A positive regulatory element in the interleukin-2 (IL-2) promoter, designated the antigen receptor response element-2, is essential for the induction of IL-2 gene expression upon the binding of an inducible multiprotein complex of proteins known as nuclear factor of activated T cells. In the current study, we demonstrated that the winged-helix transcription factor IL-2 enhancer binding factor (ILF) is constitutively expressed in both resting and activated Jurkat cells and binds to two adjacent sequence motifs immediately downstream of the binding site for NFAT. One of these elements has a high degree of homology with consensus binding sites for a variety of winged-helix DNA binding proteins, and the second site functions to modulate ILF binding. Mutagenesis of each of the two sequence elements required for ILF binding decreased IL-2 promoter activity when assayed in transfection assays. Although ILF bound constitutively to the IL-2 promoter, it was not detected as a component of the NFAT complex. These results suggest that important regulatory sequences in the IL-2 promoter are bound by ILF and that this binding may be involved in the control of IL-2 gene expression.

Induction of IL-2 expression is a pivotal event in early T-lymphocyte activation. Binding of the T cell receptor complex to its cognate antigen results in protein kinase C activation and elevated intracellular calcium levels with subsequent activation of IL-2 mRNA synthesis (1). Studies of the IL-2 promoter (2, 3) have revealed a positive regulatory element situated between −288 and −255 relative to the transcription start site that is essential for increased IL-2 expression upon the activation of T-lymphocytes (2). This purine-rich site, designated the antigen receptor response element 2 (ARRE-2), is bound by an inducible multiprotein complex known as the nuclear factor of activated cells (NFAT) following the activation of T-lymphocytes (4, 5).

T cell-specific activation of the IL-2 gene expression via the ARRE-2 site is a complex phenomenon regulated by a variety of proteins. Several members of the AP-1 and NFAT families of transcription factors participate in NFAT complex formation (6–10). NFATp and NFATc represent the first identified members of a family of transcription factors closely related to Rel proteins (9–14). Integration of these proteins into the NFAT complex is achieved by two diverse mechanisms. NFATp is present in the cytoplasm in resting T cells and translocates to the nucleus upon immunologic activation in a manner reminiscent of the transcription factor NF-κB (11). In contrast, NFATc is newly synthesized upon T cell activation (10). The two pathways are intertwined because both activation events are blocked by the immunosuppressive agent cyclosporin A (10, 11). Identification of a functional AP-1 site directly adjacent to the binding site for the NFAT proteins demonstrated additional transcription factors are able to regulate the activity of the NFAT enhancer element (7). Jun, JunB, Fos, and Fra-1 have all been demonstrated to be present in NFAT complexes isolated from activated T cells (6–8). The involvement of multiple NFAT and AP-1 family members in the NFAT complex suggests that this complex may in fact represent a closely related collection of multi-protein transcriptional activators.

Constitutively expressed transcription factors have also been proposed to play a role in NFAT complex formation (15–19). Two such constitutive factors NF90 and NF45 have been purified and cloned (20, 21) and found to bind to the same purine sequences as the NFAT proteins. While these factors appear to represent novel genes, sequence analysis of NF45 reveals homology to the prokaryotic σ-54 transcription factor. Other proteins including E1f-1, a member of the Ets family of transcription factors (22), and ILF, a member of the winged helix family of DNA binding proteins (23, 24), have also been demonstrated to bind to the IL-2 ARRE-2 site. Winged-helix or forkhead proteins share a conserved 100-amino acid DNA binding domain and represent a family of transcription factors that participate in a number of processes that regulate cellular gene expression (25–30). Constitutively expressed factors such as ILF that bind to the ARRE-2 region may help to modulate the rapidity and tissue specificity of IL-2 mRNA induction.

In this study, we present evidence that ILF binds constitutively to the ARRE-2 element in the IL-2 promoter. The optimal in vitro binding site for ILF closely resembles the non-consensus AP-1 site in ARRE-2 indicating that sequences recognized by ILF and the multi-protein NFAT complex overlap. The 3′ portion of the ILF binding site comprised of purine-rich sequences is not required for NFAT binding but is important for maximal IL-2 promoter activity. These results suggest that ILF may have a positive role in regulating IL-2 gene expression, although ILF is not a component of the multi-protein NFAT complex. Thus, ILF may be important both to prevent constitutive expression from the IL-2 promoter and to maintain the
IL-2 promoter in a configuration that allows NFAT and AP-1 to activate IL-2 gene expression.

MATERIALS AND METHODS

PCR Analysis of ILF mRNA Levels—Jurkat cells were maintained in RPMI media with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and 2 mM glutamine. T cells were activated by stimulation with 25 ng/ml phorbol myristate acetate (PMA) and 2 µM ionomycin for 30–120 min. When used, cyclosporin A was added to a concentration of 100 ng/ml. Total RNA was prepared from cells with the RNAZol reagent (Biotex Laboratories) using the manufacturer’s protocol. 10 µg of RNA was used as a template for cDNA synthesis primed by 1 µg of random hexamer oligonucleotides (Pharmacia Biotech Inc.) and catalyzed by avian myeloblastosis virus reverse transcriptase (Promega) in the presence of 4 mM dNTPs. The 4 µl of the cDNA synthesis reaction was used as a template in a 100-µl PCR reaction containing 50 mM KCl, 10 mM Tris-HCl, pH 8.9, 0.1% Triton X-100, 1.5 mM MgCl2, 0.8 mM dNTPs, and 50 pmol of each specific primer. Primers specific for ILF, IL-2, TATA-box binding protein (TBP), and NFATc human cDNAs contained the following sequences and nucleotide numbers. ILF sense (917–941): 5'-GGAGGATCCTGCAAGGACCGCGG-3'; ILF antisense (2206–2183): 5'-GGCA-CCAGAGTATGATGGT-3'; IL-2 sense (552–533): 5'-GGGATATCAATACGATCC-3'; IL-2 antisense (423–400): 5'-TTGGTCCA-TGCTGTGATG-3'; TBP sense (565–541): 5'-AGGCAGAGGACTATACAGGACCC-3'; TBP antisense (239–268): 5'-ATGCCACCAAGCAGCTCTTTCA-GTCCT-3'; NFATc sense (613–591): 5'-GGTCTGGGAGACAGCCA-GGGAGACT-3'; NFATc antisense (563–593): 5'-GATCGCTGTACAAATCTGTGTC-3'.

Predicted PCR product sizes using these primers are 240 bp (IL-2), 334 bp (TBP), and 600 bp (NFATc). ILF primers amplify PCR products of different sizes, for each transcript (ILF-1, 1289 bp; ILF-2, 1711 bp; ILF-3, 411 bp). PCR reactions were generally denatured for 5 min at 95 °C followed by 25–40 cycles of 55 °C annealing, 72 °C extension, and 95 °C denaturation (1 min each step). The 20 µl of each PCR reaction was analyzed by agarose gel electrophoresis followed by ethidium bromide staining. PCR reactions were allowed to progress for the lowest cycle number of 25 cycles at which ethidium bromide-stained PCR product could be visualized by UV light.

Preparation of ILF and Other Proteins—ILF was expressed in bacteria as a fusion protein with glutathione S-transferase (23). GST-ILF constructs contained either 145 amino acids including the ILF (amino acids 210–357) fused to GST which included the forhead domain (amino acids 1–251) or 445 amino acids (amino acids 210–655) (23). The fusion proteins were expressed in E. coli in the absence of [35S]methionine. The fusion proteins were purified by glutathione-agarose chromatography (31). Factor Xa (Promega) was used to cleave the GST moiety and inactivated using 1 mM Pefabloc (Boehringer Mannheim).

NFATc cDNAs used for CASTing experiments (32, 33) were transcribed and translated in vitro using the TNT T7 polymerase system (Promega). In vitro translation of CREB, Jun, or NFATc proteins into pGEM-3 or -4 was also performed in a coupled polymerase system (Promega). ILF, NFATc, CREB, Jun, and NFATc proteins containing a hemmagullatin epitope (amino acid sequence YPYDVPDYA) were translated using the T7 polymerase system (Promega). Proteins were allowed to bind double-stranded 75-bp fragments containing a degenerate 35-bp core for 20 min at room temperature. Beads coated with an anti-hemagglutinin monoclonal antibody were used to isolate proteins and bound DNA fragments. PCR primers 5'-GGGATCCGGAAGCCATCGGCTGTTTC-3' and 5'-purine mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCTGTTTC-3'; core mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'; 3' purine mutant, 3'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'; double core/purine mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'.

Immunoblotting of Gel-shifted Complexes from Electrophoretic Mobility Shift Assays—Gel retardation analysis using Jurkat nuclear extract was performed as described above. Reactions were scaled up to a 100-µl size. Gels were exposed to x-ray film for 2 h and the shifted complexes subsequently purified. Gel slices containing either NFAT complex or ILF were placed in the wells of an SDS-polyacrylamide gel with 50 µl of 5 × protein loading dye layered on top. Gels were subjected to electrophoresis and proteins transferred to nitrocellulose with immunoblotting performed as described previously.

Cyclic Amplification and Selection of Targets (CASTING)—Determination of the optimal binding sites of DNA binding proteins using degenerate oligonucleotides using the so-called CASTING procedure was performed as described previously (32). ILF and NFATc proteins containing a hemmagullatin epitope (amino acid sequence YPYDVPDYA) were expressed in bacteria (E. coli) and subjected to electrophoresis and proteins transferred to nitrocellulose with immunoblotting performed as described previously.

Determination of IL-2 Promoter Activity by Transient Transfection—The IL-2 promoter from −340 to +47 was cloned upstream of the phenylalaninocaproic acid transfection (CAT) gene (2, 3). Mutant IL-2 promoter plasmids were constructed using the Sculptor in vitro mutagenesis system (Amersham Corp.) according to the manufacturer’s protocol. Mutant IL-2 promoters extending from (296 to 257 had the following underlined alterations in the ARRE-2: wild type, 5'-GGAGG-GAAAACCTGTTCATACAGAGGCTGTTTC-3'; AP-1/purine mutant, 5'-GGAGG-GAAAACCTGTTCATACAGAGGCTGTTTC-3'; AP-1/core mutant, 5'-GGAGG-GAAAACCTGTTCATACAGAGGCTGTTTC-3'; and 3' purine mutant, 5'-GGAGG-GAAAACCTGTTCATACAGAGGCTGTTTC-3'. Use of poly(dI-dC) proved optimal for generating NFAT binding activity, whereas the presence of poly(dA-dT) enhanced ILF binding activity. Binding reactions were performed at room temperature and were loaded and run on 4.5% polyacrylamide gels (0.5 × TBE). Antibodies used in electrophoretic mobility shift assays were purified using protein A-Sepharose chromatography, with 2 ml of rabbit serum used to load a 1-ml-protein A column, washed with 20 ml of 1.5 M glycine, 3 mM NaCl, pH 8.9, eluted with 0.1 M citric acid, pH 3.0, into 50 µl of 1 M Tris, pH 8.9, and dialyzed into phosphate-buffered saline.

Oligonucleotide sequences (sense strand) for IL-2 ARRE-2 oligonucleotides with (mutated sequences underlined) are as follows. Wild type −5'-GGAGG-GAAAACCTGTTCATACAGAGGCTGTTTC-3'; AP-1/purine mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCTGTTTC-3'; core mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'; 3' purine mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'; double core/purine mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'.

The blot at a 1:5000 dilution. All incubations were performed at room temperature. Immunoreactive proteins were visualized using enhanced chemiluminescence detection reagents (Amersham Corp.). Blots were generally exposed to x-ray film for 10–60 s.

6. Retardation Analysis—Oligonucleotides corresponding to the IL-2 ARRE-2 (3) were annealed and radiolabeled with [32P]ATP and polynucleotide kinase. Conditions for binding of bacterially expressed ILP to DNA were as follows: 50 mM NaCl, 10 mM Tris, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 5 mM MgCl2, 1 µg of poly(dG-dC), 5 µg of GST-ILF, 0.1–0.5 ng of radiolabeled oligonucleotides (20,000 cpm) in a 20-µl reaction. Binding conditions and protein analyses using gel retardation with nuclear extracts were identical using 1 µg of poly(dI-dC) or 1 µg of poly(dA-dT) used instead of poly(dG-dC). Use of poly(dI-dC) proved optimal for generating NFAT binding activity, whereas the presence of poly(dA-dT) enhanced ILF binding activity. Binding reactions were performed at room temperature and were loaded and run on 4.5% polyacrylamide gels (0.5 × TBE). Antibodies used in electrophoretic mobility shift assays were purified using protein A-Sepharose chromatography, with 2 ml of rabbit serum used to load a 1-ml-protein A column, washed with 20 ml of 1.5 M glycine, 3 mM NaCl, pH 8.9, eluted with 0.1 M citric acid, pH 3.0, into 50 µl of 1 M Tris, pH 8.9, and dialyzed into phosphate-buffered saline.

Oligonucleotide sequences (sense strand) for IL-2 ARRE-2 oligonucleotides with (mutated sequences underlined) are as follows. Wild type −5'-GGAGG-GAAAACCTGTTCATACAGAGGCTGTTTC-3'; AP-1/purine mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCTGTTTC-3'; core mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'; 3' purine mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'; double core/purine mutant, 5'-GATGGGGAATCATCAGAGAGAGCATCACAGGCGTCTTCGCTGTTTC-3'.
Previously we have demonstrated that the transcription factor ILF when produced as a fusion with glutathione S-transferase was capable of binding to the IL-2 ARRE site (23). To further characterize the constitutive binding activity, nuclear extract prepared from nonactivated Jurkat cells was incubated with the radiolabeled wild-type IL-2 ARRE-2 probe (Fig. 2B). In addition to the predominant constitutive binding activity, a nonspecific faster mobility gel-retarded complex that was present in some Jurkat nuclear extract preparations was also detected (Fig. 2B). This latter complex bound to the wild-type IL-2 ARRE-2 probe in addition to mutant probes in the 5′, core, or 3′ purine regions (data not shown). Incubation of Jurkat nuclear extract with affinity purified rabbit polyclonal antiserum to ILF resulted in a supershift of the majority of the slower mobility binding activity with no change in the amount of the nonspecific binding activity (Fig. 2B, lane 2). A similar supershifted band was not seen when preimmune antiserum was incubated with the extract (Fig. 2B, lane 3) or when ILF antiserum was incubated with the probe alone (Fig. 2B, lane 4).

Mutations in the 5′ purine region should preclude the binding of several ARRE-2 binding proteins present in resting T cells including NFATp (9), in addition to the NF45 and NF90 proteins (20, 21). ILF antiserum also supershifted the majority of the slower mobility complex bound to an IL-2 ARRE-2 probe mutated in the 5′ purine region when used in gel retardation analysis with nuclear extract prepared from nonactivated Jurkat cells (Fig. 2C, lane 2). Again the ILF antibody did not alter the intensity of the nonspecific binding activity (Fig. 2C, lane 2). Therefore, the constitutive binding activity that is reactive with ILF antiserum is distinct from previously described proteins present in nonactivated T-lymphocytes.

Recombinant and Endogenous ILF Exhibit Similar Binding Properties to the IL-2 Promoter—Next it was important to determine whether bacterially produced ILF had the same binding specificity for the IL-2 ARRE-2 as the endogenous cellular protein. Gel retardation analysis with IL-2 probes containing mutations in the 5′ purine, core, and 3′ purine regions in the IL-2 ARRE-2 were assayed using recombinant ILF (Fig. 3A, lanes 1–4). A probe containing a mutation of the 5′ purine (Fig. 3A, lane 6) did not alter ILF binding as compared with the wild-type probe (Fig. 3A, lane 5), whereas probes containing mutations of either the core sequences (Fig. 3A, lane 7) or the 3′ purine sequences (Fig. 3A, lane 8) markedly decreased ILF binding. The amounts of wild-type and mutant IL-2 probes that were bound by ILF were quantitated using PhosphorImage scanning in three separate experiments, and the results are expressed graphically (Fig. 3B). Although mutation of the 5′ purine region had only slight effects on ILF binding to the IL-2 probe with a reduction to 84% of wild type, mutation of the core and 3′ purine regions resulted in marked diminution of ILF binding giving values of 17 and 23% of the wild type levels, respectively (Fig. 3B). A probe containing mutations in both the core and 3′ purine regions reduced ILF binding to 2.9% of wild type (data not shown). These results indicate that both endogenous and recombinant ILF protein require the core and 3′ purine element for its binding.

ILF and NFATc Have Optimal DNA Binding Sites Closely Resembling Their Target Sequences in the IL-2 ARRE-2—It was important to determine whether the sequences in the IL-2 promoter were bound by ILF were similar to the optimal ILF binding sequences. For that reason, we used degenerate oligonucleotides to select for sequences optimally bound by ILF (32, 33). As a control, we also used degenerate oligonucleotides to determine the optimal binding site for NFATc. These proteins that contained both 3′ influenza hemagglutinin sequences were in vitro translated in rabbit reticulocyte lysate, bound to de-
generate oligonucleotides, and immunoprecipitated with 12CA5 antibody that can recognize the hemagglutinin-tagged proteins. This was followed by repeated cycles of PCR and protein binding prior to the isolation of these DNAs and the DNA sequence analysis. Using this analysis, the consensus ILF binding site determined from the alignment of 19 independent sequences was TGTTTAC. As depicted in Fig. 4, each of the first five nucleotides (TGTTT) derived by site selection was conserved in 79–89% of the aligned sequences. This sequence was present in the core region of the IL-2 ARRE-2 and mutations of the sequence reduced ILF binding greater than 5-fold. The ILF consensus sequence was also highly related to binding sites seen in promoters bound by other winged-helix proteins (30). The consensus NFATc sequence determined by site selection was an octamer with the sequence TGGAAAAT (Fig. 4). This consensus sequence was even more highly conserved than that obtained for ILF with the GGAAA motif present in 100% of sequences.

The most striking aspect of the NFATc sequence determined in this analysis was its strong homology to the IL-2 NFAT binding site. Thus the use of site selection defined optimal ILF and NFAT binding sites and demonstrated that these sequences were present in the IL-2 ARRE-2 region.

Gel retardation analysis using both Jurkat nuclear extract (Fig. 2) and recombinant ILF (Fig. 3) indicated that the core element in the IL-2 ARRE-2 was critical for ILF binding and the 3’ purine sequence also influenced the degree of ILF binding. Next, the role of the 3’ purine sequence on regulating ILF binding was analyzed when the core sequence was mutated to the optimal ILF binding sequence as determined by CASTING. Gel retardation was performed with recombinant ILF protein using oligonucleotides containing either the wild-type ARRE-2, this oligonucleotide with a mutation of the 3’ purine sequences, the ARRE-2 sequence with the ILF CASTING consensus in the core region (lane 4), or probe alone (lane 5).
Purine Regions Are Essential for IL-2 Gene Expression—To assess the role of the ILF binding site on IL-2 gene expression, we performed site-specific mutagenesis on the elements in the ARRE-2 that were required for ILF and NFAT binding. The sequence of this region and the base pair changes introduced are indicated (Fig. 6A). These individual mutations were each inserted into an IL-2 promoter construct fused to CAT, and the gene expression of these constructs was analyzed following transfection into PMA and ionomycin-treated Jurkat cells. Mutation of either the 5′ purine or the core region sequences reduced IL-2 promoter activity dramatically to 9.1 and 17.4% of wild type levels, respectively (Fig. 6C, lanes 2 and 5). These results in conjunction with those of Figs. 2 and 3 indicate that the core sequence is critical for ILF binding and that the 3′ purine sequences modulate this binding.

The Core and 3′ Purine Regions Are Essential for IL-2 Gene Expression—To assess the role of the ILF binding site on IL-2 gene expression, we performed site-specific mutagenesis on the elements in the ARRE-2 that were required for ILF and NFAT binding. The sequence of this region and the base pair changes introduced are indicated (Fig. 6A). These individual mutations were each inserted into an IL-2 promoter construct fused to CAT, and the gene expression of these constructs was analyzed following transfection into PMA and ionomycin-treated Jurkat cells. Mutation of either the 5′ purine or the core region sequences reduced IL-2 promoter activity dramatically to 9.1 and 17.4% of wild type levels, respectively (Fig. 6B). The deleterious effect of the core site mutation on IL-2 promoter activity could be explained by changes in the AP-1 binding site resulting in the lack of formation of a multimeric NFAT complex (6–10). Due to the overlapping AP-1 site, it is not possible to assess the importance of ILF binding to the core region by mutagenesis. The role of ILF binding on IL-2 gene expression could best be tested by analyzing mutations in the 3′ purine region since this region does not alter the binding of the NFAT complex comprised of both NFAT and AP-1 factors. Mutation of the 3′ purine site decreased IL-2 promoter expression to 10.5% of wild type (Fig. 6B) indicating that this conserved element that is required for ILF binding is essential for maximal IL-2 gene expression. Mutations of the IL-2 promoter upstream of the ILF binding approximately 3-fold (Fig. 5, lane 4). These results in conjunction with those of Figs. 2 and 3 indicate that the core sequence is critical for ILF binding and that the 3′ purine sequences modulate this binding.

NFAT binding site extending between −344 and −300 did not alter IL-2 gene expression (data not shown). Finally, we determined whether transfection of an expression vector containing ILF into Jurkat cells was able to activate IL-2 CAT gene expression. Transfection of increasing amounts of the ILF expression vector resulted in activation of IL-2 gene expression (Fig. 6C, lanes 4–6) as compared with increasing amounts of the expression vector alone (Fig. 6C, lanes 1–3). The level of activation by ILF ranged from 3- to 6-fold in three independent experiments and was dependent on the ARRE-2 site (data not shown). These data suggest that ILF plays a positive role on activating IL-2 gene expression.

Analysis of ILF mRNA Levels in Jurkat T Cells—Three composite ILF cDNAs corresponding to alternatively spliced transcripts have been assembled based on PCR analysis of Jurkat
ILF is Not a Component of the NFAT Complex—Finally, we wished to determine whether ILF was a component of the NFAT complex. This would distinguish between two alternative models for ILF action. ILF could bind to the IL-2 ARRE-2 in the absence of the NFAT complex and precede NFAT binding or alternatively ILF could be the first protein binding to the ARRE-2 followed by the formation of a multi-protein NFAT complex containing ILF as a member. To determine if ILF was a component of the NFAT complex, we assayed the immunoreactivity of the NFAT complex with ILF antisera in gel retardation experiments (Fig. 9A). Nuclear extract was prepared from both nonactivated and PMA and ionomycin-treated Jurkat cells and used in gel retardation analysis with the IL-2 ARRE-2 probe. In contrast to an earlier extraction procedure used in this study, this extract was prepared according to a protocol to optimize the extraction of NFAT proteins (38) and resulted in a variety of proteins that bound nonspecifically to the ARRE-2 probe. ILF formed a major gel-retarded species that bound to the ARRE-2 probe with Jurkat nuclear extract prepared from nonactivated cells (Fig. 9A, lane 1). The NFAT complex was the predominant species in nuclear extract prepared from PMA and ionomycin-treated Jurkat cells (Fig. 9A, lane 4). The addition of ILF antibody supershifted the constitutive ILF gel-retarded complex (Fig. 9A, lane 2) but did not alter the mobility of the NFAT complex (Fig. 9A, lane 5). In contrast, the addition of Jun antibody inhibited the binding of the NFAT complex (Fig. 9A, lane 6). The addition of preimmune rabbit sera did not alter the mobility of either the ILF or NFAT complexes (Fig. 9A, lanes 3 and 7). These results indicate that ILF antibody did not alter the mobility of the NFAT complex bound to the IL-2 promoter.

An additional approach was also used to analyze whether ILF was a component of the NFAT complex (Fig. 9B). Gel retardation analysis was performed using the IL-2 ARRE-2 probe with nuclear extract prepared from PMA and ionomycin-treated Jurkat cells, and the NFAT complex was isolated from the polyacrylamide gel following electrophoresis and autoradiography. Gel slices containing the NFAT activity were then subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with either ILF, NFAT, TBP or Jun antibodies (Fig. 9B). In addition, nuclear extract prepared from nonactivated Jurkat cells was used in gel retardation analysis with the IL-2 ARRE-2 probe to isolate the ILF gel-retarded species. This complex was immunoblotted with either ILF or TBP antisera as a control for the sensitivity of the Western blot analysis. The TATA-box binding protein (TBP) antibody was used to rule out nonspecific association of other nuclear pro-
teins with the NFAT and ILF gel-retarded complexes. The same quantity of Jurkat nuclear extract used in the gel retardation assays was also analyzed by Western blot analysis and was designated as the input fraction (Fig. 9B). Using this procedure, we could analyze by immunologic means for the presence of ILF in the NFAT complex. Immunoblotting of the NFAT complex revealed the presence of NFATc (Fig. 9B, panel 1) and Jun (Fig. 9B, panel 2) but not ILF (Fig. 9B, panel 3) or TBP (Fig. 9B, panel 4). In contrast, the ILF antibody detected ILF in a gel-retarded complex using nonactivated Jurkat extracts (Fig. 9B, panel 3), and this complex was not reactive with TBP antibody (Fig. 9B, panel 4). These results indicate that ILF bound to the IL-2 ARRE-2 in nuclear extract prepared from nonactivated Jurkat cells but was not a component of the NFAT complex.

Finally, we determined whether the addition of recombinant ILF produced as a GST fusion and cleaved with factor X resulted in alterations of NFAT binding to the IL-2 ARRE-2 oligonucleotide. A partially truncated ILF protein was used in these studies to differentiate the bacterially produced ILF from endogenous ILF present in Jurkat extract. Gel retardation analysis with the recombinant ILF demonstrated that it bound to the IL-2 oligonucleotide and generated a species corresponding to intact ILF protein in addition to several degradation products (Fig. 9C, lane 2). Extracts prepared from Jurkat cells treated with PMA and ionomycin resulted in the generation of the inducible NFAT complex in gel retardation analysis (Fig. 9C, lane 3). The addition of increasing amounts of recombinant ILF to the gel retardation assays with extract prepared from activated Jurkat cells resulted in the loss of binding of the NFAT complex and appearance of a gel-retarded species indicative of recombinant ILF (Fig. 9C, lanes 4 and 5). In contrast there was no change in the appearance of NFAT complex following the addition of increasing amounts of GST protein (Fig. 9C, lanes 6 and 7). These results further demonstrate that ILF and NFAT were not able to form a complex on the IL-2 ARRE-2 site.

The addition of the Jun antibody to the gel retardation in Fig. 9A completely disrupted the binding of the NFAT complex and also slightly decreased ILF binding. To address whether ILF might interact directly with either Jun or NFATc, cDNAs encoding these proteins were in vitro translated in rabbit reticulocyte lysate in the presence of [35S]methionine (Fig. 9D, lanes 1–3). CREB was also in vitro translated for use as a negative control. In addition, ILF and Jun cDNAs that contained carboxyl-terminal influenza hemagglutinin sequences and could be recognized by the 12CA5 monoclonal antibody were also in vitro translated in rabbit reticulocyte lysate in the absence of [35S]methionine. The presence of these unlabeled proteins was confirmed by Western blot analysis (data not shown). The unlabeled Jun and ILF proteins were incubated with each of the [35S]methionine-labeled proteins followed by immunoprecipitation with 12CA5 antibody and autoradiography. This analysis indicated that ILF was not able to co-immu-
The regulation of IL-2 gene expression is dependent on multiple cis-acting sequences in its promoter element that bind both constitutive and inducible cellular factors (1–4, 6, 7, 9–11, 19, 20, 22, 39, 40). A critical region required for induction of IL-2 gene expression in response to T cell activation is an element located between −288 and −255 relative to the transcription start site known as ARRE-2 (2). This site is bound by an inducible protein complex known as NFAT that is present in activated T cell nuclear extract and is required for IL-2 mRNA synthesis (1, 4, 6, 8–11, 29). The NFAT complex is comprised of a group of proteins known as NFAT in addition to the Jun and Fos proteins (6–11). The NFAT proteins are comprised of four highly related members, and various spliced forms include NFATc, NFATp/NFAT1, NFAT3, and NFATx/NFAT4 (9–14) that have homology in their 300-amino acid Rel homology domain (41). In contrast to other Rel proteins that form homodimers on heterodimers on their target sequences, NFAT proteins bind as monomers to their recognition sequences (41). The stability of NFAT protein binding to the ARRE-2 site is dependent on a non-consensus AP-1 site with the sequence TGTTTCA immediately downstream of the NFAT binding site (6–9, 22). A variety of members of the Jun/Fos family have been demonstrated to be present in the NFAT protein complex including Jun, Fos, JunB, JunD, and Fra-1, and their binding to the non-consensus AP-1 site is essential for the activation of IL-2 gene expression (6–9, 22).

Activation of IL-2 gene expression is dependent on NFAT translocation from the cytoplasm to the nucleus in response to an increase in intracellular calcium and activation of the Jun family through a protein kinase C-dependent pathway (1, 4, 5, 10, 11). The translocation of the NFAT proteins occurs following dephosphorylation of these proteins by the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin (42). Although the regulation of IL-2 gene expression in acti-

antibody (lanes 1 and 4), in the presence of ILF polyclonal antibody (lanes 2 and 5), Jun antibody (lane 6), or preimmune rabbit sera (lanes 3 and 7). B, NFAT and ILF gel-retarded complexes were isolated following electrophoresis and autoradiography from both activated and nonactivated Jurkat nuclear extract. Each of the gel-retarded complexes was subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis with NFAT, Jun, ILF, or TBP antibody. The antibodies used are indicated at the top of each panel, and the Jurkat nuclear extract input used in the gel retardation analysis and the gel-retarded complexes (ILF or NFAT) used in the Western blot analysis are indicated at the bottom of each panel. The NFAT gel-retarded complex was analyzed with NFAT antibody (panel 1), Jun antibody (panel 2), ILF antibody (panel 3), or TBP antibody (panel 4). The ILF complex was analyzed with NFAT antibody (panel 1) or TBP antibody (panel 4). C, gel retardation with an IL-2 ARRE-2 probe was performed with either 3.0 μg of GST (lane 1) and ILF (lane 2) or nuclear extract (5.0 μg) prepared from PMA and ionomycin-treated Jurkat cells (lane 3). In addition, 1 or 3 μg of ILF (lanes 5 and 6) or GST (lanes 7 and 8) were added to the nuclear extract prior to gel electrophoresis. D, coupled in vitro transcription and translation was performed in rabbit reticulocyte lysate in the presence of [35S]methionine for complete cDNAs coding for Jun (lanes 1, 4, and 7), CREB (lanes 2, 4, and 6), and NFATc (lanes 3, 6, and 9). Both ILF (lanes 4 and 6) and Jun (lanes 7–9) cDNAs that contained carboxyl-terminal influenza hemagglutinin sequences were translated in rabbit reticulocyte lysate in the absence of [35S]methionine. Both the [35S]methionine-labeled and unlabeled proteins (10 μl) were incubated prior to immunoprecipitation with the 12CA5 monoclonal antibody that recognizes the influenza hemagglutinin sequences following SDS-polyacrylamide gel electrophoresis and autoradiography.

**FIG. 9.** ILF is not associated with the NFAT complex. A, gel retardation analysis was performed in nuclear extract prepared from nonactivated (lanes 1–3) or PMA and ionomycin-treated Jurkat cells (lanes 4–7) using a wild-type IL-2 ARRE-2 probe in the absence of nprecipitate with Jun or NFATc (Fig. 9D, lanes 4–6), whereas Jun was able to co-immunoprecipitate with Jun as these proteins can form homodimers (Fig. 9D, lane 8). These results support the fact that ILF cannot directly interact with components of the NFAT complex.

**DISCUSSION**

The regulation of IL-2 gene expression is dependent on multiple cis-acting sequences in its promoter element that bind both constitutive and inducible cellular factors (1–4, 6, 7, 9–11, 19, 20, 22, 39, 40). A critical region required for induction of IL-2 gene expression in response to T cell activation is an element located between −288 and −255 relative to the transcription start site known as ARRE-2 (2). This site is bound by an inducible protein complex known as NFAT that is present in activated T cell nuclear extract and is required for IL-2 mRNA synthesis (1, 4, 6, 8–11). The NFAT complex is comprised of a group of proteins known as NFAT in addition to the Jun and Fos proteins (6–11). The NFAT proteins are comprised of four highly related members, and various spliced forms include NFATc, NFATp/NFAT1, NFAT3, and NFATx/NFAT4 (9–14) that have homology in their 300-amino acid Rel homology domain (41). In contrast to other Rel proteins that form homodimers on heterodimers on their target sequences, NFAT proteins bind as monomers to their recognition sequences (41). The stability of NFAT protein binding to the ARRE-2 site is dependent on a non-consensus AP-1 site with the sequence TGTTTCA immediately downstream of the NFAT binding site (6–9, 22). A variety of members of the Jun/Fos family have been demonstrated to be present in the NFAT protein complex including Jun, Fos, JunB, JunD, and Fra-1, and their binding to the non-consensus AP-1 site is essential for the activation of IL-2 gene expression (6–9, 22).

Activation of IL-2 gene expression is dependent on NFAT translocation from the cytoplasm to the nucleus in response to an increase in intracellular calcium and activation of the Jun family through a protein kinase C-dependent pathway (1, 4, 5, 10, 11). The translocation of the NFAT proteins occurs following dephosphorylation of these proteins by the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin (42). Although the regulation of IL-2 gene expression in acti-
vated T-lymphocytes has been studied in detail, much less is known about the cellular factors that bind to the IL-2 promoter in resting T-lymphocytes. Two polypeptides designated NF45 and NF90 have similar DNA binding specificity to NFAT and bind to the ARRE-2 site in resting T-lymphocytes (20, 21). DNase I footprinting and gel retardation assays have demonstrated changes from constitutive to inducible factor binding to ARRE-2 in the IL-2 promoter upon T cell activation (15–18). These results suggest that constitutive factors that are likely different from NFAT and Jun/Fos bind to the IL-2 promoter and may be important for the subsequent induction of IL-2 gene expression.

To identify constitutive factors that are bound to the IL-2 ARRE-2 site, gel retardation analysis was performed using an IL-2 ARRE-2 probe in the presence of nuclear extract prepared from nonactivated Jurkat cells. This analysis indicated that ILF was able to bind specifically to the IL-2 promoter and that the binding specificity of ILF in Jurkat nuclear extract correlated with the binding properties seen with recombiant ILF. Site selection and mutagenesis were performed to define the sequences in the IL-2 promoter responsible for ILF binding. ILF binding is dependent on sequences that correspond to a degenerate AP-1 binding site in the IL-2 promoter and that have a high degree of homology with a consensus binding site for other members of the winged-helix family of DNA binding proteins. For example, the sequences in the IL-2 promoter TGTTTCA that are critical for ILF binding are similar to the optimal binding site sequences TGTTTAC for ILF and other winged-helix family members (30). The two 3′ nucleotides in this consensus binding sequence vary considerably between different winged-helix binding proteins. In addition, 3′ purine-rich sequences flanking the ILF binding site in the IL-2 promoter are also critical for ILF binding.

Mutations in either the core or 3′ purine regions of the IL-2 ARRE-2 site that are required for ILF binding reduce IL-2 gene expression to approximately 10% of wild-type levels. Mutations in the core region have previously been shown to decrease IL-2 gene expression by disrupting Jun and Fos assembly into the NFAT complex (6, 7). However, other DNA binding proteins have not been demonstrated to require the 3′ purine region for binding to the IL-2 promoter, and the detrimental effects of mutations of this region on IL-2 gene expression are likely due to decreased ILF binding to the promoter. This suggests that ILF may be a positively acting transcription factor involved in regulating IL-2 gene expression. However, our inability to demonstrate that ILF was a component of the NFAT complex suggests that its mechanism of action was not likely due to association with either NFAT or Jun or Fos. The predominant ARRE-2 binding activity detected in gel retardation assays with nuclear extract prepared from resting T cells was immunoreactive with ILF antibody and had similar DNA binding specificity to bacterially produced ILF. Previous studies describing ARRE-2 binding activity in nuclear extract prepared from resting T cells have predominantly detected nonspecific DNA binding. We were able to detect specific ILF-immunoreactive activity using the binding conditions and nuclear extract preparations described in this study. In vivo footprinting of the IL-2 promoter indicates the presence of a footprint over the ARRE-2 in resting T cells (17). This footprint undergoes activation-specific changes likely corresponding to replacement of constitutively bound factors such as ILF by the NFAT multi-protein complex.

ILF mainly binds to the IL-2 promoter in resting T cells during a period of transcriptional silence. The temporal binding pattern of ILF would be consistent with a role on recruiting cellular factors to the IL-2 promoter that are required for rapid transcriptional activation by other factors upon T cell activation. For example, ILF binding in resting T cells may help to recruit basal transcription factors to the IL-2 promoter enhancing the eventual formation of the transcriptional initiation complex. ILF binding activity can also be detected in nuclear extract prepared from activated Jurkat extract. In order for ILF transcriptional activation to occur, the NFAT complex must bind to the IL-2 promoter thereby replacing ILF. ILF may have no functional role in activated T cells when NFAT catalyzes IL-2 transcriptional activation or the interchangeable binding of ILF and the NFAT complex may occur in activated T cells to regulate IL-2 transcriptional levels.

The mechanism by which ILF functions to modulate IL-2 gene expression remains unclear. A number of DNA binding proteins including members of the winged-helix family of transcription factors have been shown to induce topological changes in their target sequences including DNA bending (30). Bending of DNA can in theory influence transcription by altering DNA binding of factors or by inducing interaction between DNA-bound proteins. However, we were unable to detect ILF binding of IL-2 ARRE-2 DNA (data not shown). Winged-helix factors have also been demonstrated to regulate chromatin remodeling. One study reveals that mutation of the winged-helix protein HNF-3 site in the hepatocyte-specific serum albumin enhancer results in alterations in chromatin structure as well as inhibiting serum albumin expression (29). In addition, structural studies of the winged-helix domain of the transcription factor HNF-3y demonstrates similarity with histone-like domains (27). Thus, it is reasonable to hypothesize that ILF may alter the chromatin structure of the IL-2 gene in T cells to facilitate transcriptional activation by the NFAT proteins. Since examination of chromatin structure of the IL-2 promoter strongly suggests the presence of a DNase I-hypersensitive site immediately upstream of the IL-2 ARRE-2 region (15), it is possible that the binding of factors such as ILF may be responsible. Further studies will be required to determine the mechanism by which ILF regulates IL-2 gene expression.

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