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The Immediate-Early Gene Product Egr-1 Regulates the Human Interleukin-2 Receptor β-Chain Promoter through Noncanonical Egr and Sp1 Binding Sites

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The interleukin-2 IL-2 receptor β-chain (IL-2Rβ) is an essential component of the receptors for IL-2 and IL-15. Although IL-2Rβ is constitutively expressed by lymphocytes, its expression can be further induced by a number of stimuli, including phorbol 12-myristate 13-acetate (PMA). We have now characterized factors that bind to an enhancer region located between nucleotides −170 and −139 of the human IL-2Rβ promoter. Both Sp1 and Sp3 bound to the 5′ portion of this region, whereas a PMA-inducible factor (PIF) mainly bound to its 3′ portion and bound to the Sp binding motifs as well. In Jurkat T cells, induction of PIF DNA binding activity was rapidly induced, required de novo protein synthesis, and was sustained at a high level for at least 23 h. Interestingly, PIF was constitutively activated in human T-cell leukemia virus type 1-transformed MT-2 cells. In this paper, we demonstrate that PIF is Egr-1 based on its recognition by anti-Egr-1 antisera in gel mobility shift assays, even though the IL-2Rβ DNA binding motif differed substantially from the canonical Egr-1 binding site. In addition, Egr-1 bound to the Sp binding site. In Jurkat cells, both sites were required for maximal IL-2Rβ promoter activity, and in HeLaS3 cells, transfection of Egr-1 could drive activity of a reporter construct containing both sites. Moreover, Sp1 and Egr-1 could form a complex with kinetics that correlated with the production of Egr-1 in Jurkat cells upon PMA stimulation. Thus, Sp1 and Egr-1 physically and functionally cooperate to mediate maximal IL-2Rβ promoter activity.

The interleukin-2 receptor β-chain (IL-2Rβ) is a critical signaling component of high- and intermediate-affinity IL-2Rs (28, 42, 47). Like the common cytokine γ chain, IL-2Rβ is also a component of the receptor for IL-15, a cytokine that shares a number of biological actions with IL-2 (3, 17). Recent evidence derived from gene-targeting experiments has shown that mice deficient in IL-2Rβ exhibit dysregulated T-cell activation and autoimmunity, indicating that IL-2Rβ plays vital roles in maintaining normal immune responses (45).

IL-2Rβ is constitutively expressed in resting T cells (12, 37), B cells (4, 37, 52), natural killer (NK) cells (38), monocytes (14, 37), dendritic epidermal cells (46), neutrophils (11, 50), and large granular lymphocyte-like cells in decidua during early pregnancy (40). Stimulation of T cells with a number of stimuli can greatly increase the levels of both IL-2Rβ mRNA and protein; such stimuli include the mitogen phytohemagglutinin (PHA) (20); anti-CD3 (unpublished observations), anti-CD28, (7), and anti-CD2 plus anti-CD28 (7) antibodies; the protein kinase C activator phorbol 12-myristate 13-acetate (PMA [unpublished observations]); IL-2 (reference 38 and unpublished observations); and IL-4 (6). However, the molecular mechanisms by which the IL-2Rβ gene expression is regulated are not well understood. We previously delineated the regions critical for basal and PMA-inducible activities of the human IL-2Rβ promoter and identified three enhancer-like regions. Among them, the −56 to −34 and −170 to −139 enhancer regions were active in T-cell lines but not in nonlymphoid HeLaS3 or MG63 cells, correlating with the activity of the IL-2Rβ promoter in these cells. The −56 to −34 enhancer contains an Ets binding site (EBS) that binds two Ets family proteins, Ets-1 and GA-binding protein (GABP). Although the integrity of this site is critical for both enhancer and promoter activities (30), the EBS in the −56 to −34 region is not sufficient for IL-2Rβ promoter activity, since a 5′-deletion reporter construct containing nucleotides −61 to +97 is not active. Therefore, like many other cellular promoters (31, 48), the IL-2Rβ promoter appears to be coordinately regulated by multiple elements.

Egr-1 (early growth response protein 1) cDNA was first isolated as an immediate-early gene by differential screening of cDNA libraries made from BALB/c 3T3 cells stimulated by serum in the presence of cycloheximide (27, 43). In addition to its induction by mitogens, Egr-1 is induced by other stimuli, including signals for development, differentiation, tissue or radiation injury, and neuronal excitation (15). Egr-1 has been shown to be a sequence-specific DNA binding protein that binds to the 5′-GCG(C/G)GGGCG-3′ motif (8). Like Sp family proteins, Egr-1 also contains Zn finger structures (9, 43) that are critical for its DNA binding activity (5). Depending upon the presence of other DNA binding sites nearby the Egr-1 site, it can either positively or negatively regulate gene transcription in response to stimulation, because Egr-1 contains both transactivation and repression domains (16).

We have now characterized the −170 to −139 enhancer region of the human IL-2Rβ promoter and found that Sp1, Sp3, and Egr-1 bind to this region. We also demonstrate that both Sp and Egr-1 binding sites at the −170 to −139 enhancer region are required for maximal IL-2Rβ promoter activity.

MATERIALS AND METHODS

Tissue culture, plasmids, oligonucleotides, and antibodies. Peripheral blood lymphocytes (PBLs) were isolated from normal donors by standard methods. Jurkat E6.1 cells (provided by A. Weiss) and MT-2 cells (provided by I. Miyoshi) were maintained in RPMI 1640 media supplemented with 100 U of penicillin per ml, 100 U of streptomycin per ml, 2 mM glutamine, and 10% fetal bovine serum.

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HeLaS3 cells (American Type Culture Collection, Rockville, Md.) were grown in Dulbecco's modified Eagle's medium supplemented as mentioned above.

The pBLCAT2 and J0CAT reporter vectors and the construction of the wild-type pTKβ−170/−139 and JMXHβ-548/+97 plasmids were previously described (30). To mutate Sp1 and Egr-1 in the pTKβ−170/−139 construct (see Fig. 6A), both strands of the −170 to −139 sequence with the designated mutations were synthesized with *Hin*dIII and *Bam*HI sites, respectively, at the 5′ ends of the top and bottom strands. After phosphorylation with T4 polynucleotide kinase (Amersham-U.S. Biochemicals) and ATP (Pharmacia, Piscataway, N.J.), each pair of oligonucleotides was annealed and cloned between the *Hin*dIII and *Bam*HI sites of pBLCAT2. To mutate the Sp1 site at −169 to −155, the Egr-1 site at −155 to −143, or both sites in the JMXHβ−548/+97 construct, the primers 5′-TGTGT GTGTGTGCCGatCCCAGCGTAGGAGGC-3′, 5′-CCGCCCCCAGCGTAGtc tGCAGATCTTTATCTG-3′, and 5′-CCGatCCCAGCGTAGtctGCAGATCTT TATCTG-3′ (mutated nucleotides are underlined and in lowercase), respectively, were used in conjunction with the Morph site-specific plasmid DNA mutagenesis kit (5 Prime→3 Prime, Inc., Boulder, Colo.). All of the mutant constructs were confirmed by sequencing.

An Egr-1 eukaryotic expression vector, p930, was previously described (16) and kindly provided by Vikas P. Sukhatme (Harvard Medical School). The control plasmid pcDNA3.1Neo was obtained from Invitrogen (Carlsbad, Calif.).

Anti-Egr-1 antiserum R5232 was kindly provided by Vikas P. Sukhatme and used as previously described (5). All other antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

Transient transfection and CAT assays. Jurkat cells were transfected with DEAE-dextran (Pharmacia) as described previously (30). For IL-2Rβ enhancer and promoter constructs, 5 and 10 μg, respectively, of plasmid DNA purified with Qiagen plasmid purification kits (Qiagen Inc., Chatsworth, Calif.) were used. HeLaS3 cells were seeded in 100-mm-diameter petri dishes, transfected with 5 μg each of reporter and effector plasmid DNAs with Lipofectamine reagent (Gibco-BRL, Gaithersburg, Md.), and maintained in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum. Twenty-four hours after transfection, the cells either were not treated (controls) or were treated with 10 ng of PMA (Sigma, St. Louis, Mo.) per ml. The cells were harvested 48 h after transfection, and protein concentrations were determined with the Protein Assay Reagent (Bio-Rad). Chloramphenicol acetyltransferase (CAT) assays (18) were performed with equal amounts of protein extracts. For enhancer and promoter constructs, the reaction mixtures were incubated at 37°C for 1 and 3 h, respectively. CAT activities were quantitated by analyzing the thin-layer chromatography plates (J. T. Baker Inc., Phillipsburg, N.J.) with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

EMSA and methylation interference assay. Nuclear extracts for electrophoretic mobility shift assays (EMSAs) were prepared as described previously (30). The concentrations of nuclear proteins were quantitated with the Bio-Rad protein assay reagent. Ten micrograms of nuclear extracts and 15,000 cpm of ^{32}P-labeled probes were used in each reaction mixture as previously described (30). For competition assays, nuclear extracts were preincubated for 20 min with 25 ng of unlabeled oligonucleotide as indicated, radiolabeled probe was added, and then the mixture was incubated for another 15 min. For supershift assays, prior to addition of the radiolabeled probe, the nuclear extracts were preincubated on ice for 30 min with 2 μl of antibody as indicated.

For methylation interference assays, probes ^{32}P end labeled at either the top or bottom strands were prepared as described previously (30). Typically, 500,000 cpm of probes and 30 μg of nuclear extracts were used in each binding reaction mixture. Methylation interference assays were performed according to the method of Baldwin (2).

Southwestern assay, immunoprecipitation, and western blotting. For Southwestern assays, 30 μg of nuclear extracts was denatured in Laemmli sample buffer and separated on 7.5% sodium dodecyl sulfate gels. After proteins were transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, Mass.), the blots were incubated in blocking buffer (20 mM HEPES [pH 7.9], 60 mM KCl, 1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol [DTT], 5% nonfat dry milk) at 4°C for 1 h and then incubated at 4°C for 1 h in binding buffer (20 mM HEPES [pH 7.9], 60 mM KCl, 1 mM EDTA, 0.5 mM DTT, 5 μg of salmon sperm DNA per ml) containing 400,000 cpm of ^{32}P-labeled oligonucleotides per ml. The blots were then washed in washing buffer (20 mM HEPES [pH 7.9], 60 mM KCl, 1 mM EDTA, 0.5 mM DTT) at room temperature for 30 min, and the results were visualized by autoradiography.

For immunoprecipitation and Western blotting, 10×10^6 to 20×10^6 cells were washed once with cold phosphate-buffered saline and lysed in lysis buffer [50 mM Tris (pH 7.0), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride] at 4°C for 30 min. The immunocomplexes were precipitated with 5 μg of anti-Sp1 antiserum and recombinant protein G-agarose (Bethesda Research Laboratories, Gaithersburg, Md.), resolved on 8% polyacrylamide gels (Novex, San Diego, Calif.), and blotted onto Immobilon-P membranes. The membranes were then blotted with anti-Egr-1 or anti-Sp1 antiserum and developed by enhanced chemiluminescence (ECL System; Amersham).

In some of the immunoprecipitation experiments, prior to addition of antiserum, lysates were incubated with or without ethidium bromide on ice for 30 min and cleared by centrifugation at 4°C for 5 min to remove DNA contamination from the total cell lysate (26).

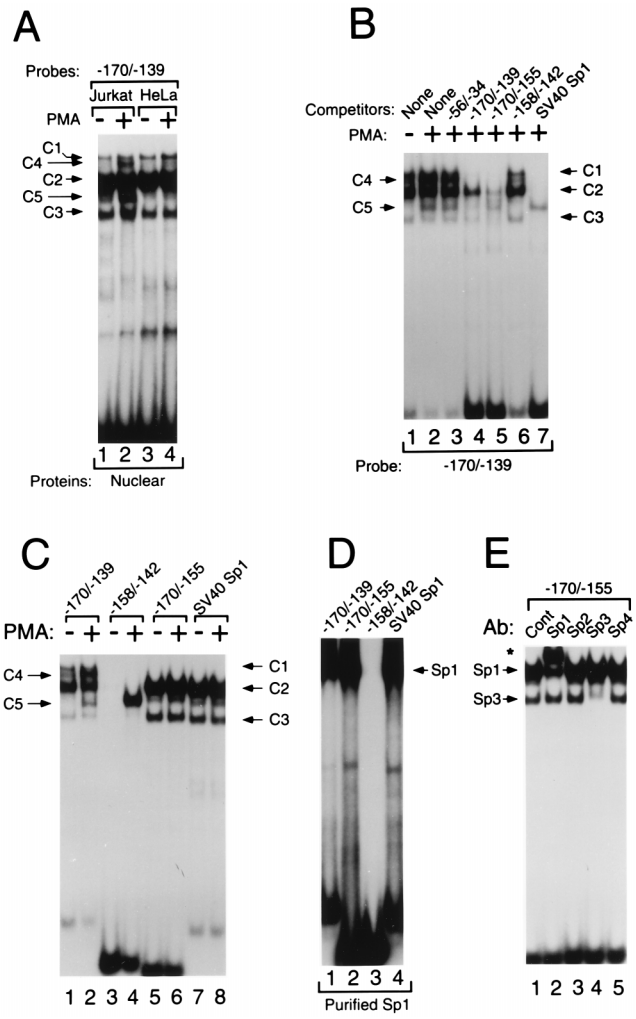

FIG. 1. EMSAs of nuclear proteins binding to −170 to −139, −170 to −155, and −158 to −142 oligonucleotides. (A) Radiolabeled −170 to −139 probe was incubated with nuclear extracts from Jurkat (lanes 1 and 2) or HeLaS3 (lanes 3 and 4) cells either untreated (lanes 1 and 3) or treated with 10 ng of PMA per ml for 1 h (Jurkat) or 5 h (HeLaS3). (B) Prior to addition of the radiolabeled −170 to −139 probe, the nuclear extracts from Jurkat cells either untreated (lane 1) or treated with PMA (lanes 2 to 7) were incubated either with no competitor (lanes 1 and 2) or with 25 ng of the following unlabeled competitors: −56 to −34 (lane 3), −170 to −139 (lane 4), −170 to −155 (lane 5), −158 to −142 (lane 6), and SV40 Sp1 (lane 7). (C) Radiolabeled −170 to −139 (lanes 1 and 2), −158 to −142 (lanes 3 and 4), −170 to −155 (lanes 5 and 6), and SV40 Sp1 probes were incubated with the nuclear extracts from Jurkat cells either untreated (lanes 1, 3, 5, and 7) or treated with 10 ng of PMA per ml for 1 h (lanes 2, 4, 6, and 8). (D) Radiolabeled −170 to −139 (lane 5), −170 to −155 (lane 6), −158 to −142 (lane 7), and SV40 Sp1 (lane 8) probes were incubated with 0.5 footprinting unit of purified human Sp1 protein (Promega, Madison, Wis.). (E) The nuclear extracts were preincubated with either unrelated antibody (lane 1) or anti-Sp1 (lane 2), anti-Sp2 (lane 3), anti-Sp3 (lane 4), or anti-Sp4 (lane 5) antibody prior to addition of radiolabeled −170 to −155 probe. The supershifted complex by anti-Sp1 antibody is indicated by an asterisk.

RESULTS

Sp1, Sp3, and PIF bind to the −170 to −139 enhancer region. To identify nuclear proteins that could bind to the −170 to −139 enhancer region, we performed EMSAs with a ^{32}P-labeled −170 to −139 probe and nuclear extracts from Jurkat and HeLa cells. As shown in Fig. 1A, major (C2) and minor (C1 and C3) complexes were detected in the nuclear extracts from the unstimulated Jurkat (lane 1) and HeLaS3

(lane 3) cells. Consistent with the ability of the −170 to −139 enhancer region to respond to PMA stimulation in Jurkat but not in HeLa cells (30), treatment of the cells with PMA induced formation of two new complexes, C4 and C5, in the nuclear extracts from Jurkat cells (lane 2) but not in those from HeLa cells (lane 4). PMA treatment did not affect the complexes C1, C2, and C3 (lane 2 versus lane 1). Induction of the C4 complex by PMA was somewhat variable, because C4 was sometimes detected in nuclear extracts from untreated Jurkat cells.

To confirm the specificity of these complexes and to examine if they bound to distinct regions of the −170 to −139 probe, we performed competition experiments. As expected, the −170 to −139 oligonucleotide competed with the formation of all of the complexes (Fig. 1B, lane 4), although for reasons unclear, the competition of C2 was less effective. Interestingly, the −170 to −155 oligonucleotide markedly diminished the formation of the C1, C2, C3, and C4 complexes but was less effective in inhibiting formation of C5 (lane 5), whereas the −158 to −142 oligonucleotide competed with the formation of the C4 and C5 complexes but did not affect C1, C2, and C3 (lane 6). An oligonucleotide corresponding to the simian virus 40 (SV40) Sp1 site abolished the formation of the C1, C2, C3, and C4 complexes but had no effect on the formation of the C5 complex (lane 7), suggesting that Sp1-like factor(s) might be involved in complexes C1 to C4 but not C5. An unrelated oligonucleotide corresponding to the −56 to −34 region of the IL-2Rβ promoter that is known to bind to Ets family proteins did not affect any of the complexes (lane 3 versus lane 2). When used in EMSAs, the −158 to −142 oligonucleotide exhibited a single PMA-inducible complex of much greater intensity than was seen with the −170 to −139 probe (Fig. 1C, lane 4 versus lane 2). The pattern of DNA complexes formed with Jurkat nuclear extracts with the −170 to −155 oligonucleotide (Fig. 1C, lanes 5 and 6) was indistinguishable from that with the SV40 Sp1 probe (lanes 7 and 8). Thus, the −170 to −139 region appeared to have two subregions, with complexes C2 and C3 binding to the −170 to −155 region and complex C5 binding primarily to the −158 to −142 region. However, the facts that the −170 to −155 oligonucleotide could partially inhibit C5 formation (Fig. 1B) and that a complex comigrating with C5 could be detected when the −170 to −155 oligonucleotide was used as a probe (Fig. 1C, lane 6) suggest that both regions could independently contribute to formation of C5. C1 and C4 formation appeared to require both subregions, because neither complex was detected except with the full-length −170 to −139 probe.

Consistent with the 5′ portion of the −170 to −139 region containing an Sp1 binding motif, both the −170 to −139 and −170 to −155 oligonucleotides could bind purified human Sp1 with a pattern indistinguishable from that seen with the SV40 Sp1 probe (Fig. 1D, lanes 1 and 2 versus lane 4). In contrast, the −158 to −142 oligonucleotide did not bind Sp1 (lane 3). Three additional closely related Sp1 family proteins (Sp2, Sp3, and Sp4) have been identified (19, 25). We therefore performed EMSAs with the −170 to −155 probe and antibodies specific for all four Sp1 family proteins to determine which were involved in the Sp complexes. As shown in Fig. 1E, an anti-Sp1 antibody supershifted most of the major complex, which migrated with a slower mobility (lane 2 versus lane 1), and an anti-Sp3 antibody blocked the complex with a faster mobility (lane 4 versus lane 1), whereas antibodies to Sp2 (lane 3) or Sp4 (lane 5) had no effect. These data establish that both Sp1 and Sp3 bind to the −170 to −155 region, the PMA-inducible factor (PIF) in the C5 complex binds to the −158 to −142 region, and the PMA-inducible C4 complex may contain both Sp1 family protein(s) and PIF.

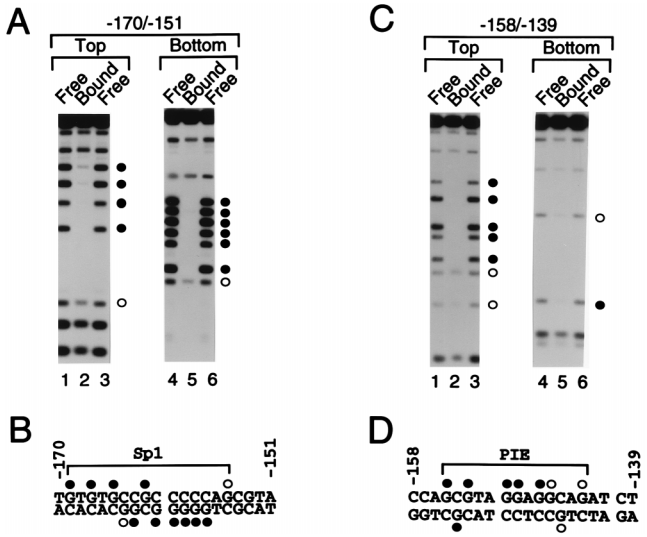

FIG. 2. Methylation interference analyses. Double-stranded −170 to −151 (A) and −158 to −139 (C) probes were end labeled on the top (lanes 1 to 3) or bottom (lanes 4 to 6) strands and partially methylated with dimethyl sulfate. EMSAs were performed with nuclear extract from Jurkat cells treated with PMA and the methylated probes. For both panels A and C, free (lanes 1, 3, 4, and 6) and bound (lanes 2 and 5) probes were excised in situ and eluted from the gel, treated with piperidine (Fisher Scientific), lyophilized, resolved on a 12% sequencing gel, and autoradiographed. The results from panels A and C are summarized in panels B and D, respectively. Strong interference with binding due to methylation of G residues is indicated by solid circles, and weak interference with binding is indicated by open circles.

Compared to the full-length −170 to −139 probe, more intense binding activities were seen with the −170 to −155 and −158 to −142 probes (Fig. 1C). To further define the DNA binding sequences of these nuclear complexes, we performed methylation interference assays. Consistent with the data obtained from the EMSAs in Fig. 1, the methylation interference pattern obtained with a −170 to −151 probe revealed that the nuclear factors in C2 (Fig. 2A, lanes 2 and 5 [summarized in Fig. 2B]) and C3 (data not shown) indeed contacted the Sp1 motif (5′-GTGTGCCGCCCCCAG-3′) between −169 and −155, whereas the methylation interference pattern obtained with a −158 to −139 probe indicated that PIF contacted the 5′-GCGTAGGAGGCAG-3′ region between −155 and −143 (Fig. 2C, lanes 2 and 5; summarized in Fig. 2D).

As expected, the wild-type −158 to −142 oligonucleotide (Fig. 3A) efficiently competed for PMA-inducible DNA binding activity to a −158 to −142 probe (Fig. 3B, lane 3 versus lane 2). Consistent with the data from the methylation interference assays, mutation of either the GCG residues at −155 to −153 into CAC (lane 4 versus lane 2) or GAG residues at −149 to −147 into TCT (lane 5 versus lane 2) of the −158 to −142 oligonucleotide abolished its ability to compete for PIF binding to the wild-type probe, and neither of these mutant oligonucleotides could bind PIF (Fig. 3C, lanes 3 and 4 versus lane 2). The DNA binding motif for PIF differs from the consensus motifs of other known PIFs, including AP-1 (TGA CTCA) and NF-κB (GGGRNNYYCC) (1). As expected, oligonucleotides corresponding to AP-1 (lane 6), NF-κB (lane 7), and NF-AT (lane 8) binding sites did not compete for PIF binding. Furthermore, in contrast to the ability of antibodies to c-Fos and c-Jun to compete for NF-AT binding (10, 35), a number of antibodies that specifically recognize c-Fos, c-Jun

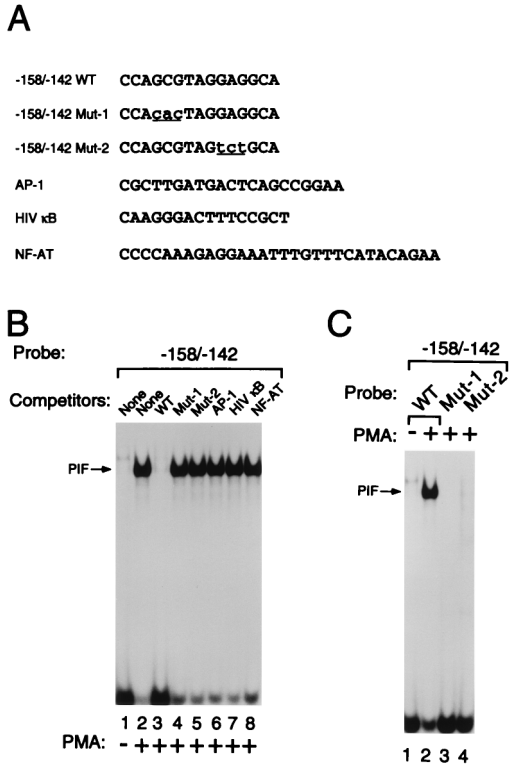

FIG. 3. PIF is different from AP-1, NF-κB, and NF-AT. (A) Sequences of the wild-type and mutant −158 to −142 oligonucleotides (mutations are underlined and in lowercase), and AP-1, HIV κB, and NF-AT oligonucleotides. The NF-AT sequence is from the murine IL-2 promoter (31). (B) Prior to addition of the radiolabeled −158 to −142 probe, the nuclear extracts from Jurkat cells either untreated (lane 1) or treated with PMA (lanes 2 to 8) were incubated either with no competitor (lanes 1 and 2) or with 25 ng of the following unlabeled competitors: wild type (WT; lane 3) or mutant (lanes 4 and 5) −158 to −142 oligonucleotides or oligonucleotides corresponding to AP-1 (lane 6), HIV κB (lane 7), or NF-AT (lane 8) sites. (C) EMSA was performed with radiolabeled wild-type (WT; lanes 1 and 2) and mutant (Mut-1 in lane 3, Mut-2 in lane 4) −158 to −142 oligonucleotide probes and nuclear extracts from Jurkat cells either untreated (lane 1) or treated with PMA for 2 h (lanes 2 to 4).

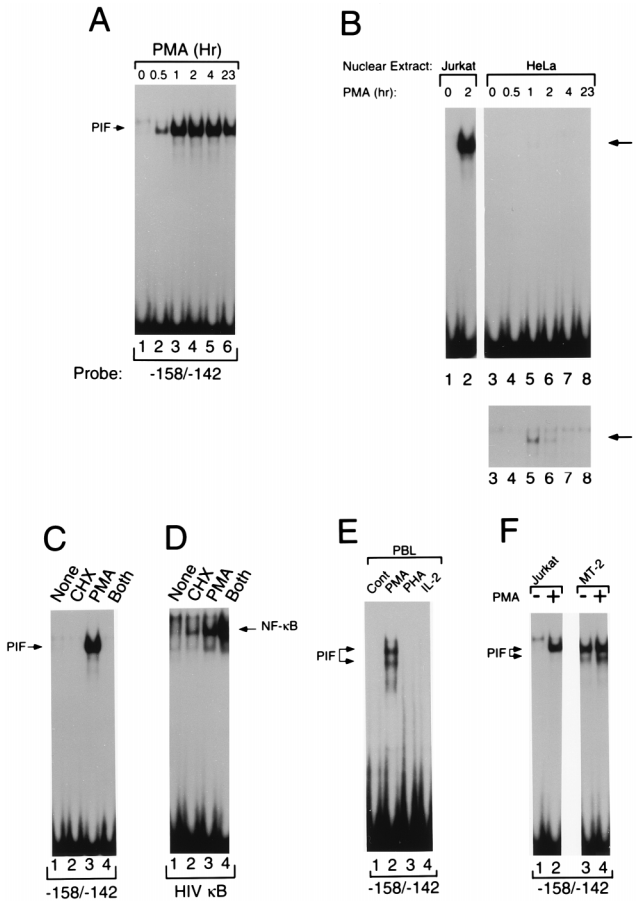

FIG. 4. PIF is induced in various cell types, and the induction in Jurkat cells is abolished by cycloheximide. (A) Radiolabeled −158 to −142 probe was incubated with nuclear extracts from Jurkat cells either untreated (lane 1) or treated with PMA for 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), or 23 h (lane 6). (B) Radiolabeled −158 to −142 probe was incubated with the nuclear extracts from Jurkat cells either untreated (lane 1) or treated with PMA for 2 h (lane 2) or was incubated with the nuclear extracts from HeLaS3 cells either untreated (lane 3) or treated with PMA for 30 min (lane 4), 1 h (lane 5), 2 h (lane 6), 4 h (lane 7), and 23 h (lane 8). The lower panel is a longer exposure of the upper lanes 3 to 8, and PMA-inducible DNA binding activities are indicated by arrows. (C) Radiolabeled −158 to −142 probe was incubated with the nuclear extracts from Jurkat cells either untreated (lane 1) or treated with 20 μg of cycloheximide per ml for 1 h (lane 2), PMA for 1 h (lane 3), or both cycloheximide and PMA for 1 h (lane 4). (D) Radiolabeled HIV κB probe was incubated with the same Jurkat nuclear extracts used in panel B. (E) Radiolabeled −158 to −142 probe was incubated with nuclear extracts from PBLs either untreated (lane 1) or treated for 2 h with 10 ng of PMA per ml (lane 2), 1 μg of PHA per ml (lane 3), or 2 nM IL-2 (lane 4). (F) Constitutive PIF binding activity in HTLV-1-transformed MT-2 cells. Radiolabeled −158 to −142 probe was incubated with nuclear extracts from Jurkat (lanes 1 and 2) or MT-2 (lanes 3 and 4) cells.

proteins, or Fos or Jun family proteins did not affect PIF binding activity (data not shown), diminishing the possibility that AP-1 family proteins are involved in the PIF complex.

PIF binding activity is rapidly induced by PMA and de novo protein synthesis is required for its production. A kinetic analysis revealed that PIF DNA binding activity was detectable as early as 30 min after treatment of Jurkat cells with PMA (Fig. 4A, lane 2), was maximal between 1 and 2 h (lanes 3 and 4), and was sustained at a high level for at least 23 h (lane 6). Interestingly, PIF DNA binding activity was only very weakly and transiently detected in HeLa nuclear extracts at 1 h (Fig. 4B, lane 5) and 2 h (lane 6) following PMA treatment, but at a much lower level than that seen with Jurkat nuclear extracts (lane 2). The transient weak activation of PIF in HeLaS3 cells was not sufficient to activate a CAT reporter construct in response to PMA in these cells with either of the −158 to −142 (not shown) or −170 to −139 (30) regions.

Because PIF was rapidly induced, we investigated whether de novo protein synthesis was required for its induction or whether like NF-κB, it was present in a latent form in the cells. Jurkat cells were treated with PMA in the presence or absence of the protein synthesis inhibitor cycloheximide, and nuclear extracts were prepared. Cycloheximide did not induce PIF (Fig. 4C, lane 2) but abolished the ability of PMA to induce PIF DNA binding activity (lane 4 versus lane 3). In contrast, as previously reported (1), cycloheximide not only induced (Fig. 4D, lane 2) but in fact superinduced NF-κB binding activity by PMA (lane 4 versus 3). Thus, de novo protein synthesis is essential for the production of PIF.

PIF binding activity is induced by PMA but not by PHA or IL-2 in normal PBLs and is constitutively expressed in HTLV-1-transformed MT-2 cells. In addition to its induction in Jurkat T cells, PIF binding activity was induced by PMA in normal PBLs (Fig. 4E, lane 2 versus lane 1). However, whereas a single major PIF complex was detected in Jurkat nuclear extracts (Fig. 4F, lane 2), two complexes were detected in the nuclear

extracts from PBLs isolated from three independent donors (Fig. 4E, lane 2, and data not shown), and the upper complex comigrated with the Jurkat PIF complex (data not shown). The basis for the added complex in PBLs is unclear and requires further investigation. Neither complex was induced by PHA (lane 3) or IL-2 (lane 4). These results indicate that unlike NF-κB, PIF binding activity appears to be mainly induced by PMA.

Many cellular genes are activated in HTLV-1-infected T cells by a 40-kDa viral transactivator Tax protein of HTLV-1 (32, 51). Tax activates transcription of these genes by modulating the DNA binding activity of transcription factors, including AP-1, ATF/CREB, and NF-κB. Some of these PMA-inducible transcription factors, like c-*fos* and NF-κB, are constitutively expressed at high levels in HTLV-1-transformed MT-2 cells and are known to be activated by Tax in these cells (51). We therefore examined whether PIF DNA binding activity was affected in MT-2 cells. Constitutive PIF DNA binding activity was detected in MT-2 nuclear extracts (Fig. 4F, lane 3), and it was also modestly increased after PMA treatment (lane 4 versus lane 3), suggesting that HTLV-1 protein(s) might activate PIF via a protein kinase C-dependent pathway.

Determination of the molecular mass of PIF. To further characterize PIF, we performed Southwestern assays with the −158 to −139 probe. Two proteins migrating at 82 and 60 kDa, respectively, were detected with a wild-type (Fig. 5A, lane 2) but not mutant (lane 4) probe in nuclear extracts from Jurkat cells after PMA stimulation (lane 2) and from unstimulated MT-2 cells (data not shown). No proteins were detected in the nuclear extracts from unstimulated Jurkat cells (lane 1). A constitutively expressed 120-kDa protein was detected by both wild-type (Fig. 5A, lanes 1 and 2) and mutant (lanes 3 and 4) probes, indicating that it binds to the sequences unrelated to the PIF site.

PIF is Egr-1. The rapid induction of PIF and the requirement of de novo protein synthesis for its DNA binding activity suggested that PIF was encoded by an immediate-early gene. Because PIF contains 82- and 60-kDa DNA binding proteins (Fig. 5A), we investigated whether PIF was related to the Zn finger-containing Egr family of transcription factors, which are immediate-early gene products induced by a variety of stimuli, including PMA, and which have a molecular mass of 80 to 82 kDa (5, 44). In EMSAs with Jurkat nuclear extracts, an Egr-1 binding site (EBS-1; CGCCCCCGC) (5) bound to a PIF binding activity (Fig. 5B, lane 4 versus lane 3) that comigrated with the PIF seen with the −158 to −142 probe (lane 2). Although the core DNA binding sites differed (5′-GCGTAGGAGGCA G-3′ for PIF and 5′-CGCCCCCGC-3′ for Egr-1), the unlabeled −158 to −142 and EBS-1 oligonucleotides nevertheless inhibited PIF binding activity (Fig. 5C, lanes 2 and 3 versus lane 1 and lanes 5 and 6 versus lane 4), with the −158 to −142 oligonucleotide being less efficient (lanes 2 and 6 versus lanes 3 and 5). These results indicate that Egr family protein(s) are likely involved in the formation of the PMA-inducible DNA complex. This notion was further supported by the fact that anti-Egr-1 antisera either abolished (Fig. 5D, lanes 3 and 6) or supershifted (data not shown) PMA-inducible DNA binding activity to either the −158 to −142 or EBS-1 probe, whereas antisera specific for other Egr family proteins, including Egr-2, Egr-3, and Wilm's tumor antigen, did not affect protein binding to either probe (not shown). These results establish that the PIF that bound to the −158 to −142 region in the human IL-2Rβ promoter is Egr-1, and we therefore redenote the −158 to −142 region PIF as an Egr-1 site. Although the consensus DNA binding site for Egr-1 was defined as GCGKGG GCG (8), our data show that Egr-1 can recognize a DNA sequence very different from the consensus. Because a 60- to 62-kDa truncated form of Egr-1 has also been reported (15) and the anti-Egr-1 antiserum abolishes PIF binding to the −158 to −142 probe (Fig. 5D), we speculate that the 60-kDa

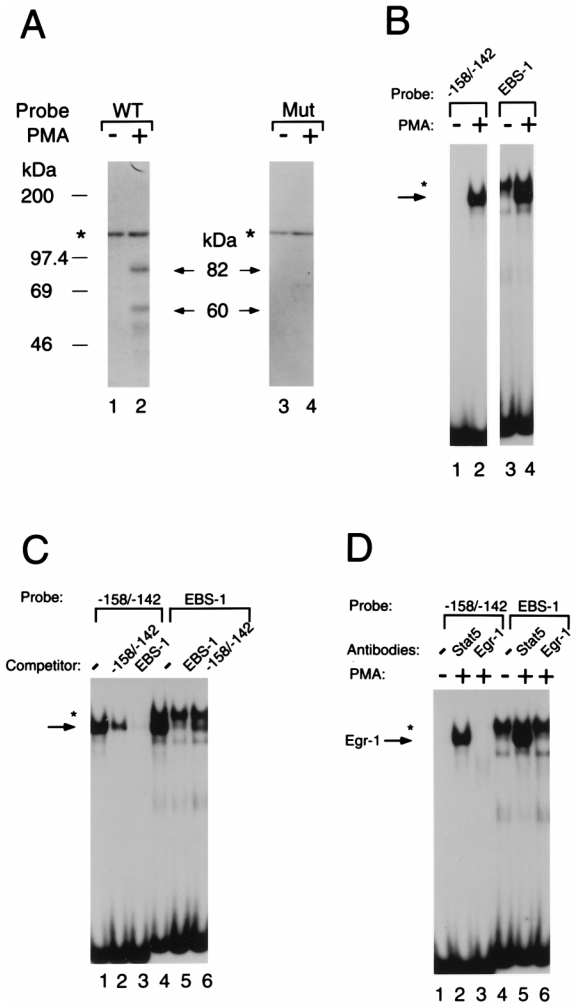

FIG. 5. Characterization of PIF. (A) Southwestern analysis with the −158 to −139 probe. Nuclear extracts from Jurkat cells either untreated (lanes 1 and 3) or treated with PMA for 1 h (lanes 2 and 4) were analyzed by Southwestern assay with either radiolabeled wild-type (lanes 1 and 2) or mutant (lanes 3 and 4) −158 to −139 probes. A 120-kDa band constitutively present in the nuclear extracts is indicated by an asterisk. Molecular masses were determined by comparisons with the mobilities of prestained molecular markers (Amersham). (B) PIF comigrates with Egr family protein(s). Results are from EMSA with radiolabeled −158 to −142 (lanes 1 and 2) or canonical Egr binding motif (EBS-1; lanes 3 and 4) probes and nuclear extracts from Jurkat cells either unstimulated (lanes 1 and 3) or stimulated with PMA (lanes 2 and 4). (C) Inhibition of PIF DNA binding activity by Egr DNA binding site. Results are from EMSA with radiolabeled −158 to −142 (lanes 1 to 3) and the canonical Egr binding motif (EBS-1; lanes 4 to 6) probes and nuclear extracts from Jurkat cells either unstimulated (lanes 1 and 4) or stimulated with PMA (lanes 2, 3, 5, and 6). The extracts were incubated with either the unlabeled −158 to −142 probe (lanes 2 and 6) or the Egr binding site (EBS-1; lanes 3 and 5) prior to addition of the radiolabeled probes. (D) PIF is recognized by anti-Egr-1 antiserum. Results are from EMSA with radiolabeled −158 to −142 (lanes 1 to 3) and the canonical Egr binding motif (EBS-1; lanes 4 to 6) probes and nuclear extracts from Jurkat cells either unstimulated (lanes 1 and 4) or stimulated with PMA (lanes 2, 3, 5, and 6). Prior to addition of the radiolabeled probes, the extracts were incubated either with anti-Stat5 (Stat5; lanes 2 and 5) as a control antiserum or with anti-Egr-1 (Egr-1; lanes 3 and 6). In panels B to D, a constitutive DNA binding activity was detected by the EBS-1 probe, as indicated by asterisks.

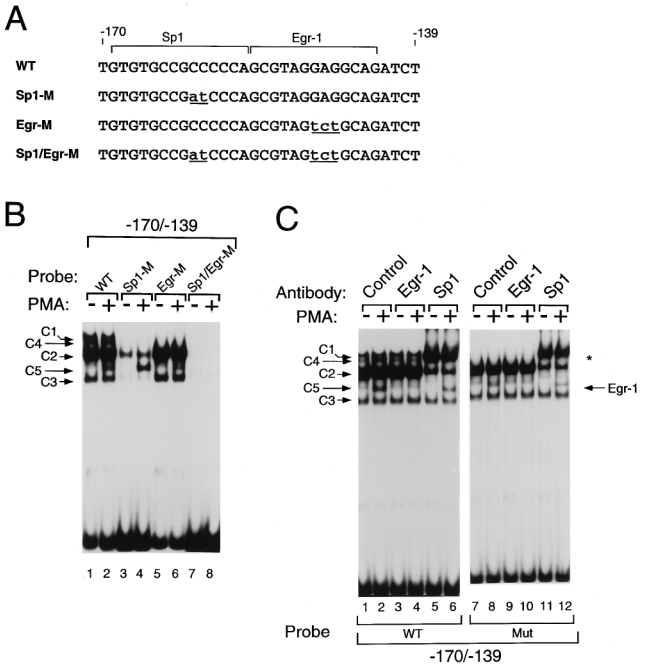

FIG. 6. Egr-1 binds to both Sp1 and Egr-1 sites at the −170 to −139 region. (A) Sequences of wild-type (WT), Sp1 mutant (Sp1-M), Egr-1 mutant (Egr-M), and Sp1 plus Egr-1 mutant (Sp1/Egr-M) −170 to −139 oligonucleotides, showing the Sp1 and Egr-1 motifs, and mutated nucleotides (underlined in lowercase). (B) EMSA with radiolabeled wild-type (WT; lanes 1 and 2), Sp1 mutant (Sp1-M; lanes 3 and 4), Egr-1 mutant (Egr-M; lanes 5 and 6), and Sp1 plus Egr-1 mutant (Sp1/Egr-M; lanes 7 and 8) probes and nuclear extracts from Jurkat cells either unstimulated (lanes 1, 3, 5, and 7) or stimulated with PMA (lanes 2, 4, 6, and 8). (C) EMSA with radiolabeled wild-type (WT; lanes 1 to 6) and Egr-1 mutant (Mut; lanes 7 to 12) −170 to −139 probes and nuclear extracts from Jurkat cells either unstimulated (lanes 1, 3, 5, 7, 9, and 11) or stimulated with PMA (lanes 2, 4, 6, 8, 10, and 12). Prior to addition of the probes, the extracts were incubated either with control (lanes 1, 2, 7, and 8), anti-Egr-1 (Egr-1; lanes 3, 4, 9, and 10), or anti-Sp1 (Sp1; lanes 5, 6, 11, and 12) antiserum. The anti-Sp1 supershifted complexes are indicated by an asterisk.

protein detected by Southwestern blotting (Fig. 5A) might be a truncated form of Egr-1.

The Sp site also binds to Egr-1, and the integrity of both Sp and Egr-1 sites is essential for IL-2Rβ promoter activity. We next evaluated how mutation of the Sp and/or Egr-1 binding sites (Fig. 6A) affected the formation of each complex with the −170 to −139 probe. Mutation of the Sp site abolished the formation of complexes C1, C3, and C4 and greatly diminished that of C2 (Fig. 6B, lanes 3 and 4), whereas that of C5 was actually increased, suggesting that Sp1 and/or Sp3 have higher DNA binding affinities to the wild-type oligonucleotide and probably interfere with the binding of Egr-1. Mutation of the Egr-1 site abolished the C1 and C4 complexes, but as expected, had no effect on the C2 and C3 complexes. Interestingly, this mutation had no significant effect on the PMA-inducible C5 complex (lanes 5 and 6), suggesting that in addition to the Egr-1 site at −158 to −142, this factor likely recognizes other sequences within the −170 to −139 region, consistent with our earlier observation that both the −158 to −142 and −170 to −155 oligonucleotides could inhibit C5 formation (Fig. 1B). As expected, mutation of both Sp and Egr-1 sites abolished all of the complexes (lanes 7 and 8).

To determine whether the C4 and C5 complexes both contained Egr-1 and whether the PIF bound to the −170 to −139 probe containing a mutation at the Egr-1 site was also Egr-1, we performed EMSA with anti-Egr-1 antiserum. As shown in Fig. 6C, the anti-Egr-1 antiserum abolished the formation of both the C4 and C5 complexes (lane 4 versus lane 2). In addition, the PMA-inducible complex bound to the Egr-1 mutant −170 to −139 probe was also recognized by anti-Egr-1 antiserum (lane 10 versus lane 8), suggesting that Egr-1 may bind to the Sp site on the basis of the similarity between the Egr-1 and Sp sites. In contrast, when all three probes were used (Fig. 6C), anti-Sp1 antiserum only supershifted the C2 complex and had no effect on Egr-1 binding. Together with the results of Fig. 1, these data demonstrate that the Sp site not only binds to Sp1 and Sp3 but also binds to Egr-1.

In the context of the −170 to −139 region with the heterologous thymidine kinase promoter, mutation of the Sp1 site diminished basal enhancer activity but had less of an effect on the fold induction by PMA; mutation of both sites was required to significantly diminish PMA-inducible enhancer activity (Fig. 7A). In the context of the −548 to +97 IL-2Rβ promoter, the greatest effect was also seen when both Sp1 and Egr-1 sites were simultaneously mutated (Fig. 7B), indicating that both of the Sp1 and Egr-1 sites are required for maximal IL-2Rβ promoter activity.

Since much less endogenous Egr-1 was detected in HeLaS3 cells than in Jurkat cells (Fig. 4B), we next evaluated whether overexpression of Egr-1 in HeLaS3 cells could drive expression of the IL-2Rβ constructs. Indeed, the pTKβ−170/−139 reporter construct was activated by Egr-1, and activity was diminished when both of the Sp1 and Egr-1 sites were mutated (Fig. 7C). Interestingly, there was very little, if any, PMA inducibility whether the cells were cultured in the presence of 10% (data not shown) or 0.5% (Fig. 7C) serum, indicating that overexpression of Egr-1 in HeLaS3 cells obviated the need for PMA treatment. In contrast, no induction was seen with the −548 to +97 IL-2Rβ promoter construct, even when both Ets-1 and GABPs were also coexpressed (data not shown), suggesting that the additional lineage-restricted factor(s) are required for IL-2Rβ promoter activity.

In view of the importance of both Sp and Egr-1 sites at the −170 to −139 region for IL-2Rβ promoter activity and the fact that Sp1 can form complexes with either itself or with other transcription factors (see Discussion), we investigated whether Sp1 could form a complex with Egr-1 to regulate the IL-2Rβ promoter. In coimmunoprecipitation experiments, we found that Egr-1 could be precipitated by anti-Sp1 antiserum in response to PMA stimulation (Fig. 8A, lanes 2 to 4 versus lane 1), and the amount of Egr-1 coimmunoprecipitated correlated with the expression level of Egr-1 in the cells (lanes 5 to 8). To evaluate the possibility that coimmunoprecipitation of Egr-1 by anti-Sp1 antiserum required the presence of DNA, total cell lysates were incubated with ethidium bromide prior to immunoprecipitation to eliminate DNA-dependent protein-protein interactions (26). Anti-Sp1 antiserum was still able to efficiently coprecipitate Egr-1 proteins even after treatment of the lysates with ethidium bromide (Fig. 8B, lanes 3 and 4), although the amounts of Egr-1 coprecipitated by anti-Sp1 antiserum were somewhat smaller than that seen in the lysate without ethidium bromide treatment (lanes 3 and 4 versus lane 2). These results suggest that both DNA-independent and -dependent interactions of Sp1 and Egr-1 exist and also imply that the Sp1–Egr-1 complex can simultaneously occupy both Sp and Egr-1 sites at the −170 to −139 region, thus regulating IL-2Rβ promoter activity.

DISCUSSION

We have characterized a PMA-inducible regulatory element in the human IL-2Rβ promoter region and have shown that it

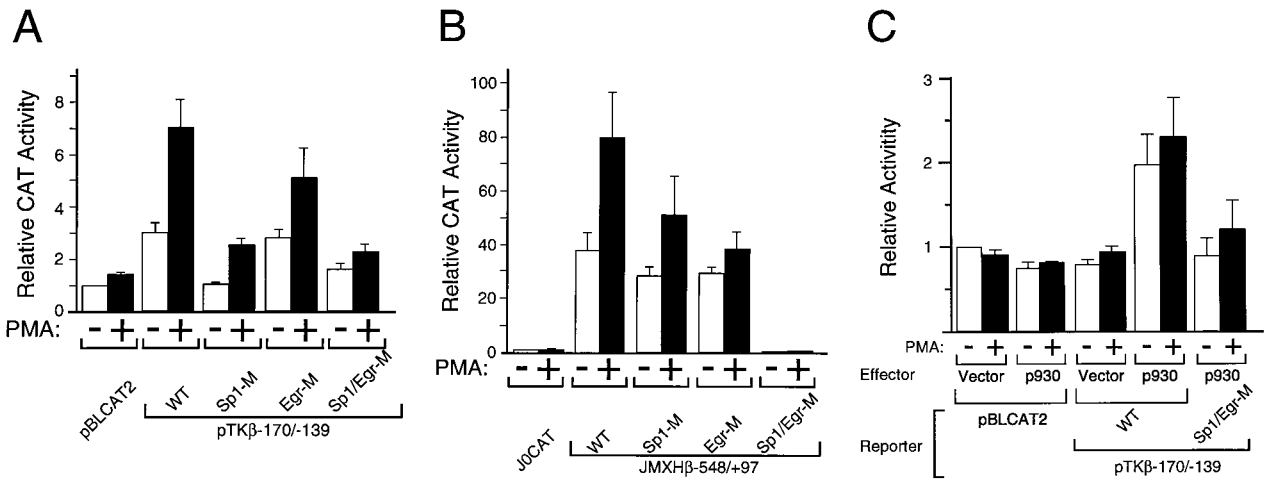

FIG. 7. Both Sp1 and Egr-1 sites are required for maximal promoter activity. (A) The enhancer CAT reporter plasmid pBLCAT2 and pTKβ−170/−139 constructs (WT; Sp1 mutant [Sp1-M], Egr-1 mutant [Egr-M], and Sp1 plus Egr-1 mutant [Sp1/Egr-M]) were transfected into Jurkat cells. (B) The promoter CAT reporter plasmid J0CAT and JMXHβ−548/+97 constructs (WT; Sp1 mutant [Sp1-M], Egr-1 mutant [Egr-M], and Sp1 plus Egr-1 mutant [Sp1/Egr-M]) were transfected into Jurkat cells. In both panels, the cells were either not treated (open bars) or were treated with PMA (solid bars) overnight before being harvested for CAT assays. Data represent the means ± standard errors derived from three independent experiments. The activities of pBLCAT2 (A) and J0CAT (B) without PMA treatment are assigned a value of 1.0; the activities of other constructs are expressed as relative fold increases. (C) Cotransfections of HeLaS3 cells were performed with the following combinations: pBLCAT2 plus either pcDNA3.1neo (Vector) or p930, wild-type pTKβ−170/−139 (WT) plus either pcDNA3.1neo (Vector) or p930, and pTKβ−170/−139 construct mutated at both Sp1 and Egr-1 sites (Sp1/Egr-M) plus p930. The cells were either not treated or treated with PMA overnight before harvest and measurement of CAT activities. Data represent the means ± standard errors derived from three independent experiments. The activity of pBLCAT2 plus pcDNA3.1neo without PMA treatment is assigned a value of 1.0.

is controlled by Sp1 family proteins and the immediate-early gene product Egr-1. Sp1 was originally identified as a ubiquitously expressed mammalian transcription factor that binds to GC box promoter elements and activates the SV40 early promoter (13). It has also been shown to be important for the promoter activity of many other viral and cellular genes. At least in some cases, Sp1 activates transcription by cooperative interaction either with itself (41) or with other transcription factors, such as the E2 protein bound to the bovine papillomavirus enhancer (29), Tat protein bound to the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (21, 22), NF-κBp65 bound to the HIV-1 enhancer (34), and Rb protein bound to the retinoblastoma control element in c-*fos*, c-*myc*, and transforming growth factor β1 genes (49). In the IL-2Rβ promoter, a functional Sp1 binding site located at the −169 to −155 region is required mainly for basal promoter activity. Interestingly, Sp1 is also physically associated with Egr-1 in vivo upon PMA stimulation. Because both Sp1 and Egr-1 can simultaneously bind to the −170 to −139 region, it is likely that Sp1-Egr-1 complex formation plays a role in regulating IL-2Rβ promoter activity in response to PMA.

Our observation that either Sp1 or Egr-1 can bind to an Sp binding site in the IL-2Rβ promoter region is similar to a report (39) that the ZIP (zinc finger protein binding) region in the IL-2 promoter binds independently to either Sp1 or Egr-1 proteins. However, in addition to the Sp binding site, the IL-2Rβ promoter has an adjacent Egr-1 binding site just downstream of the Sp site at the −170 to −139 region, which only binds to Egr-1. Mutation of either the Sp or Egr-1 site alone at the −170 to −139 region diminished IL-2Rβ promoter activity, whereas simultaneous mutation of both sites abolished promoter activity. Given that we have previously shown that the EBS in the −56 to −34 region is essential but not sufficient for promoter activity (30), it is clear that both the −170 to −139 region and the EBS at −56 and −34 are required, but neither of them alone is sufficient for IL-2Rβ promoter activity. Thus, inducible expression of the IL-2Rβ promoter is most likely regulated by functional cooperation between both the Sp1 and Egr-1 sites in the −170 to −139 region and the EBS in the −56 to −34 region.

Although only a few genes are known to be regulated by Egr-1 in hematopoietic cells, the ability to induce Egr-1 expression in response to mitogens or differentiation stimuli is well correlated with proliferation of B lymphocytes or differentiation of monocytes. In mature B cells, anti-μ antibodies induce Egr-1 expression and promote cell proliferation, whereas in immature B cells, these antibodies do not induce Egr-1 expression, inhibit cell proliferation, and thereby eventually cause cell death (36). In U937 and HL-60 myeloid leukemia cells, PMA induces Egr-1 expression and causes monocytic differentiation (23, 24), whereas dexamethasone blocks Egr-1 expression and inhibits differentiation (23). Furthermore, Egr-1 antisense oligonucleotides can prevent macrophage differentiation induced by PMA, whereas constitutive expression of Egr-1 favors macrophage differentiation in HL-60 cells but prevents granulocyte differentiation induced by dimethyl sulfoxide in these cells (33).

The role of Egr-1 in fibroblasts and certain hematopoietic cells has been more extensively studied; however, little is known about the role played by Egr-1 and/or other Egr family proteins in T cells. The coordinate regulation of both the IL-2 (39) and IL-2Rβ genes in T cells by Egr-1 and Sp1 suggests that these factors may coordinately influence expression of a range of T-cell-specific genes during T-cell activation. The optimal targets for the Egr-1 site have not been defined by a DNA binding site selection assay, and we now show here that the Egr-1 site in the IL-2Rβ promoter binds well to Egr-1 despite the fact that the site is markedly different from the canonical site (8). Therefore, we hypothesize that other noncanonical Egr sites will be identified in the promoter regions of other T-cell-specific genes that play a role in regulating gene expression in response to mitogen stimulation. Interestingly, PHA does not induce Egr-1, but some of the cytokines produced by the cells in response to mitogen or antigen stimula-

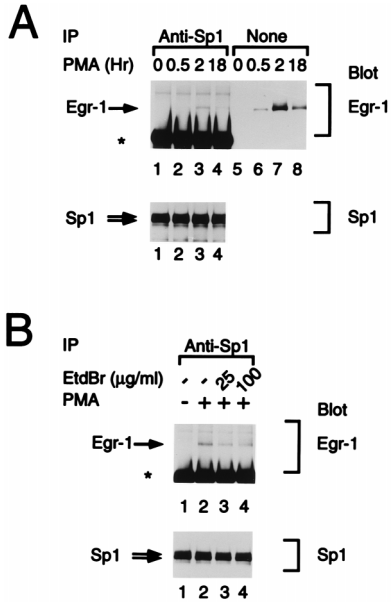

FIG. 8. Association of Egr-1 with Sp1 in Jurkat cells upon PMA stimulation. Jurkat cells were either untreated (lanes 1 and 5) or treated with PMA for 30 min (lanes 2 and 6), 2 h (lanes 3 and 7), or 18 h (lanes 4 and 8). Cells were then lysed, and lysates were immunoprecipitated (IP) with anti-Sp1 (lanes 1 to 4). In lanes 5 to 8, aliquots of lysates were blotted with anti-Egr-1 as controls for the amount of Egr-1 induced at the different time points. (B) Total cell lysates were prepared from Jurkat cells either untreated (lane 1) or treated with PMA for 2 h (lanes 2 to 4). The lysates were either untreated (lanes 1 and 2) or treated with 25 (lane 3) or 100 (lane 4) μg of ethidium bromide (EtdBr) per ml as described in Materials and Methods. Western blotting was performed with antisera specific for Egr-1 or Sp1 and developed by enhanced chemiluminescence. Egr-1 and Sp1 proteins are indicated by arrows, and the immunoglobulin heavy chain is indicated by asterisks.

tion, like IL-1 and tumor necrosis factor alpha, induce Egr-1 expression (15). Therefore, it is possible that Egr-1 is induced by IL-1 and tumor necrosis factor alpha during T-cell activation and serves as an important mediator that regulates expression of a broad range of genes.

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