Elf-1 Contributes to the Function of the Complex Interleukin (IL)-2–responsive Enhancer in the Mouse IL-2 Receptor α Gene

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Summary

Lymphocytes regulate their responsiveness to IL-2 through the transcriptional control of the IL-2Rα gene, which encodes a component of the high affinity IL-2 receptor. In the mouse IL-2Rα gene this control is exerted via two regulatable elements, a promoter proximal region, and an IL-2–responsive enhancer (IL-2rE) 1.3 kb upstream. In vitro and in vivo functional analysis of the IL-2rE in the rodent thymic lymphoma-derived, $CD4^-CD8^-$ cell line PC60 demonstrated that three separate elements, sites I, II, and III, were necessary for IL-2 responsiveness; these three sites demonstrate functional cooperation. Site III contains a consensus binding motif for members of the Ets family of transcription factors. Here we demonstrate that Elf-1, an Ets-like protein, binds to site III and participates in IL-2 responsiveness. In vitro site III forms a complex with a protein constitutively present in nuclear extracts from PC60 cells as well as from normal $CD4^-CD8^-$ thymocytes. We have identified this molecule as Elf-1 according to a number of criteria. The complex possesses an identical electrophoretic mobility to that formed by recombinant Elf-1 protein and is super-shifted by anti–Elf-1 antibodies. Biotinylated IL-2rE probes precipitate Elf-1 from PC60 extracts provided site III is intact and both recombinant and PC60-derived proteins bind with the same relative affinities to different mutants of site III. In addition, by introducing mutations into the core of the site III Ets-like motif and comparing the corresponding effects on the in vitro binding of Elf-1 and the in vivo IL-2rE activity, we provide strong evidence that Elf-1 is directly involved in IL-2 responsiveness. The nature of the functional cooperativity observed between Elf-1 and the factors binding sites I and II remains unresolved; experiments presented here however suggest that this effect may not require direct interactions between the proteins binding these three elements.

IL-2 is a T cell–derived cytokine implicated in the regulation of growth and differentiation of a variety of cells expressing IL-2 receptors. The high affinity IL-2 receptor (IL-2R)[1] consists of three distinct subunits, the IL-2Rα, -β, and -γ chains, which are assembled into a signaling complex after their interaction with IL-2 (for reviews see references 1–3). Lymphocytes control their level of IL-2 responsiveness by regulating the expression level of IL-2R. This is achieved via transcriptional regulation of the IL-2Rα gene, and in certain cells the IL-2Rβ gene; the IL-2Rγ subunit is constitutively expressed (4–6). Resting lymphocytes do not express the IL-2Rα gene. Transcription of the gene is induced by signals from the antigen receptor, but in the absence of any other stimuli, these signals only trigger a transient wave of IL-2Rα synthesis in T lymphocytes. Maximal and sustained IL-2Rα transcription depends on IL-2 itself, which thus acts as a positive feedback regulator of IL-2R expression and IL-2 responsiveness. The level of IL-2Rα gene expression is also modulated by other extracellular stimuli, notably IL-1 and TNF (7–9). In $CD4^-$ $CD8^-$ thymocytes, IL-1 has a similar effect as antigen in mature T cells in that it acts synergistically with IL-2 to increase IL-2Rα transcription (6, 10).

[1] *Abbreviations used in this paper:* IL-2R, IL-2 receptor; IL-2rE, IL-2–responsive enhancer; PRR, promoter-proximal positive regulatory region; STAT, signal transducers and activators of transcription.

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The rodent thymic lymphoma-derived cell line PC60 is growth factor independent and resembles early thymic T cell precursors in that it expresses neither CD4 nor CD8 antigens. This line was the first in which induction of IL-2Rα expression by IL-2 was reported (11) and is a well-characterized model system in which to analyze the IL-2 responsiveness of this gene (6, 12). As in normal $CD4^-CD8^-$ thymocytes, IL-1 and IL-2 act synergistically to induce IL-2Rα transcription (9). In a manner similar to antigenic stimulation in mature T cells, in PC60 IL-1 induces only a transient wave of IL-2Rα expression, and it primes cells to become IL-2 responsive. Transcriptional control of the IL-2Rα gene is exerted by two separate regulatory regions in the 5′ flanking region, one of which is promoter proximal and required for IL-1 inducibility and the other a more distal IL-2–responsive enhancer (IL-2rE) (6). Promoter-proximal positive regulatory regions (PRRs in the terminology proposed by John et al. [13]), located between positions −54 to −584 in the mouse, and −244 to −276 (PRRI) and −137 to −64 (PRRII) in the human IL-2Rα genes, respectively, are required for the rapid appearance of IL-2Rα mRNA after IL-1 exposure of PC60 or EL4 (14) cells, or PMA treatment of human T cell leukemias (12, 13, 15). Several DNA-binding factors were implicated in exerting effects on transcription via these elements, including NF-kB, Elf-1, and HMG-I(Y) (12–14, 16). Transcriptional stimulation by IL-2, on the other hand, operates via IL-2rE. The position of this enhancer in the mouse gene was mapped, by transient transfection experiments, to a 48-nucleotide stretch, 1.3 kb upstream of the transcriptional start site (12). It corresponds to a DNase I hypersensitive site (DH2), that appears in the chromatin of normal mouse T cells upon stimulation with Con A and IL-2 (10), and can increase transcription in response to IL-2 stimulation both in its normal context of 2.5 kb of IL-2Rα 5′ flanking region as well as when inserted upstream of a non-orthologous promoter. Recently, the human homologue of the mouse IL-2rE has been identified in a region ~4 kb upstream of the transcription start site of the human IL-2Rα gene (17, 18). The mouse IL-2rE contains three separable *cis*-acting elements, named sites I, II, and III; mutations in any one of these three elements abolishes IL-2 responsiveness of reporter constructs containing the IL-2rE, demonstrating functional cooperativity between the sites (12) (see Fig. 1 *A* for summary). By identifying the proteins which bind to these DNA elements it will be possible to characterize the nature of some of the downstream transducers of the IL-2 signaling pathway.

Sites I and II of the IL-2rE contain sequence elements potentially recognized by signal transducers and activators of transcription (STAT) proteins (19), some of which have recently been shown to be activated by IL-2 (20, 21). In PC60, IL-2rE function most likely also depends on the binding of IL-2 activated STAT5 to both sites I and II (Meyer, M., P. Reichenbach, V. Schindler, E. Soldaini, M. Nabholz, manuscript submitted for publication). Site III contains a consensus binding site for members of the Ets-like family of transcription factors, distinguished by a conserved DNA binding domain which recognizes sequences containing a GGAA/T core (22). Ets-like proteins have been shown to be involved in the regulation of genes important for several different biological processes including lymphocyte differentiation, T cell activation, growth control, viral infection cycles, and transformation (22). Ets-like proteins often interact with other transcription factors and accessory proteins forming complexes both in the presence and absence of DNA (23–27).

The aim of this study is to elucidate which, if any, of the Ets-like proteins bind to site III and form part of the complex of proteins generating the response of the IL-2Rα gene to IL-2. Using several experimental approaches, we demonstrate that site III specifically binds the Elf-1 transcription factor. A direct correlation between the effect of site III point mutations on Elf-1 binding in vitro and on the in vivo activity of the corresponding IL-2rE forms, strongly implies Elf-1 as the transcription factor involved in the IL-2 inducibility of the IL-2Rα gene. Experiments designed to elucidate the molecular basis of the cooperativity observed between the three sites making up the IL-2rE suggest that this phenomenon does not rely on the formation of direct interactions between Elf-1 and the factors binding to sites I and II.

Materials and Methods

Cells and Culture Conditions. The PC60.21.14 cell line, further referred to as PC60, and its culture conditions have been described previously (9, 28, 29). Human recombinant interleukins were the kind gifts of Glaxo IMB S.A. (Geneva, Switzerland). IL-1β, further referred to as IL-1, was used at final concentrations of 1 ng/ml; IL-2 was used at 100 units/ml. Spleenic T cells were prepared by fractionation on nylon wool columns according to *Current Protocols in Immunology* (30). $CD4^-CD8^-$ thymocytes were prepared and cultured as described previously (31). The purity of $CD4^-CD8^-$ thymocytes, determined by flow cytometry, was 98%.

Plasmids and Preparation of Mutant Constructs. The reference plasmid for transient transfection experiments, pGβAcβG1D, the wild-type IL-2Rα/reporter construct, pwt1βG1, in which the segment from nt −2539 to +93 of the IL-2Rα 5′ flanking region is fused to a β-globin reporter gene, and the mutants used for the experiments described in Fig. 1, *B* and *D* have been described previously (12, 32). Mutations within the IL-2rE sequence were introduced into the pwt1βG1 plasmid using the Chameleon Mutagenesis Kit (Stratagene, Zürich, Switzerland). Plasmids were prepared using Qiagen Plasmid kits, and the validity of all the constructs was confirmed by DNA sequencing using a T7 polymerase sequencing kit (Amersham Rahn, Zürich, Switzerland).

Transfection of PC60 Cells and Reporter Gene Assays. PC60 cells were cultured in the presence of IL-1 for 3 d and then transiently transfected with DNA constructs using the DEAE-dextran method (33). Each transfection included a defined ratio between the reference plasmid and a IL-2Rα/reporter construct. After transfection, cells were split into two equal aliquots. One aliquot was grown for an additional two days in the presence of IL-1 and IL-2, while the other was cultured in the presence of IL-1 alone. The transcripts from the IL-2Rα/reporter gene and from the reference plasmid were then measured by RT PCR as described

previously (12, 32). Results from this assay were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunny Vale, CA). Signals due to the IL-2Rα/reporter gene constructs were normalized by comparing them to signals generated from the reference plasmid. To determine the level of IL-2 induction, transcriptional activity generated from the reporter construct in the presence of both IL-1 and IL-2 was divided by the activity observed in cells cultured in the presence of IL-1 alone.

Electrophoretic Mobility Shift Assays (Bandshifts). Nuclear extracts were prepared essentially as described by Schreiber et al. (34): 1×10^6 cells were rinsed in PBS and lysed in 200 μl of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 2% NP-40). The lysate was centrifuged, the supernatant discarded, and the proteins extracted from the nuclear pellet in buffer B (high salt buffer: 20 mM Hepes, pH 7.9, 0.4 M KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT). Both buffers A and B contained protease (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF) and phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_3). Binding reactions were performed in a final volume of 20 μl in binding buffer (10 mM Tris, pH 7.5, 100 mM KCl, 10% glycerol, 1 mM DTT, 1 μg/ml BSA, 1 μg dIdC, 0.5 μg sonicated salmon sperm DNA) containing 1 μg of cell nuclear extract or 1 μg of recombinant protein, 1×10^6 cpm of an end-labeled PCR fragment (spanning bases −1384 to −1290 of the 5′ flanking region of the IL-2Rα gene, Fig. 1 *B*) or SIII oligonucleotide probe (see Table 1). Reactions were incubated on ice for 20 min and then separated on 4.5% non-denaturing polyacrylamide gels in 0.3× TBE. For competition experiments, unlabeled PCR fragments or oligonucleotides were premixed with the radiolabeled probe before the addition of the proteins; for antibody supershift experiments the antibodies were preincubated with the nuclear proteins for the specified time before the addition of the radiolabeled probe.

Oligonucleotides (see Table 1) were obtained from MWG-Biotech (Ebersberg, Germany) or Microsynth (Balgach, Switzerland). Annealed oligonucleotides were stored in 10 mM Tris, pH 7.5, 50 mM NaCl. As a nonspecific competitor for band shift assays, a double-stranded oligonucleotide with the sequence 5′-AGAGTTAGCTTGCGGTTCCCAGG-3′ was used.

DNaseI Footprint Analysis. The probe for in vitro footprinting assays was obtained by subcloning the 188-bp SauIIIA–HindII fragment of the IL-2Rα 5′ flanking region into Sma I–BamHI–digested pUC18. To label the non-coding strand this plasmid was linearized with EcoRI and the resulting sticky end filled in with a nucleotide mixture containing [^{32}P]ATP. After digestion with PstI the labeled IL-2Rα fragment was purified on a non-denaturing 8% polyacrylamide gel and recovered by electroelution. For each footprint reaction 2 ng of probe and, where indicated, a 100-fold excess of competitor was incubated with 35 μg of nuclear extract in 27 μl of buffer (33 mM Hepes, pH 6, 0.1 mM EDTA, 50 mM KCl, 0.6 mM DTT, 4 mM $MgCl_2$, 0.4 mM 2-ME, 0.1 mg/ml poly-dIdC, containing several protease inhibitors, listed above) for 30 min on ice. The samples were prewarmed for 1 min at 20°C and 0.1 unit of Dnase I (Pharmacia, Duebendorf, Switzerland) in 2 μl 25 mM $CaCl_2$ and 10 mM Hepes, pH 7.6, was added. After 1 min at 20°C the reaction was stopped and the DNA extracted, denatured, and fractionated on a 6% sequencing gel.

Table 1. *IL-2rE Mutants*

Mutation	Sequence
	-1336 Core -1302
wt	GAGAGTTGAGCAACTTCCTGATATGTGAGACTCAG
M10	--TCTG----------
M11	------GTGT----------
M12	----------ACCA----------
M13	----------GGAA----------
M14	----------GTCGC----------
Δ15	----------ΔΔΔΔΔΔΔΔΔΔ----
SIII	----------
C1	-----A----------
C2	-----C----------
C3	-----G----------
C4	-----A----------
C5	-----A----------
S1	---GA----------
S2	----------CA----------
I5	----------
	TTAAC

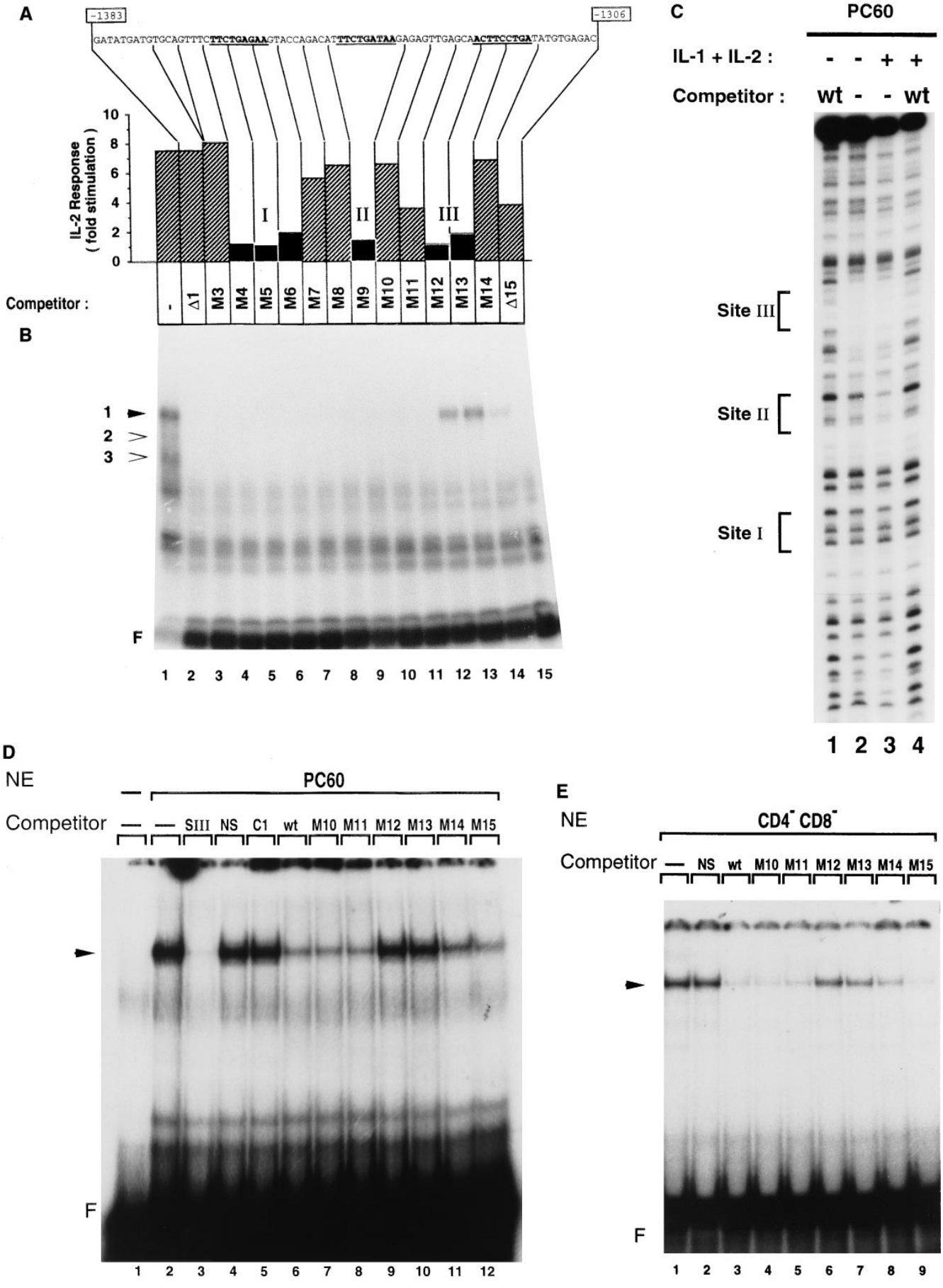
Recombinant Proteins and Antibodies. Recombinant AcNPV baculoviruses expressing rElf-1 and rEts-1 proteins (35, 36), and anti–Ets-1/2 (no. 8 [37]), anti–Pu-1 (no. 65 [38]) and anti–Fli-1 (no. 61 [39]) antisera have been described previously. Anti–Elf-1 polyclonal antibodies (C-20) were purchased from Santa Cruz Biotechnology (CA), and HRP-coupled goat anti–rabbit antibodies from Biorad Laboratories (CA).

Affinity Precipitation of IL-2rE–bound Proteins. Biotinylated IL-2rE probes were obtained as follows: (*a*) wild type and probes carrying mutations that abolish the activity of a single enhancer site were made by amplifying the corresponding reporter plasmid (M4 for site I, M9 for site II, M12 for III, see Table 1 and reference 12 for sequences) with primers spanning the segment between nucleotides −1402 (oligonucleotide A) and −1286 (biotinylated oligonucleotide B) of the IL-2Rα 5′ flanking region. (*b*) Probes in which more than one site was inactivated were obtained in three steps essentially as described in Ho et al. (40) and explained here for the example of a probe in which all three enhancer sites are destroyed. (*i*) The plasmid carrying the mutation in site I (M4) was amplified with oligonucleotide A and a 3′ oligonucleotide C′, covering site II with the changes destroying this site. Simultaneously the plasmid with a mutation in site III (M12) was amplified with a 5′ primer (C) complementary to oligonucleotide C′ and oligonucleotide B. (*ii*) The resulting PCR products were gel-purified, denatured, and annealed with each other. (*c*) The annealing mixture was used as template for an extension reaction, and the resulting full-length IL-2rE fragments amplified with primers A and B. PCR fragments were purified and quantified by densitometric analysis of the corresponding bands on agarose gels.

IL-2rE–binding proteins recovered from whole PC60 cell extracts were isolated after lysis of 2.0×10^6 cells/ml in buffer C (50 mM Tris-HCl, pH 8.0, 0.5% NP-40, 150 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_3, 1 mM PMSF, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin). Aliquots of extract from 2.0×10^6 cells were incubated with 1 μg biotinylated probe in buffer C at 4°C for 1 h. The probe and proteins bound were recovered by incubation with 30 μl streptavidin-agarose beads (Sigma Chemie, Buchs, Switzerland). Beads were washed twice in buffer C and then boiled in gel loading buffer (2% SDS, 10% glycerol, 80 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue, 5% 2-mercaptoethanol). Eluted proteins were separated by SDS-PAGE on 10% gels and transferred to Immobilon-P membranes (Millipore, Volketswil, Switzerland). Elf-1–specific bands were revealed with a 1/500 dilution of anti–Elf-1 antibody by ECL (according to the manufacturer's instructions, Amersham Rahn, Zürich, Switzerland).

Results and Discussion

Site III of the Mouse IL-2rE Forms an In Vitro Complex with at Least One Specific Protein. To identify the proteins that


A
-1383
GATATGATGTGCAGTTTCTTCTGAGAAGTACCAGACATTTCTGATAAAGAGAGTTGAGCAACTTCCTGATATGTGAGAC
-1306
IL-2 Response (fold stimulation)
10
8
6
4
2
0
I
II
III
Competitor :
-
Δ1
M3
M4
M5
M6
M7
M8
M9
M10
M11
M12
M13
M14
Δ15
B
1
2
3
F
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C
PC60
IL-1 + IL-2 :
- - + +
Competitor :
wt - - wt
Site III
Site II
Site I
1 2 3 4
D
NE
PC60
Competitor
— — SIII NS C1 wt M10 M11 M12 M13 M14 M15
F
1 2 3 4 5 6 7 8 9 10 11 12
E
NE
CD4- CD8-
Competitor
— NS wt M10 M11 M12 M13 M14 M15
F
1 2 3 4 5 6 7 8 9

bind to the IL-2rE, and may therefore be implicated in the regulation of IL-2rE activity, we performed bandshift assays with a probe containing the entire IL-2Rα enhancer (nucleotides −1384 to −1290). This probe forms a number of complexes with proteins extracted from IL-1–primed and IL-2–induced PC60 nuclei (Fig. 1 *B*). To determine which of these complexes is due to specific DNA-binding proteins recognizing functionally important sites within the IL-2rE, we used a DNA competition-based assay. In this assay DNA fragments harboring specific mutations defining the sites required for IL-2rE enhancer activity are used as competitors to disrupt the formation of the IL-2rE complexes (Fig. 1 *B*). Three of the IL-2rE complexes were inhibited by the addition of an excess of unlabeled DNA, but only the complex with the lowest mobility (complex 1) results from an interaction with a protein specifically binding a discrete region of the IL-2rE. Mutations M12 and M13 strongly impair the capacity of the corresponding DNA fragments to compete for the formation of this specific complex. The flanking mutations M11 and M14 result in a relatively lower, but still significant, reduction in the competitive efficiency of the IL-2rE DNA. M12 and M13 abolish IL-2rE activity and define site III; the striking correlation of this in vivo effect with the effect on competitor efficiency in vitro suggests that the specific bandshift complex is due to one or several proteins participating in the control of the IL-2rE activity.

On a probe spanning the entire IL-2rE (nucleotides −1472 to −1268), PC60 extracts produce a specific footprint extending from the 5′ end of site III to several bases downstream, essentially covering the Ets-like protein consensus binding motif (Fig. 1 *C*, lanes *2* and *3*). Detection of this footprint is abolished by the addition of unlabeled probe (Fig. 1 *C*, lanes *1* and *4*). The protein complex that gives rise to the footprint is present in nuclear extracts from both unstimulated and IL-1–primed and IL-2–stimulated cells (compare Fig. 1 *C*, lanes *2* and *3*). The same is true for the protein(s) forming the site III–specific complex detected in bandshift assays (data not shown). (Note that under the conditions used here we could not detect PC60 proteins binding specifically to site I or II. However, using other techniques we have shown that IL-2–induced STAT5 proteins bind to these sites but not to site III [Meyer, M., P. Reichenbach, V. Schindler, E. Soldaini, M. Nabholz, manuscript submitted for publication].)

SIII, a shorter IL-2rE probe spanning nucleotides −1336 to −1302, contains all the necessary sequence information to allow the formation of the site III–specific complex with extracts from PC60 (Fig. 1 *D*) or normal $CD4^-CD8^-$ thymocytes (Fig. 1 *E*). The single, specific SIII complex demonstrates the same sensitivity to mutant IL-2rE fragments in competition assays as that of the complete IL-2rE probe–site III–specific complex (compare Fig. 1, *B*, *D*, and *E*).

The Ets-like Transcription Factor Elf-1 Is Present in the Site III–specific Complex. Site III contains a consensus binding sequence for members of the Ets-like family of transcription factors. The two mutations, M12 and M13, which define site III and abolish competition for the site III–specific complex, destroy this consensus binding site. Mutation M14, which has no significant effect on enhancer activity and reduces competition in band shifts only to a minor extent (compare Fig. 1, *A* and *B*), results in a sequence that can still bind some Ets-like proteins. To determine which, if any, of the numerous Ets family members participates in the formation of the site III–specific bandshift complex several approaches were employed.

First, using recombinant Elf-1, an Ets-like protein, and Ets-1 itself in bandshift assays, we found that Elf-1 forms a complex with the SIII probe that migrates with a mobility identical to that of the complex generated in PC60 extracts. Recombinant Ets-1 binds to the SIII probe but results in the formation of a faster migrating complex (Fig. 2 *A*). The observation that the complex generated by PC60 nuclear extracts comigrates with that formed by recombinant Elf-1 suggests that there is no requirement for additional proteins.

Second, we tested the capacity of antibodies raised against the Ets-like proteins Elf-1, Ets-1/2, Fli-1 (Fig. 2, *B* and *C*) and Pu-1 to affect the mobility of the site III–specific bandshift complex. The anti–Elf-1 antibodies supershifted the complex formed by non-induced, as well as primed and IL-2 induced nuclear extracts from PC60 (Fig. 2, *B* and *C*; the appearance of the additional nonspecific

Figure 1. Identification of a DNA-binding protein that recognizes a critical element in the IL-2rE of the mouse IL-2Rα gene. *A* shows a summary of the published results (12) of the functional analysis of the mouse IL-2rE. The histogram shows the effect of mutations in the IL-2rE on the IL-2 inducibility of constructs containing 2.5 kb of the IL-2Rα 5′ flanking region. Mutations are either substitutions (M) or deletions (Δ) (see Table 1). The presence of the three separate elements, site I, II, and III, in the enhancer is required for IL-2 responsiveness. The Ets-like consensus site that overlaps with site III and the STAT consensus binding motifs in sites I and II are underlined. (*B*). Bandshift competition analysis of the complexes formed between a probe containing the entire IL-2rE (nucleotides −1384 to −1290) and nuclear extracts isolated from IL-1–primed, IL-2–induced PC60 cells. The competitors are unlabeled IL-2rE fragments used at a 200-fold molar excess which contain the mutations indicated in *A*. Competitors containing the mutations defining site III do not compete for the formation of complex 1 which is marked with a filled arrowhead. Empty arrowheads point to complexes 2 and 3, which are competed by all competitors. *F*, free probe. (*C*) DNase I footprint analysis of the IL-2rE in vitro. DNase I protection analysis of the IL-2rE was performed with nuclear extracts isolated from either untreated or IL-1–primed and IL-2–induced PC60 cells as indicated. The reactions were performed either in the presence or absence of unlabeled IL-2rE probe (wt) as detailed. The positions of the three sites (sites I, II and III) delineated in *A* were determined by fractionating a sequencing reaction on the same gel (data not shown). (*D* and *E*) A site III probe (SIII) is sufficient to allow formation of the IL-2rE–specific complex corresponding to complex 1 in *B* from PC60 (*D*) and $CD4^-CD8^-$ thymocytes (*E*). The radiolabeled probe SIII spanning nucleotides −1336 to −1302 of the IL-2rE forms a specific complex, indicated with a filled arrowhead, with nuclear extracts isolated from IL-1–primed, IL-2–induced PC60 cells. The SIII-specific complex was subjected to competition analysis with the following unlabeled probes: SIII, unlabeled SIII; NS, a nonspecific oligonucleotide of the same length as SIII; C1, a mutant form of SIII (see Table 1) which contains a mutation in the core of the Ets-like binding site; M9 to M15, mutant forms of the IL-2rE (see *A* and Table 1).

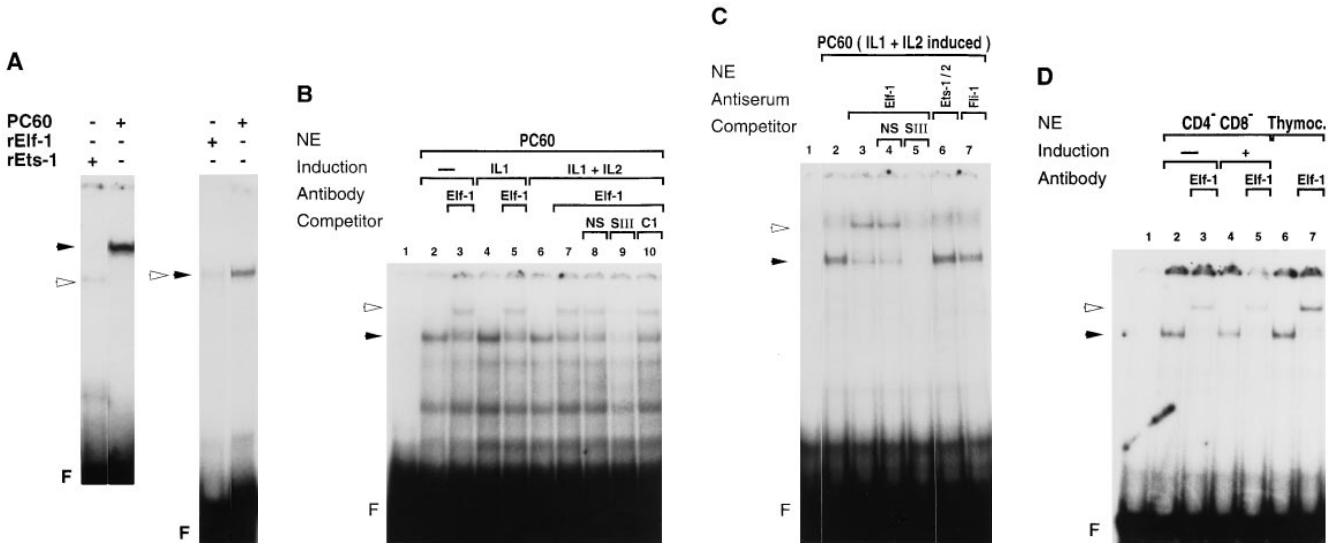
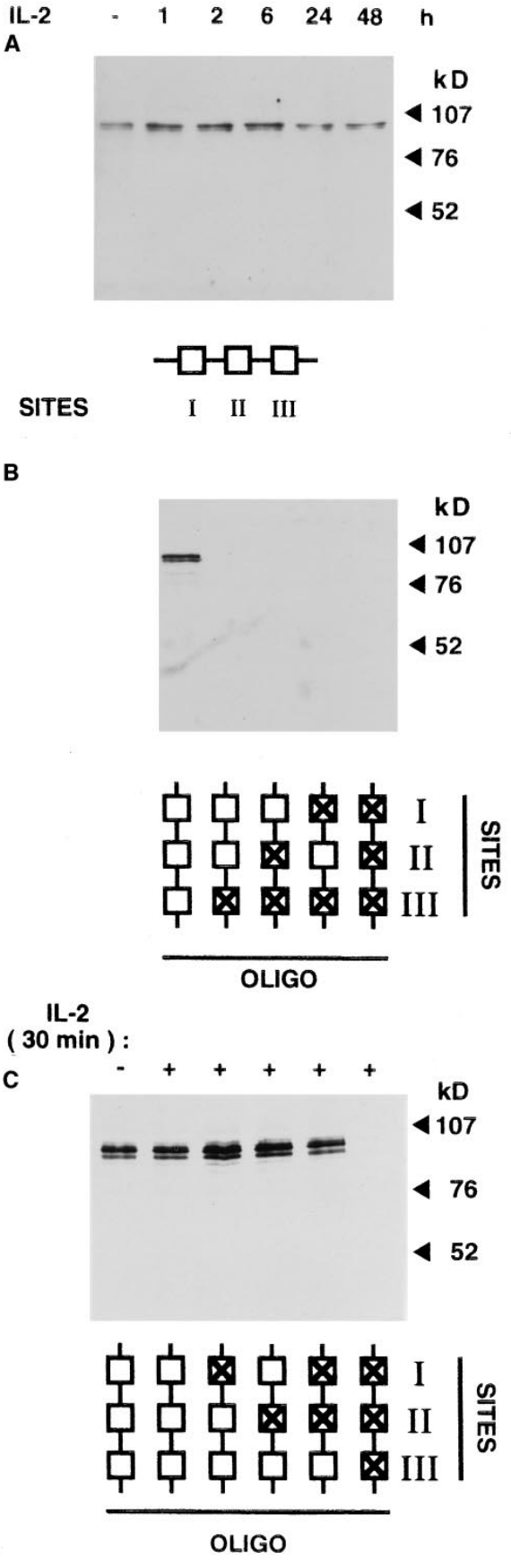


Figure 2. Elf-1 generates the IL-2rE site III–specific complex in vitro. *A* shows a comparison of the relative electrophoretic mobilities of the site III–specific complexes formed by recombinant Ets-1 (rEts-1), Elf-1 (rElf-1), and IL-1–primed, IL-2–induced PC60 nuclear extracts (*PC60*) and the SIII probe. The complexes formed by the recombinant proteins and PC60 extracts are indicated with empty and filled arrowheads, respectively. F, unbound probe. (*B*, *C*, and *D*). The effect of antibodies against different Ets-like proteins on the electrophoretic mobility of the site III–specific complex. The SIII probe was incubated with nuclear extracts from PC60 cells (*B*, *C*) or from unfractionated or $CD4^-CD8^-$ thymocytes (*D*), in the presence or absence of antibodies against Elf-1, Ets-1/2 or Fli-1 as indicated. PC60 cells were either untreated, IL-1 primed alone, or IL-1 primed and IL-2 induced as indicated. $CD4^-CD8^-$ thymocytes were either freshly isolated or stimulated for 2 d with PMA, ionomycin, IL-1, and IL-2. Filled and empty arrowheads mark the site III–specific complex in the presence or absence of antibodies, respectively. In *B* binding reactions were performed at room temperature, whereas in *C* and *D* incubations were performed on ice. 1 μl and 2.5 μl of the anti–Elf-1 antibodies was used to produce the supershift visible in *B* and *C*, respectively. *B* illustrates that the site III–specific supershifted complex is competed by the SIII probe itself (*SIII*) but not by a nonspecific competitor (*NS*) or by a probe containing a mutation in the site III Ets core (*C1*, see Table 1 and Fig. 1 *D*). F, Unbound probe.

higher mobility bands in Fig. 2 *B* results from incubation at room temperature. These bands are not present when the incubations are performed on ice as in Fig. 2 *C*) or with $CD4^-CD8^-$ thymocytes (Fig. 2 *D*). The observation that the proportions of the complexes supershifted varies between experiments suggests that the anti–Elf-1 antiserum only partially supershifts the site III specific complex as a result of the relatively low affinity of this reagent. Anti–Elf-1 antibodies also supershift the site III specific complex formed with the complete IL-2rE probe (data not shown). Antibodies against Ets-1 and Ets-2, Fli-1 (Fig. 2 *C*), and Pu-1 (data not shown) do not affect the mobility of the site III–specific complex.

Third, the nature of the protein(s) which could be precipitated from PC60-derived nuclear extracts by biotinylated IL-2rE oligonucleotide probes was investigated. IL-2rE probes containing all three sites precipitate a protein recognized by the anti–Elf-1 antiserum and whose molecular mass, 91 kD, corresponds to that of Elf-1 (Fig. 3 *A*). The Elf-1 doublet observed on Western blots has been reported previously (41), and the relative levels of the two protein species remain unchanged after IL-1 priming or IL-2 induction (data not shown). As expected from the bandshift experiments, the presence in PC60 nuclei of Elf-1 active for DNA binding does not depend on IL-2 stimulation, although sometimes a small increase is seen in extracts from IL-2–treated cells (Fig. 3, *A* and *C*). Experiments with biotinylated IL-2rE probes containing mutations in sites I, II, or III clearly show that Elf-1 binding to IL-2rE is abolished by a mutation in site III, whereas mutations in sites I or II have no effect on the amount of Elf-1 protein precipitated (Fig. 3, *B* and *C*). The human IL-2rE contains an additional Ets-like protein-binding site overlapping a STAT consensus binding motif in site I (17, 18). Fig. 3 *B* demonstrates that the murine site I does not bind Elf-1, in agreement with the inability of site I to compete for the site III–specific complex (Fig. 1 *B*). Transfection experiments in human lymphoid cell lines suggest that Elf-1 binding to site I may produce a negative effect on the activity of the IL-2rE enhancer (17). If this effect is important in the regulation of the human IL-2Rα gene expression in normal lymphocytes, it suggests the presence of subtle differences in the transcriptional control of the homologous mouse and human genes.

Finally, we compared the effect of point mutations in the core of the site III Ets-like consensus site both on the ability of recombinant Elf-1 to bind and on the formation of the site III–specific complex by PC60 extracts. Binding site selection studies have identified the consensus sequences recognized by human and mouse Elf-1 as being A(A/t)(C/a)CCGGAAGT(A/S) (42) and A(A/T)(A/c)CCGGAAGT(G/T)(G/A) (3) respectively. Both contain the core GGAA (underlined) characteristic of Ets-like binding sites; site III of the IL-2rE (AAT*CAGGAAGT*TG (in italics nucleotides identical in all three motifs) is very similar to these sequences. There is complete concordance in the behavior



of recombinant Elf-1–derived and PC60-derived Elf-1 site III complexes in terms of their sensitivities to the various mutations present within the core sequence (Fig. 4, *A* and *B*). Note, in particular, the effect of the change GGAA to GGA*T* (mutant C5), which is not expected to affect binding of Ets-1 but does, as previously described (35, 42), result in a strong reduction of the binding of recombinant Elf-1 as well as of the site III–binding protein in PC60 extracts.

The Effects of Site III Mutations On Elf-1 Binding In Vitro Correlate with Those on IL-2rR Activity In Vivo. The data presented above demonstrate that the protein in PC60 extracts binding to site III of the IL-2rE is Elf-1. Although previous mutational analyses suggest that Elf-1 is required for IL-2rE inducibility, they do not rule out the possibility that another protein, not detectable in in vitro binding experiments, is responsible for site III function in vivo. To obtain further evidence that Elf-1 is indeed the protein upon which site III function depends in vivo, we compared the effect of mutations in the core of the Elf-1 binding site on the in vivo IL-2 responsiveness of the IL-2rE. This was measured using an accurate PCR-based reporter assay described previously (12, 32). Comparison of the results obtained from these assays (Fig. 5) with those obtained in the bandshift competition experiments (Fig. 4, *A* and *B*) shows good agreement between these two parameters. Mutations C1 and C3 very strongly affect Elf-1 binding in vitro and reduce the IL-2 responsiveness of the IL-2rE about fivefold. Mutations C2 and C4 result in a slightly weaker effect on both Elf-1 binding and IL-2rE activity, and mutation C5, which competes for Elf-1 binding more strongly than the other core mutants, reduces enhancer inducibility twofold. As mentioned above this mutation reduces the binding of Elf-1 but should have little effect on the binding of Ets-1 or Ets-2. This change therefore results in a core element still recognized by Elf-1 but at a much reduced efficiency. Two additional mutations, S1 and S2, which alter the nucleotides flanking the Ets-like core sequence, almost completely abolish IL-2rE activity. Note that S2 overlaps with M14 which itself has little effect on Elf-1 binding and enhancer inducibility. Together these data lend strong support to the hypothesis that Elf-1 participates in the IL-2 response of the mouse IL-2rE. In the recently identified human IL-2rE the individual sites required for enhancer activity, in-

Figure 3. Elf-1 is precipitated from PC60–derived nuclear extracts by biotinylated IL-2rE oligonucleotide probes containing an intact site III. The ability of various biotinylated IL-2rE oligonucleotide probes to precipitate Elf-1 from PC60-derived nuclear extracts was investigated. The nature of the IL-2rE probe used to precipitate the protein is indicated below the lane(s); a blank or crossed box, respectively, serves to illustrate the presence of either a wild-type or a mutant sequence at the designated site of the IL-2rE enhancer (the mutant forms of sites I, II, and III contain the sequences M4, M9, and M12, respectively, which destroy IL-2rE enhancer activity, see Fig. 1 *A* and Table 1). In *A* and *C* nuclear extracts were prepared from cells that had been IL-1 primed and then IL-2 induced for the specified time periods. In *B*, all extracts were prepared from cells that had been IL-1 primed and IL-2 induced for 30 min. The blots were probed with anti–Elf-1 antibodies and molecular mass markers are indicated.

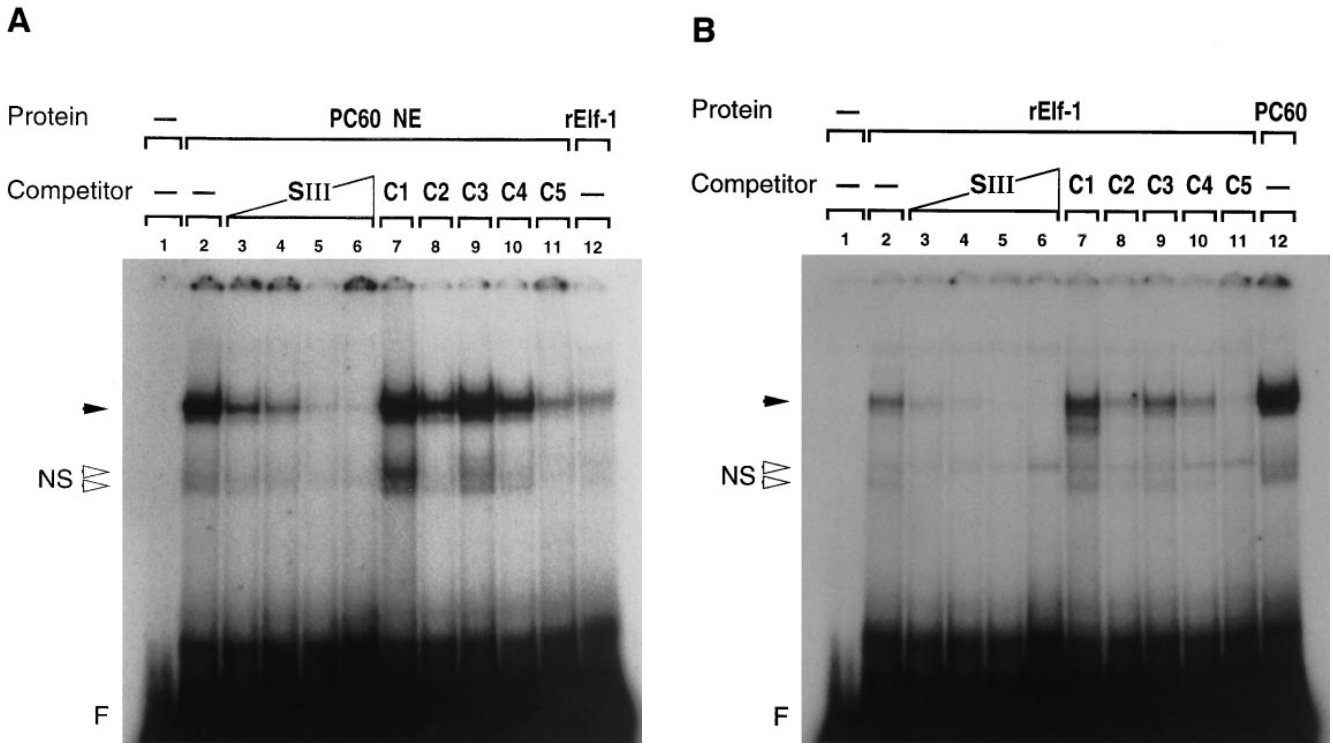


Figure 4. A comparison of the sensitivity of the PC60 nuclear extract and recombinant Elf-1–derived site III–specific complexes to different competitors. IL-1–primed, IL-2–induced PC60 nuclear extracts (PC60 NE, *A*) or recombinant Elf-1 proteins (rElf-1, *B*) and the SIII probe were incubated in the presence or absence of the indicated unlabeled competitors: SIII, C1 to C5, see Table 1. The SIII competitor was added at a 50-, 150-, 500-, and 1,500-fold molar excess to the labeled SIII as illustrated by the increasing gradients, the other competitors were used at a 1,500-fold molar excess. The final DNA concentrations of all the reactions were adjusted by addition of an appropriate amount nonspecific competitor (NS, see Table 1). The site III–specific complex is highlighted with a filled arrowhead; two nonspecific complexes (NS) formed in incubations with the recombinant Elf-1 protein are indicated with empty arrowheads. Lane *12* contains the site III complex formed by either recombinant Elf-1 (*A*) or PC60 extracts (*B*) in order to allow comparison of their relative mobilities. F, Unbound probe.

cluding site III, are conserved (17, 18), and John et al. (18) has provided evidence suggesting that the human gene is also regulated by Elf-1.

There is evidence implicating Elf-1 in the regulation of a number of lymphocyte specific genes (IL-2 [43], GM-CSF [24], CD4 [41], IL-3 [23], IgH3′ [44] and terminal transferase [45]). The case of the IL-2Rα gene is interesting as this gene contains two (or, in man, three) Elf-1 sites, forming part of both the promoter proximal and distal control elements. Elf-1 DNA-binding activity in PC60 cells is not affected by IL-2 (Fig. 3 *A*). Thus, the simplest model for the molecular basis of the IL-2rE's IL-2 inducibility is that enhancer activity depends on the binding of other IL-2–activated proteins (most likely STAT5 [Meyer, W., P. Reichenbach, V. Schindler, E. Soldaini, M. Nabholz, manuscript submitted for publication]) to sites I and II and that Elf-1 remains constitutively present on site III. Constitutive binding of Elf-1 to regulatory elements implicated in stimulation of transcription has been described previously in studies of the PHA and PMA inducible IL-3 gene (23). Experiments to detect possible posttranslational modifications of Elf-1 controlling its capacity to contribute to enhancer activity have so far been unsuccessful. Elf-1 activity may also depend on interactions with other inducible factors binding in its immediate vicinity. In this context it is interesting that mutations M11 and Δ15 flanking the Elf-1 binding site result in a moderate reduction of IL-2rE activity; these regions contain potential binding sites for NFIL-6 and AP-1. Note, however, that the site III specific complex formed by PC60 extracts has the same electrophoretic mobility as that formed with recombinant Elf-1, indicating that the PC60 complex does not contain additional proteins.

The Nature of the Functional Cooperativity between the Three Sites Making Up the IL-2rE Remains Unresolved. One of the striking features resulting from the study of the mouse IL-2rE is that mutations in any one of the three sites that make up this enhancer abrogate IL-2 responsiveness. The observation that the distances between the three IL-2rE sites (∼20 nucleotides or two helical turns from center to center) are conserved between mouse and man suggests that evolutionary conservation of the relative orientations of proteins binding to them is important, possibly because of a requirement for direct interactions between the factors binding to the three sites. However, the insertion of five nucleotides in mutant I5, which changes the relative orientation of site III with respect to sites I and II, results in only a minor ef-

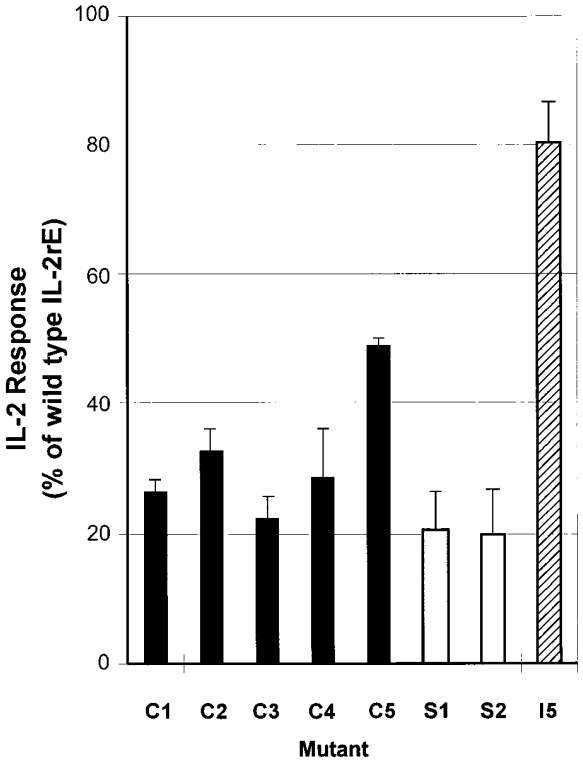


Figure 5. The effects of different site III mutations on the IL-2 inducibility of IL-2rE. The effects of site III mutants on the activity of the IL-2rE were investigated in transiently transfected PC60 cells. 2.5 kb of the mouse IL-2Rα gene 5′ flanking region, containing either the wild-type or the indicated mutant form of the IL-2rE (see Table 1), was fused to the rabbit β-globin gene. These plasmids were transiently transfected into IL-1–primed PC60 cells together with a reference plasmid (for details see reference 12). The transfected cells were either cultured in IL-1 alone or with IL-1 and IL-2. 2 d later the relative amounts of reporter gene mRNA were measured and the IL-2 response of each plasmid determined (see Materials and Methods). For each IL-2rE mutation, with the exception of C1, the assay was repeated with at least two independently derived mutants and in every instance the assay was performed twice with all constructs. IL-2 responses are plotted as percent of the response of the wild-type construct in the same experiment. Wild-type responses varied from 5.2- to 8.0-fold induction. For each mutant the mean response ± SD is given. Shading of the histogram bars distinguishes different classes of mutations: (*black*) mutations in the Elf-1 core motif; (*gray*) mutations flanking the core motif; (*diagonally hatched*) 5 nucleotide insertion between sites II and III.

fect on IL-2 inducibility, arguing against direct protein–protein interactions (Fig. 5). Using biotinylated IL-2rE oligonucleotide probes, we demonstrated that the presence or absence of sites I and II has no affect on the amount of Elf-1 protein precipitated from PC60-derived nuclear extracts (Fig. 3 *C*; probing the same membranes with anti-STAT5 antiserum reveals that STAT5 binds to site I and II [Reichenbach, P., manuscript in preparation]) again arguing against direct protein–protein interactions. However, this possibility needs to be explored further using additional approaches.

The simplest model consistent with our findings is that the different IL-2rE binding proteins cooperate by interacting independently with components of the transcription initiation complex, as has been described previously in in vitro studies of transcriptional regulation by the *Drosophila* proteins Bicoid and Hunchback (46) and in the control of the c-*fos* gene (47). But our data do not rule out more complex models involving additional proteins or cofactors needed for an efficient stimulation of transcription by the IL-2rE.

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