MOLECULAR AND CELLULAR BIOLOGY, Mar. 1995, p. 1786–1796 Vol. 15, No. 3
0270-7306/95/$04.00+0

Regulation of Cell-Type-Specific Interleukin-2 Receptor α-Chain Gene Expression: Potential Role of Physical Interactions between Elf-1, HMG-I(Y), and NF-κB Family Proteins

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Received 29 September 1994/Returned for modification 16 November 1994/Accepted 21 December 1994

The interleukin 2 receptor α-chain (IL-2Rα) gene is rapidly and potently induced in T cells in response to mitogenic stimuli. Previously, an inducible enhancer between nucleotides −299 and −228 that contains NF-κB and CArG motifs was identified. We now report the characterization of a second essential positive regulatory element located between nucleotides −137 and −64 that binds Elf-1 and HMG-I(Y). This element had maximal activity in lymphoid cells, paralleling the cell type specificity of Elf-1 expression. Transcription from the IL-2Rα promoter was inhibited when either the Elf-1 or the HMG-I(Y) binding site was mutated. Coexpression of both proteins activated transcription of the −137 to −64 element in COS-7 cells. Elf-1 physically associated with HMG-I and with NF-κB p50 and c-Rel in vitro, suggesting that protein-protein interactions might functionally coordinate the actions of the upstream and downstream positive regulatory elements. This is the first report of a physical interaction between an Ets family member and NF-κB family proteins. These findings provide significant new insights into the protein-protein and protein-DNA interactions that regulate cell-type-specific and inducible IL-2Rα gene expression and also have implications for other genes regulated by Elf-1 and NF-κB family proteins.

The interaction of interleukin 2 (IL-2) and IL-2 receptors (IL-2R) critically regulates the magnitude and duration of the T-cell immune response (42, 70). Resting T cells and large granular lymphocytes express intermediate-affinity IL-2R, which consist of the IL-2R β (25 64, 76) and γ (68) chains. γ is encoded by the gene that is defective in X-linked severe combined immunodeficiency (56) and is now referred to as the common γ chain (γ_c), on the basis of its also being shared by the IL-4, IL-7, and IL-15 receptors (21, 32, 33, 55, 62). With cellular activation, the IL-2R α-chain (IL-2Rα) gene is induced (39, 54), converting intermediate-affinity receptors to high-affinity ones (42 70). Thus, whereas IL-2Rβ and γ_c are essential molecules whose dimerization is required for IL-2 signaling (52, 53), IL-2Rα expression is an essential determinant of the acquisition by a cell of full responsiveness to IL-2. Consequently, IL-2Rα transcription is tightly regulated (reviewed reference 38).

Transcription of the IL-2Rα gene is potently induced within 1 h following mitogenic stimulation of normal peripheral blood T cells (41). The gene is also rapidly induced by phorbol 12-myristate 13-acetate (PMA) in Jurkat T cells, which have been extensively used to delineate regulatory regions of the human IL-2Rα promoter (2, 3, 7, 43, 75). Previous studies have demonstrated that inducible IL-2Rα expression is at least partially regulated by a potent enhancer located between nucleotide positions −299 and −228 relative to the major transcription start site (44), herein termed positive regulatory region I (PRRI) (Fig. 1A). This enhancer contains NF-κB and CArG motifs, binding sites which play important roles in activation of the IL-2Rα gene in response to the transactivator protein (Tax) of human T-cell lymphotropic virus type I (HTLV-I), PMA, and tumor necrosis factor alpha (2, 3, 8, 43, 46, 75). The sequence of this enhancer, however, is not well conserved in the murine IL-2Rα gene (67), and furthermore, internal deletions within the IL-2Rα promoter suggested the presence of other positive regulatory elements more proximal to the promoter (unpublished observations).

We now report the characterization of a transcriptional regulatory element located between nucleotides −137 and −64 (PRRII) (Fig. 1A). This element contains binding sites for at least two DNA-binding proteins: an Ets family protein, Elf-1 (72), and the nonhistone chromatin-associated proteins, HMG-I(Y) (14, 30, 48). Deletion or mutation of the binding sites for these proteins profoundly reduced IL-2Rα gene transcription, even in the presence of an intact upstream enhancer (PRRI), and overexpression of Elf-1 and HMG-I together in COS-7 cells activated transcription from PRRII. Elf-1 specifically bound to NF-κB1 (p50) and c-Rel in vitro, suggesting that these protein-protein interactions may mediate the transcriptional coordination between PRRI and downstream PRRII. The characterization of PRRII has, therefore, significantly enhanced our understanding of the molecular regulation of the IL-2Rα gene and opens up new avenues for understand-

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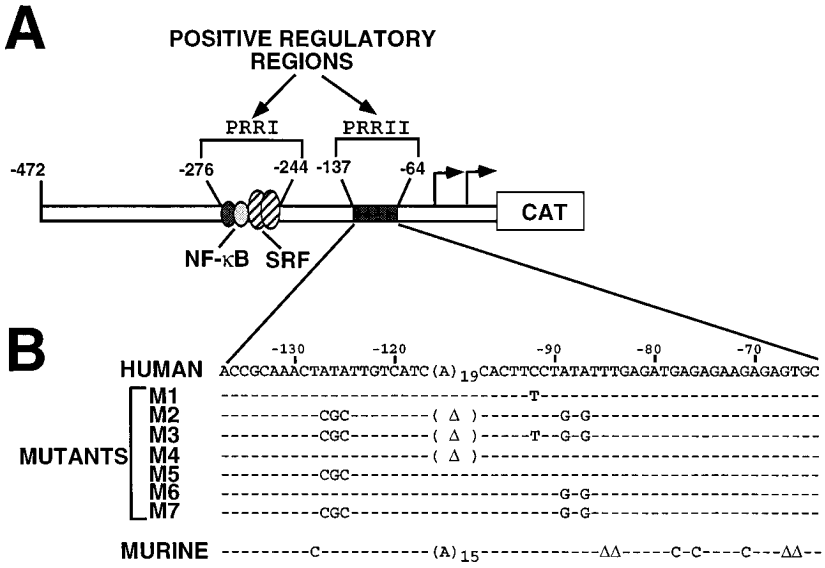

FIG. 1. (A) Schematic diagram of the human IL-2Rα5′ regulatory region between nucleotides −472 and +109, including the upstream and downstream positive regulatory regions (PRRs). The upstream enhancer (PRRI) was originally defined as −299 to −228, but a smaller fragment from −276 to −244 spanning the NF-κB and CArG motifs exhibits similar activity. SRF can bind to the CArG motif in PRRI (75). The newly identified −137/−64 positive regulatory element is designated PRRII. (B) Sequences of the human −137 to −64 region, Elf-1 and HMG-I(Y) mutants (M1 to M7), and the corresponding region from the murine IL-2Rα gene. Locations of mutations: M1, Elf-1 site; M2, all three HMG-I(Y) sites; M3, Elf-1 and all three HMG-I(Y) sites; M4, major HMG-I(Y) site; M5, HMG-I(Y) binding site between −128 and −123; M6, putative HMG-I(Y) binding site between −90 to −85. M7, double mutant containing the M5 and M6 mutations. –, nucleotide identical to that in the human sequence; Δ, deletion.

ing IL-2Rα expression in response to different physiological stimuli.

MATERIALS AND METHODS

Cell culture. Jurkat E6.1, MT-2, and Kit 225 T cells were cultured in RPMI (Gibco-BRL), and GM637 and COS-7 cells were cultured in Dulbecco modified Eagle medium (Gibco-BRL), each containing 10% fetal bovine serum and 100 U of penicillin per ml, 100 U of streptomycin per ml, and 2 mM glutamine. Human peripheral blood mononuclear cells were isolated from buffy coats or leukapheresis of healthy donors by density gradient centrifugation. Purified T cells were isolated from peripheral blood mononuclear cells with nylon wool (26) and cultured in RPMI–10% fetal bovine serum for 48 h prior to stimulation with PMA (20 ng/ml) or PMA (20 ng/ml) plus ionomycin (4 μg/ml).

Nuclear extracts, antibodies, and purified protein. Nuclear extracts were prepared (10) and protein concentrations were determined with a Bio-Rad protein assay kit. R4641 anti-human Ets-1 (45), anti-Ets-2 (18), and anti-HMG-I(Y) antibodies 1 and 2 have been described previously (11, 16). The anti-human Elf-1 antiserum was produced by immunizing rabbits with a glutathione *S*-transferase (GST)–Elf-1 fusion protein.

Electrophoretic mobility shift assays (EMSAs). Binding reaction mixtures (20 μl) contained 2 μl of in vitro-translated proteins or 2 μg of T-cell nuclear extracts, 10,000 cpm of probe (0.1 to 0.2 ng), 2 μg of poly(dI-dC) in 10 mM Tris HCl (pH 7.5), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 50 mM KCl, 1.25 mM dithiothreitol, 1.1 mM EDTA, and 15% glycerol. Following incubation on ice for 30 min, DNA-protein complexes were analyzed on 5% polyacrylamide gels (acrylamide/bisacrylamide ratio, 40:1) run in Tris-borate buffer at 150 V for 2.5 h at room temperature. In some experiments, 2 μl of specific antiserum was incubated with protein for 30 min on ice prior to the

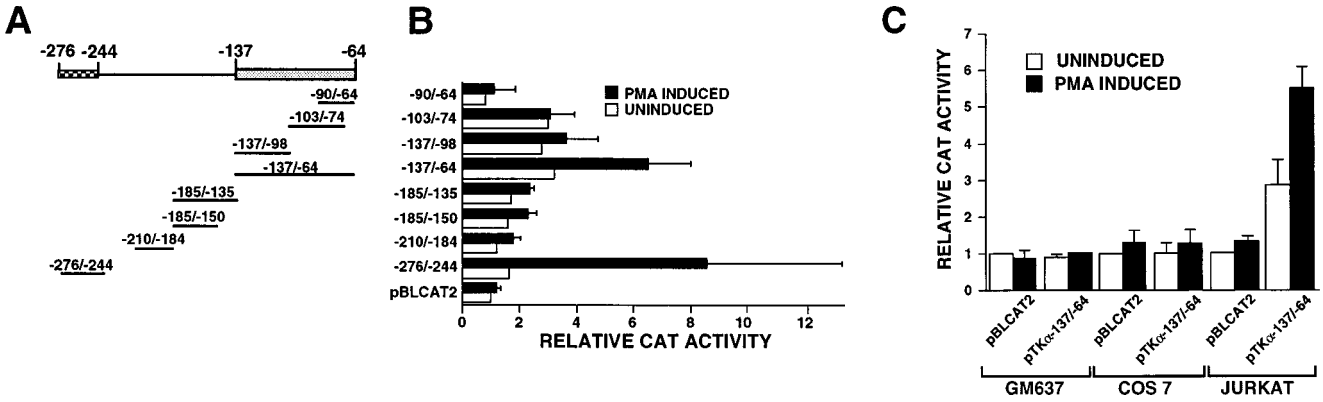

FIG. 2. Identification of a novel positive regulatory element in the IL-2Rα promoter. (A) Schematic of the −276 to −64 region and fragments used to analyze the region between −210 and −64. (B) Summary of CAT assays in Jurkat cells performed with the constructs shown in panel A. The activity of pBLCAT2 without PMA treatment was assigned a value of 1, and the activities of the other constructs are expressed as fold increase over this activity. (C) Summary of CAT assays using the control vector pBLCAT2 and pTKα−137/−64 in GM637, COS-7, and Jurkat cells (means and standard errors of the means for two independent experiments for GM637 and COS-7 cells and three assays for Jurkat cells). Successful transfection in GM637 and COS-7 cells was confirmed by the fact that pTKκB3 (45) and pSV2CAT constructs exhibited significant activity (data not shown).

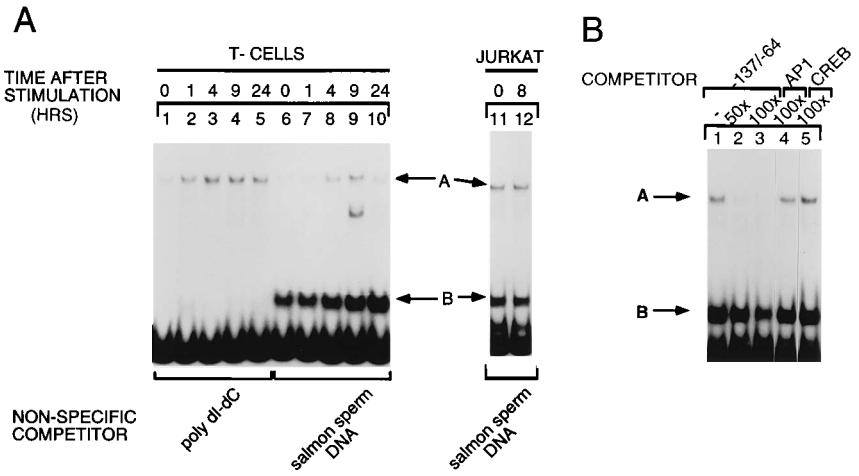

FIG. 3. Inducible binding of nuclear proteins to the ^{32}P-labeled −137/−64 probe in normal T cells. (A) Binding reaction mixtures contained 2 μg of poly(dI-dC) (lanes 1 to 5) or 1 μg of sonicated salmon sperm DNA (lanes 6 to 12) and nuclear extracts. Extracts were prepared from T cells (lanes 1 to 10) or Jurkat cells (lanes 11 and 12) induced with 20 ng of PMA per ml for the indicated periods. A more detailed time course confirmed the lack of inducibility of complexes A and B in Jurkat cells. (B) Specific binding of complexes A and B. Induced Jurkat nuclear extracts were incubated with a 0-, 50-, or 100-fold molar excess of unlabeled −137/−64 oligonucleotide or a 100-fold molar excess of either the AP1 digonucleotide or the CREB oligonucleotide and sonicated salmon sperm DNA prior to addition of probe.

addition of probe. After an additional 15-min incubation, samples were analyzed on gels. For competition assays, excess unlabeled oligonucleotides were incubated with protein for 30 min on ice; a ^{32}P-labeled DNA probe was then added, and the incubation continued for a further 15 min on ice.

Oligonucleotides, plasmids, and in vitro mutagenesis. Double-stranded oligonucleotides PRRI, PRRII, and M1 to M7 and all the subfragments depicted in Fig. 2A were synthesized (DNA/RNA model 392 synthesizer; Applied Biosystems Inc.) with *Hin*dIII (5′) and *Bam*HI (3′) ends and then cloned between these sites in pBLCAT2 (47).

IL-2Rα mutant M1 was generated by PCR mutagenesis (51) of the −472 to +109 wild-type IL-2Rα promoter construct (60) using an oligonucleotide, 5′-AAAAAAAAAACACTTTCTATATTTGAGATGAG-3′, which changes the C at position −92 to a T (underlined). IL-2Rα M4 (deletion of the −116 to −96 region) was generated with the Bio-Rad Muta-Gene kit and the oligonucleotide 5′-AACTATATTGTCATCCTTCCTATATTTGAG-3′. Both mutants were confirmed by DNA sequencing. The double-stranded oligonucleotide competitors in Fig. 5B were as follows (the GGAA, CRE, and AP-1 motifs are underlined; mutated bases are boldfaced): MSV LTR, 5′-AGCTTCTCGGAGAGCGGAAGCGCGCG-3′ (24); E74, 5′-AGCTTCTCTAGCTGAATAACCGGAAGTAACTCATCG-3′ (77); NFAT, 5′-AGCTTAGAAAGGAGGAAAAACTGTTTCATACAGG-3′ (72); mNFAT, 5′-AGCTTAGAAA**CG**T**CC**TAAAACTGTTTCATACAGG-3′ (72); CREB, 5′-TCGAGCTCCTAGCCTGACGTCAGAGAGAGAGC-3′ (50); PEA3, 5′-AGCTTCGAGCAGGAAGTTCGG-3′ (80); mPEA3, 5′-AGCTTCGAGCAA**G**AAGTTCGG-3′ (80); AP1, 5′-CGCTTGATGAGTCAGCCGGAA-3′ (35).

The Elf-1 expression vector pcDElf-1 has been described previously (72). All of the Elf-1 deletion mutants were generated by PCR and cloned between the *Bam*HI and *Xba*I sites of pcDNA3 (Invitrogen). The NF-κB p50 construct corresponded to the 1,680-bp *Hin*dIII-*Xba*I fragment (encoding amino acids 1 to 502) of the KBF1 cDNA (31). The c-Rel, RelA (p65), and serum response factor (SRF) cDNAs have been reported previously (4, 57, 61). The HMG-I expression plasmid pRc/CMVHMG-I was constructed by inserting an *Xba*I-*Hin*dIII fragment from pBSHMG-I (30) between these sites in pRc/CMV (Invitrogen).

Transfection and CAT assays. Cells were transfected with DEAE-dextran (65). Transfections of IL-2Rα enhancer or promoter constructs were performed with 5 or 10 μg of DNA, respectively. Cotransfection experiments with COS-7 cells were performed with Lipofectamine (Gibco-BRL) according to the manufacturer's instructions by using 5 μg of reporter and 3 μg of each expression plasmid. The total DNA in the cotransfection experiments was kept constant with pUC13 DNA. Following transfection, cells were maintained in medium for 24 to 30 h and then were treated with 0 or 20 ng of PMA per ml for 12 to 14 h. Cell extracts were prepared by three cycles of freeze-thawing in 0.25 M Tris-HCl, pH 7.5. Extracts were clarified at 65°C for 10 min and assayed for protein concentrations with the Bio-Rad protein assay kit. Equal amounts of protein were used in chloramphenicol acetyltransferase (CAT) assays. Acetylated forms of chloramphenicol were separated by thin-layer chromatography and quantitated with a Molecular Dynamics PhosphorImager.

In vitro transcription and translation reaction. In vitro transcription and translation reactions were carried out with the Eukaryotic in vitro translation kit or the TNT transcription/translation kit (both from Promega, Madison, Wis.). For the former, pCDneo-Elf-1 (72) pBSHMG-I and pBSHMG-Y (17, 30) plasmids were linearized at *Xho*I, *Xba*I, and *Bam*HI sites, respectively. For the latter, plasmids were not linearized.

Methylation and ethylation interference assays. pTKα−137/−64 was linearized at either end with *Hin*dIII or *Bam*HI and labeled with Klenow (New England Biolabs), by using [α-^{32}P]dATP and [α-^{32}P]dGTP (both at 3,000 Ci/mmol and both from Amersham). The labeled insert was released by digesting with *Bam*HI or *Hin*dIII as appropriate and gel purified. Typically, 0.5×10^6 cpm of end-labeled probe and 25 μg of nuclear extracts were used in binding reactions. Methylation and ethylation interference assays were performed as described elsewhere (1, 66).

DNase I footprinting assay. Isolated −137 to −64 fragments, radiolabeled at either the bottom strand 5′ *Hin*dIII site or the top strand 3′ *Bam*HI site, were incubated with recombinant HMG-I at various molar ratios of protein to DNA and digested with DNase I as described previously (59). The optimal enzyme concentration and digestion time for each preparation were determined empirically. DNA was fractionated on a 9% sequencing gel (acrylamide/bisacrylamide ratio, 37.5:1) with a buffer containing 90 mM Tris, 90 mM borate, and 2 mM EDTA (pH 8.3) (1× TBE).

GST retention and c-Rel and HMG-I association assays. The GST-Elf-1-Tag plasmid was constructed by inserting the myc-tag epitope (15) between amino acids 5 and 6 of the Elf-1 sequence by PCR mutagenesis (51), and this Elf-myc-tag cDNA was cloned between the *Sma*I and *Xho*I sites of pGEX 2Tplink (created by inserting the polylinker 5′-GATCCCGGGGCGGCCGCTCGAGG-3′ between the *Bam*HI and *Eco*RI sites of pGEX 2T [Pharmacia-LKB]). The recombinant plasmids were verified by DNA sequencing. GST-Elf-Tag protein was made per the manufacturer's instructions (Pharmacia-LKB). GST fusion proteins on beads (15 μl) were incubated for 2 h at 4°C with 3 μl of ^{35}S-labeled in vitro-translated protein in 300 μl of NETN binding buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 0.1% bovine serum albumin (BSA) and 0 or 400 μg of ethidium bromide per ml (34). The beads were washed five times in binding buffer, and the proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For Fig. 10A, 10^7 MT-2 cells were lysed in 1 ml of 50 mM Tris (pH 8.0)–120 mM NaCl–0.5% Nonidet P-40–protease inhibitors for 15 min on ice. Cellular lysates were clarified by centrifugation at 17,680 × *g* for 10 min at 4°C, and 400 μl of lysate was incubated with 30 μl of GST–Elf-1 Sepharose beads in 300 μl of NETN buffer. The GST retention assay was performed as described above, and the specifically bound proteins were separated on an 8 or 10% Tris-glycine minigel (Novex) and Western blotted (immunoblotted) with anti-NF-κB p50 or c-Rel antiserum by enhanced chemiluminescence (Amersham). Fifty microliters of c-Rel (1–300) coupled to agarose (Santa Cruz Biotechnology) or HMG-I coupled to agarose via its C terminus was incubated with 3 to 5 μl of ^{35}S-labeled in vitro-translated Elf-1 proteins in 300 μl of NETN containing 1 mg of BSA per ml for 2 h. The beads were then processed as described above for the GST retention assay, and coprecipitated proteins were visualized by autoradiography. All other reactions were resolved on 8 to 16% Tris-glycine minigels. The Elf (207–303) reaction for the c-Rel coprecipitation assay was resolved on an 18% Tris-glycine minigel.

RESULTS

Identification of a new positive regulatory element in the IL-2Rα gene. To investigate the existence of important elements downstream of PRRI, we cloned a series of IL-2Rα fragments (Fig. 2A) upstream of the thymidine kinase promoter in pBLCAT2 (47) and assayed their abilities to promote transcription in Jurkat T cells (Fig. 2B). These studies revealed that the −137 to −64 construct (pTKα−137/−64) exhibited significantly higher levels of transcription in unstimulated and PMA-stimulated Jurkat cells than did the control plasmid, pBLCAT2 (Fig. 2B). Truncation of the −137 to −64 region diminished transcriptional activity, and therefore we assigned −137 and −64 as the boundaries of this novel IL-2Rα positive regulatory region (PRRII) (Fig. 1A), the sequence of which is shown in Fig. 1B. Since IL-2Rα expression is restricted principally to lymphoid cells, it was noteworthy that pTKα−137/−64 was not transcriptionally active in two nonlymphoid cell lines, GM637 and COS-7 (Fig. 2C); thus, its expression appeared to exhibit cell type specificity.

Identification of nuclear factors that bind to PRRII. Factors binding to PRRII were identified by EMSAs with nuclear extracts from unstimulated and PMA-stimulated normal and Jurkat T cells (Fig. 3A). When poly(dI-dC) was used as the nonspecific competitor, only complex A was observed (lanes 1 to 5), whereas in the presence of sonicated salmon sperm DNA (lanes 6 to 12) or poly(dG-dC) (data not shown), the faster-mobility complex B was also detected. Complex A was constitutively expressed in Jurkat cells (Fig. 3A, lanes 11 and 12). In contrast, it was only weakly detected in resting normal T cells but increased following mitogenic stimulation and then declined by 24 h (Fig. 3A). Complex B was also constitutively present in Jurkat cells (lanes 11 and 12). In normal T cells, it was modestly induced and continued to increase for at least 24 h (lanes 6 to 10). Both complexes were specific, as demonstrated by the ability of unlabeled −137/−64, but not AP1 or CREB, oligonucleotides to inhibit their formation (Fig. 3B, lanes 4 and 5 versus 3). The additional band at the 9-h time point in T-cell nuclear extracts (Fig. 3A, lane 9) was variably detected, and its identity is unknown. Complexes A and B were also induced in normal T cells stimulated with PMA and ionomycin, and analogously to the situation for Jurkat, they were constitutively expressed in three other lymphoid cell lines tested (MT-2 and Kit-225 T cells and YT-1 natural killer-like cells) (data not shown).

Complex A contains Elf-1. To identify the nucleotides contacted in complex A, we performed methylation (Fig. 4A) and ethylation (Fig. 4B) interference assays. Methylation of the bottom strand G residues at positions −91 and −92 (Fig. 4A, lane 4) strongly diminished binding activity (Fig. 4A, lane 4). The ethylation interference assays confirmed these results and additionally showed strong protection of nucleotides −88, −90, −93, −94, −95 and weaker protection of nucleotides −85 and −86 on the bottom strand (Fig. 4B, lane 4) and −87 and −89 on the top strand (lane 2). The protected nucleotides (Fig. 4C) span a GGAA purine-rich motif (−91 to −94), suggesting that complex A contained an Ets family protein. Members of this family have been shown to regulate a number of lymphoid-tissue-specific genes (22, 28, 78; for a review, see references 6, 49, and 63).

In order to identify the Ets family protein contained in complex A, we performed antibody supershift and oligonucleotide competition analyses. Antibodies to Ets-1 and Ets-2 did not block or supershift complex A (Fig. 5A, lanes 3 and 4).

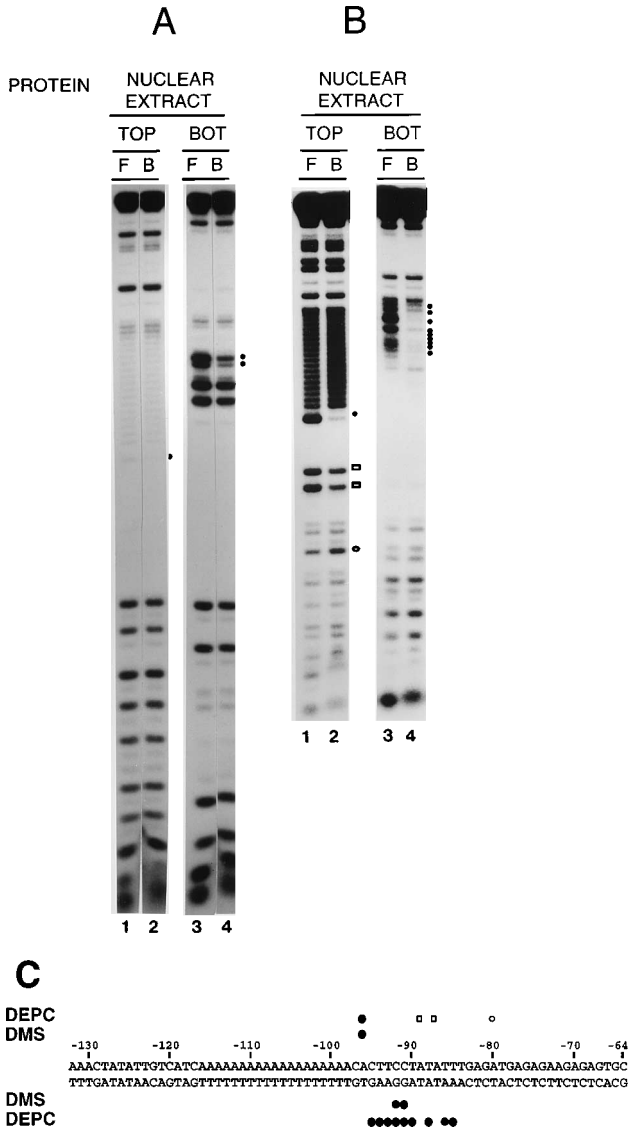

FIG. 4. Methylation and ethylation interference analyses of complex A. Double-stranded −137 to −64 probes labeled on the top (lanes 1 and 2) or bottom (lanes 3 and 4) strand were partially methylated (A) with dimethyl sulfate (DMS) or ethylated with diethylpyrocarbamate (DEPC) (B) and Jurkat nuclear extracts bound. Free (F) and bound (B) probes from complex A were excised from the gel, treated with piperidine, lyophilized, resolved on 9% denaturing gels, and autoradiographed. (C) Summary of the data from panels A and B. Strong (solid circles) and weak (squares) interferences are indicated. The A residue at position −80 is hyperethylated by DEPC (open circle).

Furthermore, a polyomavirus enhancer (PEA3) oligonucleotide capable of binding Ets-1 and Ets-2 did not inhibit complex A formation (Fig. 5B, lane 8). The Moloney murine sarcoma virus long terminal repeat (MSV LTR) (lane 2) and NFAT (lane 4) oligonucleotides competed to a small extent, while the *Drosophila* E74 oligonucleotide (lane 3) blocked complex A formation. CREB (lane 6), AP1 (lane 11), and mutant NFAT (lane 5) oligonucleotides did not compete. This specificity pattern suggested that the T-cell-specific protein Elf-1, which is closely related to the *Drosophila* E74 protein (72, 79), was the most likely Ets family protein binding to PRRII in vitro. In support of this hypothesis, an antiserum specific for Elf-1 inhibited complex A formation (Fig. 5C, lane 2 versus lane 1).

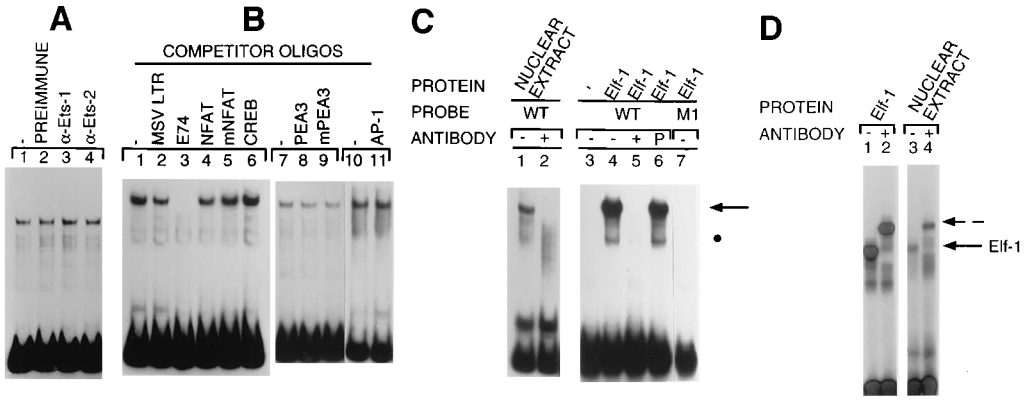

FIG. 5. Elf-1 is a component of complex A. (A) Binding reaction mixtures containing 2 μg of nuclear extracts from T cells that had been stimulated with PMA (20 ng/ml) plus ionomycin (4 μg/ml) were preincubated with no antibody (lane 1), preimmune serum, anti-Ets-1 (α-Ets-1) antibody R4641, or anti-Ets-2 (α-Ets-2) antibody prior to the addition of a ^{32}P-labeled −137/−64 probe. (B) Competition for complex A binding by various transcription factor binding sites. Prior to the addition of probe, T-cell nuclear extracts were preincubated without competitor oligonucleotides (oligos) (lanes 1, 7, and 10) or with 50 ng of unlabeled MSV LTR, E74, wild-type or mutant (m) NFAT oligonucleotide, CREB, wild-type or mutant PEA3 oligonucleotide, or an AP-1 oligonucleotide (lane 11). (C) T-cell nuclear extracts were preincubated with salmon sperm DNA and either no antibody or an anti-Elf-1 antibody prior to the addition of the wild-type (WT) −137 to −64 probe. Binding reaction mixtures containing unprogrammed lysate (lane 3) or Elf-1 cRNA-programmed lysate (lanes 4 to 7) were preincubated with no antibody, preimmune serum (P), or anti-Elf-1 antibody prior to the addition of M1 mutant or wild-type −137 to −64 probe. The upper Elf-1 complex (arrow) comigrates with complex A from nuclear extracts; a faster-migrating Elf-1 specific complex is often seen with in vitro-translated Elf-1 (dot). (D) In vitro-translated Elf-1 protein or T-cell nuclear extracts were preincubated with an anti-Elf-1 monoclonal antibody (lanes 2 and 4) prior to the addition of wild-type −137 to −64 probe. Dashed arrow, new supershifted Elf-1 complex.

Additionally, EMSAs using Elf-1-programmed rabbit reticulocyte lysates yielded a major complex that comigrated with complex A (Fig. 5C, lane 4). A second faster-mobility complex, which is also related to Elf-1 (and perhaps represents a degradation product of Elf-1), since its formation was inhibited by the antiserum to Elf-1 (lane 5) but not by preimmune serum (lane 6), was often seen. Furthermore, a monoclonal antibody to Elf-1 can supershift the Elf-1 complex to a new complex (Fig. 5D). Methylation and ethylation interference assays of both complexes seen with in vitro-translated Elf-1 protein yielded results identical to those obtained with complex A from nuclear extracts (data not shown). As expected, mutation of the GGAA sequence (M1) abrogated Elf-1 binding activity (lane 7). The above data together indicate that complex A contains Elf-1.

Complex B contains HMG-I(Y). The IL-2Rα promoter contains several AT-rich sequences (29, 40, 67), the longest of which is 19 bp long (−116 to −98) and is contained within PRRII. Several lines of evidence indicated that complex B (Fig. 3) contained HMG-I(Y) proteins, low-molecular-weight (11,000 to 12,000) high-mobility group proteins that bind in the minor groove of AT-rich sequences. First, complex B was not readily detected when poly(dI-dC) was used as a nonspecific competitor (Fig. 3A), a feature exhibited by HMG-I(Y) (58). Second, in vitro-translated HMG-I and HMG-Y bound to PRRII (Fig. 6A, lanes 2 and 3). Third, purified recombinant human HMG-I protein yielded a complex that comigrated with complex B (Fig. 6B, lane 4 versus lane 1). Finally, an antiserum to HMG-I(Y), but not preimmune serum, supershifted complex B (Fig. 6B, lane 2 versus lane 3). In fact, two supershifted ternary complexes were formed, with purified HMG-I or nuclear extracts, with the lower one approximately superimposed on complex A (lane 2 versus lane 3 and lane 5 versus lane 6). A second polyclonal antiserum to HMG-I(Y), which yields only one supershifted complex with purified HMG-I(Y) (16), allowed us to visualize the disappearance of complex A. In the case of the lymphotoxin gene (16), this antibody supershifts the faster-mobility complex containing HMG-I(Y) and blocks the formation of more slowly migrating complexes. The effect of this antiserum on complex A suggests that this complex contains not only Elf-1 but also HMG-I(Y) or protein(s) antigenically similar to HMG-I(Y) (Fig. 6C, lane 2 versus lane 1). Taken together, these results suggest that HMG-I(Y) can exist as a cocomplex with Elf-1 in complex A. The addition of control preimmune serum to the binding reaction mixtures did not alter the EMSA binding pattern seen with nuclear extract alone (Fig. 6C, compare lanes 3 and 1).

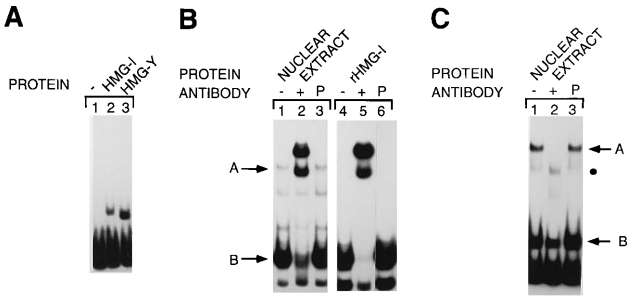

FIG. 6. HMG-I(Y) binds to PRRII. (A) EMSAs using unprogrammed (−) or HMG-I- or HMG-Y-programmed lysates. (B) Jurkat nuclear extracts or purified recombinant human HMG-I (rHMG-I) was preincubated with no antibody, anti-HMG-I(Y) antibody 1, or preimmune serum (P) prior to the addition of probe. Purified rHMG-I comigrated with complex B from nuclear extracts. Similar results were obtained when either poly(dG-dC) or salmon sperm DNA (data not shown) were used. (C) EMSAs were performed as for panel B with salmon sperm DNA as the nonspecific competitor. Binding reaction mixtures were incubated with no antibody, anti-HMG-I(Y) antibody 2, or preimmune serum. The position of the supershifted HMG-I(Y)–DNA–antibody ternary complex is indicated (dot).

To define the DNA sequences in PRRII contacted by HMG-I(Y), we performed DNase I footprinting using purified HMG-I protein and the −137/−64 oligonucleotide (Fig. 7). Under the conditions used, two AT-rich regions between −128 to −123 (region A in Fig. 7A) and the poly(A·T) stretch between nucleotides −116 and −98 (region B in Fig. 7A and B) were protected. To confirm that these sites were contacted by HMG-I(Y), we performed EMSAs with wild-type and mu-

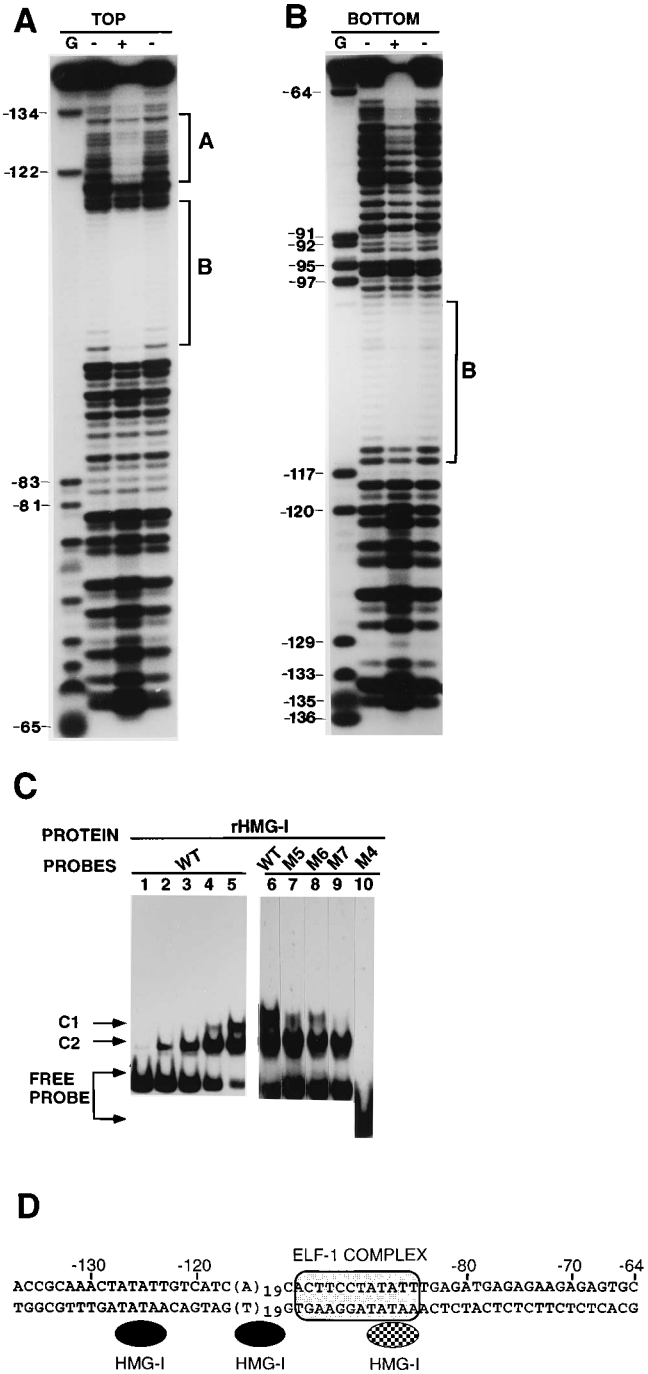

FIG. 7. DNase I footprint analysis of recombinant human HMG-I (rHMG-I) bound in vitro to double-stranded −137 to −64 probes labeled on the top (A) or bottom (B) strand. Reactions were performed at a 20:1 protein/DNA molar ratio. G, Maxam-Gilbert G lanes; −, free DNA; +, DNA plus HMG-I. The two protected regions (A and B) are indicated (C) EMSAs of wild-type (WT) and mutant (Fig. 1B) ^{32}P-labeled −137/−64 probes. Binding reaction mixtures in lanes 1 to 5 contained 10, 25, 50, 75, and 100 ng of rHMG-I protein, respectively, and lanes 6 to 10 each contained 75 ng of rHMG-I protein. The M4 probe exhibited faster mobility since it is shorter than the other probes. (D) Schematic representation of HMG-I(Y) binding sites identified by DNase I footprinting (solid ellipses; −116 to −98 and −128 to −123) and a third possible site suggested by EMSAs with oligonucleotides mutated at these sites (stippled ellipse; −90 to −84). HMG-I(Y) bound with highest affinity to the −116 to −98 region.

tant −137/−64 oligonucleotides. The sequences of the mutant oligonucleotides used in this study are depicted in Fig. 1B. Titrations of purified HMG-I suggested that two complexes of different affinities could form with the −137/−64 oligonucleotide (Fig. 7C, lanes 1 to 5). Complex C2 represented a protein-DNA interaction of high affinity, since it was apparent with the addition of only 10 ng of purified protein, while retarded complex C1 was observed only at higher concentrations of HMG-I, indicating lower-affinity interactions. The formation of complex C1 was affected by mutation of the TATATT sequences located either at nucleotides −128 to −123 (M5) or at nucleotides −90 to −85 (M6), and mutation of both these sites simultaneously (M7) resulted in the complete abrogation of complex C1. Deletion of the −116 to −98 poly(A · T) run (M2) inhibited the formation of both complexes, C1 and C2, suggesting that this is the major HMG-I(Y) binding site. Thus, we believe that there are three potential binding sites for HMG-I(Y) in PRRII (Fig. 7D) that have various degrees of affinity for the protein.

Elf-1 and HMG-I(Y) can interact in the absence of DNA. Since it appeared that both Elf-1 and HMG-I(Y) were contained in complex A, we investigated if Elf-1 could associate with HMG-I(Y), which was covalently coupled to agarose beads. Full-length Elf-1 [Elf (1–619)] efficiently bound to HMG-I, deletion of the N-terminal 99 amino acids [Elf (100–619)] did not diminish this interaction, while removal of amino acids 1 to 343 [Elf (344–619)] greatly decreased binding to HMG-I (Fig. 8). An extremely weak signal was seen with Elf (344–619), but its significance is unclear. A construct containing residues 1 to 303 retained the ability to interact; however, deletion of the conserved Ets homology domain between amino acids 207 and 303 (37) resulted in a total abrogation of interaction with HMG-I (lanes 9 and 10). In agreement with the major interaction surface mapping to the Ets domain of Elf-1, constructs that contained amino acids 100 to 304 (lanes 11 and 12), 151 to 303 (lanes 13 and 14), and 207 to 303 (lanes 15 and 16) were all able to efficiently interact with HMG-I. As a control for specificity of the Elf-1–HMG-I interaction, ^{35}S-labeled in vitro-translated IL-7 receptor did not bind to HMG-I (data not shown). We have also found that GST–Elf-1 can specifically retain a Gal4–HMG-I fusion protein, but not Gal4, in the absence of contaminating DNA (unpublished data). Taken together, these results suggest that Elf-1 can interact with HMG-I in the absence of DNA through its conserved Ets domain (residues 207 to 303). The role of HMG-I(Y) in facilitating the binding of other transcription factors has been previously shown (12, 71); however, we found no evidence for an increase in Elf-1 binding to PRRII when HMG-I was added (data not shown).

The Elf-1 and HMG-I(Y) binding sites are essential, and together Elf-1 and HMG-I(Y) can transactivate the −137/−64 element in COS-7 cells. We next investigated the role of HMG-I(Y) and Elf-1 in activating transcription from PRRII, using cotransfection experiments with COS-7 cells. As shown in Fig. 9A, HMG-I or Elf-1 alone weakly transactivated pTKα−137/−64; however, together they potently increased transcription of this reporter construct. This synergistic effect required intact binding sites for Elf-1 and HMG-I(Y), as demonstrated by diminished activity of constructs mutated at either or both of these sites (pTKαM1, pTKαM2, and pTKαM3; see Fig. 1B for sequences of the mutants). Additionally, we also tested the abilities of Elf-1 and HMG-I to transactivate a mutant construct, M4 or one in which only the two HMG-I(Y) sites identified by DNase I (−128 to −123 and −116 to −98) were mutated, and obtained results similar to those with M2 (data not shown). Elf-1 bound to mutants M2 and M4 at levels

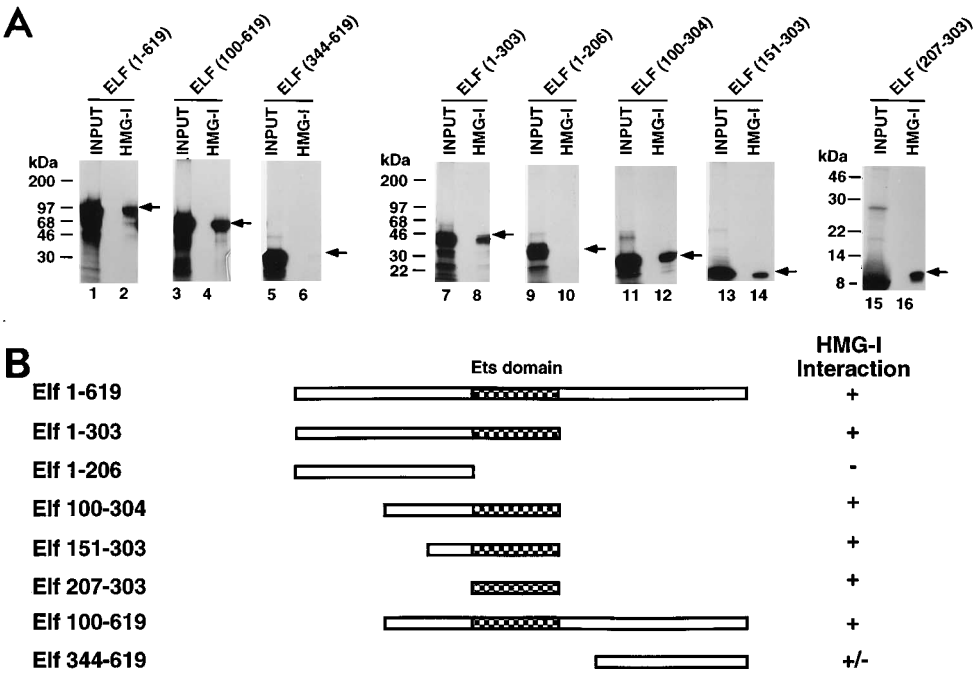

FIG. 8. The Ets domain of Elf-1 mediates its interaction with HMG-I. ^{35}S-labeled N- and C-terminal deletion mutants of Elf-1 were produced by in vitro transcription-translation reactions and incubated with HMG-I coupled to Sepharose beads. (A) The bound Elf-1 proteins were analyzed by SDS-PAGE. Odd lanes, amounts of input proteins; even lanes, bound proteins. The protein products expected in each case are indicated (arrows). (B) Schematic diagram of the Elf-1 derivatives and summary of the interaction results. +, specific interaction; −, no specific interaction; +/−, very weak interaction.

similar to those for the wild-type −137/−64 oligonucleotide (data not shown). These results suggest a functional cooperativity between Elf-1 and HMG-I in the activation of transcription from PRRII.

Corresponding to these results with COS cells, the Elf-1 and HMG-I(Y) binding sites were required for maximal activity of the −472 to +109 IL-2Rα promoter construct in Jurkat T cells. Mutation of either the Elf-1 binding site (IL-2RαM1) (Fig. 1B) or the dominant HMG-I(Y) binding site (IL-2RαM4) (Fig. 1B) significantly decreased the overall levels of transcription (Fig. 9B). Analogous mutations in pTKα−137/−64 also resulted in decreased activity (data not shown). The fact that mutation of the Elf-1 site profoundly diminished IL-2Rα promoter activity, even when the −276 to −244 upstream enhancer (PRRI) was intact, indicates the existence of important functional interactions between Elf-1 and factors that bind to PRRI.

Elf-1 interacts with NF-κB p50 and c-Rel proteins in the absence of DNA. Since both PRRI and PRRII are essential for IL-2Rα promoter activity, we speculated that these regions functionally coordinate with each other via interactions between Elf-1 and a factor(s) that binds to PRRI. As shown in Fig. 10A, GST–Elf-1 bound NF-κB p50 and c-Rel contained in MT-2 T-cell extracts but not the p105 precursor form of p50. We obtained similar results with in vitro-translated proteins (Fig. 10B, lanes 6, 9, and 12) and additionally demonstrated that GST–Elf-1 did not interact with NF-κB p65 (lane 3) or SRF (lane 15) at the concentrations used in these experiments. The interactions of Elf-1 with NF-κB p50 and c-Rel were not mediated by contaminating DNA, since similar results were obtained when experiments were performed in the presence of ethidium bromide. We consistently observed a small increase in the amount of NF-κB p50 retained by GST-Elf columns in the presence of HMG-I, consistent with HMG-I(Y) acting as "molecular glue" between Elf-1 and NF-κB p50 (data not shown). We confirmed the interaction of Elf-1 with c-Rel but not p65 by coprecipitation experiments (Fig. 10C). These results are provocative in view of the suggestion that c-Rel and NF-κB p50 synergize to activate the IL-2Rα promoter during T-cell activation (69).

To identify the region of Elf-1 necessary for interaction with c-Rel, N- and C-terminal deletion constructs were labeled with ^{35}S-methionine by in vitro transcription-translation and evaluated for their ability to associate with c-Rel (amino acids 1 to 300) coupled to agarose beads. As shown in Figure 10D, full-length Elf-1 protein [Elf (1–619)] associated with c-Rel (lanes 1 and 2). Deletion of either the N-terminal 100 amino acids (lanes 3 and 4) or the C-terminal half of the protein (lanes 5 and 6) did not abrogate interaction with c-Rel. Truncated Elf-1 proteins that contained only residues 100 to 304 (lanes 9 and 10) or 207 to 303, the Ets domain (lanes 11 and 12), could still bind, while deletion of the Ets domain (lanes 7 and 8) prevented interaction with c-Rel. Thus, the highly conserved Ets domain of Elf-1 appears to be necessary and sufficient for its interaction with c-Rel as well as with HMG-I.

DISCUSSION

We have delineated a novel regulatory element (PRRII) essential for IL-2Rα gene expression. Unlike the previously defined −299 to −228 upstream enhancer (PRRI), PRRII is more strictly conserved between mice and humans (Fig. 1) (67). Elf-1 and HMG-I(Y) bound to PRRII in an inducible manner. Although inducible Elf-1 binding to other promoter regions has been observed (22, 78, 81), to our knowledge this is the first demonstration of mitogen-induced binding of HMG-I(Y) to DNA. This finding may be physiologically sig-

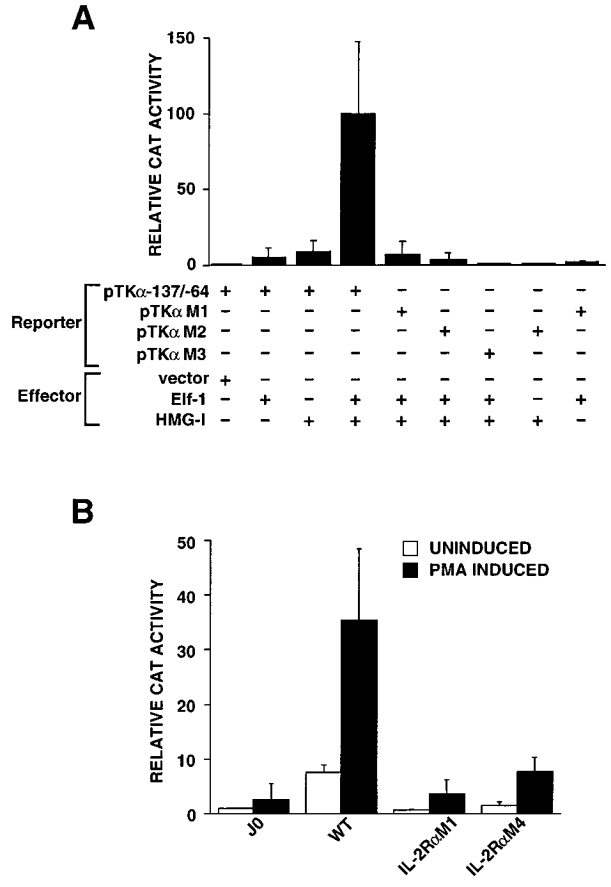

FIG. 9. Functional significance of Elf-1 and HMG-I(Y) for IL-2Rα gene regulation. (A) Elf-1 and HMG-I(Y) are required for maximal transcription from the −137/−64 element in COS-7 cells. The data are means and standard errors of the means derived from three independent experiments. The relevant reporter and effector plasmids are indicated. The activity of pTKα−137/−64 cotransfected with the empty vector was assigned a value of 1, and all other values are expressed as fold increase over this value. In addition the activities of pTKαM1, pTKαM2, and pTKαM3 cotransfected with the empty vector were subtracted from the values obtained for cotransfection with Elf-1 and HMG-I for each of these reporter plasmids. (B) The Elf-1 and HMG-I(Y) sites are required for IL-2Rα expression in Jurkat cells. Cells were transfected with wild-type (WT) or mutant promoter −472 to +109 IL-2Rα–CAT reporter constructs. J0, control plasmid JYMCAT-0. The M1 and M4 mutations in IL-2RαM1 and IL-2RαM4 are defined in Fig. 1B. The data are means and standard errors of the means for three independent experiments. The activity of J0 without PMA was assigned a value of 1, and all other activities are expressed as fold increase over this value.

nificant given that transcription of the human HMG-I(Y) gene is stimulated by PMA plus ionomycin (17).

Whereas HMG-I(Y) proteins are widely expressed (14), Elf-1 is lymphoid tissue specific (72), in agreement with its playing an important role in regulating the cell-type-specific expression of the IL-2Rα gene. Although Elf-1 is implicated in the regulation of other T-cell-specific genes (22, 78, 81), this study is the first to demonstrate direct activation of a gene by overexpression of Elf-1. Interestingly, transfection of Elf-1 alone was insufficient to activate transcription from PRRII in COS-7 cells, even though these cells contain endogenous HMG-I(Y), suggesting that a threshold level of HMG-I(Y) is required. The synergy between Elf-1 and HMG-I(Y) is consistent with the fact that many Ets family proteins activate transcription in conjunction with other proteins (9, 13, 27, 73).

In contrast to the situation with the beta interferon gene promoter, in which HMG-I(Y) proteins facilitate the binding of transcriptional activator proteins to DNA (12, 71), we have found no evidence for augmented Elf-1 binding to PRRII by HMG-I(Y). However, both Elf-1 and HMG-I(Y) can be detected in the same complex in EMSAs and can interact with each other in the absence of DNA. The Ets domain of Elf-1 was necessary and sufficient to mediate its interaction with HMG-I. Thus, the interaction with HMG-I(Y) may allow Elf-1 to maintain a transcriptionally active conformation. Another function of HMG-I(Y) in this scenario could be to act as molecular glue in bridging interactions between Elf-1 and NF-κB and perhaps with other DNA-binding proteins that bind in the immediate vicinity. Furthermore, its ability to bend DNA (36) could also facilitate protein-protein interactions, thereby promoting the formation of an active transcription complex.

PRRI contains a κB site that plays an important functional role for IL-2Rα expression in normal peripheral blood lymphocytes (46; data not shown) and in HTLV-I-transformed MT-2 cells (75). We have shown that deletion or mutation of the Elf-1 and HMG-I(Y) binding sites in PRRII greatly diminished promoter activity even when the highly inducible PRRI was intact, in agreement with the possibility that protein-protein interactions coordinate the activities of the PRRI and PRRII. In keeping with this idea, we have shown that Elf-1 can interact with NF-κB p50 and c-Rel, both of which have been implicated in the inducible expression of the IL-2Rα gene (69). Furthermore, the interaction of Elf-1 with NF-κB p50 is modestly enhanced by the presence of HMG-I(Y), which has also been shown to associate with NF-κB p50 (12, 71). Therefore, Elf-1 may be involved in the selective binding and stabilization of specific NF-κB family proteins to PRRI during the course of T-cell activation. Elf-1 can interact with NF-κB p50 but not its precursor p105, suggesting that the interaction interface is masked in p105.

Elf-1 is the first Ets family protein now known to interact with members of the NF-κB family of proteins, a finding that may have direct implications for other Elf-1 regulated genes (37). Mapping of the interaction surface of Elf-1 with c-Rel revealed that, as with HMG-I(Y), the Ets domain was necessary and sufficient to mediate this interaction. Therefore, the highly conserved Ets domain may be a common interaction surface generating multiple protein-protein interactions. In keeping with this theme, we hypothesize that other specific Ets family proteins may also interact with NF-κB family proteins, thus providing a mechanism for generating further diversity in inducible gene expression in these situations. Indeed, our preliminary studies with the Ets domain of another member of this family of proteins indicates that it can also interact with NF-κB family proteins in vitro.

Thus, the mitogen-stimulated expression of the IL-2Rα gene requires coordinated interactions between multiple regulatory proteins. The model depicted in Fig. 11 is consistent with our data and is somewhat analogous to models proposed for the T-cell receptor α-chain gene and beta interferon genes, in which HMG proteins architecturally facilitate the formation of stereospecific enhancer complexes (5, 19, 20, 23, 74). In contrast to these other systems, however, cell-type-specific regulation and inducible IL-2Rα regulation involve interactions not only between adjacent proteins within a single transcriptional regulatory domain but also between two spatially separated transcriptional regulatory domains, presumably resulting in the formation of a highly specific nucleoprotein complex. It seems likely that PRRII contributes more towards basal promoter activity and cell type specificity of expression whereas PRRI

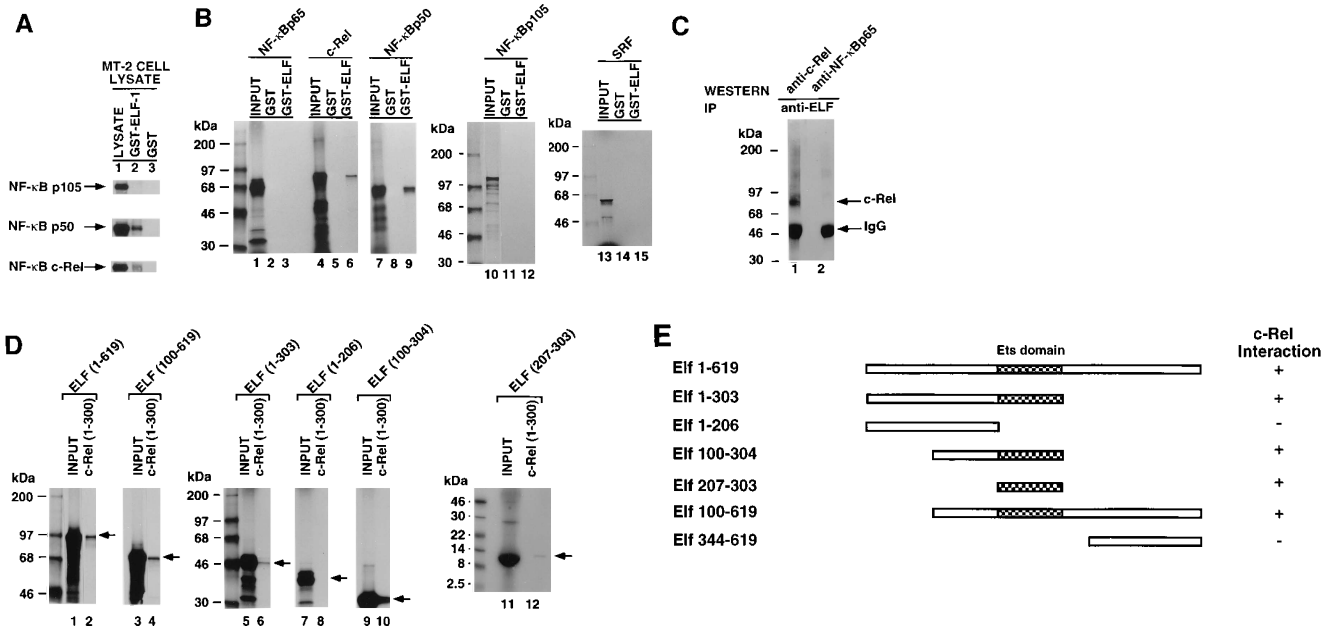

FIG. 10. Elf-1 physically associates with NF-κB p50 and c-Rel but not with NF-κB p65, p105, or SRF. (A) MT-2 T-cell extracts were incubated with GST–Elf-1 or GST immobilized to glutathione-Sepharose beads. Lane 1 contains one-fifth of the input cell extract. The specifically retained products were visualized with a c-Rel antibody or an anti-N-terminal NF-κB p50 antibody, which can immunoprecipitate both p50 and its precursor, p105. (B) In vitro-translated ^{35}S-labeled NF-κB p50, NF-κB p65, NF-κB p105, c-Rel, and SRF were incubated with GST or GST–Elf-1 immobilized on glutathione-Sepharose beads. Similar results were obtained in control experiments performed in the presence of 400 μg of ethidium bromide per ml. (C) MT-2 T-cell extracts were immunoprecipitated (IP) with a monoclonal antibody to Elf-1, and the coprecipitated proteins were analyzed by Western blotting (immunoblotting) using antibodies to c-Rel or NF-κB p65. The positions of the immunoglobulin G (IgG) heavy-chain and c-Rel bands are indicated. Similar experiments to detect the coprecipitation of NF-κB p50 with Elf-1 were difficult to evaluate since the NF-κB p50 band was expected to be superimposed on the heavy-chain signal. (D) Identification of the domain of interaction of Elf-1 with c-Rel. In vitro-translated ^{35}S-labeled deletion mutants of Elf-1 were incubated with c-Rel protein (amino acids 1 to 300) coupled to agarose. The bound Elf-1 proteins were analyzed by SDS-PAGE. Odd lanes, amounts of input proteins; even lanes, bound proteins. The expected protein products are indicated (arrows). (E) Schematic diagram of the Elf-1 derivatives and summary of the interaction with c-Rel. +, specific interaction; −, no specific interaction.

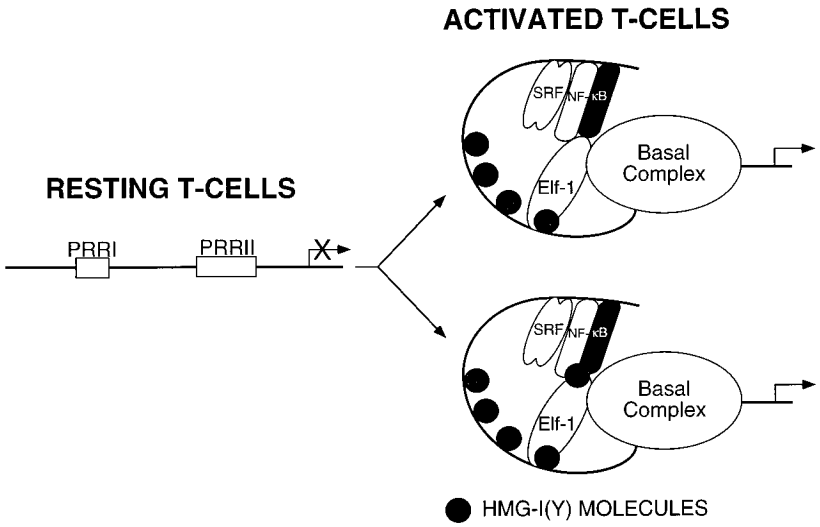

FIG. 11. Model of the IL-2Rα promoter before (resting T cells) and after (activated T cells) mitogen stimulation depicting direct interactions between NFκB, Elf-1, and HMG-I(Y). Two schematics are depicted for the activated state; the upper schematic depicts direct Elf-1–NFκB interactions, whereas the lower schematic additionally shows the possibility that HMG-I may enhance the Elf-1–NF-κB p50 interaction. It is possible that both models depicting the activated state exist at the same time. In addition to the three HMG-I(Y) binding sites depicted in Fig. 7C, we have evidence for a fourth site between nucleotides −183 and −153; hence, four HMG-I(Y) molecules are shown contacting DNA. Although no HMG-I(Y) is shown bound to the promoter in resting T cells, we believe that there is some binding to the major HMG-I(Y) site in these cells.

more potently contributes to the inducibility of the IL-2Rα gene. This hypothesis is consistent with the results in Fig. 9B, showing that mutation of the Elf-1 site significantly diminishes transcriptional activity but has a minimal effect on the PMA inducibility of the construct. Since the IL-2Rα gene is not expressed in unstimulated normal T cells, we hypothesize that the constitutive activity of PRRII in Jurkat cells reflects the endogenous levels of Elf-1 and HMG-I(Y) in Jurkat cells whereas no Elf-1 is found in resting T cells. It will be interesting to determine if the two- to threefold inducibility of PRRII in Jurkat cells results from posttranslational modification(s) of Elf-1. PRRII also appears to play a role in coordinating and amplifying signals received from other parts of the promoter since a point mutation in the Elf-1 binding site is sufficient to abrogate transcription from the IL-2Rα promoter. In this regard, it is relevant that Elf-1 can bind TFIIB in vitro (unpublished observations). Thus, the identification of this novel regulatory region of the IL-2Rα promoter further elucidates the molecular mechanisms that operate to ensure the precise expression of the high-affinity IL-2 receptor.

ACKNOWLEDGMENTS

We thank Roy Pollock, Brian Safer, Robert Adelstein, and Kuan-Teh Jeang for critical comments; Nancy Rice for anti-p50 antiserum and the c-Rel cDNA; Narayan Bhat for the anti-Ets-2 antisera; Alex Bassuk for pcDNA1 Elf (100–619) and pcDNA1 Elf (100–304); Bradley Cobb for the anti-Elf-1 monoclonal antibody; Alain Israel for the NF-κB1 (p50) cDNA; Richard Treisman for the SRF cDNA; Craig Rosen for the RelA (p65) cDNA; Mark Nissen for the HMG-I agarose beads; Takashi Uchiyama for Kit-225 cells; Arthur Weiss for Jurkat E6.1 cells; I. Miyoshi for MT-2 cells; Junji Yudoi for YT cells; and Yumi Imai for Cos-7 cells. This work was supported in part by an NIH grant, RO1 AI29673, to J.M.L.

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