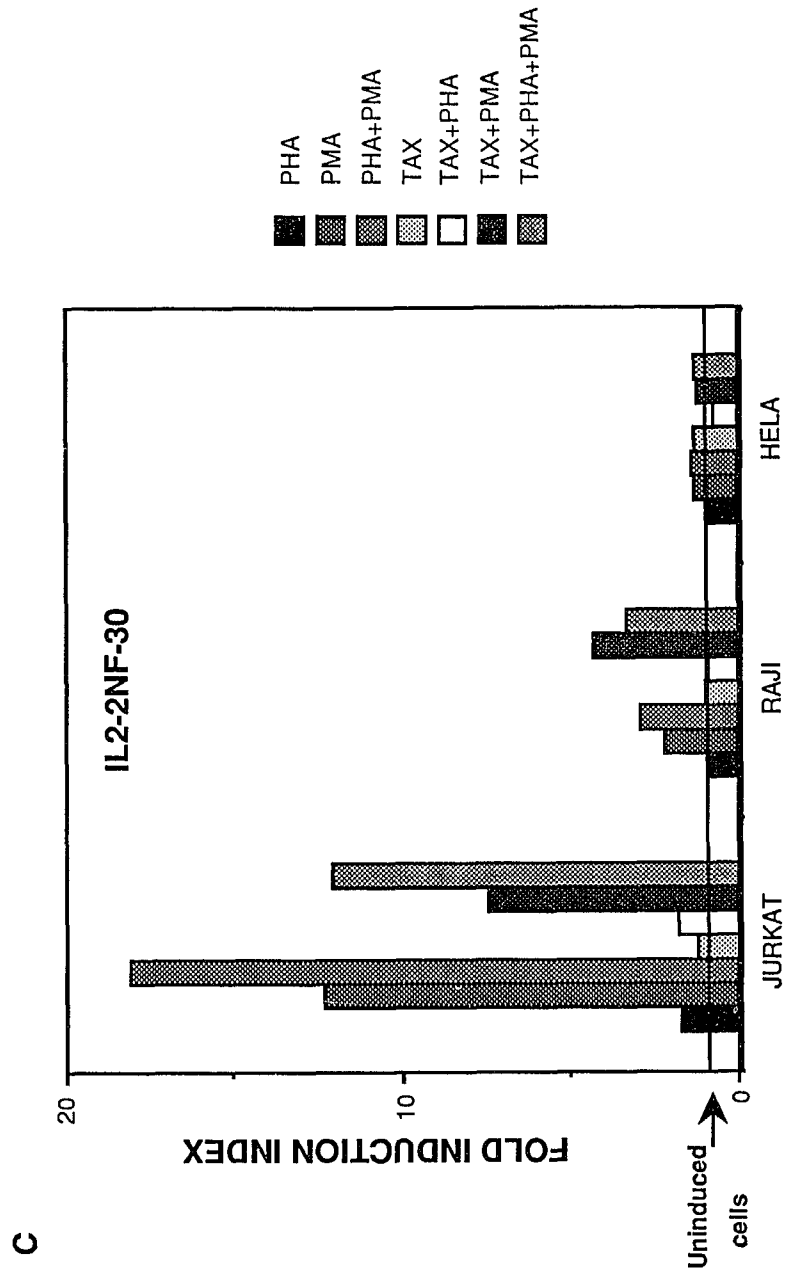


Fig. 10 Binding of an inducible nuclear complex to the IL2-2NF sequence and mutant versions.

(A) Binding to the IL2-2NF sequence -211 to -187 wild type and mutated versions was tested. Nuclear extracts were prepared from Jurkat cells induced with 1 ug/ml PHA plus 50 ng/ml PMA and from uninduced cells. The probes were ³²P labeled to the same specific activity. The different probes are indicated at the top of the figure and the culture condition at the bottom. The sequence of the probes are:

HIV-kB:	gatcc AGGGACTTCC
IL2-kB:	gatcc AAAGAGGGATTTCACCT
IL2-2NF:	gatcc ACAAAGAGGATTTCACCTACATCC
IL2-M5:	gatcc ACAAAG <u>ACTCGAAACAG</u> CTACATCC
IL2-M6A:	gatcc ACATTCAGGGATTTCACCTACATCC
IL2-M6B:	gatcc ACAAAGAGGGATTTCACC <u>AGG</u> ATCC
IL2-M8:	gatcc ACAAAGAGGGATTTCACC <u>CTT</u> ATCC
IL2-M9:	gatcc ACAAAGAGGGATTTCACCTACT <u>AGC</u>

Probe:

IL-2 M6B
IL-2 M6A
IL-2 M5
IL-2 NF
IL-2 2NF
HIVAB

1 2 3 4 5 6

IL-2 M8

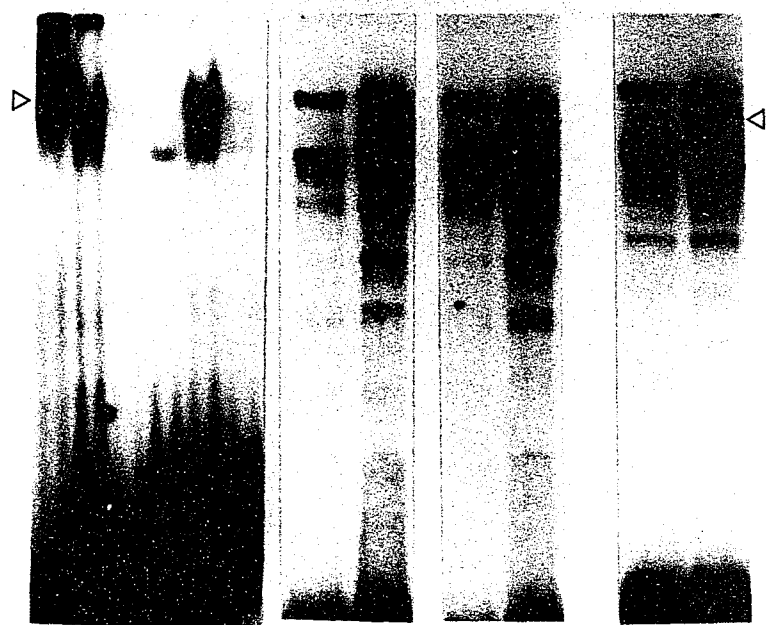
IL-2 M9

IL-2 2NF

7 8

9 10

11 12



PHA+PMA

Fig. 10A

(Fig. 10B) Competition for the binding of the inducible complex. The binding of this complex is competed by the various kB-like elements. Gel retardation assay were performed using ^{32}P labeled IL2-2NF with the same extracts as in A. The competitors were used at 100 fold molar excess. The sequence of the competitors are:

IL2-kB:	gatcc AAAGAGGGATTTACCT
IL2-2NF:	gatcc ACAAAGAGGGATTTACCTACATCC
IL2-RW:	garcc ACCTACATCCATTC
IL2-RW2:	gatcc TTTCACCTACATCCATTCAGTCAGT
IL2-RW3:	gatcc TTTCACCTACATCCAT <u>ATAGTC</u> <u>TTT</u>

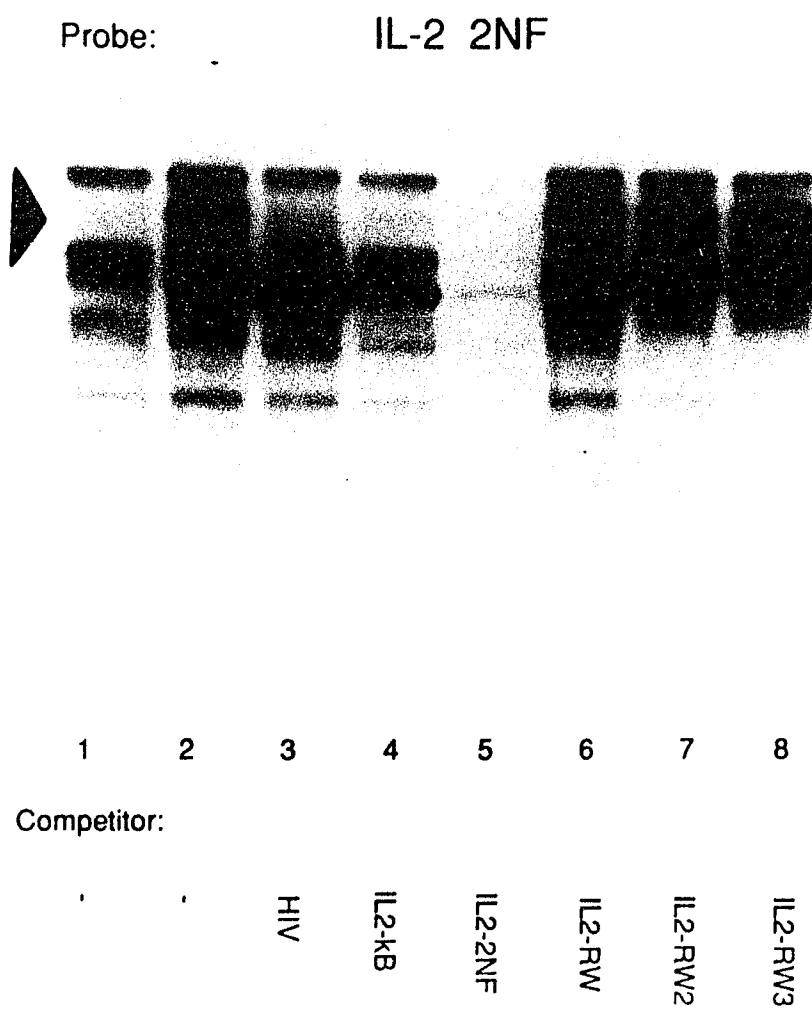


Fig. 10B

Discussion

Our previous study shows that NF-kB is the factor binding to the NFkB-like element at position -206 to -195 in the human Interleukin-2 (IL-2) promoter. Serfling and colleagues working in the murine IL-2 promoter with the sequence -212 to -188 show the binding of a T-cell specific factor, and the enhancer activity of this sequence. The low affinity of NF-kB for the human IL2-kB-element as well as the results obtained by Serfling and co-workers, prompted us to investigate the function of the sequence immediately 3' to the NF-kB-element, referred in this study as RW. The human and murine IL-2 promoters differ within this sequence by three base changes at positions -212, -209, and -191.

Site-directed mutagenesis of the IL-2 promoter indicated that the three nucleotides immediately downstream from the NF-kB site have a regulatory role, inhibiting promoter function by as much as 25-50%. This is possible if the flanking sequence contributes to the binding of NF-kB. To test this notion an oligonucleotide was synthesized encompassing downstream sequence corresponding to that of RW and tested for its ability to bind nuclear factors(s) in gel retardation assays. The assumption, as previously indicated, was that NF-kB could not recognize this sequence and, therefore, no binding would be detected. Surprisingly, a complex did interact with RW. Thus, this present study was focused towards determining the identity of the factor(s) recognizing RW, which we have called RW-BF.

The RW-BF complex binds in a sequence-specific manner as demonstrated by the binding assays. Competition experiments using either random oligonucleotides or oligonucleotides containing mutated, or truncated version of RW show that the binding cannot be abrogated. In addition, complex formation was not detected when the mutated oligonucleotides as probes themselves were used in the gel retardation assays. The complex was induced by mitogens such as PMA and PHA; however, no synergistic effect was detected when cells were stimulated by both PHA and PMA. This observation is significant since interaction between NF-kB and RW-BF is proposed and NF-kB is mainly activated through signals derived from PMA stimulation. Unlike NF-kB, induction of RW-BF is sensitive to cycloheximide, and insensitive to tax activation. In addition, CsA inhibitory effect on protein binding to the IL2 promoter is not seen with RW-BF. Moreover, UV-crosslinking studies show that the complex is composed of two polypeptides of molecular size corresponding to 40 and 95 kilodaltons. It might be said that the larger protein is the dimer of the small polypeptide; this is unlikely given the conditions used for the SDS/PAGE. This complex consisting of two proteins would explain why we have been unsuccessful in our attempts to purify RW-BF (chapter IIIB). We observed the loss of binding activity during the purification even when active nuclear extracts were applied directly to an affinity column. It is probable that the protein complex was dissociated during the purification and consequently no binding function could subsequently be detected.

Transient transfection experiments in Jurkat cells using RW concatamers in the TKCAT vector indicate that this sequence does not represent an enhancer element. This finding is similar to the results obtained for the kB-like element of the IL-2. This is in contrast to the high level of activity seen with the putative kB motif in the same vector (Pierce et. al., 1988). This difference could be explained by difference in affinity of the factor for the various kB-like motifs (Chapter II). Nevertheless, LINF although demonstrating enhanced affinity for NF-KB through extension of its 3' end by three nucleotides remained unable to behave as an enhancer element in transfection assays.

The results of Serfling as well as the data obtained from transient transfection experiments in Jurkat, Raji, and Hela cells demonstrate that multimerized IL2-2NF (similar sequence as that used by Serfling) constructs are able to direct transcription of a heterologous promoter in a lymphocyte-specific manner. The T-cell specific factor identified by Serfling and colleagues by DNase protection assay binding within the mouse IL2-2NF may correspond to the complex formed by the interaction between NF-kB and RW-BF; however, this T-cell binding specificity cannot be demonstrated under the gel retardation assay conditions used by either laboratory.

Interestingly, binding to the human IL2-2NF and to the mutated version indicate that point mutation within the kB-like element or the RW site interfere with the

binding of the inducible complex. However, while the various kB-like motifs abrogated the binding of the inducible complex, the RW sequence did not. To elucidate this finding, as well as to determine how the binding of NF-kB and RW-BF results in transactivating activity the purification and cloning of RW-BF were undertaken.

Purification of RW-BF

Abstract

The Interleukin 2 (IL-2) promoter enhancer comprises several sequence elements essential for gene expression. We have attempted the purification of a nuclear DNA binding complex, designated RW binding factor (RW-BF), that interacts with the sequence at position -197 to -183 upstream of the transcription initiation site on this promoter. Chromatographic approaches including ion exchange, gel filtration, glycerol gradient, as well as sequence-specific DNA affinity column have been used. Our findings indicate that our methodology is unable to reach detectable amounts of this protein. There is a plausible explanation for the results. RW-BF may be a complex which dissociates during the purification manipulations and, therefore, its binding activity cannot be detected by gel retardation assays. We have evidence which suggest this conclusion.

Introduction

Interleukin 2 is a gene regulated at the level of transcription. A great deal of effort has been directed towards the identification of regulatory sequences within its promoter elements as well as the characterization of nuclear proteins interacting with the consensus elements. Our laboratory has identified a DNA binding protein that has IL-2 regulatory function, not of its own but rather through possible interaction with Nuclear Factor-kappa B (NF-kB). Serfling and colleagues, working with the mouse IL-2 promoter, have identified a T-cell specific factor binding the sequence that we have previously characterized as an NF-kB binding element (Hoyos et. al., 1989). The sequence used in their studies corresponds to -212 to -188. This element is identical to the human IL-2 promoter sequence except for three base changes at positions -211, -209, and -191. Detailed analysis of the sequence -197 to -183 indicated the presence of a binding protein which is sensitive to cycloheximide but which is not T-cell specific although binding in some cells is hardly detectable. The work by this group as well as our own suggests that the sequence -211 to -187 is an enhancer element. Because the sequences -211 to -194 and -197 to -183 do not have enhancer function on their own and these elements interact with the complexes NF-kB and RW-BF respectively, we propose that an interaction between NF-kB and RW-BF results in the transactivating function seen with the sequence -211 to -187. Purified proteins would be useful to elucidate this interaction.

Materials and Methods

Preparation of nuclear extracts:

Nuclear extracts were prepared from Molt 2 cells at 4°C according to Dignam (1983). All the buffers used included antipain at 0.3 ug/ml, 5 mM phenylmethylsulfonyl (PMSF), 0.3 ug/ml leupeptin, and 1 mM dithiothreitol (DTT). The protein yield from 2×10^9 cells was 60 mg as measured by the Bio-Rad protein assay.

Oligonucleotides:

The oligonucleotides were synthesized on an Applied Biosystem DNA 380B Synthesizer and purified as described (Bohnlein et. al., 1988). The sequence of the oligonucleotides are: gatcCACCTACATCCATTC, referred to as RW; gatccAGGGACTTTCC, termed HIV-kB.

Gel retardation assays:

The assay was performed as previously described (Bohnlein et. al., 1986, Scheidereit et. al., 1987) with the following modifications: the various chromatographic fractions (2-5 ul) were preincubated with binding buffer (4% Ficoll, 20 mM Hepes pH 7.9, 60 mM KCl, 2 mM DTT, and 0.1 mg/ml BSA) for 10 min at room temperature in the presence of 1 ug of Poly (dl-dC). This was followed by incubation for 20 min with 0.2 - 0.6 ng of double stranded oligonucleotide label with

Klenow used to fill-in the ends, in a final volume of 20 μ l. The complexes are separated from free probe in a 6% native polyacrylamide gel electrophoresis, containing 0.25X TBE. The electrode buffer system was 0.25X TBE.

Chromatographic fractionation:

Hydroxyapatite Chromatography:

Hydroxyapatite was performed as previously described (Lenardo et. al., 1988; Yano et. al., 1987) with some modifications. Hydroxyapatite (Bio-Rad) was rehydrated as indicated by the company and 2 ml in a 3 cc syringe barrel and equilibrated with buffer D (20 mM Hepes pH 7.9, 0.2 mM EDTA, 0.1 M KCl, 20% v/v glycerol). Nuclear extract (6 mg/ 0.6 ml) was applied by gravity to the column. The column was washed with 5 column volumes of buffer D and then eluted with 2 column volumes each of buffer D containing a step gradient of KCl, as follows: 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, and 1 M. Fractions of 0.5 ml were collected.

Heparin-Agarose Chromatography:

Heparin-Agarose chromatography (Sigma) was performed as for Hydroxyapatite gel.

Bio-Rex 70 Chromatography:

Bio-Rex 70 chromatography was performed as previously described (Kawakami et. al., 1988) with some modifications. Bio-Rex 70 (Bio-rad) was equilibrated in buffer D as recommended by the company and 2 ml of the matrix was packed in a 2.5 x 25 cm column. 16 mgs/1.6 ml of nuclear extract was applied by gravity to the column. The column was eluted in step-wise gradient manner with 3 column volumes of buffer D containing 0.3 M, 0.6 M, and 1 M KCl, respectively. The flow rate was 30 ml/min, and fractions of 5 ml were collected.

Phosphocellulose Chromatography:

The chromatography was performed as described previously (Lenardo et. al., 1988; Karsenty et. al., 1991) with some modifications. Cellulose phosphate P11 was activated and then equilibrated in buffer D as recommended by the supplier (Whatman), 4 ml of the matrix was packed in a 2.5 x 25 cm column. 16 mg/ 1.6 ml of nuclear extract was applied by gravity to the column. Step wise gradient was performed using 3 column volumes of buffer D containing successively 0.3 M, 0.6 M, and 1 M KCl. The flow rate was 0.3 ml/min and fractions of 3 ml were collected.

DE-52 Chromatography:

DE-52 chromatography was performed as described (Kawakami et. al., 1988) with some modifications. Pre-swollen DE-52 cellulose (Whatman) was activated and equilibrated in buffer D following the procedure recommended by the supplier. 16

mg/ 1.6 ml of nuclear extracts was applied by gravity to the column and elution was done as described for phosphocellulose chromatography.

Gel filtration chromatography:

Nuclear proteins (10 mg) were fractionated on Sephadex G-200 column, (26 x 60 cm) equilibrated in buffer D. Eighty fractions of 4.0 ml each were collected at a flow rate of 2 ml/min.

Glycerol gradient:

Molt 2 nuclear extract was centrifuged for 16 hrs at 40,000 rpm (SW50.1 rotor) through a 10-30% glycerol gradient in buffer D (Baeuerle et. al., 1988; Ghosh et. al., 1990). A total of 24 fractions of 200 ul each were collected from the top.

DNA affinity chromatography:

The affinity column was prepared as indicated by Kadonaga and Tjian (1986) using the RW sequence. One milliliter of matrix was equilibrated in buffer D containing 0.1% NP-40. Crude extract (10 mg/1 ml) was combined with 100 ug of Poly (dI-dC) and incubated for 10 min at 4°C. The mixture was then applied to the column and allowed to stand for 10 min.. The column was washed four times with 2 column volumes of buffer D. Elution was conducted with 1.5 column volumes of buffer D containing 0.2 M, 0.4 M, 0.6 M, 0.8 M, and 1 M KCl. For each of these elution steps the buffer was allowed to stand in the matrix for 10 min after which

the fraction was collected. The first ml eluted during the regeneration of the matrix was also collected and tested for binding to RW.

Results

We have followed the purification of the complex, termed RW-BF, by gel retardation assays using the oligonucleotides described in materials and methods as the probes. Protein elution from the columns was monitored by U.V. 280 nm absorption. Several pilot experiments were performed to determine what would be the best steps to follow for the purification of RW-BF.

Hydroxyapatite chromatography (Lenardo et. al. 1988) was used because it has been demonstrated to be efficient in the removal of nucleic acids and nonspecific contaminants that bind to DNA at high salt concentration from nuclear preparations. The results of three independent experiments indicated that none of the fractions collected, including the effluent, had binding activity (data not shown).

Heparin-Agarose is a matrix that binds a small number of proteins in a complex mixture and thus has been used as a first chromatographic step for purification (Preifelder, 1982). This column was used as a mean to remove contaminants from the crude extract. The column was eluted as indicated in Materials and Methods. Our results showed that, as with hydroxylapatite, we had lost binding activity (data not shown).

Bio-Rex 70 is a weak cation exchanger that was used for the purification of RW-BF. In two previous experiments we had tested this matrix using a continuous and a step elution KCl gradients. No RW-BF binding activity was recovered from the collected fractions. We then tried to recover binding activity by eluting the proteins from the column with a set of three broad step elution buffers consisting of 0.3 M, 0.6 M, and 1 M KCl. RW-BF bound to the column since no activity was detected in the effluent and RW-BF binding activity was eluted with 0.6 M KCl in fraction 4 (see fig. 11A). As shown in Table 1, the specific activity of this fraction was low, and the protein recovery was poor. An experiment was performed to determine if by combining different fractions, two at a time, additional RW-BF activity could be recovered. Fig. 11A shows no binding or enhancement of binding with any of the combination tested. Although the recovery from the column was low, this column gave the very first indication that RW-BF binding activity could be recovered after chromatography.

Phosphocellulose is another cation exchanger which was tested for purification of RW-BF (Kawakami et. al., 1988; Lenardo et. al., 1988). Activity was recovered in fraction #5 (see fig. 11B) after elution with 0.6 M KCl. As demonstrated in Table 1, the specific activity of this fraction was similar to that of the crude extract.

Realizing that the cation chromatography was not giving encouraging results, we decided to test an anion exchange column. DE-52 was chosen. The objectives of

using this column were to remove nucleic acids that we thought were interfering with the binding of RW-BF to the matrices and the elimination of nonspecific DNA binding proteins. The results indicate that one third of the binding activity was recovered in one fraction containing the effluent, with no activity detected in any of the other fractions (data not shown).

The next approach taken was gel filtration. Sephadex G-200 separates proteins of molecular weights ranging from 5,000-800,000. The nuclear extract was first concentrated using a centriprep-10 mini-concentrator of 10,000 MW C.O. (Amicon) and the solution was ultracentrifuged at 70,000 rpm (TLA-100.3 rotor) for 40 min, at 4°C. Upon fractionation (fig. 12) three observations were made. First, a major peak eluted in fractions 55 to 60. This peak consisted of incompletely digested fragments of nucleic acids as demonstrated by the OD reading at 260 nm, and agarose gel electrophoresis showing fragments of about 300 bp after ethidium bromide stain. The fragments resulted from treatment of the extract with nucleases done to eliminate nucleic acids contaminating the extract. Second, although extensive binding to RW was detected in fractions 11-36, the electrophoretic mobility of the bands was faster than that expected for RW-BF (data not shown). Only fraction 14 to 17 showed a very modest RW-BF binding activity of the expected mobility. Third, we tested all the fractions for binding to HIV-kB as a control for column function. It is known that HIV-kB interacts with NF-kB, a complex consisting of two subunits. The results showed a distinct binding

confined to fractions 9 to 16 (fig. 12). This demonstrated that Sephadex G-200 column, under condition used for RW purification, was able to resolve the NF-kB complex in an intact active form.

One additional method was used to semi-purify the extract before the use of an affinity chromatography. Zonal sedimentation through a continuous 10-30% glycerol gradient of the nuclear extract was performed. The fractions were tested for binding and the results are illustrated in fig. 13A. Gel retardation assay of the fractions showed RW-BF binding activity in fractions 5 to 16. These fractions also showed some additional bands of faster mobility. All the fractions were also tested for binding to the HIV-kB, as a control probe; this binding activity was confined to fractions 6-11 (fig. 13B).

From this gradient a pellet was collected that once resuspended had binding activity (see fig. 13a, lane 20); however, no binding to the HIV-kB probe was detected in this pellet (see fig. 13b, lane 20). This finding suggests that the RW-BF complex is forming aggregates possibly due to a high degree of its hydrophobicity.

DNA affinity chromatography was performed as indicated in materials and methods. The crude extract was directly applied to the column. Most of the binding activity was eluted with 0.8 M KCl as shown in fig. 14A. Unfortunately, the protein is labile, and binding activity is gradually lost with each successive run on this column. Analysis by SDS/PAGE of the recovered fractions (fig. 14B) revealed

the absence of proteins as detected by silver stain (equal volume from each fraction was loaded on the gel). Table 1 shows that affinity chromatography was the only means by which we could obtain a significant recovery of RW-BF binding activity (Table I).

To test the specificity of the RW-BF binding activity eluting with 0.8M KCl, a cold inhibition experiment was done. An aliquot of the fraction was preincubated for 10 min with a 50 fold molar excess of RW. Fig. 14A (lane 9) shows that binding to RW was abrogated under this conditions.

SDS/PAGE analyses of the fraction collected did not show the presence of any band showing enrichment. On the contrary, no protein was detected after the fraction W2. The band seen represents BSA added after the fractionation to preserve the stability of the proteins (fig. 14B).

RW-BF Purification

<u>Column</u>		<u>Unit Deff.*</u>	<u>mg</u>	<u>Vol</u>	<u>Units</u>	<u>S.A.</u>	<u>Fold Purif.</u>	<u>% Yield</u>
Bio-rex	Crude	10 u/ul	16	1.6	16000	1000	-	100
	fraction 4	0.14 u/ul	2.9	3.5	500	172	0.17	0.31
Phospho-cellulose	Crude	10 u/ul	16	1.6	16000	1000	-	100
	fraction 5	0.14 u/ul	0.5	3.6	514	1028	1	3.2
DE-52	Crude	3 u/ul	16	1.6	4,800	300	-	100
	Effluent	0.14 u/ul	4.7	7.3	514	109	0.3	11
Glycerol gradient	Crude	10 u/ul	16	1.6	160000	1000	-	100
	fraction 10	0.1 u/u/	ND	0.2	ND	ND	ND	ND
Sephadex G-200	Crude	8 u/ul	10	1.3	10000	1000	-	100
	fraction 15	0.01 u/ul	ND	4	ND	ND	ND	ND
DNA-affinity	Curde	0.25 u/ul	0.6	1.5	375	625	-	100
	0.8 KCl	0.125 u/ul	0.008	1.5	188	23500	38	50

* Units of binding activity are arbitrarily and independently defined for each chromatographic column. A unit represents the binding activity seen in the active fraction(s) and based on it determining the units present in the crude extract that was used for each column.

ND : Not determined.

Table

Figures

Fig. 11. Cation-exchange Chromatography.

(A). Gel retardation assay of Bio-rex 70 column chromatography fractions with the RW labeled oligonucleotide. Each fraction (7 ul) was incubated with 1 ug of poly (dI-dC), and subjected to electrophoresis as described in Materials and Methods. Lane 1 indicates the positive control for this assay with (30 ug, 20 U) Molt 2 crude extract and 1 ug of poly (dI-dC). The arrow head indicates the specific retarded band formed by RW. Lanes 2-6 show binding with the fraction number indicates; lanes 7-12 show binding to the fractions that were combined.

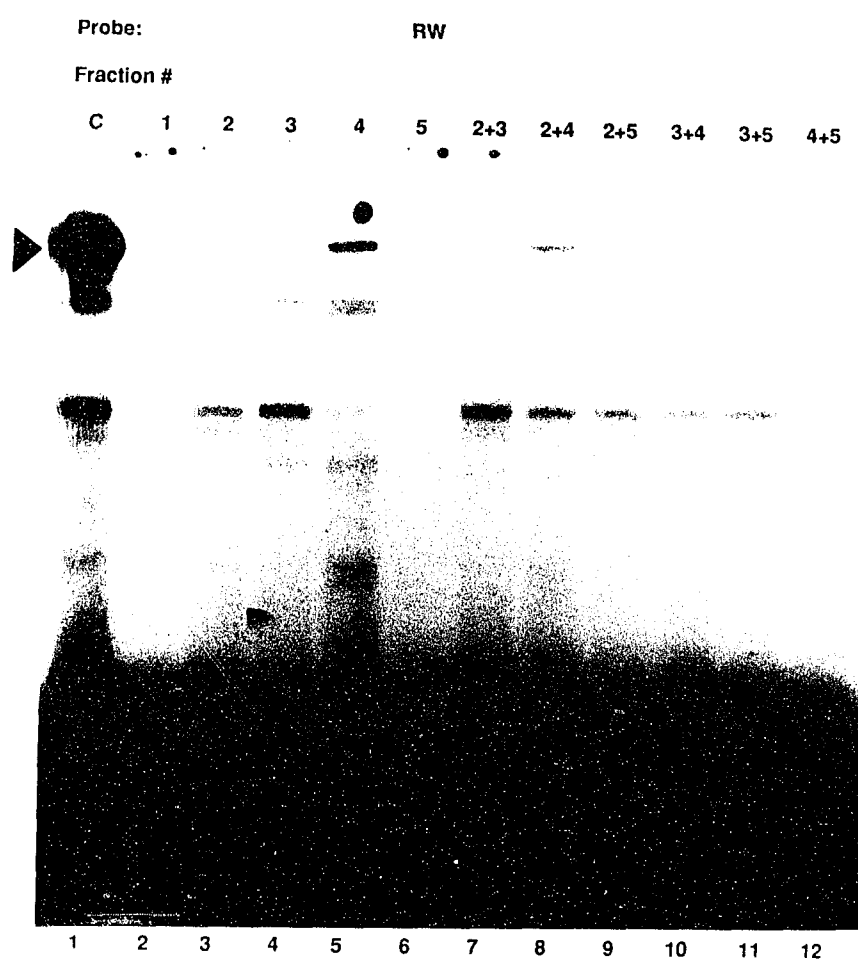


Fig. 11A

(Fig. 11B) Gel retardation assay of phosphocellulose column chromatography fractions with RW. The assay was carried out as described for (A). Lane 2 is binding to the fraction eluting with the void volume of the column.

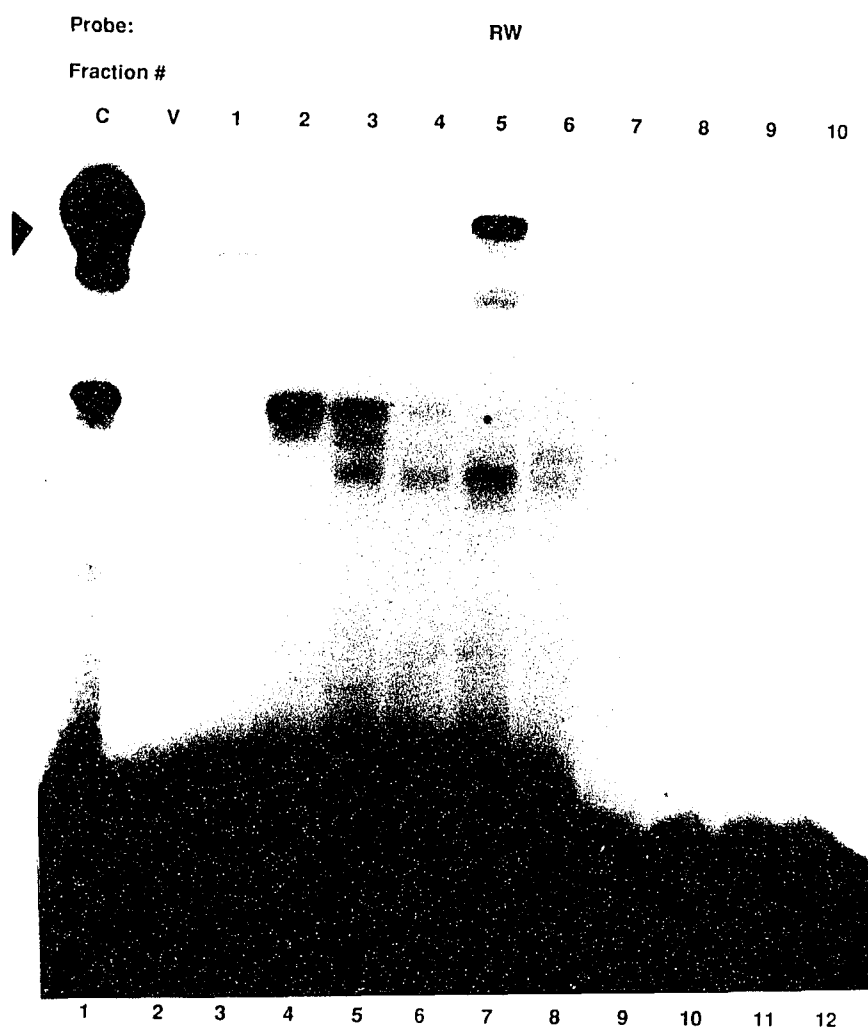


Fig. 12. Gel filtration elution profile, and RW-BF binding activity of the fraction.

The elution profile is based on elution of proteins from the Shephadex G-200 column as measured by 280 nm reading. The binding activity of the fractions was determined by gel retardation assays. A fraction was positive for binding activity if a shift characteristic of RW-BF was detected even if it was hardly detectable.

	Binding Activity										
Fraction #	8	9	10	11	12	13	14	15	16	17	18
RW	-	-	-	-	-	-	+	+	+	+	-
HIV-kB	-	+	+	+	+	+	+	+	+	-	-

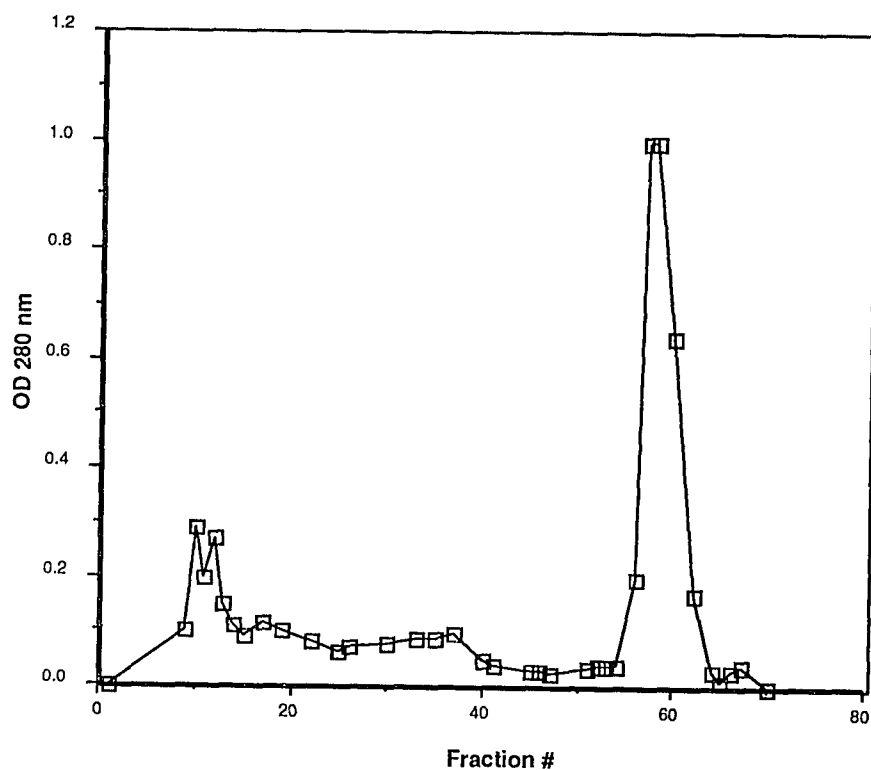
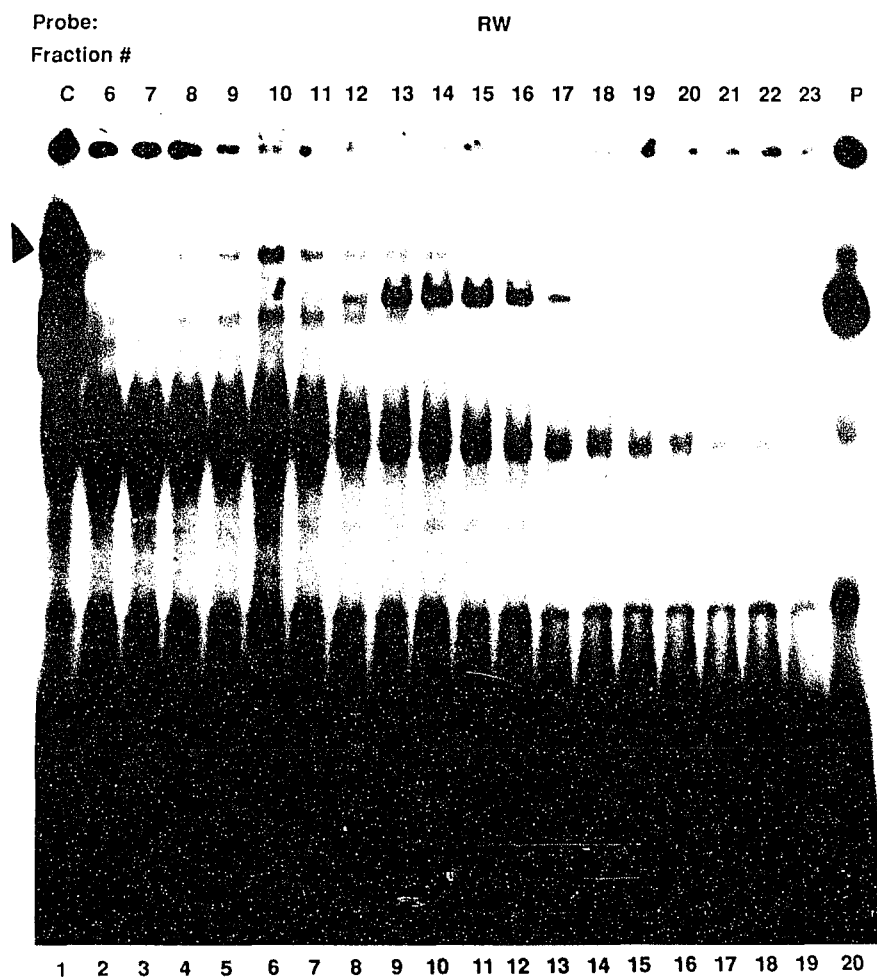


Fig. 13. Glycerol gradient analysis.

The gradients were prepared and run as indicated in Materials and Methods. (A) Gel retardation assay of the glycerol fractions with RW as probe. Each fraction (10 ul) was incubated with 1 ug of poly (dI-dC), and the free and bound probe were separated as in figure 11A. Lane 1 shows binding of RW with (30 ug) of Molt 2 crude extract, lanes 2-19 represent binding to fractions 6 to 23, lane 20 represents binding to the pellet recovered after the centrifugation and which was resuspended in buffer D (10 ul out of the 50 ul were use for the binding assay).



(Fig. 13B) Same as (A) except that HIV-kB is used as a control probe.

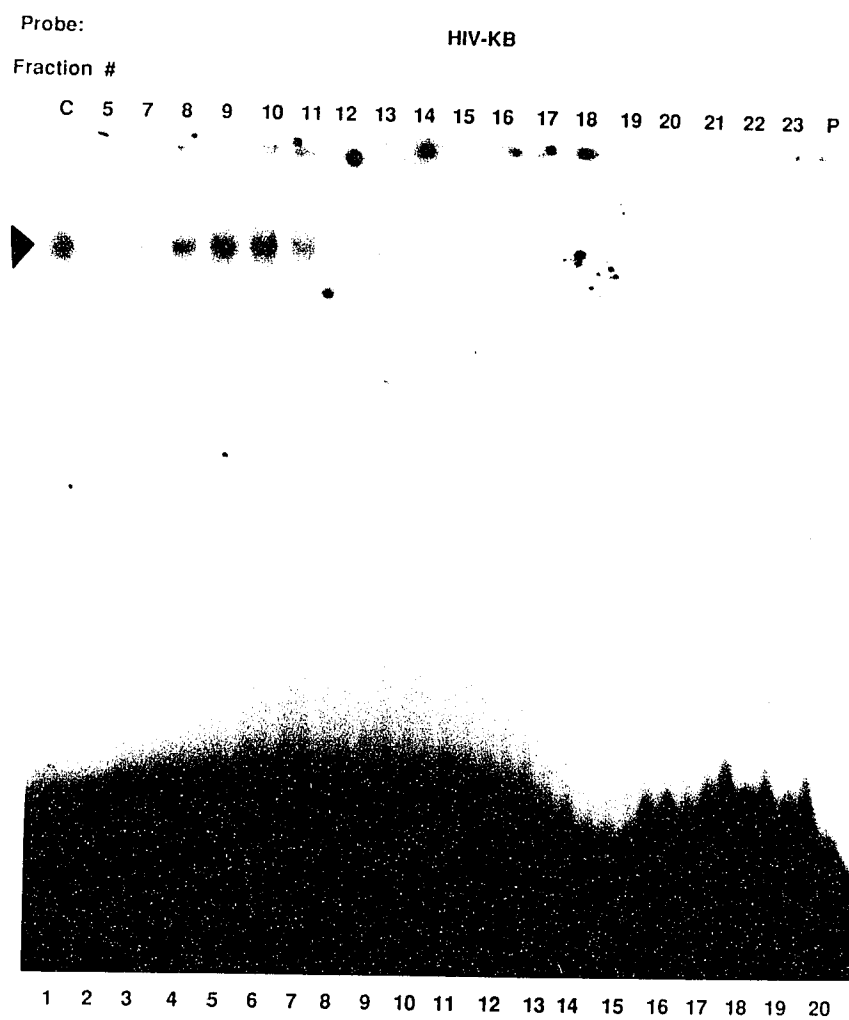
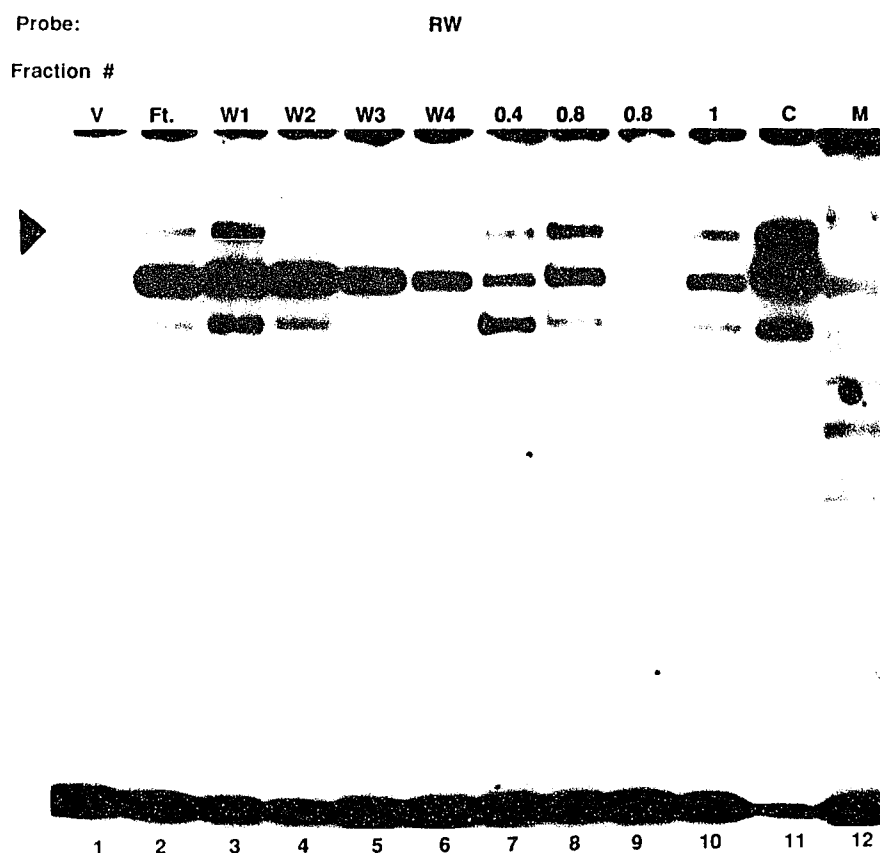
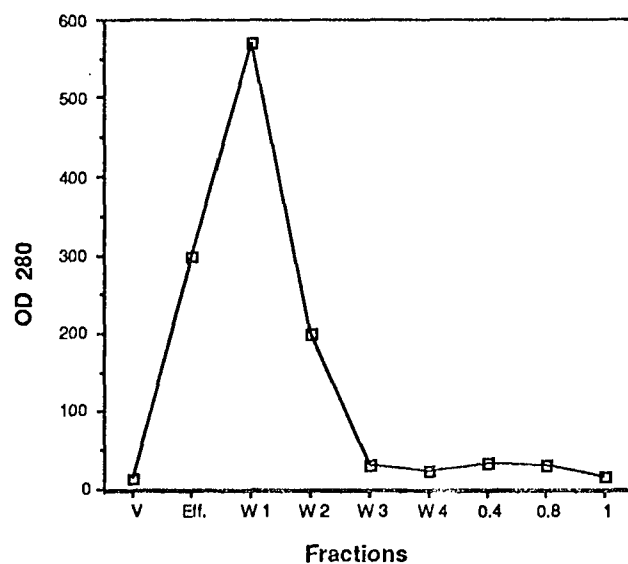


Fig. 14. DNA Affinity column chromatography.

(A) Gel retardation assay of affinity column chromatography using RW as probe. Each fraction (10 ul) was incubated with probe and separated as described in Materials and Methods. Lane 1 is probe alone, lane 2 shows binding to the flow through, lanes 3-6 show binding in each successive wash, lane 7,8, and 10 indicate binding to the material eluting with 0.4 M, 0.8 M, and 1 M KCl respectively, lane 11 shows control binding with (30 ug) Molt 2 crude extract, lane 9 shows the ability of 50X molar excess cold RW to compete for binding, and lane 12 shows labeled 1 Kb DNA ladder.



(Fig. 14B) Protein analysis of the affinity chromatography fractions. An aliquot (20 μ l) of each fraction was mixed with 2X sample buffer (0.0625 M tris pH 6.8, 3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and boiled at 100°C for 5 min before loading on a 7.5% SDS/PAGE, Laemmli (1970). The 65 Kd band present in all the lanes is BSA which was added to the fractions to preserve protein stability. Protein silver staining was performed as previously described, Maniatis (1989).



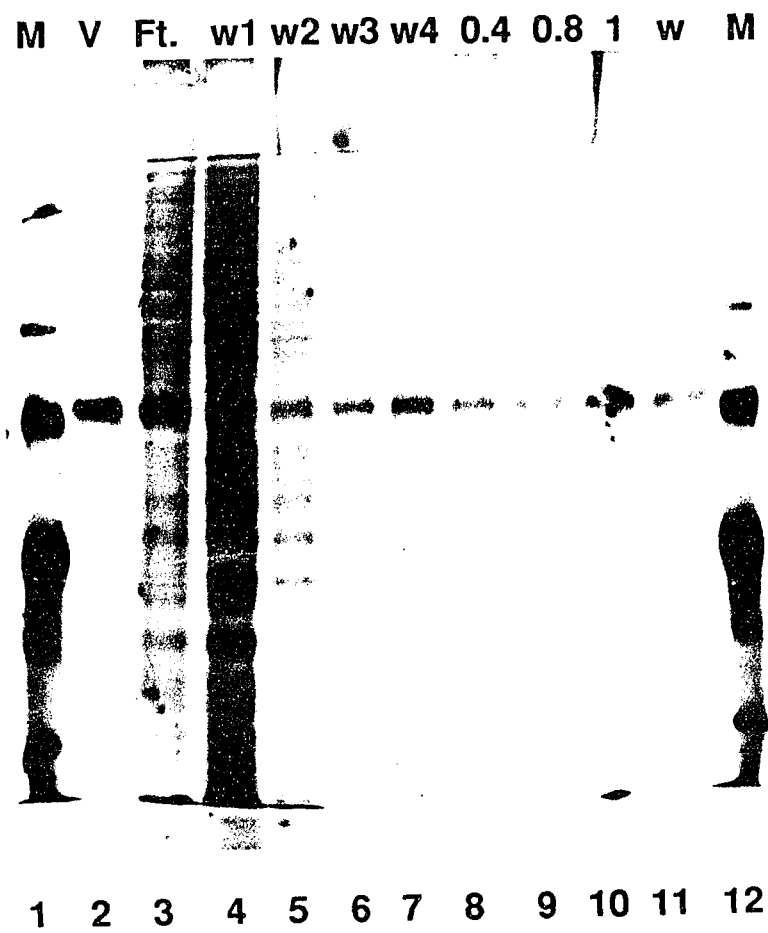


Fig. 14B

Discussion

We have attempted to purify the complex binding to the IL-2 sequence -197 CACCTACATCCATTC -183 by conventional methodologies. These consisted of ion-exchange and gel filtration as preliminary steps that would result in a semi-purified extract. This mixture would then have been applied to an affinity column and the active fraction applied successively until complete purification is achieved. This is a strategy that with some variations, depending on the type of matrices used, has resulted in the successful purification of several nuclear factors by different laboratories.

Hydroxyapatite is a crystalline form of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, to which proteins bind by an unknown mechanism. In the purification of RW-BF we used this column in order to resolve proteins from a crude extract using a 0.1 to 1 M KCl step elution gradient with increments of 0.1 M. Gel retardation assays using the fraction collected did not detect binding activity to RW in any of the fractions obtained from three different experiments. We determined that RW-BF was binding to the column given that no binding activity was observed in the effluent.

Two independent experiments were performed exactly as for hydroxyapatite using heparin-agarose column. This matrix also failed to resolve the complex. RW-BF could not be detected in any of the fractions. The protein did bind to the column

since no binding activity was observed in the effluent. Nevertheless, desorption or detection of the protein could not be accomplished.

There are several possible explanations as to why these columns failed to purify RW-BF. One, the complex may bind tightly to the matrix and 1 M KCl was not sufficient to elute it from the column; two, the matrix could cause conformational changes in the protein rendering it undetectable by our assays; three, subunits making up RW-BF may dissociate from the complex during the chromatography; four, the complex may have been slowly diluted out during elution from the column in amounts undetectable by the assay. We did not rule out any of these possibilities.

With the conditions used neither hydroxyapatite nor heparin-agarose resulted in resolution and detection of RW-BF binding activity; therefore, ion-exchange chromatography was tested for the purification of RW-BF. The first experiment was performed using Bio-Rex 70, a cation exchanger, using the same eluting conditions utilized for hydroxyapatite and heparin-agarose matrixes. The experiment gave no recovery of RW binding activity. Thinking that desorption of the protein in an active form could occur with a narrower KCl gradient, we repeated the experiment using a 0.1 M to 1 M KCl continuous gradient. Again, no RW-BF binding activity was detected. A broad step gradient was used next. It consisted of three different

buffers 0.3 M, 0.6 M and 1 M KCl. With this gradient RW-BF activity was eluted with 0.6 M KCl. These results suggested that the lack of RW-BF activity observed with hydroxyapatite, heparin agarose, and this matrix could be explained by the complex being slowly washed out of the column under the elution conditions initially employed.

Although RW-BF binding activity was recovered, it was weak (see Table 1). It was speculated that RW-BF activity would be lost if RW-BF is a complex consisting of subunits which were dissociated during the fractionation. An experiment conducted to test this hypothesis did not support it. We were unable to recover binding activity by combinations of two fractions at a time. All the activity that was detected was in fraction 4 alone and no enhancement of binding activity was seen with any of the combinations that included fraction 4. However, the inability to reconstitute an active complex by combining two fractions is inconclusive for two reasons: one, RW-BF may consist of subunits that are separated into more than two fractions; second, the complex, if reconstituted, may not be active under the conditions used. Neither of these possibilities were ruled out.

Another explanation for the loss of RW-BF binding activity when using the Bio-Rex 70 matrix is that the complex, although eluted from the column with 0.6 M KCl, was unable to interact with RW and therefore was weakly detected by gel retardation assays. This inability of the complex to bind the sequence could result from conformational changes caused by the interaction of Bio-Rex 70 with the protein(s). Proteins may denature under these conditions on this column

(Kohanski's unpublished observations).

Phosphocellulose, another cation exchanger, was therefore tested. The data showed that most of RW-BF activity also eluted with 0.6 M KCl. Comparison of Phosphocellulose with Bio-Rex 70 indicated that both columns resulted in the loss of most of the RW-BF protein. However, phosphocellulose was better than Bio-Rex 70 in that the protein that was recovered from this matrix had the same specific activity as that of the crude nuclear preparation; this is in contrast to the protein eluted from the Bio-Rex 70 matrix which had very low specific activity.

DE-52 is an anion exchanger that was tested next to determine if it would be more effective than phosphocellulose for the purification of RW-BF. As stated in results, it was expected that the use of this column would result in the collection of a single fraction containing most of RW-BF in an active form from which nucleic acids and their nonspecific binding proteins had been removed. The data suggests that the only fraction with RW-BF activity, the effluent, had only 11% of the total RW-BF units and 1/3 the specific activity. DE-52, although better than Bio-Rex 70 for RW-BF purification, was not as efficient as phosphocellulose.

Altogether, the ion-exchange chromatography indicated that to resolve RW-BF we needed a broad KCl gradient, and that elution from the columns could be accomplished with 0.6 M KCl. Furthermore, of the three ion-exchange columns used for RW-BF purification, phosphocellulose was the most effective; much of the

protein was lost but whatever was recovered from this column retained its original specific activity. In addition, the loss of RW-BF binding activity after chromatography may be explained by RW-BF losing activity through dissociation of its subunits.

If the components of the complex dissociated during the ion-exchange chromatography due to changes caused by the eluting salt gradients, then RW-BF could possibly be resolved by gel filtration. The data obtained from Sephadex G-200 utilized to test this idea showed that the binding activity of RW-BF could be weakly detected in fractions 14 to 17. Binding of NF- κ B, used as control in this experiment, was detected in fractions 9 to 16. The resolution of these two nuclear proteins within approximately the same fractions was not surprising since on gel retardation assays these two complexes have the same mobility indicating similarity in molecular size. However, there are two observations which are difficult to explain: one is the very weak binding activity of RW-BF; the second is the additional bands seen binding to RW in fractions 11 to 36. The very weak binding activity of RW-BF could be explained by a complex rendered unstable during the resolution. The reasons for this instability may be caused by a change in conformation or by the lack of a cofactor (described below). The extra bands seen in fractions 11 to 36 could represent the binding of the individual subunits that make up the RW-BF complex. If these additional bands do represent the binding of the independent subunits then the components of the complex seem to be held

together by weak forces. This is suggested by the results showing NF-kB binding activity. This complex is composed of two subunits, the p50 and the p65, each of which can interact independently with the DNA binding motif (Ghosh et. al., 1990; Nolan et. al., 1991). In our assay we see no evidence for the independent binding of either of the subunits, we see only the binding of the NF-kB complex. This may suggest that the forces acting on the NF-kB complex as a whole are stronger than those acting on RW-BF and therefore RW-BF could be easily dissociated. However, it is also possible that a cofactor(s), present in the crude extract, stabilizes the subunits of RW-BF and this cofactor(s) were diluted out during the chromatography since it is not present in the eluting buffer. This washing out of the cofactor(s) may result in the recovery of an unstable complex, if at all.

Not being able to obtain a good semi-purified RW-BF preparation from either ion-exchange or gel filtration chromatography, a glycerol gradient was tested hoping to obtain RW-BF activity within a few fractions which could then be further processed. The results showed that RW-BF activity was present in 16 of the 24 fraction and in the pellet. This gave indications that the complex is probably highly hydrophobic and so forms aggregates. Although the complex was more stable in this gradient, this method was not practical for the purification of RW-BF since the activity was distributed over half of the fractions. These 16 fractions also showed the presence of additional bands by gel retardation assay which was reminiscent of what was seen with Sephadex G-200 column. This finding gives additional

support to the hypothesis that RW-BF consist of subunits that were dissociated during the chromatography. The contribution of a cofactor in maintaining protein stability was not resolved by this method.

Ultimately, significant purification of RW-BF could only be achieved by the use of DNA affinity chromatography. Attempts to further increase the degree of purification of the active fractions by consecutive application to the column resulted in decrease in RW-BF activity with each successive application. This indicated that the complex is labile. This was not surprising since the modest binding activity recovered by cation exchange was lost within a few hours of collection. Thus, RW-BF could be semi-purified by DNA affinity chromatography; however, under the conditions of chromatography the complex was unstable, possibly due to the lack of cofactor(s).

The cloning of RW-BF

Abstract

We have isolated two cDNA clones by screening 2×10^6 plaques from a HUT78 lambda gt11 library, which is a PMA induced T lymphoma cell line expressing IL2, using the Interleukin-2 promoter sequence -197 to -185 (RW) as probe. The sequences of the clones show 99.5% identity with DNA binding protein B (Dbpb) and 95 % identity with YB-1, a 42 kD nuclear protein that recognizes the CCAAT-box. The specificity of binding to RW by the bacterial expressed protein can best be demonstrated by dot blot analysis. We think that this protein may represent the DNA binding subunit of a complex termed RW-BF which interacts with the above mentioned sequence in gel retardation assays. At present, there is no direct evidence for the identity of Dbpb as a subunit of the RW-BF complex.

Introduction

Gene transcription regulation is mediated by transcription factors composed of a DNA-binding and an activation domain which interact with regulatory elements on the promoter of the genes that they are modulating. Many regulatory factors have been cloned by taking advantage of their ability to interact with specific DNA motifs. cDNA libraries have been cloned in expression vectors such as lambda gt11 which allows transfer of the expressed proteins to nitrocellulose filters. The screening for these proteins can be done with labeled fragments containing the specifically-recognized sequence (Muller et. al., 1988; Singh et. al., 1988; Lenz et. al., 1990; Yan et. al., 1991).

We have used this method to identify nuclear proteins interacting with the IL-2 promoter sequence -197 to -183 (Chapter IIIA). Two identical clones were isolated. The cDNA, when used as probe in Northern blot analysis of Jurkat cells mRNA, identified a constitutively-expressed message of approximately 2.5 kilobases.

Materials and Methods:**Isolation of cDNA from HUT78 expression library:**

The PMA induced HUT78 library in lambda gt11 vector was obtained from **Clontech**. The library was plated on 9 cm dishes (Falcon) at a density of 1×10^5 plaque forming units (pfu) per plate on E. coli Y1090 for 3.5 hours at 42°C. The fusion proteins were induced by overlaying a dry nitrocellulose filter impregnated with 10 mM IPTG (isopropyl thiogalactoside) on the soft agar over night at 37°C. For binding site screening the membranes were first rinsed in TBST (50 mM Tris pH 7.9, 150 mM NaCl, 0.05% Tween-20), blocked in TBST containing 20% Fetal Calf serum and 100 ug/ml Sperm salmon DNA for 1 hour at room temperature and subsequently incubated with radiolabeled RW for 3 hours at room temperature. The membranes were washed in three changes, 10 minutes each, of TBST and exposed to X-ray film. All positive signals were plaque-purified three times.

Lambda DNA preparation:

Lambda DNAs were prepared from liquid lysate. The bacteriophage particles were collected by ultracentrifugation and the DNA extracted as indicated by Maniatis (1989). The DNA was redissolved in TE pH 8 and analyzed after EcoRI digestion by agarose gel electrophoresis.

Induction of the fusion protein:

Escherichia-coli strain Y1090 was infected with clones lambda gt11 #1 and #2 and were grown at 30°C until an OD of 0.45 was reached. At this point the cultures were incubated for 15 minute at 44°C and induced with 10 mM IPTG for 1 hour at 37°C. The bacteria were centrifuged at 12,000g for 30 seconds at 4°C and resuspended in lysogen extraction buffer (50mM Tris pH 7.5, 1 mM EDTA pH 8, 5 mM DTT, 50 ug/ml PMSF). After lysis by freezing in liquid N₂ the mixture was centrifuged at 12,000g for 30 minute and dialyzed on Millipore filter. (142 mm, type VS, 0.025-micron pore size). The supernatant was used, Maniatis (1989).

Gel retardation assays:

The assay was performed as previously described (Bohnelein et. al., Scheiderer 1987) with the following modifications: Bacterial cell crude extract (10 ul) were pre-incubated with 1 ug of poly dl-dC in binding buffer (4% Ficoll, 20 mM Hepes pH 7.9, 60 mM KCl, 2 mM DTT, 0.1 mg/ml; BSA) for 10 minute at room temperature in the presence or absence of competitors. The probe,, 0.2-0.6 ng of radiolabeled double stranded oligonucleotide was subsequently added and incubated for 20 minutes at room temperature, in a final volume of 20 ul. The complexes were separated from free probe in a 6% native polyacrylamide gel electrophoresis containing 0.25X TBE. The electrode buffer system was 0.25X TBE.

DNA sequencing and computer analysis:

cDNA was excised from lambda gt11 phage by EcoRI digestion and subcloned into M13 p19. Sequencing reactions were performed by single-stranded dideoxynucleotide sequencing with a Sequenase Kit (UBS) Sequence analysis was performed with the program VAX-gcg-FastA of the **GenBank**.

Northern hybridization:

For Northern analysis 20 ug of mRNA per sample was size separated on 1% agarose gel in the presence of 2.2 M formaldehyde, Maniatis (1989). RNA was transferred by capillary action with 10X SSC to Zetabind membrane (Cuno Inc.). Hybridization condition was 50% formamide, 5X SSC, 5X Denhardt's, 10% Dextran sulfate, 0.001% SDS, 100 ug/ml Salmon sperm DNA and 2.0×10^6 cpm/ml of radiolabeled cDNA at 42°C overnight.

Isolation of mRNA from Jurkat cells:

RNA was isolated as previously described. Briefly, $2-3 \times 10^7$ Jurkat cells were disrupted by vortexing in TKM buffer (10 mM Tris pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 1% Triton-X100). The nuclei were removed by centrifugation. The supernatant was extracted twice with phenol/chloroform and ethanol precipitated.

Dot blot analysis:

One ug of bacterial extract, in 1 ul, were spotted on to nitrocellulose and allowed to air dry. The individual spots were cut out and incubated in 200 ul of TBST containing 20% fetal calf serum and +/- competitor for 30 minutes at room temperature. Subsequently, radiolabeled probe was added and incubation continued for 1 hour at room temperature. After washing in three changes of TBST, the spots were air dried and exposed to X-ray film. The assay was performed on 24 macrowell plates (Falcon).

Results and Discussion

In pursuing the identification of the factor(s) binding to the sequence -197 to -183 (RW) a HUT-78 cDNA lambda gt11 library was screened using monomeric synthetic oligonucleotide corresponding to the RW sequence as probes. Two clones were isolated which were plaque-purified three times (fig. 15A). The size of the inserts correspond to 900 and 1100 base pairs (bp), as indicated by Eco RI digestion of the phage DNA (Fig. 15B).

DNA sequence analyses show the two clones to be identical. Comparison of the nucleotide sequence of the isolated cDNAs with those of the **GenBank** data base using the program VAX-gcg-FastA, identified DNA binding protein B (Dbpb) as a protein sharing 99.5% identity with the clones over a region covering 1100 bases (fig. 16). In addition, over 90% identity is observed with two other related but different CCAAT-box binding proteins, namely YB-1 and EFIA.

Dbpb was first identified by Ishii and coworkers (1988) when screening a placenta cDNA expression library using a 530 bp fragment from the epidermal growth factor receptor enhancer (EGF), and a 120 bp fragment from the promoter region of the human c-erb gene. In this study, Northern analysis of the of the isolated clone using Hela cells total RNA, demonstrated two bands or approximately 2.5 and 4.0 kb. In contrast, our analysis of the clones using Jurkat mRNA, shows a single,

noninducible, and abundant band of about 2.5 kb (fig. 17). The additional band in Ishii's study may represent differences in the cell lines, the use of total versus mRNA, or differences in stringency of washing and thus represent a crossreactive message. In addition, the study also indicated that in contrast to IL2 mRNA which is induced by mitogens and is sensitive to CsA (Kranke et. al., 1984; Briekel et. al., 1987; Elliot et. al., 1984; Weiss et. al., 1984; Larsson et. al., 1980; Truneh et. al., 1985), the message of the clones is not induced by mitogens such as PHA, PMA, PHA plus PMA, or PMA plus anti-CD28. Furthermore, the presence of CsA has no effect on the transcription of this message. The constitutive expression of the mRNA indicates that the expression of encoded cDNA gene may be regulated posttranscriptionally. This is suggested by the mitogen inducibility of RW-BF binding activity (chapter IIIA, fig. 7A), and the sensitivity of binding to cycloheximide treatment (chapter IIIA, fig. 7D)

To demonstrate the ability of the cDNA encoded protein to specifically interact with the RW sequence, gel retardation assays were performed using crude lysogenic bacterial cells extracts. The assays were unsuccessful in identifying a shift unique and specific to the clone lysogens. Two possible explanation for these results are: first, the threshold of sensitivity of the gel retardation assay is above the affinity of the bacterial expressed protein for RW; second, the protein binds to RW but produces a shift similar to that seen with the negative controls.

Therefore, the specificity of binding was tested using a different method (Materials and Methods). The same concentration and volume of each sample was spotted. In addition, the buffer conditions for binding were the same as those used for the cDNA library screening. Using this method we were able to demonstrate the specificity of binding of the protein encoded by clones 1 and 2. As seen in fig 18, no binding was detected to either one of the negative controls, and binding to RW could be inhibited only by the specific RW sequence.

As indicated above, the nucleotide sequence of the clones share over 90% identity with YB-1 (Didier et. al., 1988) which binds to the MHC class II genes, and EF1A (Ozer et. al., 1990) which binds to the Rous sarcoma virus. Both of these proteins bind to the CCAAT-box. Therefore, it was important to determine the ability of the encoded cDNA proteins to interact with the various CCAAT-box DNAs. Furthermore, it was important to determine the ability of the RW sequence and the CCAAT-box oligonucleotides to crosscompete. To address these questions two different CCAAT-box oligonucleotides were synthesized. CCAAT-box 1 is derived from the CCAAT-box motif of the adenovirus which is recognized by the NF-1 transcription factor, and CCAAT-box 2 from the alpha-globin gene (Jones et. al., 1987). Gel retardation assay were performed using RW, CCAAT-box 1 and CCAAT-box 2 and bacterial extracts from lysogen for clone 2, which has a longer insert. As negative control, crude extract from lambda vector lysogen is used.

The two CCAAT-box oligonucleotides show an identical shift pattern to that of RW and to that of each other. Therefore, data from the CCAAT-box 2 is presented since it demonstrates stronger binding than that of CCAAT-box 1 (fig 19). The unique band is specifically competed by 50 fold molar excess of CCAAT-box 2 (lane 3), but not by the same fold excess of RW or the RW mutants RWH and RW-6B (lanes 4, 5, and 6) respectively. However, if 100 fold molar excess is used, the CCAAT-box 2 competes 100%, CCAAT-box 1 competes approximately 70%, RW less than 30%, and RWH or RW-6B not at all (data not shown). Although RW binding presents the same pattern as that of the CCAAT-box oligonucleotides, at a 50 fold molar excess inhibition of binding to the unique band is hardly detected (lane 10); a minor effect can be seen when using 100 fold molar excess of RW (data not shown). However, RW does compete specifically at 50 fold molar excess for two bands not unique to the lysogenic clone. In addition, the CCAAT-box oligonucleotides do not cross-compete for the unique band that also binds to RW.

Together, the data indicates that the dDNA expressed protein (Dbpb) binds to RW and to the various CCAAT-box oligonucleotides. Binding to the CCAAT-box oligonucleotides is specific since they cross-compete for binding to each other; however, competition is always better by the self DNA. This observation has been previously been described for the various CCAAT-box oligonucleotides (Gai et. al., 1991). Moreover, the protein does bind to RW but with less specificity, requiring over 100 fold molar excess of RW to inhibit binding. In addition, the CCAAT-box

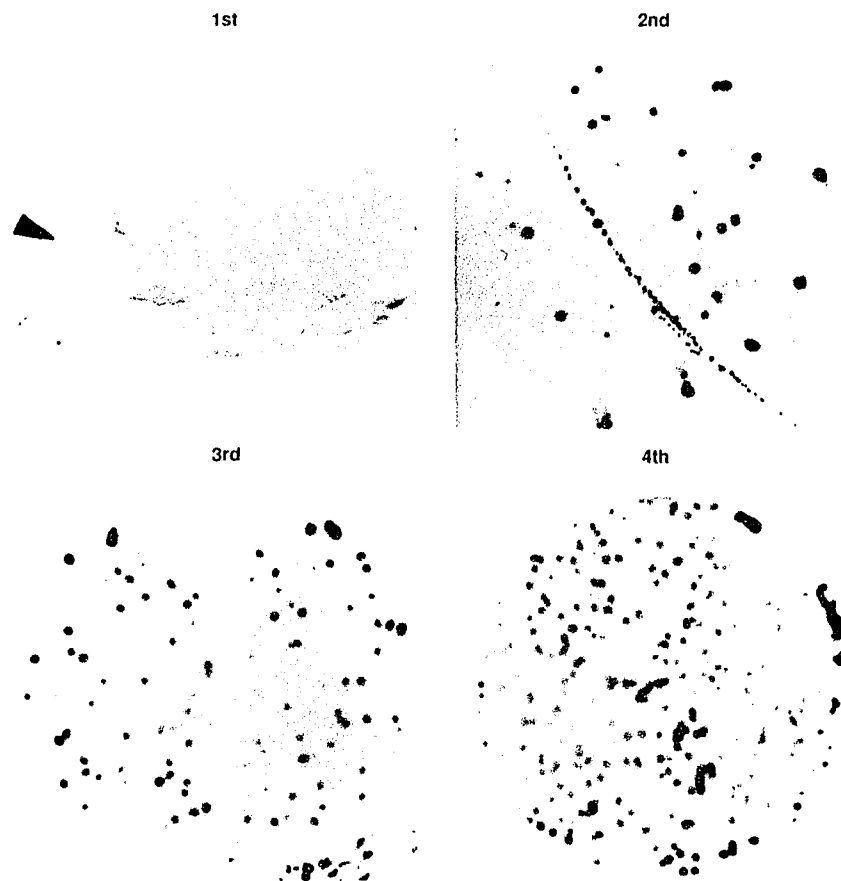
oligonucleotides do not cross-compete for binding to RW and vice versa. This may be explained by the lack of a true CCAAT-box element within the RW sequence.

In contrast to the gel retardation assays, the dot blot analysis of the binding to RW demonstrated specific binding to RW which was competed by as low as 10 fold molar excess. This could be explained by conditions in the dot blot assay that facilitated this binding, or by the presence in the fetal calf serum used in this assay of a factor that interacts with bacterial expressed protein rendering this complex specific for interaction with RW. This explanation might have been ruled out if CCAAT-box oligonucleotides had been used in cross-competition dot blot assays.

At present we cannot say that RW-BF is the cDNA encoded protein (Dbpb). The various CCAAT-box oligonucleotides do not bind or compete for binding to RW-BF in Jurkat cell extracts (data not shown). It is possible that the cDNA encoded protein is the 40 kD subunit of RW-BF (chapter IIIA, fig 8). The ability of a Dbpb peptide antibody to recognize the RW-BF would resolve this question.

Fig. 15 Isolation of cDNA clones from HUT78 expression library.

(A) Screen of HUT78 lambda gt11 expression library with radiolabeled RW. Purification of the positive shown by the arrow on the first screen by first, second and third plaque purification.



(Fig. 15B) Determination of the size of the insert cDNA clones. cDNAs were excised from lambda gt11 by EcoRI digestion. The insert were separated on 0.8% agarose gel. Lane 1 shows clone 1, lane 2 indicates the 1Kb ladder marker, and lane 3 identifies clone 2.

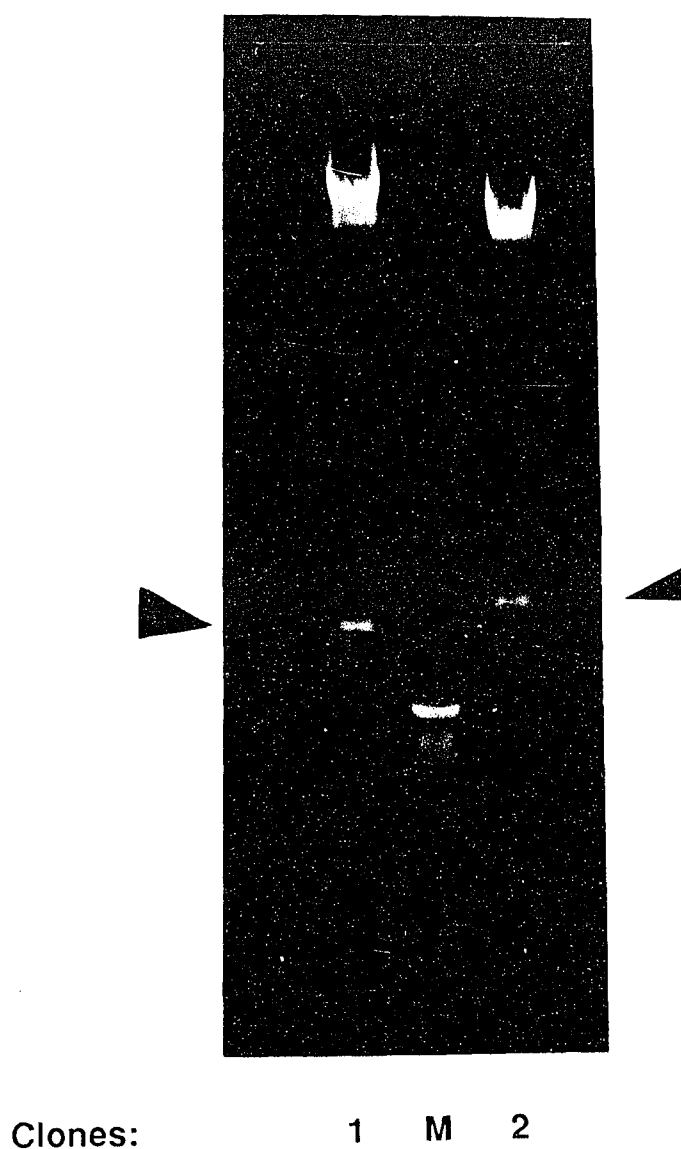


Fig. 16 Comparison of the cDNA nucleotide sequence with DNA binding protein B (Dbpb).

Dbpb shows 99.5% identity with the isolated cDNA sequence. An asterisk indicates the absence of the nucleotide in the sequence. The lines represent identity between the two sequences. When the sequence is different, the nucleotide is indicated.

```

Dbpb:  GAATTCGGGG  GACCCCAGAG  AGCCCTGAGC  AGCCCCACCG  CCGCCGCCGG  CCTAGTTACC  60
cDNA:  -----C--  *****  ****-----  --A-----  -----C  GG-----
Dbpb:  ATCACACCCC  GGGAGGAGCC  GCAGCTGCCG  CAGCCGGGCC  CAGTCACCAT  CACCGCAACC  120
cDNA:  -----
Dbpb:  ATGAGCAGCG  AGGCCGAGAC  CCAGCAGCCG  CCCGCCGCCG  CCCCCGCCGC  CCCCGCCCTC  180
cDNA:  -----
Dbpb:  AGC*GCCGCCG  ACACCAAGCC  CGGCACTACG  GGCAGCGGCG  CAGGGAGCGG  TGGCCCCGGC  240
cDNA:  ---T-----  -----
Dbpb:  GGCCTCACAT  CGGCGGCGCC  TGCCGGCGGG  GACAAGAAGG  TCATCGCAAC  GAAGGTTTGT  300
cDNA:  -----*-----
Dbpb:  GGAACAGTAA  AATGGTTCAA  TGTAAGGAAC  GGATATGGTT  TCATCAACAG  GAATGACACC  360
cDNA:  -----
Dbpb:  AAGGAAGATG  TATTTGTACA  CCAGACTGCC  ATAAAGAAGA  ATAACCCAG  GAAGTACCTT  420
cDNA:  -----
Dbpb:  CGCAGTGTAG  GAGATGGAGA  GACTGTGGAG  TTTGATGTGT  TTGAAGGAGA  AAAGGGTGCG  480
cDNA:  -----
Dbpb:  GAGGCAGCAA  ATGTTACAGG  TCCTGGTGGT  GTTCCAGTTC  AAGGCAGTAA  ATATGCAGCA  540
cDNA:  -----
Dbpb:  GACCGTAACC  ATTATAGACG  CTATCCACGT  CGTAGGGGTC  CTCCACGCAA  TTACCAGCAA  600
cDNA:  -----
Dbpb:  AATTACCAGA  ATAGTGAGAG  TGGGGAAAAG  AACGAGGGAT  CGGAGAGTGC  TCCCGAAGGC  660
cDNA:  -----*-----
Dbpb:  CAGGCCCAAC  AACGCCGGCC  CTACCGCAGG  CGAAGGTTCC  CACCTTACTA  CATGCGGAGA  720
cDNA:  -----
Dbpb:  CCCTATGGGC  GTCGACCACA  GTATTCCAAC  CCTCCTGTGC  AGGGAGAAGT  GATGGAGGGT  780
cDNA:  -----
Dbpb:  GCTGACAACC  AGGGTGCAGG  AGAACAAGGT  AGACCAAGTGA  GGCAGAATAT  GTATCGGGGA  840
cDNA:  -----
Dbpb:  TATAGACCAC  GATTCCGCAG  GGGCCCTCCT  CGCCAAAGAC  AGCCTAGAGA  GGACGGCAAT  900
cDNA:  -----
Dbpb:  GAAGAAGATA  AAGAAAATCA  AGGAGATGAG  ACCCAAGGTC  AGCAGCCACC  TCAACGTCGG  960
cDNA:  -----
Dbpb:  TACCGCCGCA  ACTTCAATTA  CCGACGCAGA  CGCCAGAAA  ACCCTAAACC  ACAAGATGGC  1020
cDNA:  -----
Dbpb:  AAAGAGACAA  AAGCAGCCGA  TCCACCAGCT  GAGAATTCGT  CCGCTCCCGA  GGCTGAGCAG  1080
cDNA:  ----- 1043
Dbpb:  GGC GGGGCTG  AGTAAATGCC  GGCTTACCAT  CTCTACCATC  ATCCGGTTTA  GTCATCCAAC  1140

Dbpb:  AAGAAGAAAT  ATGAAATTCG  AGCAATAAGA  AATGAACAAA  AGATTGGAGC  TGAAGACCTA  1200

Dbpb:  AAGTGCTTGC  TTTTGGCCCG  TTGACCAGAT  AAATAGAACT  ATCTGCATTA  TCTATGCAGC  1260

Dbpb:  ATGGGGTTTT  TATTATTTTT  ACCTAAAGAC  GTCTCTTTTT  GGTAATAACA  AACGTGTTTT  1320

Dbpb:  TTAAAAAGC  CTGGTTTTTC  TCAATACGCC  TTTAAAGGTT  TTTAAATTGT  TTCATATCTG  1380

Dbpb:  GTCAAGTTGA  GATTTTTAAG  AACTTCATTT  TTAATTTGTA  ATAAAAGTTT  ACAACTTGAT  1440

Dbpb:  TTTTTCAAAA  AA  1452

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Fig. 17 Northern blot analysis of the cDNA.

Message RNA (20 ug each) was isolated from Jurkat cells uninduced (-); stimulated with 1 ug/ml PHA (H); 50 ng/ml PMA (M); PHA plus PMA (P); anti-CD28 (9.3) plus PMA and 1 ug/ml cyclosporin A, CsA (CsA, 9.3, M); anti-CD28 plus PMA (9.3, P).

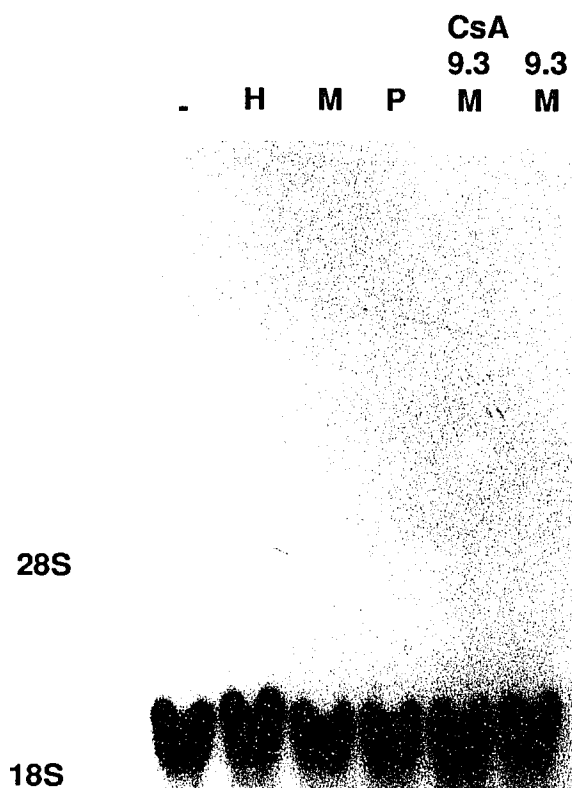


Fig. 18 Specificity of binding to RW by the bacterial expressed protein encoded by the isolated cDNA.

C, represents binding to bacterial extract; lambda, illustrates binding of bacterial extracts lysogenic for lambda; clones 1 and 2, represent binding of bacterial extracts of cells lysogenic for clone 1 and 2 respectively. The assay was performed as indicated in material and methods. Competition was performed using the indicated oligonucleotides at 100-fold molar excess, or at the indicated molar excesses shown for RW

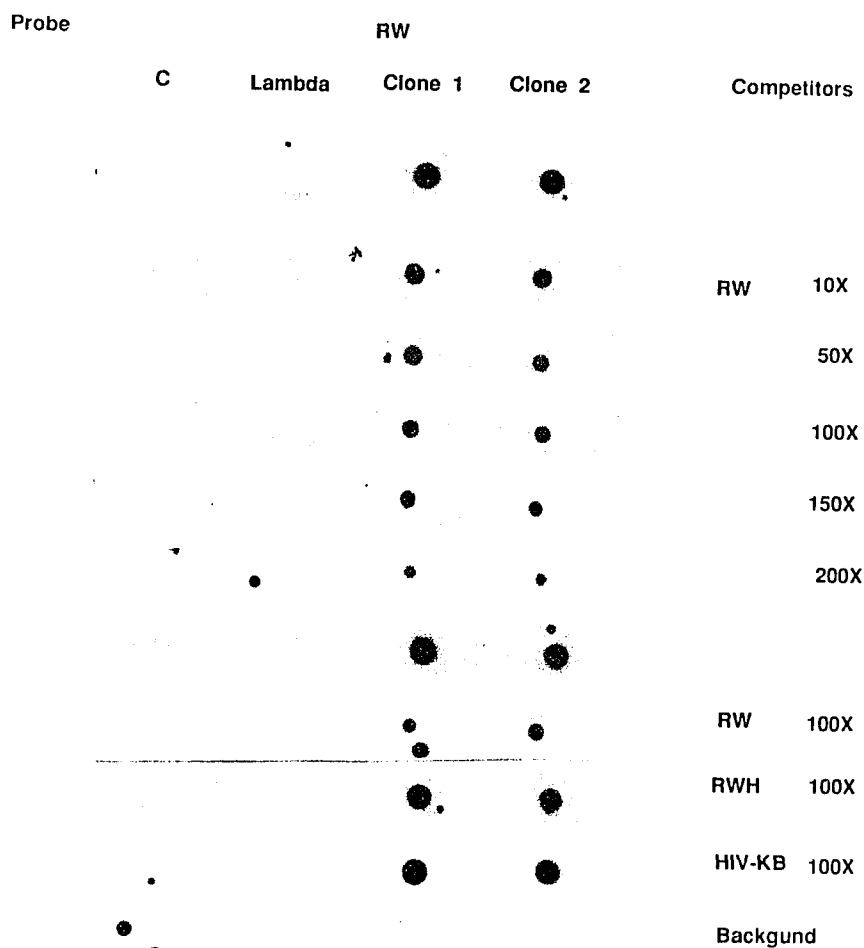


Fig. 19 Different specific complexes are seen interacting with the CCAAT-box and the RW oligonucleotides in lysogenic bacterial crude extracts.

The probe for lanes 1 to 6 is the CCAAT-box 2 oligonucleotide; for lanes 7 to 12, RW is the probe. Lanes 1 and 7 illustrate binding seen with extracts from lysogenic bacteria for lambda; all the other lanes show binding using cells lysogenic for clone 2. Competition assays were performed using the competitors at a 50 fold molar excess. The sequence of the oligonucleotides used as competitors and probes are:

CCAAT-box 1:	gatcc	TGAAGCCAATATGA
CCAAT-box 2:	gatcc	GATCGCCAGCCAATGA
RW:	gatc	CACCTACATCCATTC
RW6B:	gatc	CACC <u>AGG</u> ATCCATTC
RWH:	agctt	ACCTACATCCATTC

Probes:	CCAAT-2				RW							
Competitors:	-	-	CCAAT	RW	RWH	RW6B	-	-	CCAAT	RW	RWH	RW6B
	1	2	3	4	5	6	7	8	9	10	11	12

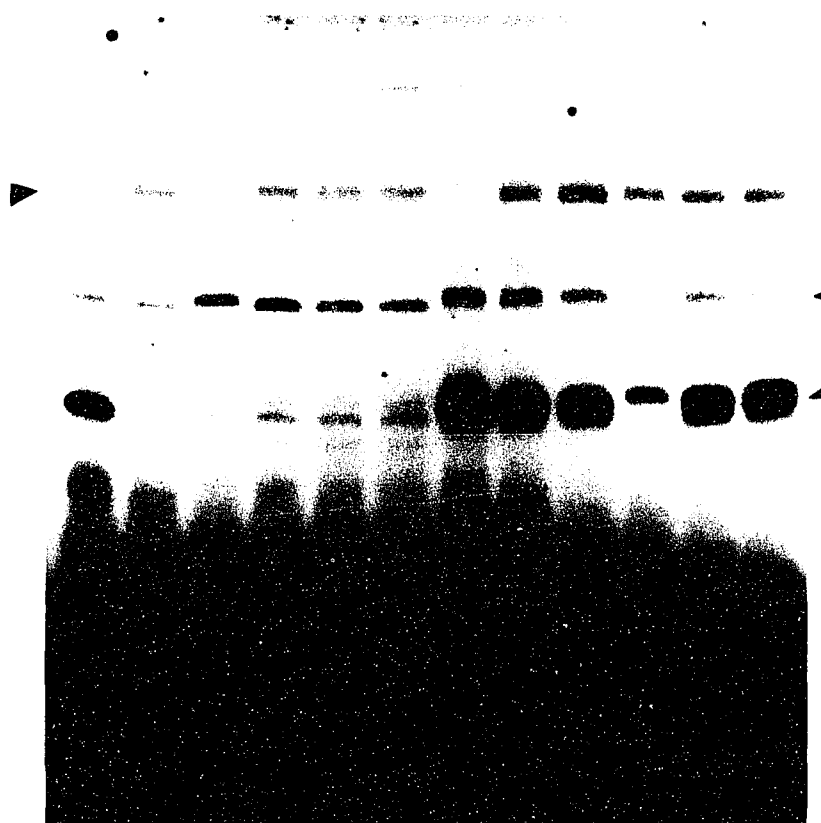


Fig. 19

General Discussion

Interleukin-2 is regulated at the level of transcription. Several factors important in the regulation of this gene have been identified and carefully analyzed. Although Interleukin-2 is T-cell specific, the only transcription factor able to exert major regulatory function on IL-2 gene expression in a T-cell specific manner is NF-AT.

We sought to identify and characterize IL-2 T-cell specific transcription factors(s). This search led to the identification of the element -206 to -195 which shares 7 out of 10 base similarity with the NF-kB motif which at the time of this study was thought to be lymphoid specific (Sen and Baltimore, 1986). Analysis of this sequence by site-directed mutagenesis demonstrated that sequence to be important in IL2-gene regulation. This is in contrast to deletion analysis done by another laboratory (Durand et. al. 1988) which showed that this sequence could be deleted without affecting promoter function. An explanation for this observation is that in the presence of a consensus sequence there is an interaction between the transacting factor and the cis-element that may exert a regulatory function through modulation, enhancing, or restriction of the expression of the gene. In the absence of this regulatory element the gene is expressed in a lax manner and as the result of complementation of the other elements on the promoter. A detailed analysis of this deletion was not undertaken by the investigators; the main objective of this analysis had been to identify regions which abrogated promoter

activity.

Having demonstrated the importance of the consensus element in terms of IL-2 gene function, the identification of the transacting nuclear factor was carried out. Gel retardation assays were used to show through competition experiments that the protein interacting with the putative NF-kB motif could also interact with the IL2-NFkB-like element. This assay also demonstrated that the protein interacting with the IL-2 sequence behaved identically to NF-kB transcription factor under conditions that are characteristic for NF-kB (chapter II). Additional evidence to ascertain the identity of the factor as NF-kB came from UV-crosslinking studies which showed polypeptides of similar molecular sizes to those characteristic for NF-kB. Although there is no definite proof that NF-kB is the nuclear protein regulating IL-2 gene expression **in-vivo**, (an NF-kB-like factor may be regulating IL-2), all the indirect evidence presented by this investigation suggests that NF-kB does have a regulatory role on IL-2.

NF-kB is not T-cell specific. Further studies on this protein prompted Baltimore to describe it as a "promiscuous complex". It regulates several genes in various cell types (Leonardo and Baltimore, 1989). Because NF-kB displays a lower affinity for the IL2-kB-like element than for the kappa light chain enhancer or the HIV-LTR ,the contribution of the flanking sequences was analyzed. This study shows importance only of the nucleotides at the 3' end. Interestingly, the 10 to 12 bases

flanking the 3' end of the IL2-kB-like element were found to interact with a nuclear protein distinct from NF-kB and this complex was termed RW-BF for right ward binding factor.

RW-BF is novel in that it interacts with a sequence not previously identified as a binding motif; furthermore, based on the criteria for differentiating it from NF-kB, it is a novel factor regulating IL-2 gene expression. More important, it is mitogen-inducible and abundant in T-cells in contrast to B-cells such as Raji, or Hela (an epithelioid cell line). In Raji and Hela cells the binding of the complex is induced but detected at a 1/20 fold of that seen with T-cell.

The identification of a possible T-cell specific factor within this region (-212 to -187) of the IL-2 promoter was not surprising. Serfling and colleagues, working with the murine IL-2 promoter element, identified a T-cell specific factor binding the corresponding sequence on the mouse IL-2 promoter. Comparison of the sequence of the murine IL-2 and the human IL-2 within -212 to -188 shows three nucleotide differences at -212, -209, and -192. What is interesting about the murine study is that the binding sequence behaves as an enhancer element which is lymphoid specific and most active in T-cells. The same element in the human IL-2 behaves similarly to the murine counter element. However, in contrast to the murine sequence which interacts with one T-cell specific factor, the human element is recognized by two different nuclear proteins, namely NF-kB and RW-

BF. We propose that these two factors interact, and the product of this interaction is able to bind and activate the -212 to -187 element in a T-cell specific manner.

Evidence for this interaction was to be pursued in two different ways: differential footprinting using a series of mutated and wild-type IL2-CAT constructs; as well as **in-vitro** transcription of these constructs. For the **in-vitro** transcription assays nuclear extracts from uninduced T-cells was to be supplemented with purified NF-kB, RW-BF, and a combination of these factors. NF-kB has been purified (Kawakami et. al. 1988) and the subunits cloned (Ghosh et. al., 1990; Kieran et. al., 1990; Nolan et. al., 1991; Ruben et. al., 1991). RW-BF, on the other hand, needed to be either purified or cloned.

The purification of RW-BF was undertaken using Jurkat T-cells crude nuclear extracts and several chromatographic columns. Unfortunately, the results indicated that under the conditions used, RW-BF could only be semipurified using DNA-affinity column. Furthermore, the protein, after the manipulations, was labile and lost binding activity within a few hours. Thus, the purification was pursued no further.

The cloning of the protein was the next alternative. To accomplish this a HUT78 cDNA expression library in lambda gt11 was screened using a radiolabeled monomeric RW sequence. Two identical clones were identified and the nucleotide

sequence analysis showed the clones to have 99.5% identity with the transcription factor DNA binding protein B or Dbpb (Sakura et. al. 1988). This protein is 95% identical to YB-1, and EF1A both CCAAT-box binding proteins (Didier et. al., 1988; Ozer et. al., 1990). The protein expressed the clones binds specifically to the RW sequence, although there is no true CCAAT-box element within this RW element. The protein does however bind specifically to two different CCAAT-box oligonucleotides. Interestingly, in competition assays, there is no true cross competition among these oligonucleotides in that binding is competed best by self peptides. Additionally, the bands that are competed specifically by CCAAT-box oligonucleotides and RW are of different mobilities. These observations could be explained by different conformational or oligomerization states of the protein. The Dbpb and the CCAAT-box binding proteins do not have any of the previously described DNA-binding domains, rather the proteins contains a series of regions of positive and negative charges (Tafui et. al. 1990). It is likely that different domains of the protein interact with the various motifs which may result in conformational changes affecting its mobility. Interaction with the different sequences may depend on the oligomeric state of the protein which would be reflected by differences in mobility.

At present, definite proof for Dbpb as a true component of the RW-BF complex is pending. An anti-peptide antibody to Dbpb should be able to establish the relationship between Dbpb and RW-BF. Interaction of the antibody with RW-BF

and the consequent change in its mobility in gel retardation assays or through its depletion of RW-BF from nuclear extract could prove that Dbpb is part of the RW-BF complex.

In conclusion, the contribution of this thesis work to the general understanding of IL-2 transcriptional regulation has been the identification of NF-kB and RW-BF as transcription factors having a functional role in the regulation of IL-2 gene expression. Of greater importance, but one for which not much evidence was obtained, is the hypothetical interaction between NF-kB and RW-BF to establish a T-cell specific complex that is then able to contribute to the regulation of IL-2 in a T-cell specific manner. Whether this interaction results in the complex that is detected in the murine system remains to be determined.

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