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Characterization of Antigen Receptor Response Elements within the Interleukin-2 Enhancer

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T-cell activation and induction of interleukin-2 (IL-2) expression in human T lymphocytes require both interaction of foreign antigen with the T-cell antigen receptor and protein kinase C (PKC) stimulation. Agents such as phorbol 12-myristate 13-acetate (PMA) that stimulate PKC augment the effects of antigen but are not sufficient for IL-2 activation. By analysis of deletion mutants, we identified three DNA sequences extending from −73 to −89, −217 to −255, and −263 to −279, designated IL-2 sites A, D, and E, respectively, that are required for maximal induction of IL-2 expression. One of these regions, site E, interacted with a protein (NF-IL-2E) present only in the nuclei of cells which have been stimulated. The other two sequences interacted with a protein (NF-IL-2A) that is constitutively expressed in T cells. When multiple tandem copies of either the E site or the A site were placed upstream of the gamma-fibrinogen promoter, they activated expression via this promoter in response to signals initiated at the antigen receptor but not following PMA stimulation. For this reason, we denoted them antigen receptor response elements. The uncoupling of antigen receptor and PKC requirements in these studies indicates that these signal pathways are, at least in part, distinct and integrated at the level of the gene.

Exposure of T lymphocytes to foreign antigen in the proper histocompatibility context induces a complex series of events that lead to cellular division and differentiation. T-cell activation requires both binding of antigen to its receptor at the cell surface and activation of protein kinase C (PKC) (36, 37, 38). The signals generated by these events lead to an increase in intracellular calcium (16, 34, 35), activation of the phosphoinositol pathway (12, 33), and, eventually, the activation of sets of genes in a temporally ordered fashion. The "immediate early" genes include c-*fos* and c-*myc*, while the interleukin-2 (IL-2), IL-2 receptor, and gamma-interferon genes are "early" in that they are activated only slightly later (26). Transcripts from the c-*fos* gene can be detected within 15 min, and c-*myc* transcripts can be detected by 30 min (18); IL-2 and gamma-interferon are first detected by ribonuclease mapping between 30 and 45 min (D. B. Durand, P. J. Utz, and G. R. Crabtree, unpublished data). The "late" genes, activated after several hours, include genes involved in differentiated T-cell function and cell division. These classes of genes vary in their requirements for activation; for example, c-*fos* can be activated by phorbol 12-myristate 13-acetate (PMA) alone (11; Durand et al., unpublished), while IL-2 requires both PKC and antigen receptor stimulation for its expression (37, 38). By studying IL-2 gene activation, we hoped to identify the molecules that convey the signals from the antigen receptor to the target genes.

The human T-cell line Jurkat has provided a useful model of IL-2 gene regulation, because it mimics physiologic T-cell activation and IL-2 expression at several levels. IL-2 expression is controlled largely at the transcriptional level in both Jurkat and peripheral blood T cells (19, 20, 32). In Jurkat, as in normal peripheral blood T cells, the IL-2 gene is not expressed prior to stimulation and activation requires both antigen receptor binding and PKC activation (37, 38). In both Jurkat and peripheral blood T lymphocytes, a nuclease-hypersensitive site is induced approximately 85 base pairs (bp) 5′ to the initiation site of transcription following stimulation with phytohemagglutinin (PHA) and PMA (28). In PHA- and PMA-stimulated Jurkat cells, a 274-bp fragment extending from 52 to 326 bp upstream of the IL-2 transcription initiation site acts as an enhancer element in that it will function in either orientation to activate the expression of a heterologous promoter (6). Fujita et al. (8) have shown that IL-2 sequences between −127 and −319 function independently of orientation to activate the beta-interferon promoter in the murine T-cell line EL-4 following PMA stimulation. Furthermore, after binding of a monoclonal antibody to the Jurkat antigen receptor in the presence of PMA, the −52 to −326 sequence activates expression from a heterologous promoter but fails to do so in a mutant Jurkat cell line lacking cell membrane antigen receptor expression (6). These data indicate that sequences between 52 and 326 bp upstream of the IL-2 transcriptional initiation site receive signals originating at the antigen receptor. In this paper, we demonstrate that multimers of two 30-bp oligonucleotides from the IL-2 enhancer convey enhanced transcription in response to signals from the antigen receptor. Surprisingly, these sequences bind different proteins; one of these is present only in nuclear extracts of stimulated T lymphocytes, while the other is constitutively expressed.

MATERIALS AND METHODS

Plasmid construction. The *Xmn*I-to-*Dra*I (A fragment) and *Dra*I-to-*Ssp*I (B fragment) duplication clones were constructed by isolating the fragments, ligating with *Sph*I linkers, digesting with an excess of *Sph*I enzyme, and repurifying the fragments. The gamma-fibrinogen CAT clone (6) was opened at the *Sph*I site, treated with alkaline phosphatase, and ligated to the A and B fragments in various molar ratios. Candidate clones were selected by the colony hybridization technique, using fragment A and B random-primer labeled

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probes. Clones were then mapped by restriction enzyme analysis and sequenced by the Hattori-Sahaki plasmid-sequencing protocol (13).

The internal deletion mutants were made by digesting the IL-2 CAT plasmid (28) with *Cla*I and *Bam*HI and isolating the *Cla*I-to-*Bam*HI fragment containing the ampicillin resistance gene. The IL-2 CAT plasmid was cut with *Xmn*I and *Bam*HI, and the fragment containing the IL-2 promoter region was isolated and ligated to the *Cla*I-to-*Bam*HI fragment (see above) in the presence of a *Cla*I linker. After purification of the vector from the *Cla*I linker, a *Cla*I digest was performed, and the vector (designated pIL-2 CAT) was closed with T4 DNA ligase. The resulting pIL-2 CAT is essentially a 5′ deletion mutant with IL-2 sequences extending from the *Xmn*I site (converted to a *Cla*I site) at −326 to the 5′ nontranslated region at +47 (converted to a *Hin*dIII site).

Deletions were generated from the *Cla*I and *Hin*dIII ends by opening the vector at either site, treating with BAL 31 for various time periods, phenol extracting, treating with the Klenow fragment of DNA polymerase enzyme, and closing the vector by ligation. The *Cla*I and *Hin*dIII BAL 31 mutants were then characterized on nondenaturing polyacrylamide gels, followed by analysis of end-labeled fragments on denaturing polyacrylamide gels. Appropriate *Cla*I and *Hin*dIII BAL 31 mutants were then cut with *Xho*I and *Xmn*I, and the complementary bands were eluted from agarose gels and ligated. Clones were then analyzed on agarose gels and sequenced by the Hattori-Sahaki method (13), using oligonucleotide primers. Each clone has one *Xho*I linker (CCTCGAGG) except for ID 59/45, which has two linkers.

The pIL-2/L plasmid was constructed by first partially digesting pJD204 (5) with *Bam*HI. The single-cut vector was gel purified, the sticky ends were filled in with the Klenow fragment of DNA polymerase, and a *Cla*I linker was inserted. The resulting vector, with a *Cla*I site inserted at the *Bam*HI site 5′ to the luciferase gene, was called pSV2-CLUC. To provide further versatility of this vector, we introduced the *neo* gene from pSV2neo. To ensure a unique *Hin*dIII site in the final vector, pSV2neo was digested with *Hin*dIII and the ends were filled in with the Klenow fragment. The *Nde*I-to-*Bam*HI fragment of this vector (pSV2neo-HIII) was isolated, filled in with the Klenow fragment, and ligated to *Aat*II-cut, T4 polymerase-treated pSV2CLUC (pSV2CLUCneo). The pIL-2/L vector was made by ligating the *Cla*I-to-*Sca*I and *Sca*I-to-*Hin*dIII fragments of pSV2CLUCneo to the *Hin*dIII-to-*Cla*I IL-2 fragment from pIL-2 CAT.

The multimers of IL-2 sequences were constructed by digesting the Fib CAT construct (6) with *Sph*I, treating with alkaline phosphatase, and ligating the linearized plasmid to the *Eco*RI-to-*Sph*I polylinker from pUC19. The resulting plasmid was digested with *Nru*I, phenol-chloroform extracted, digested with *Sac*I, and then treated with T4 DNA polymerase and closed with T4 DNA ligase. The product, pγ42-CASI, was digested with *Sma*I, treated with alkaline phosphatase, and ligated overnight to 40 ng of a given oligonucleotide that had been previously kinased and treated with T4 DNA ligase. The multimers were digested with *Kpn*I and *Hin*dIII, end labeled, and analyzed on denaturing polyacrylamide gels. Clones of interest were then sequenced by the Maxam and Gilbert method (22) to determine the number and orientation of the oligonucleotides.

Transfection. Jurkat cells (generously provided by Richard Robb) were transfected by the DEAE-dextran/chloroquine method as described previously (6) except that the DEAE-dextran and chloroquine incubation was at 37°C (on a rotary shaker at 75 rpm) and the DEAE-dextran concentration was 0.1875 mg/ml.

Stimulation and cell harvest. Jurkat T cells were stimulated for 8 h at approximately 40 h after transfection with 2 μg of PHA and 50 ng of PMA per ml or with other reagents as described previously (6).

Following stimulation, the cells were centrifuged at 200 × *g* for 5 min and suspended in 10 ml of phosphate-buffered saline at 4°C. The cell suspension was divided in half in 15-ml conical tubes. The cells for CAT assay were sedimented and suspended in 1 ml of Tris-EDTA-NaCl (6) and transferred to a 1.5-ml Eppendorf tube. The cells were then pelleted in an Eppendorf 5415 centrifuge at 200 × *g* for 6 min. After aspiration of the supernatant, 150 μl of 25 mM Tris base (pH 7.8) was added, and the cells were frozen on dry ice while the harvest of the luciferase extract was continued. Cells for the luciferase assay were sedimented, suspended in 1 ml of phosphate-buffered saline, and transferred to a 1.5-ml Eppendorf tube. After being spun down, the cells were suspended in 50 μl of 100 mM potassium phosphate (pH 7.8) with 1 mM dithiothreitol. The CAT and luciferase extracts were then freeze-thawed three times (dry ice to 37°C bath with moderately vigorous vortexing for 30 s after each thaw cycle). Cell debris was then spun down in the Eppendorf Microfuge at 4°C for 15 min. The extract supernatant was transferred to another Microfuge tube, and the volume was precisely measured. Extracts that were not immediately assayed were quick-frozen on dry ice and stored at −80°C.

CAT and luciferase assays. The CAT assay was performed as described previously (6), except that assays were performed on one-half of the extract for 4 h in the presence of 2 μl of [^{14}C]chloramphenicol.

The luciferase assay was performed in duplicate, using 15% of the extract per assay performed as described previously (5), by using a Monolight 2001 Luminometer (Analytical Luminescence, San Diego, Calif.).

Preparation of nuclear extracts. Nuclear extracts were prepared as described by Ohlsson and Edlund (25). All steps were performed at 0 to 4°C. The cells were harvested by centrifuging at 200 × *g* for 6 min, washed once with phosphate-buffered saline, and centrifuged once more at 200 × *g* for 6 min. The cell pellet was suspended in three times its volume in buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.8], 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]). After a 10-min incubation on ice, the cells were broken by homogenization in a Teflon Dounce homogenizer and checked by microscopy for lysis of cells. The cell homogenate was then centrifuged at 2,000 rpm for 10 min (Sorvall RT6000B rotor). The crude nuclear pellet was suspended in 6 ml of buffer A, and 4 M ammonium sulfate (pH 7.9) was added to a final concentration of 0.3 M. The nuclei were lysed by gentle rocking at 4°C for 30 min. The DNA was pelleted at 42,000 rpm for 60 min at 4°C in a Beckman 50 Ti rotor. The supernatant was recovered, and solid ammonium sulfate was added up to 0.2 g/ml. The precipitated proteins were collected by centrifugation at 20,000 rpm for 15 min at 4°C in a Beckman 50 Ti rotor. The protein pellet was suspended in 0.3 to 1 ml of buffer C (50 mM HEPES [pH 7.8], 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10% glycerol). The protein suspension extract was cleared by centrifugation at 10,000 rpm for 10 min and dialyzed against buffer C for 4 h at 3°C, and aliquots were frozen in liquid nitrogen and stored at −80°C.

When extracts were prepared from stimulated cells, cultures were treated with PHA (5 μg/ml) and PMA (50 μg/ml) for 2 h at 37°C.

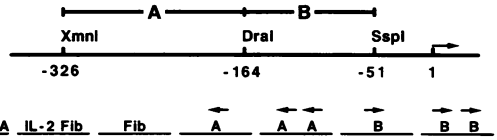
Gel mobility shift assay. The gel mobility shift assay was performed essentially as described (7, 29, 30). The binding reactions were carried out in a final volume of 15 μl consisting of 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, and 5 μg of poly(dI-dC). Various amounts of extracts were added with 0.1 to 0.5 ng of end-labeled DNA fragments at room temperature for 60 min. The DNA-protein complexes were then analyzed on 4% polyacrylamide gels (acrylamide:bisacrylamide, 30:1) in low-ionic-strength buffer (6.7 mM Tris hydrochloride, 3.3 mM sodium acetate, 1 mM EDTA [pH 7.5]).

Footprint analysis. The footprint analysis procedure was based on previous methods, with modifications (3, 9, 17). Binding reaction mixtures consisted of the following components in a final volume of 50 μl: 25 mM HEPES (pH 7.8), 50 mM KCl, 0.05 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5% glycerol, 2.5 μg of poly(dI-dC), 2 to 5 ng of end-labeled DNA fragment, and up to 25 μl of nuclear extract. Various amounts of extract were preincubated with poly(dI-dC) in the reaction mixture for 20 min at room temperature, and then the probes were added and the incubation was continued for an additional 60 min at room temperature. Following addition of $MgCl_2$ to a final concentration of 5 mM, DNase I was added and digestion was carried out for 60 s at either room temperature or 0°C. The amount of DNase I added was determined empirically. The reactions were stopped by adding 100 μl of stop buffer (100 mM Tris [pH 8.0], 100 mM NaCl, 1% sodium dodecyl sulfate, 10 mM EDTA, 100 μg of proteinase K per ml, 100 μg of tRNA per ml). The reaction mixture was incubated at 37°C for 15 min and then was extracted successively with 150 μl of phenol, 150 μl of phenol-chloroform (1:1), and 150 μl of chloroform and ethanol precipitated. The fragments were analyzed on 6% polyacrylamide-urea gels.

RESULTS

Single copies of 5′ and 3′ IL-2 enhancer sequences lack function, whereas duplication reconstitutes inducible enhancer activity. In our previous studies, the 275-bp IL-2 sequence between −52 and −326 (relative to the transcription start site) was the smallest fragment tested that could enhance transcription in either orientation through a heterologous promoter (6). Given the precedent of duplicated functional subunits in other promoters and enhancers (14, 15) and the presence of several similar sequences within the IL-2 enhancer (6), we examined whether subfragments of the −52 to −326 sequence could activate transcription from a heterologous promoter when duplicated in tandem. Specifically, the *Xmn*I-to-*Dra*I fragment (A) (Fig. 1) was inserted in duplicate immediately 5′ to the gamma-fibrinogen promoter, which was in turn linked to the chloramphenicol acetyltransferase gene (see Materials and Methods). The same was accomplished for the *Dra*I-to-*Ssp*I fragment (B), as well as for various combinations of the A and B fragments. The gamma-fibrinogen promoter contains a single SP1 binding site and a TATA box and initiates transcription correctly both in vivo and in vitro (2). Jurkat cells were cotransfected with these constructs and an internal control vector, pRSV/L. In this vector, designed by de Wet and co-workers (5), the Rous sarcoma virus (RSV) promoter-enhancer activates the expression of the luciferase indicator gene whose product can be assayed in cell protein extracts. The extracts are incubated in the presence of luciferin and ATP, and the emitted light is measured with a luminometer. Following transfection, the cells were either stimulated with PHA and PMA or were left nonstimulated. The pRSV/L vector is expressed under both constitutive and stimulated (PHA plus PMA) conditions. Since the RSV promoter is sensitive to PHA-plus-PMA stimulation (approximately eightfold increase upon stimulation [D. B. Durand, M. R. Bush, and G. R. Crabtree, unpublished data]), unstimulated points must be compared separately from stimulated values. This poses no difficulty since none of the IL-2 expression vectors examined in these experiments had constitutive activity. Eight hours after stimulation, cell extracts were prepared and assayed for CAT and luciferase activity. Percent chloramphenicol conversions were then normalized according to the value of the luciferase activity and expressed relative to the control plasmid IL-2FibCAT. This control plasmid consists of the IL-2 enhancer fragment (−326 to −52) linked in the forward orientation to a gamma-fibrinogen promoter sequence (−54 to +36 in the gamma-fibrinogen gene) and, in turn, linked to the CAT gene (6). Certain combinations of each of the A and B fragments inducibly activated expression through the fibrinogen promoter. Certain AA, BB, and AB combinations were strongly induced upon stimulation with PHA and PMA, whereas no significant constitutive activity was seen above that of the gamma-fibrinogen promoter alone (Fig. 1). As demonstrated previously, a single copy of the A or B fragment conveyed no inducible activity (Fig. 1; 6). Further double-fragment combinations without inducible activity included BA, AA, and BA (data not shown). These may have been negative for trivial reasons such as mutations in the CAT gene or elsewhere, though the more likely explanation is that the duplicated sequences must achieve a specific spatial orientation with respect to one another for function.

Sequences within the IL-2 enhancer required for inducible expression defined by internal deletion mutations. Having demonstrated the existence of a duplicated functional subunit within the IL-2 enhancer, we sought to define further the active sequences within the A and B fragments. To do so, we constructed a series of internal deletion mutants (IDs), using CAT as the indicator gene. Each mutant was transfected into



PHA/PMA	IL-2 Fib	Fib	A	A A	B	B B	A B
+	100*	2.3	2.2	20.4	3.0	36.7	34.2
-	2.4	6.9	4.0	3.3	4.4	3.9	4.4

FIG. 1. The IL-2 enhancer consists of two functionally equivalent elements. Fragments designated A and B from the IL-2 5′-flanking region were cloned in the indicated combinations and directions at position −54 of the gamma-fibrinogen promoter which is, in turn, ligated at position +36 to the CAT gene. This promoter contains a TATA box (4) and a single SP1 binding site (J. G. Morgan et al., unpublished data) and initiates transcription correctly both in vivo and in vitro (2). Jurkat cells were cotransfected with the indicated construct, and the RSV luciferase plasmid and percent conversions were normalized for transfection efficiency and expressed relative to the wild-type enhancer fused to the fibrinogen promoter (IL-2 Fib) stimulated with PHA and PMA. As noted in Results, constructs with fragment orientations $\overrightarrow{BA}$, $\overrightarrow{AA}$, and $\overleftarrow{BA}$ gave less than 2% conversion in either stimulated or nonstimulated cells.

the Jurkat T-cell line, using pRSV/L as the internal transfection control, and 40 h later the cells were then either stimulated with PHA and PMA or left nonstimulated. Cell extracts were prepared and assayed for both CAT and luciferase activity. After correction of the raw percent [^{14}C]chloramphenicol conversion for luciferase expression, the activity of each mutant was expressed relative to the activity of the full-length pIL-2 CAT construct (100%; IL-2 sequence, +44 to −326) (see Materials and Methods). It should be noted that Fujita and co-workers (8) have shown that transcripts are properly initiated from IL-2 promoter sequences deleted 5′ to 3′ to −319. We have confirmed this initiation site by linking IL-2 sequences from −576 to +44 to the c-*myc* coding region and expressing this construct in transgenic mice (J.-P. Shaw, E. Lacy, F. Bachmann, and G. R. Crabtree, unpublished data).

None of the mutants was constitutively active. The unstimulated expression of the wild-type and mutant plasmids was equal to the background CAT activity detectable in nontransfected Jurkat cells (data not shown). The stimulated values in Fig. 2 show that mutants 317/286, 208/174, 153/121, and 116/88 have wild-type activity. In contrast, mutants 279/263, 255/217, 159/151, 89/73, 82/73, 68/63, and 59/44 show diminished activity, implying that these sites are important in the function of the IL-2 enhancer and implicating them as potential binding sites for transcriptional factors. To further verify the stimulation results, we used a second internal control, a vector with the nonmutated IL-2 sequence 5′ to the luciferase gene. The expression of this vector should be subject to the same influences as the IDs, provided that critical sequences have not been deleted. The vector was made by placing the IL-2 sequence from pIL-2 CAT into a luciferase vector (5) with two polyadenylation signals positioned 5′ to the IL-2 sequence (pIL-2/L; see Materials and Methods). De Wet and co-workers have shown that these polyadenylation sequences act as termination signals and, therefore, block readthrough transcripts because of cryptic promoters within vector sequences. This pIL-2/L construct was cotransfected with the IDs. Only the stimulated state

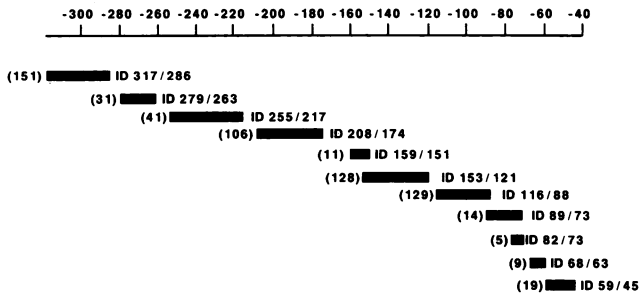


FIG. 2. Deletion analysis of the IL-2 enhancer. Each block represents an internal deletion mutation, the 5′ and 3′ deleted bases of which are given by the numbers on the right of each block. The upper line gives the positions relative to the IL-2 transcription initiation site. The results of cotransfections into stimulated Jurkat cells have been normalized for transfection efficiency and expressed as a percentage relative to the wild-type IL-2 enhancer (in parentheses to left of each block). The results in the figure are the averages of two to four determinations, using either the pRSV/L (signified by R below) or the pIL-2/L (signified by I below) internal control, that were as follows: 317/286, 169 (R) and 132 (I); 279/263, 26 (R), 34 (R), 22 (R), and 43 (I); 255/217, 39 (R), 30 (R), and 53 (I); 208/174, 128 (R), 88 (R), and 102 (I); 159/151, 12 (R) and 9 (I); 153/121, 112 (R) and 143 (I); 116/88, 141 (R) and 116 (I); 89/73, 12 (R), 13 (R), and 18 (I); 82/73, 5 (R) and 6 (I); 68/63, 11 (R) and 6 (I); and 59/45, 27 (R), 13 (R), and 18 (I).

was examined with this internal control, since pIL-2/L does not have unstimulated activity (data not shown). The levels of stimulated expression of the mutants normalized with the pIL-2/L control were comparable to those normalized with the pRSV/L control (Fig. 2, legend).

Interactions of nuclear proteins with functionally important IL-2 enhancer sequences. Having preliminarily identified several functionally important sequences, we sought to determine whether these sequences interacted specifically with nuclear proteins by preparing nuclear extracts from stimulated and nonstimulated cells and examining the sequences protected from DNase I digestion by these extracts. Five protected regions were identified within the IL-2 enhancer and were designated A to E, with A nearest the TATA box (see Fig. 8 for a schematic diagram). Four of these corresponded to sites deleted in the functionally defective mutants. Footprints were identified over sites 89/73 (IL-2A), 255/217 (IL-2D), and 279/263 (IL-2E) (footprints −65 to −93, −242 to −256, and −263 to −289, respectively; Fig. 3A, lane 4; Fig. 3B, wt). An additional footprint overlaying the 159/151 mutant was identified (−157 to −135; observed with extracts from stimulated but not from nonstimulated cells) but is not further pursued in this work (data not shown; see Discussion). These footprints were absent in the corresponding functionally defective deletion mutants (Fig. 3A and B), demonstrating the sequence specificity of these interactions. Proteins bound to sites D and E independently, since the mutant lacking the D site (DEL 13, Fig. 3B) still bound the E factor (NF-IL-2E) and the mutant lacking the E site (DEL 5, Fig. 3B) still bound the D factor (NF-IL-2D). The A site footprint occurred independently of D and E; D and E footprints were detected by using the *Cla*I-to-*Hin*fI fragment which lacks the A site, and the A footprint was detected by using the *Hin*dIII-to-*Alu*I fragment lacking the D and E sites (Fig. 3).

To relate the factors binding sites A, D, and E, we performed competition studies using nuclear extracts derived from stimulated Jurkat cells and oligonucleotides −65/−94 (A site) and −257/−286 (E site) in the gel mobility shift assay (see Materials and Methods). The NF-IL-2A retarded complex (Fig. 4, left) is shown in lane 0, whereas unbound probe is seen in lane FP. Competition of NF-IL-2A binding is seen with oligonucleotides −65/−94 (containing the A site; lane B) and −234/−259 (containing site D; lane D), whereas no competition is seen with oligomers −257/−286 (containing site E; lane A) or −2/+27 (lane C). Furthermore, when nuclear extracts (from stimulated or nonstimulated cells) were bound to the −234/−259 oligonucleotide, a complex with a mobility identical to that seen with the −65/−94 oligonucleotide was observed (data not shown), leading to the conclusion that NF-IL-2A and NF-IL-2D have similar binding specificities and may be identical proteins. In contrast, NF-IL-2E binding (Fig. 4, right) competes only with the −257/−286 (containing site E, lane A) oligonucleotide and not with several others. These results indicate that NF-IL-2E is distinct from NF-IL-2A and NF-IL-2D.

Further evidence distinguished NF-IL-2E from NF-IL-2A. When an extract from stimulated rather than nonstimulated cells was used in the footprint assay, the footprint at the E site was extended several bases upstream of the original footprint, whereas the portion of the E site footprint seen with nonstimulated extracts remained unchanged (Fig. 5A). The stimulation-specific extension of the E site footprint was not dependent on the concentration of protein used in the footprinting assay (data not shown). In contrast to

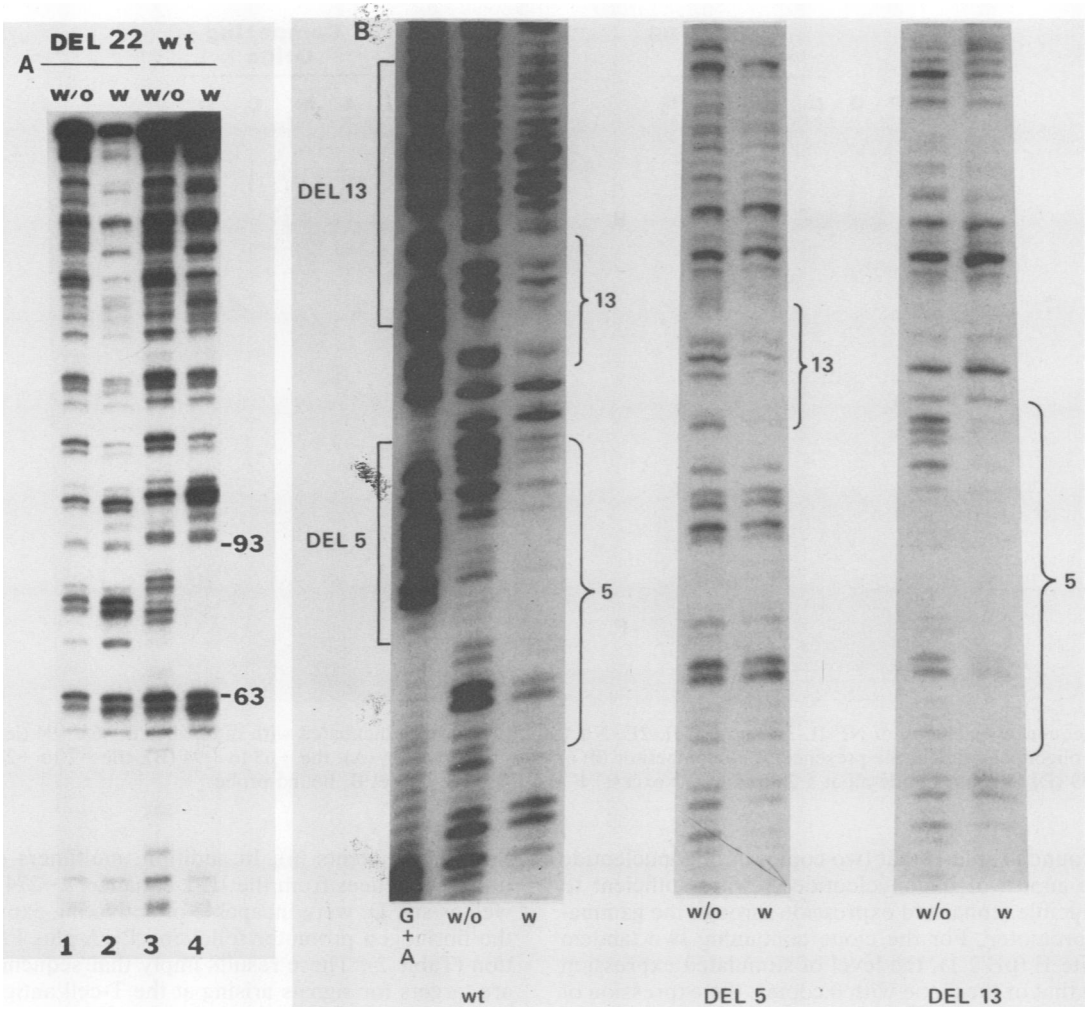


FIG. 3. Functionally defective internal deletion mutations disrupt DNA-protein interactions. DNase I protection results are shown for the wild-type (wt) and deletion (DEL) mutations for internal deletion 22 (−73 to −82) (A) and internal deletion 5 (−279 and −263) and internal deletion 13 (−255 and −217) (B). With (w) and without (w/o) refer to the presence or absence of the proteins extracted from the nucleus of activated Jurkat cells. Bovine serum albumin was added in the absence of nuclear extract (see Materials and Methods). The wild-type footprints extend between −65 and −93 (A) and between −242 to −256 and −263 to −289 (B). G+A, Sequencing reactions.

these stimulation-specific changes in the site E footprint, nuclear extracts prepared from stimulated and nonstimulated cells produced an identical footprint at site A (Fig. 5B). There were stimulation-specific changes involving other sequences (footprint from −188 to −208) at sites not determined to be functionally important in our study of deletion mutants. In addition, there were stimulation-specific DNase I-hypersensitive sites at −135 (Fig. 5B) and −158 (data not shown). These hypersensitive sites presumably reflect secondary changes in DNA conformation due to protein binding interactions at other sites.

The extension of the E site footprint in the presence of stimulated cell extracts could be explained in several ways, one of which is that it represents the binding of a stimulation-specific factor. The appearance of a factor in nuclear extracts of stimulated cells that binds to the E sequence was confirmed with the gel mobility shift assay. Using the E oligonucleotide extending from −257 to −286 (Fig. 6B), we detected a specific retarded band in extracts of cells stimulated with PHA and PMA (lane 2) but not in extracts of nonstimulated cells (lane 3). When higher protein concentrations and longer exposure times were used, a much fainter band migrating closer to the gel origin was seen in nonstimulated extracts (data not shown). Consistent with footprinting results showing no change in the footprint of the A site upon stimulation, there was little difference in the retarded bands observed when the site A oligonucleotide was incubated with either stimulated or nonstimulated nuclear extracts (Fig. 6A, lanes 2 and 3). We conclude that the NF-IL-2A and NF-IL-2E complexes involve different proteins.

Multimers of the IL-2A and IL-2E sequences have inducible enhancer activity. To provide independent evidence that sites A and E, found to be functionally important in the deletion analysis, were involved in the antigen receptor-dependent activation of the IL-2 gene, we examined the ability of these sequences to respond to signals originating at the antigen receptor. Since we knew from our experiments testing the function of one and two copies of the *Xmn*I-to-*Dra*I and *Dra*I-to-*Ssp*I IL-2 fragments that single copies of these sequences failed to enhance the expression of a linked gene, we linked tandem multiples of the A or the E oligonucleotides 5′ to the gamma-fibrinogen CAT promoter and assayed for expression after stimulation with PHA and

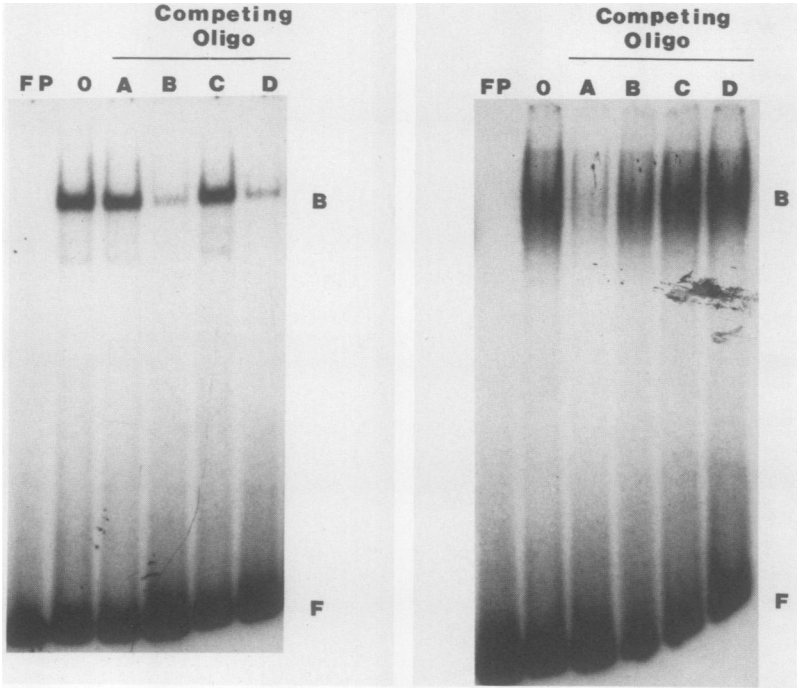


FIG. 4. Sequence specificity of NF-IL-2A and NF-IL-2E. Nuclear extracts were incubated with radiolabeled −65/−94 (left) or −257 to −286 (right) oligonucleotide in the presence of no competitor (0) or the −257 to −286 (A), the −65 to −94 (B), the −2 to +27 (C), and the −234 to −259 (D) oligonucleotide all at a 20-fold molar excess. F and FP, Free probe; B, bound probe.

PMA. We found (Table 1) that two copies of oligonucleotide E and four copies of oligonucleotide A were sufficient to provide inducible, enhanced expression through the gamma-fibrinogen promoter. For the clone containing two tandem copies of site E (pE 2.1), the level of stimulated expression was 10-fold that of the clone with 0 copies; the expression of the site A tetramer (pA 4.1) was 14-fold increased. Neither the A nor the E multimer clones had substantial constitutive activity (data not shown). In addition, a control multimer containing three tandem copies of a nonprotected sequence (Table 1) lacked stimulated activity. It is possible that the A site dimer (pA 2.1) lacked enhanced expression because of a nonproductive spacing of binding sites, since similar restrictions on fragment orientation were seen in the duplication experiments with the *Xmn*I-to-*Dra*I and the *Dra*I-to-*Ssp*I fragments (described above).

Having established that the A and E sequences responded to PHA and PMA induction, we sought to determine the nature of the membrane events that initiate this response. We have previously determined that for maximal induction, the IL-2 *Xmn*I-to-*Ssp*I (−326 to −52) enhancer fragment requires both antigen receptor binding (by monoclonal antibody C350, referred to hereafter as anti-Ti) and PKC stimulation (by PMA); neither signal alone is sufficient (6). Neither the E site nor the A site multimer responds to PMA alone, whereas both A and E site multimers respond to either anti-Ti or PHA alone (Table 2). In the presence of anti-Ti or PHA, PMA may augment the response of the E multimer slightly (Table 2). Of note is the absence of a response of the A and E site multimer clones to stimulation of Jurkat cells by an antibody directed against a Jurkat human lymphocyte antigen (HLA) determinant (anti-HLA) plus PMA; nor is there significant constitutive activity. We have shown previously that the gamma-fibrinogen promoter does not respond to any of the signal combinations used in these experiments (6). In addition, multimers of two additional sequences from the IL-2 enhancer, −274 to −291, as well as site D, were incapable of activating expression from the fibrinogen promoter following PHA-plus-PMA stimulation (Table 2). These results imply that sequences E and A are targets for signals arising at the T-cell antigen receptor, whereas neither sequence responds to PKC stimulation.

Since the A sequence was capable of activating expression by antigen receptor binding when four copies were linked, whereas a single copy of this sequence had no activity, we used the gel mobility shift assay to examine the interactions of nuclear factors with different multiples of the A oligonucleotide. A shifted complex was identified when a fragment containing a single A sequence was used in the binding assay (Fig. 7). The complex formed in the presence of a nuclear but not a cytoplasmic extract, and the addition of cytoplasmic extract to nuclear extracts did not alter binding. When a fragment containing four tandem copies of the site 12 oligonucleotide was used in the binding assay, a dramatic increase in binding was seen; essentially all of the free probe shifted in the presence of 5 μg of nuclear extract from either stimulated or nonstimulated cells. These results suggest a correlation between enhanced binding of NF-IL-2A and enhanced function in the transfection assay.

DISCUSSION

We have previously demonstrated that IL-2 sequences between 52 and 326 bp upstream of the initiation site of transcription are required for enhancement of expression from a heterologous promoter in response to signals from the T-cell antigen receptor and PKC. The present studies indicate that there are functionally related elements at the 5′ and 3′ ends of the IL-2 enhancer. Studies of the simian virus 40 (SV40) enhancer have shown that duplication of either of

two enhancer elements restores activity, while single copies of these elements have weak enhancer activity (14, 15, 39). Similarly, tandem duplication of 5′ and 3′ IL-2 fragments restores the ability to activate a heterologous promoter that is lacking when single fragments are used. These initial experiments suggested that there were functionally equivalent elements at either end of the enhancer. A series of internal deletion mutations led to the identification of several functionally important sequences within these 5′ and 3′ IL-2 fragments, as shown in Fig. 2.

Since the function of IDs may reflect a secondary effect upon binding of a factor at a distant site or a disruption of phasing of protein-binding sites (31), we sought evidence of direct protein interactions with the deleted sites, using the footprinting and gel mobility shift assays. Of five footprints identified within the IL-2 enhancer, three of the sites were found to be functionally important. Site A, D, and E mutants

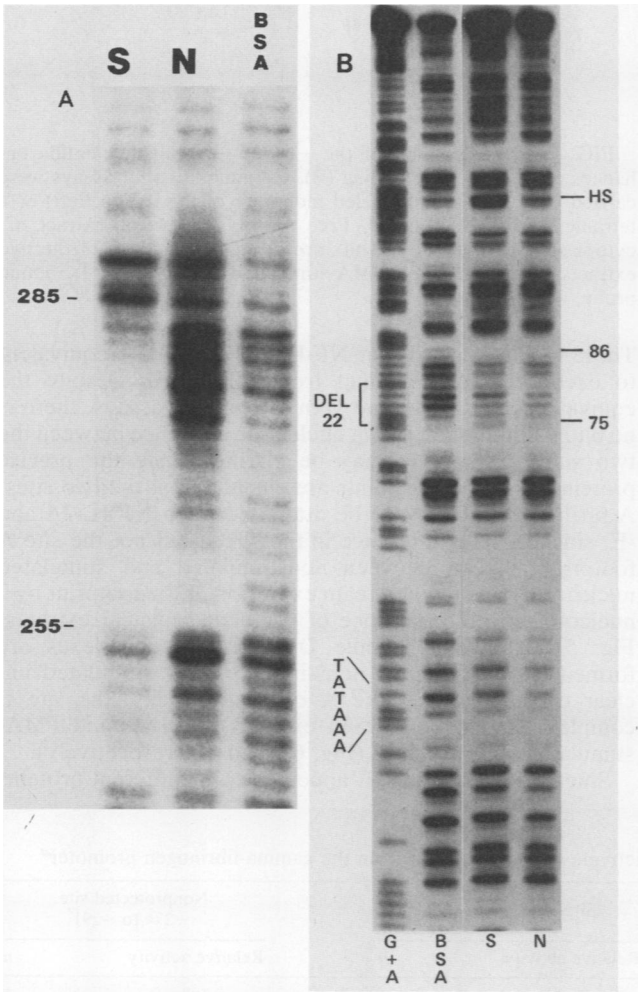


FIG. 5. The footprint spanning the −255 to −285 sequence is altered with nuclear extracts of activated compared to nonactivated T cells, while the footprint spanning the −65 to −93 sequence is unchanged upon activation. (A) DNase I protection studies, using a *Cla*I-to-*Hin*fI fragment extending from −326 to −147. (B) DNase I protection studies of the wild-type IL-2 promoter, using a *Hin*dIII-to-*Alu*I fragment extending from +48 to −239. S, Nuclear extracts from Jurkat cells stimulated with PHA and PMA; N, nuclear extracts from nonstimulated Jurkat cells; BSA, no nuclear extract was used and bovine serum albumin was the only protein present in the incubation; HS, hypersensitive site.

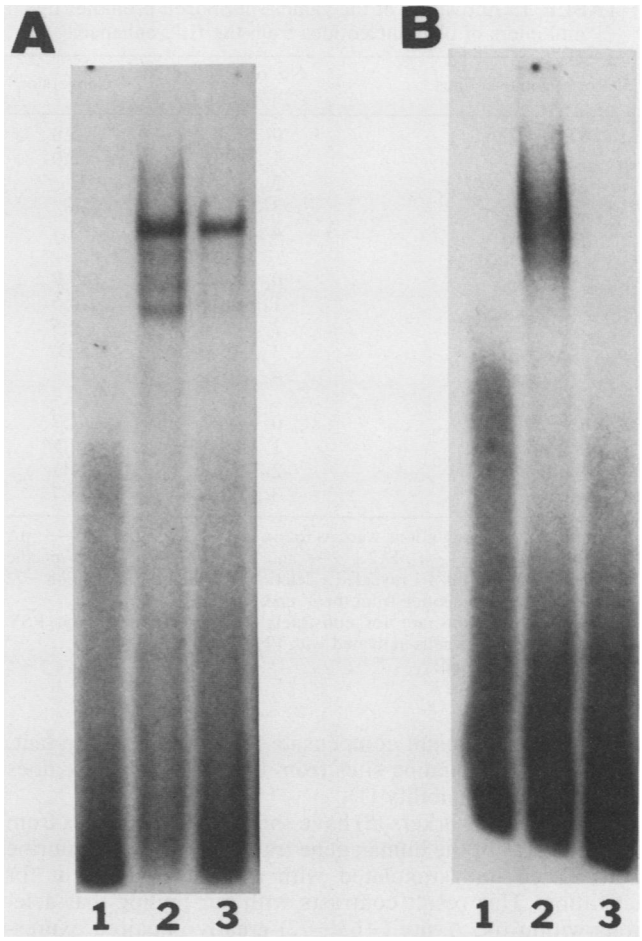


FIG. 6. The complex with the −65 to −94 region forms in extracts from activated as well as nonactivated Jurkat cells (A), whereas the complex formed with the −257 to −286 region is seen only with extracts from activated T cells (B). Lanes 1, Free probe; lanes 2, nuclear extract from activated Jurkat cells; lanes 3, nuclear extracts from nonactivated Jurkat cells.

had approximately 5, 41, and 31% control (pIL-2 CAT) activity, respectively, and each site interacted with a nuclear protein (Fig. 8). In addition, each of these nuclear factor-binding events occurred independently of the others (see Results). The nuclear factors binding these three sites were named NF-IL-2A, -D, and -E in accordance with precedents in the literature (27). There were two other footprints at sites B and C, as diagrammed in Fig. 8. As mentioned, the footprint overlaying the B site is stimulation specific and deletion of the site impairs stimulated expression in the transfection assay. Whether NF-IL-2E and NF-IL-2B are the same protein remains to be seen. Though deletion of the sequence corresponding to the C site did not result in diminished functional activity, it is possible that the protein(s) binding the site still plays a functional role not assayed in these experiments. Of significance is the lack of a footprint over the sequence similar to the consensus AP1 binding site (−178 to −185), since AP1 binding is apparently activated in the presence of PMA (21, 23). Furthermore, there is no functional impairment after deletion of this sequence (Fig. 2). Still, the fact that a deletion does not result in functional impairment cannot be taken as definitive evidence that the sequence is unimportant in IL-2 regulation,

TABLE 1. Activation of the gamma-fibrinogen promoter by multimers of oligonucleotides from the IL-2 enhancer

Site or oligonucleotides	No. of copies[a]	% Conversion[b]
E, −257 to −286	0	5.0
	1	5.0
	2	53
	3	56
	4	93
A, −65 to −94	0	5.0
	1	4.5
	2	2.5
	3	ND[c]
	4	73
−274 to −291	0	1.7
	1	ND
	2	ND
	3	3.7

[a] Multimer copy orientations were as follows: pA 1.1, ←; pA 2.1 ←→; pA 4.1, →→→→; pE 1.1, ←; pE 2.1 ←→ and pE 3.1, ←←←. Base-specific chemical cleavage of pA 4.1 revealed a deletion of a single A at position −72 in the second oligonucleotide from the 3′ end.

[b] Percent conversions are for constructs cotransfected with the RSV luciferase plasmid into cells activated with PHA and PMA.

[c] ND, Not determined.

since other sites might compensate for the deletion. In fact, deletion of AP1 binding sites from the SV40 enhancer does not diminish inducibility (1).

Fujita and co-workers (8) have shown that sequences from −319 to −127 of the human gene transfected into the murine EL-4 T-cell line stimulated with PMA are sufficient for activation. This result contrasts with our finding that deletions within the A site (−63/−72) greatly impaired expression and that multimers of the A site inducibly increase expression through the fibrinogen promoter in response to signals from the antigen receptor. The fact that the endogenous EL-4 IL-2 gene can be activated by PMA alone, whereas both normal T cells and the Jurkat T-cell line require both antigen receptor and PKC stimulation for IL-2 expression (37), implies that activation requirements are somehow less stringent in EL-4.

To examine the relative sequence specificities of these IL-2 enhancer binding factors, we performed cross-competition experiments with oligonucleotides corresponding to these and other IL-2 sequences and found that sites A and D cross-competed with each other but not with E (Fig. 4).

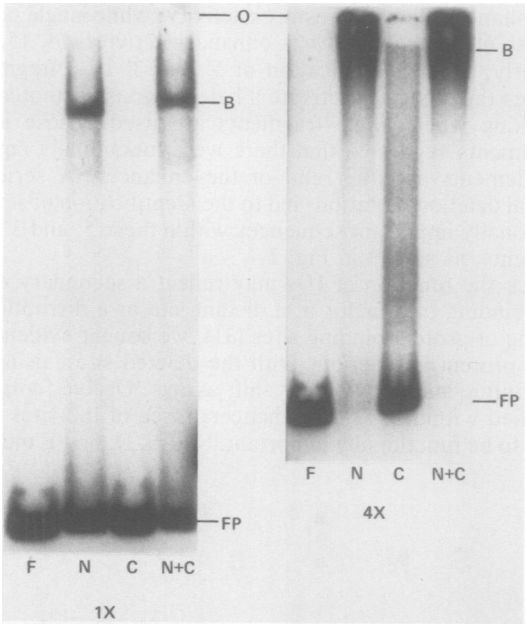


FIG. 7. Concatenation of the −65 to −94 oligonucleotide enhances nuclear protein binding (B). Gel mobility shift assays were carried out with an end-labeled monomer of the region (left) or a tetramer (right). F and FP, Free probe; N, nuclear extract; C, cytoplasmic extract; N+C, mixture of equal quantities of the two extracts from PHA- and PMA-stimulated Jurkat cells; B, bound probe; O, origin.

These results suggest that NF-IL-2A and -D are equivalent to each other and distinct from NF-IL-2E. Despite the equivalence of sites A and D in the binding assays, there is no outstanding similarity in nucleotide sequence between the two sites. This issue may be clarified once the precise protein-DNA contact points are elucidated at the two sites. A further distinction can be drawn between NF-IL-2A and -E, since, first, a difference in the site E but not the site A footprint is seen between nonstimulated and stimulated nuclear extracts (an upstream extension of the footprint by 7 nucleotides in the presence of stimulated nuclear extracts; Fig. 5A). Secondly, while DNA-protein complexes are formed after binding of nonstimulated and stimulated nuclear extracts to the site A oligonucleotides, the site E complex is seen only when extracts of PHA- and PMA-stimulated cells are used (Fig. 6A and 6B, respectively).

Since NF-IL-2A and -E appeared to be different proteins

TABLE 2. Ability of oligonucleotides from the IL-2 enhancer to activate CAT expression from the gamma-fibrinogen promoter[a]

Treatment	Site A, −65 to −94		Site E, −257 to −286		Nonprotected site, −274 to −291	
	Relative activity[b]	n	Relative activity	n	Relative activity	n
Nonstimulated	1.0	5	1.0	4	1.0	1
PHA	7.0	3	20.4	2	ND[c]	
PMA	1.5	4	0.8	3	ND	
PHA + PMA	8.2	4	36.2	4	1.8	1
Anti-Ti	8.9	2	12.4	3	ND	
Anti-Ti + PMA	8.1	2	25.1	3	ND	
Anti-HLA + PMA	0.5	2	1.3	2	ND	

[a] Actual values for the nonstimulated percent conversion ranged from 0.3 to 6% and for PHA plus PMA from 14 to 75%. The site A and nonprotected site clone contained 3 copies, while the site E clone contained 4 copies.

[b] Relative activity is the mean of *n* independent determinations of percent conversion expressed relative to the nonstimulated control.

[c] ND, Not determined.

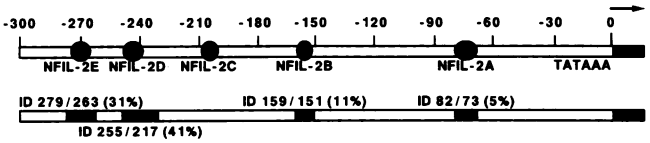


FIG. 8. The regions within the IL-2 enhancer protected from digestion by DNase I (●) and the results of the analysis of internal deletion mutations. The limits of the deletion mutations are indicated above or below each shaded box, whereas the degree of functional impairment of the internal deletion mutation is shown as a percentage relative to the wild-type enhancer.

(or protein complexes), it was possible that the corresponding sequences responded to different signals; however, we found both sequences to be responsive to antigen receptor signals and nonresponsive to PMA alone. The experiments outlined in Tables 1 and 2 represent the second approach we took to verify the significance of both the internal deletion studies and the protein-binding studies, as well as to understand the signal specificities of these sequences. Multimers of both A and E sites placed 5′ to the gamma-fibrinogen promoter responded to anti-Ti alone but not to PMA alone. The full-length IL-2 enhancer (−52 to −326) responds weakly, if at all (6), to anti-Ti alone, suggesting that in the A and E site multimers the juxtaposition of multiple anti-Ti-responsive elements overcomes this limitation, perhaps through cooperative binding. There is no constitutive activation of the gamma-fibrinogen promoter by the concatenated IL-2 sequences, in keeping with the normal lack of transcription of the IL-2 gene in the absence of stimulation. In summary, we have identified two sites within the IL-2 enhancer that respond to signals arising at the antigen receptor. Since these sequences do not respond to PMA, we conclude that the signals from the antigen receptor and PKC must be integrated at the level of the gene. We have yet to identify the elements mediating the PMA response.

Though NF-IL-2E is only found in nuclear extracts of stimulated cells and is therefore likely to be related to IL-2 gene activation, the relevance of NF-IL-2A remains unclear. Of note, concatenation of the D site, which seems to bind the same factor as the A site (see cross-competition studies, Fig. 4), did not show the D site to be responsive to anti-Ti (data not shown). This may be due to constraints on the spatial positioning of the binding sites that are possibly not fulfilled by the D site multimers. Alternatively, the A site may bind yet another factor not detected in our assays, since the range of DNA-protein interactions that can be examined in any DNA-protein-binding assay is limited. For example, a protein with a high dissociation rate or short occupancy time may not be detected. One suggestion that NF-IL-2A may in fact be playing a role in IL-2 gene activation is illustrated in Fig. 7. The construct containing a single copy of the A site lacks inducible CAT activity, and the monomer binds NF-IL-2A weakly, as shown by the limited shift from the free to the bound form in Fig. 7. In contrast, the tetramer is highly inducible in the transfection assay and appears to bind the NF-IL-2A protein with greater affinity. Nevertheless, a more thorough study of this relationship between NF-IL-2A binding and site A function is indicated. Further supporting a functional role for NF-IL-2A is the presence of an inducible nuclease-hypersensitive site in this region (28), though this finding may reflect an alteration in DNA structure rather than a DNA-protein interaction.

Fujita and co-workers (8) have noted that sequences between −273 and −255 of the human immunodeficiency virus long terminal repeat are 75% identical to part of the IL-2E sequence defined in our studies. This similarity may have functional significance, since both the IL-2 enhancer and the human immunodeficiency virus long terminal repeat are functional only in activated T lymphocytes (24). In addition, the IL-2A sequence is homologous with sequences located between −66 and −54 of the gamma-interferon gene (10) and between −70 and −80 of the IL-2 receptor gene (6). Again, since both of these genes are only expressed in activated T cells, these sequence similarities may be of functional significance. It is possible that NF-IL-2A and -E interact with control regions of several activation-dependent T-cell genes and thereby play a general role in controlling T-cell activation.

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