Title

A 275 basepair fragment at the 5' end of the interleukin 2 gene enhances expression from a heterologous promoter in response to signals from the T cell antigen receptor.

Permalink

https://escholarship.org/uc/item/4md1587h

Journal

Journal of Experimental Medicine, 165(2)

ISSN

0022-1007

Authors

Durand, DB
Bush, MR
Morgan, JG
et al.

Publication Date

1987-02-01

DOI

10.1084/jem.165.2.395

Peer reviewed

A 275 BASEPAIR FRAGMENT AT THE 5' END OF THE INTERLEUKIN 2 GENE ENHANCES EXPRESSION FROM A HETEROLOGOUS PROMOTER IN RESPONSE TO SIGNALS FROM THE T CELL ANTIGEN RECEPTOR

By DAVID B. DURAND,* MARK R. BUSH,* JOHN G. MORGAN,*
ARTHUR WEISS,‡ AND GERALD R. CRABTREE*

*From the *Department of Pathology, Stanford University Medical School,
Stanford, California 94305; and the ‡Department of Medicine, Howard Hughes Medical
Institute, The University of California, San Francisco, California 94143*

The process of T cell activation involves the interaction of antigen with the antigen receptor (Ti)[1]/T3 complex (1–4), activation of protein kinase C (5–7), and an increase in the intracellular concentration of calcium (8–11). These events must specifically regulate a limited number of genes in the nucleus, which program T cell division and differentiated T cell functions. Still, to date, little is known about the signals that must pass between the antigen receptor and the genes that are the targets of these signals. One approach to understanding these intracellular signals in T lymphocyte activation involves first defining the precise DNA targets for these signals and then using these DNA sequences to isolate the signal molecules.

The interleukin 2 gene, one of the earliest genes to be activated by these signal molecules, is expressed by T lymphocytes in a strictly regulated manner. Resting T cells make no detectable IL-2 transcripts (12); however, the combination of antigen in the presence of the appropriate antigen presenting cell leads to the activation of the IL-2 gene (12, 13). This is a primary gene activation, because no protein synthesis is required for IL-2 gene transcription (12). The kinetics of induction are quite rapid, with transcripts detectable in total cellular RNA preparations within 1 h (14, 15). Recently, we found that a nuclease hypersensitive site is induced ~85 bp 5' to the initiation site of transcription of the IL-2 gene (16). The appearance of this site coincides with the onset of IL-2 gene transcription in human peripheral blood T cells and the human T cell line Jurkat. Furthermore, at least one agent known to block IL-2 gene activation, cyclosporin A, inhibits the appearance of this site (16). We now demonstrate that the IL-2 5'-flanking region contains a 275 bp sequence that, in either orientation, enhances transcription from a heterologous promoter. Moreover, this 275 bp

[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; Ti, T cell receptor for antigen.

sequence is a target for the signal pathway originating at the T cell antigen receptor.

Materials and Methods

Plasmid Constructs.   The IL-2 CAT plasmid was made using the 632 bp Rsa I fragment of the 5' flanking region of the human IL-2 gene, which was lightly digested with BAL 31 to remove the initiator codon from its 3' end (16). The 5' overhanging ends of the resulting fragments were then filled in using Klenow enzyme, ligated to Hind III linkers, and then ligated to the chloramphenicol acetyltransferase (CAT) gene from the T_N9 transposon after digestion with Hind III enzyme. There are no simian virus 40 sequences in this plasmid except for the splice and polyadenylation sequences at the 3' end of the CAT gene (16). For these experiments we used an IL-2 CAT plasmid that has just 7 bp removed from each end relative to the original 632 bp Rsa I fragment.

The γ fibrinogen promoter construct was prepared by subcloning the 6.5 kb Xho I fragment carrying the rat γ fibrinogen upstream region and its first seven introns (17) into the Sal I site of pBR322. This plasmid was digested with Pvu II and Bst EII; the resultant 822 bp fragment was isolated and its termini were filled in using Klenow enzyme. Following attachment of synthetic Hind III linkers, the fragment was ligated onto the Hind III site of pJYMCAT (16). This plasmid consisting of γ fibrinogen sequences −792 to +36 linked to the CAT gene was linearized at the Cla I site in the polylinker containing the 5' Hind III site, and a series of 5' deletions were constructed by Bal 31 digestion, followed by filling in using Klenow enzyme and adding of synthetic Sph I linkers. One such 5' deletion mutant is referred to as Fib CAT (containing nucleotides −54 to +36 of the γ fibrinogen gene, as determined by sequencing [J. Morgan, E. Evans, L. A. Campbell, and G. R. Crabtree unpublished data]).

The IL-2 Fib CAT constructs were made by isolating the fragments indicated in Fig. 1 from IL-2 CAT; filling in 5' overhangs, where necessary, using Klenow enzyme; ligating synthetic Sph I linkers to the blunt ends; and ligating the resulting fragments in both orientations into the Sph I site of Fib CAT. The constructs containing the IL-2 Xmn I to Ssp I fragment in forward and reverse orientations are referred to as $\overrightarrow{\text{IL-2}}$ Fib CAT and $\overleftarrow{\text{IL-2}}$ Fib CAT, respectively. All enzymes were obtained from New England Biolabs (Beverly, MA). All cloning procedures were performed as previously described (16).

Transfections.   Jurkat cells were transfected by a modified DEAE-dextran method (16). The Jurkat mutant lacking T3/Ti complex surface expression was previously described (2, 3). Briefly, aliquots of 12×10^6 Jurkat cells grown in RPMI 1640 with 10% FCS and 1% penicillin/streptomycin (Difco Laboratories, Chagrin Falls, OH) (final medium) were washed once in RPMI 1640 by centrifugation (all spins of Jurkat cells were at 200 g for 5 min) and were gently resuspended in 2 ml 250 µg/ml DEAE-dextran (mol wt 5×10^5; Sigma Chemical Co., St. Louis, MO), 0.1 mM chloroquine diphosphate (Sigma Chemical Co.), and 10 µg/ml DNA in RPMI 1640 at 25°C. The aliquots were incubated at room temperature for 70 min, spun down, washed once in RPMI 1640, and then resuspended in 15 ml final medium. The cells were stimulated with various reagents, as described in Results, 8 h before harvest. Final concentrations used were PHA-P (Sigma Chemical Co.), 2 µg/ml; PMA (Sigma Chemical Co.), 50 ng/ml; C305 antibody, 1:10,000 (2); anti-HLA, 1:10,000 (W6/32) (5); and A23187 (Calbiochem Behring Corp., La Jolla, CA), 1 µg/ml. Cells were harvested 50–52 h after transfection, and the protein extracts were tested for CAT activity as described (18). The entire extract (0.4 mg protein) was incubated in the presence of 2.75 mM acetyl coenzyme A and 1 µl of [^{14}C]chloramphenicol (Amersham Corp., Arlington Heights, IL) at 37°C for 18 h. Samples were subsequently run on thin-layer chromatography plates as described (18), and the plates were exposed to x-ray film for 24–48 h at −70°C with one intensifying screen. After autoradiography, the zones containing converted and unconverted [^{14}C]chloramphenicol were cut out and scraped to allow quantitation of the percent conversion to acetylated forms. Counting was performed in Aquafluor (New England Nuclear, Boston, MA).

IL-2 Assays.   Assays were performed on the supernatants of the transfected Jurkat cells as described (19).

INDUCIBLE
N S S
TATAAA
Met

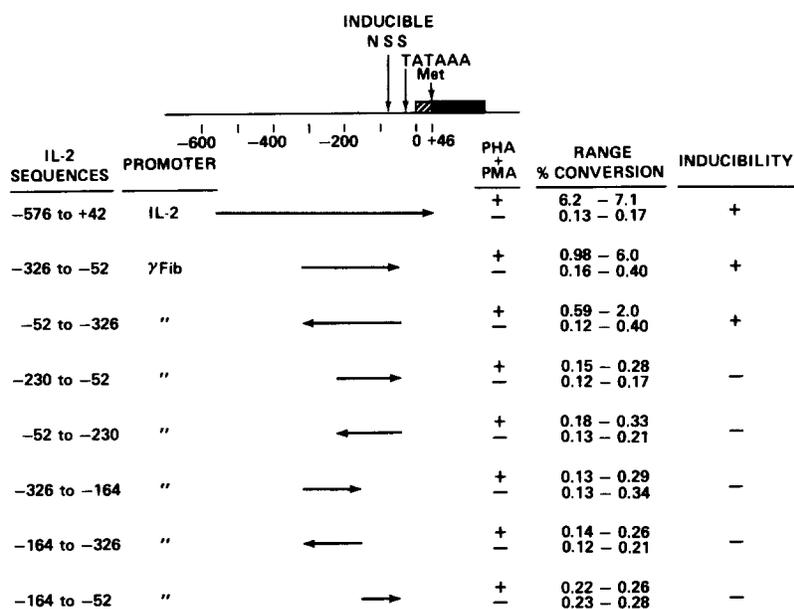| IL-2 SEQUENCES	PROMOTER		PHA + PMA	RANGE % CONVERSION	INDUCIBILITY
−576 to +42	IL-2	⟶	+ −	6.2 − 7.1 0.13 − 0.17	+
−326 to −52	γFib	⟶	+ −	0.98 − 6.0 0.16 − 0.40	+
−52 to −326	"	⟵	+ −	0.59 − 2.0 0.12 − 0.40	+
−230 to −52	"	⟶	+ −	0.15 − 0.28 0.12 − 0.17	−
−52 to −230	"	⟵	+ −	0.18 − 0.33 0.13 − 0.21	−
−326 to −164	"	⟶	+ −	0.13 − 0.29 0.13 − 0.34	−
−164 to −326	"	⟵	+ −	0.14 − 0.26 0.12 − 0.21	−
−164 to −52	"	⟶	+ −	0.22 − 0.26 0.23 − 0.28	−

FIGURE 1. The delimitation of IL-2 sequences that enhance expression from a heterologous promoter in response to PHA plus PMA treatment. A map of the IL-2 5' flanking region and first exon is shown at the top of the figure. The inducible nuclease sensitive site (NSS), the TATA box (TATAAA), and the location of the first ATG codon (Met) are indicated. The region is numbered according to the start site of transcription (at 0). The composition of the transfected constructs is indicated at left. The construct described on the first line is IL-2 CAT, which contains the IL-2 5' flanking region (−576 to +42) ligated to the CAT gene. For the other constructs shown, the given IL-2 sequences were ligated to the γ fibrinogen promoter, which in turn is ligated to the CAT gene. The arrows indicate the orientation of the IL-2 sequences relative to the γ fibrinogen promoter. Jurkat cells were transfected with the various constructs and were stimulated 42 to 44 h later with PHA and PMA, or were left unstimulated (as indicated by a plus or a minus, respectively, in the PHA + PMA column). 8 h later the CAT protein was extracted and the CAT activity of the extracts was determined. The activity is expressed as ranges of percent [^{14}C]chloramphenicol conversion, as explained in Results. Inducibility is defined by the criterion of nonoverlapping ranges between stimulated and unstimulated samples (see Results for further explanation).

Results

Identification of a 275 bp DNA Fragment from the IL-2 5' Flanking Region that Enhances Expression from a Heterologous Promoter in Transfected Jurkat Cells in Response to PHA plus PMA Stimulation.   To study the molecular signals that activate the IL-2 gene, we have begun to define the shortest sequence from the 5' flanking region of the gene that is responsive to the signals that induce endogenous IL-2 gene expression. We started our search for such sequences in the 5' flanking region of the IL-2 gene for two reasons. First, this is the most frequent location for regulatory sequences in differentiation-specific genes (20–23). More important, we have identified an inducible nuclease-sensitive site in the immediate IL-2 5' flanking region that may represent a regulatory protein binding site (16). The appearance of this nuclease sensitive site (NSS in Fig. 1) was inhibitable with cyclosporin A, a drug that inhibits the induced transcription of the IL-2 gene (24), further indicating the potential importance of this region in the regulation of the IL-2 gene (16).

To identify regulatory sequences within the IL-2 5' flanking region, we initially

linked the IL-2 sequences extending from −576 to +42 to the CAT coding region, and assayed the function of this plasmid, IL-2 CAT, in transiently transfected Jurkat cells (16). Fig. 1 shows schematically that the −576 to +42 sequence contains the inducible nuclease-sensitive site as well as a TATA box. IL-2 CAT lacks the IL-2 ATG codon for the first methionine, such that the CAT transcripts must initiate translation at the AUG of the CAT gene. Jurkat cells were transfected and were then stimulated with PHA and PMA or were left unstimulated (see Materials and Methods). 8 h later, the protein was extracted from the cells and assayed for CAT activity. This time was selected because the peak of accumulated IL-2 transcripts occurs ∼6 h after stimulation with PHA and PMA (the additional 2 h allow sufficient time for CAT translation to occur) (24). The acetyltransferase activity of the cellular extract has been shown to reflect the level of accumulated CAT transcripts in a given transfected cell line (25, 26). Since IL-2 gene expression is controlled primarily at the transcriptional level (12), acetyltransferase activity should reflect the transcriptional regulatory activity of the IL-2 5' flanking sequences in a particular construct, provided the transcriptional start site is not shifted such that an out-of-phase methionine is used.

Fig. 1 indicates that when Jurkat cells were transfected with IL-2 CAT and stimulated with PHA plus PMA (noted by a + in the PHA plus PMA column), the range of percent [^{14}C]chloramphenicol conversion was 6.2–7.1%, while for unstimulated transfected cells the range was 0.13–0.17% (ranges for three experiments). This and subsequent figures represent data pooled from multiple complete experiments, including the specific controls indicated in each of the figures. Data pertaining to a given construct transfected under specific conditions could be pooled and expressed as ranges, because the time course of the transfections, the time of CAT protein extraction and the duration of the CAT assay were kept constant in these experiments. Because the range of percent conversion for cells that underwent the transfection process in the absence of DNA (i.e., mock-transfected cells) overlapped the range of percent conversion for transfected unstimulated cells (0.12–0.23 and 0.13–0.17, respectively), we concluded that there was no detectable constitutive expression of the IL-2 CAT construct in Jurkat cells. When the mock-transfected values are subtracted from the values for DNA-transfected unstimulated cells, the difference approaches zero and the induction of stimulated transfected cells relative to unstimulated transfected cells approaches infinity. For this reason our results are expressed in terms of inducibility (+ under Inducibility in Fig. 1) or noninducibility (− under Inducibility in Fig. 1). The conclusions indicated by the inducibility column in Fig. 1 are based on the statistical test of nonoverlapping ranges. In other words, where the PHA plus PMA–positive range is greater than and does not overlap with the PHA plus PMA–negative range, the plasmid is considered inducible. Where these ranges overlap, the given plasmid is considered noninducible. Where experiments were performed once, single values are given. For IL-2 CAT, our conclusion was that the plasmid was constitutively inactive, but strongly inducible with PHA plus PMA.

To further delineate the inducing sequences, we have begun to define the shortest fragment from the IL-2 5' flanking region that will enhance transcription from an unrelated promoter. The heterologous promoter we chose is a truncated

γ fibrinogen promoter including only 22 bp 5' to the TATA box linked to the CAT gene by 36 bp of 5' untranslated region. This Fib CAT construct functions at a low constitutive level in hepatocytes, which is at least 40 times lower than the activity of longer 5' deletion mutants. In addition, Fib CAT correctly initiates transcription in vivo (Morgan et al., unpublished observations) and nuclear run-off experiments indicate that the control of γ fibrinogen expression is at the transcriptional level (Zlotnick, A., and G. Crabtree, unpublished observations). To identify a minimal control sequence, various fragments from the original 618 bp IL-2 5' flanking region (shown schematically as arrows in Fig. 1) were ligated to Fib CAT at the 5' end of the fibrinogen sequences in both orientations with respect to the γ fibrinogen promoter (i.e., IL-2 sequence, γ fibrinogen promoter; CAT coding sequence). These plasmids were transfected into Jurkat cells that were then stimulated with PHA and PMA, or were left unstimulated. As illustrated in lines 2 and 3 of Fig. 1, only the IL-2 −326 to −52 fragment (Xmn I to SspI; in both forward and reverse orientations, $\overrightarrow{IL-2}$ Fib CAT and $\overleftarrow{IL-2}$ Fib CAT, respectively) could enhance expression from the fibrinogen promoter in response to PHA plus PMA treatment. The −326 to −164 (Xmn I to Dra I), the −164 to −52 (Dra I to Ssp I), and the −230 to −52 (Dde I to Ssp I) fragments were unable to enhance expression from the fibrinogen promoter in stimulated cells (Fig. 1). It should be noted that no constitutive expression of these plasmids could be detected above the low endogenous acetyltransferase activity of Jurkat cells. In short, the 275 bp Xmn I to Ssp I fragment represents the shortest IL-2 5' flanking sequence examined that could enhance expression from a heterologous promoter in response to PHA plus PMA treatment. The shorter fragments could be interpreted as transcriptionally inactive due to a shift in the transcription initiation sites, so that strictly speaking, this 275 bp fragment represents the shortest fragment that enhances transcription in such a way as to produce a CAT mRNA that can be properly translated. As mentioned, however, both IL-2 and γ fibrinogen expression are controlled at the transcriptional level. Furthermore, Fib CAT initiates transcription correctly in vivo (Morgan et al., unpublished data), and CAT activity has reflected the level of accumulated CAT transcripts in several previously examined systems (25, 26). Finally, as discussed below, this 275 bp IL-2 fragment responds to various activation stimuli in parallel to expression of the endogenous IL-2 gene. For these reasons, it is likely that our results reflect changes at the level of transcript accumulation rather than differences in initiation start sites.

The IL-2 5' Flanking Region Confers Inducibility upon an Unrelated Promoter after Stimulation of Transfected Jurkat Cells with Anti-Ti plus PMA. PHA binds to multiple cell surface molecules on T lymphocytes, but may initiate activation by triggering the T3/Ti complex (2, 8). To ascertain that the IL-2 5' flanking region was receiving the physiologic signal from a pathway beginning at the antigen receptor, we used the antibody C305, which binds to the β chain of the antigen receptor component of the T3/Ti complex at the Jurkat cell membrane (2, 3, and A. Weiss, unpublished observations). Weiss and coworkers have shown that this antibody induces IL-2 protein expression in the presence of PMA (2). Furthermore, when presented singly to Jurkat cells, neither C305, PMA, nor PHA yields a substantial response (2). Only PHA induces detectable secreted IL-

TABLE I

Response of the Transfected IL-2 CAT Plasmid in the Jurkat T Cell Line to Membrane and/or Cytosolic Signals

Stimulus	Percent conversion (range)	Inducibility	IL-2 production (U/ml)
None*	0.13–0.23	–	0.0
Anti-Ti + PMA	0.62–2.0	+	6.4–20.4
Anti-Ti	0.16–0.40	–	0.0
PMA	0.18–0.35	–	0.0
Anti-HLA + PMA	0.23	–	0.0
Anti-HLA	0.26	–	0.0
PHA + PMA	3.38–7.07	+	23.8–77.0
PHA	0.21–0.37	–	5.2–8.8
A23187 + PMA	5.93–10.9	+	5.5–29.3
A23187	0.22–0.25	–	0.0
Mock‡	0.09–0.28		0.0

Jurkat cells were transfected with the IL-2 CAT plasmid and were subsequently treated with the stimuli noted in the far left column. CAT protein was extracted 8 h later and CAT assays were performed as described (see Materials and Methods). The CAT assay results, given as percent conversion (range), represent three to eight separate experiments, except where single values are given. These values were normalized to a mean CAT assay time of 18.2 h on the assumption that the CAT assay is linear with respect to time (18 and our unpublished observations). CAT assay times varied in these experiments from 16.2 to 22.3 h. Inducibility is defined by the criterion of nonoverlapping ranges between stimulated and unstimulated samples. IL-2 production is given in the far right column in units per milliliter.

* None refers to Jurkat cells that underwent transfection with DNA but were not stimulated.

‡ Mock refers to Jurkat cells that underwent the transfection process in the absence of DNA and without stimulation.

2 activity, and this represents $\sim^{1}/_{14}$th of the PHA plus PMA–induced activity (8). The ability of PHA alone to induce small amounts of IL-2 may reflect its interaction with cell surface molecules other than the T3/Ti complex. To examine signal specificity we transfected Jurkat cells with IL-2 CAT and stimulated the cells for 8 h with the stimuli indicated in the first column of Table I. IL-2 production, a measure of endogenous IL-2 gene expression, was monitored by assaying the supernatants at the time of cell harvest, and is expressed in units per milliliter in the fourth column. Table I shows that the range of percent conversions for the stimulus combination anti-Ti plus PMA (0.62–2.0) was greater than and did not overlap with the range of percent conversions for unstimulated transfected cells (0.13–0.23), for anti-Ti alone (0.16–0.40), or for PMA alone (0.18–0.35) in three separate experiments; hence, the combination of anti-Ti and PMA was capable of activating expression of IL-2 CAT, while anti-Ti alone and PMA alone were not. As a control for the specificity of the anti-Ti antibody, we used an antibody that recognizes an HLA antigen on the Jurkat cell membrane (6). Neither anti-HLA plus PMA nor anti-HLA alone induced IL-2 CAT expression in Jurkat. In nearly every experiment, the expression of the introduced plasmids paralleled the expression of the endogenous IL-2 gene, as demonstrated by measurements of supernatant IL-2 activity. The only exception was PHA alone, which resulted in detectable IL-2 activity in the

supernatant and no detectable CAT activity, possibly due to the sensitivity limits of the transient transfection system. We conclude from the results in Table I that the IL-2 5' flanking region is a target for the antigen receptor signal pathway.

Calcium Ionophore plus PMA Can Induce Expression of the IL-2 CAT Construct in the Jurkat Cell Line. To further dissect the IL-2 gene activation pathway distal to T3/Ti complex binding, we examined the effect of intracellular calcium changes on expression of the IL-2 CAT plasmid. Triggering of the T3/Ti complex is accompanied by an increase of cytoplasmic free calcium (8, 9). Moreover, calcium ionophores such as A23187 are able to substitute for anti-Ti antibody in endogenous IL-2 gene activation (2, 8). Although the A23187-induced calcium increase may not mimic T3/Ti activation in all respects, it is thought that A23187 acts distal to Ti binding by antigen (2, 5, 8). As shown in Table I, this distal signal provided by A23187 complements PMA in activating IL-2 CAT expression. Again, A23187 alone is an ineffective stimulus, as is anti-Ti antibody alone.

Since the PMA stock was dissolved in 95% ethanol (at 1 mg/ml), and the A23187 stock was dissolved in DMSO (at 50 mg/ml), we performed control experiments to show that the necessary signals for the activation of our plasmids are not due to the solvents, either directly or indirectly. First, neither PHA plus ethanol, A23187 plus ethanol, PMA plus DMSO, nor C305 plus ethanol induced expression of the IL-2 CAT plasmid. Second, when the reagents were added to fresh flasks and the solvents were allowed to evaporate before transfer of the transfected cells to these flasks, no difference in the PHA plus PMA, A23187 plus PMA, and C305 plus PMA signals was seen compared to addition of the reagents directly to the medium (data not shown).

The 275 bp IL-2 Fragment Enhances Expression from Linked Heterologous Promoter after Stimulation of Transfected Jurkat Cells with Anti-Ti plus PMA as well as Calcium Ionophore plus PMA. To be certain that the 275 bp fragment from the IL-2 gene was receiving the same physiologic signals as the larger 618 bp fragment in IL-2 CAT, we carried out experiments parallel to those described in Table I using, instead, the 275 bp fragment linked to the Fib CAT promoter (IL-2 Fib CAT). These results are shown in Table II and parallel those presented in Table I. As shown for IL-2 CAT, anti-Ti plus PMA, PHA plus PMA, and calcium ionophore plus PMA were capable of inducing the expression of the IL-2 Fib CAT plasmid. Anti-Ti, PMA, calcium ionophore, and anti-HLA used separately, as well as anti-HLA plus PMA were not able to induce IL-2 Fib CAT expression in Jurkat. The fibrinogen promoter alone (Fib CAT) was not induced by any of the signal combinations capable of inducing IL-2 Fib CAT, demonstrating that the induction of IL-2 Fib CAT was dependent upon the presence of the 275 bp IL-2 fragment. We conclude that the 275 bp IL-2 fragment is a target for signals from the T cell antigen receptor pathway.

Calcium Ionophore plus PMA Induces Expression of the IL-2 CAT Plasmid in a Jurkat Mutant Cell Line that Fails to Express the T3/Ti Complexes at the Cell Surface. As a test of our conclusion that the IL-2 5' flanking region is a target for signals emanating from the T cell antigen receptor, we examined the function

TABLE II

Response of the Transfected IL-2 FIB CAT Plasmid in the Jurkat T Cell Line to Membrane and/or Cytosolic Signals

Stimulus	$\overrightarrow{\text{IL-2}}$ Fib CAT		
	Percent conversion (range)	Inducibility	IL-2 production (U/ml)
None	0.18–0.32	–	0.0–0.0
Anti-Ti + PMA	0.40–0.49	+	11.5–14.5
Anti-Ti	0.20	–	0.0
PMA	0.20–0.23	–	0.0
Anti-HLA + PMA	0.20	–	0.0
Anti-HLA	0.23	–	0.0
PHA + PMA	0.69–4.87	+	38.5–42.6
A23187 + PMA	1.00	+	0.0
A23187	0.25	–	0.0
Mock	0.09–0.28		
	Fib CAT		
None	0.27–0.34	–	0.0–0.0
Anti-Ti + PMA	0.21–0.39	–	10.0–12.3
Anti-Ti	0.29	–	0.0
PMA	0.29	–	0.0
Anti-HLA + PMA	ND	–	ND
Anti-HLA	ND	–	ND
PHA + PMA	0.17–0.36	–	ND
A23187 + PMA	0.29	–	ND
A23187	0.30	–	ND
Mock	0.09–0.28		

Jurkat cells were transfected with the $\overrightarrow{\text{IL-2}}$ Fib CAT plasmid and were subsequently treated with the stimuli noted in the far left column. CAT protein was extracted and CAT assays were performed as described in Materials and Methods. The CAT assay results, given as percent conversion, are representative of at least three separate experiments, except where single values are given. These values were normalized to a mean CAT assay time of 18.2 h according to the assumption that the CAT assay is linear with respect to time (18 and our unpublished observations). CAT assay times varied in these experiments from 16.2 to 22.3 h. The percent conversion values for $\overrightarrow{\text{IL-2}}$ Fib CAT represented in the no stimulus range are 0.18, 0.20, 0.20, 0.23, 0.28, 0.32, and 0.32, whereas the values for the anti-Ti + PMA range are 0.40, 0.42, 0.44, and 0.49. Inducibility is defined by the criterion of nonoverlapping ranges between stimulated and unstimulated samples. IL-2 production is given in the far right column in units per milliliter. We obtained the same 0.0 U/ml value for the IL-2 Fib CAT transfection with A23187 + PMA stimulation upon repetition of the bioassay. Note, however, that A23187 + PMA induced 5.5–8.4 U/ml in the IL-2 CAT transfections of Jurkat (Table I) and 44.4 U/ml in the IL-2 CAT transfection of the Jurkat mutant (Fig. 2). None and mock (under the stimulus column) are as defined in Table I.

of the IL-2 CAT plasmid in a Jurkat mutant cell line (J. EMS-T3.3) that does not express cell surface T3/Ti complexes (2, 3). As illustrated in Fig. 2, only A23187 plus PMA induces IL-2 CAT expression in this mutant cell line, while neither PHA plus PMA nor C305 plus PMA can activate the IL-2 CAT plasmid (compare the A23187 plus PMA lane in Fig. 2 with the PHA plus PMA and

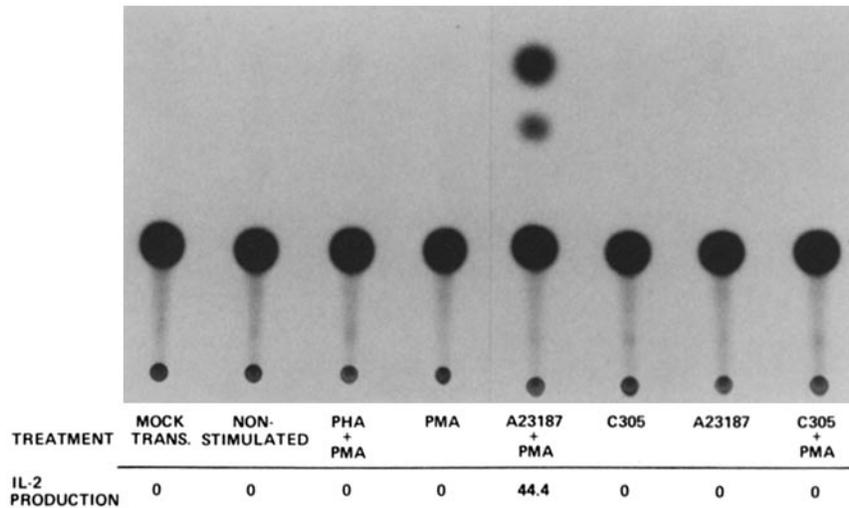| TREATMENT	MOCK TRANS.	NON-STIMULATED	PHA + PMA	PMA	A23187 + PMA	C305	A23187	C305 + PMA
IL-2 PRODUCTION	0	0	0	0	44.4	0	0	0

FIGURE 2. Calcium ionophore plus PMA, but neither PHA plus PMA nor antibody to the antigen receptor plus PMA activate the transfected IL-2 CAT plasmid in the Jurkat T3/Ti⁻ mutant. A Jurkat mutant cell line lacking T3/Ti cell surface expression was transfected with the IL-2 CAT plasmid. The cells were then stimulated with various signal combinations, and the CAT extraction and assay were performed as described in Materials and Methods. A photograph of an x-ray film exposed to the thin-layer chromatography plate for 48 h is shown. IL-2 production was assayed in the corresponding supernatants and is expressed in units per milliliter at the bottom of each lane.

C305 plus PMA lanes). In the Jurkat mutant, the expression of IL-2 CAT paralleled the expression of the endogenous gene as indicated by the IL-2 activity measurements on the bottom line of Fig. 2.

Discussion

We have identified a DNA sequence in the 5' flanking region of the IL-2 gene with several of the operational characteristics of an inducible transcriptional enhancer. This sequence, in either orientation, enhances the expression of a linked gene. Furthermore, this sequence functions when linked to a heterologous promoter and confers inducibility upon a promoter that is not normally inducible. The −326 to −52 (Xmn I to Ssp I) fragment from the IL-2 5' flanking region represents the smallest sequence examined that can enhance expression from a heterologous promoter in the Jurkat cell line.

Beyond its definition as an enhancing sequence, we have shown that this 275 bp IL-2 fragment is likely to be a target for signal transduction molecules in T lymphocytes. We have demonstrated that a monoclonal antibody against the β chain of the Jurkat T cell antigen receptor is capable of activating expression of a linked heterologous promoter through this 275 bp fragment in the presence of PMA. This result implies that the IL-2 5' flanking region contains target sequences for a signal pathway originating at the T cell antigen receptor. That A23187 plus PMA can activate the expression of IL-2 CAT in the Jurkat mutant lacking T3/Ti surface expression, whereas neither C305 plus PMA nor PHA

TABLE III

Sequence Homologies within the IL-2 5' Flanking Region

Gene segment	Position*	Sequence	Degree of ho- mology
A. IFN-γ	−66 to −54	**TGTGAAAATACGT**	11/13
IL-2	−97 to −85	**TTTGAAAATATGT**	
IL-2 receptor	−80 to −70	**TTTGAAAAATT**	9/11
B. IL-2 homology region I	−291 to −274	**AGAAAGGAGGAAAAACTG**	
IL-2 homology region II	−142 to −122	**AGAA GAGGAAAAA TG**	15/18
c-*fos* gene control element	−281 to −287	**GTGGAAA**	6/7

* Relative to the transcriptional start site.

plus PMA can, indicates that the activation of the IL-2 5' flanking region by the anti-Ti antibody as well as by PHA is dependent upon the presence of T3/Ti complexes; further, T3/Ti binding can be bypassed in the Jurkat mutant by the intracellular calcium increase provided by calcium ionophore in the activation of the IL-2 5' flanking sequence (see Fig. 2).

The presence of an inducible nuclease-hypersensitive site within this 275 bp IL-2 fragment supports the notion that this region may be the target for signal transduction molecules (see Fig. 1 and Siebenlist et al. [16]). Such nuclease-sensitive areas are believed to represent sites of specific DNA/protein interactions (27, 28). Still other sequences may be central to the regulation of the IL-2 gene as well as other T cell activation genes, which may be under coordinate regulatory control (7). These genes may share certain regulatory sequences and their corresponding signal molecules. Notably, a (33/45 bp) homology (−297 to −252) to the IFN-γ promoter (−33 to −77) (29, 30), as well as a (9/11 bp) homology (−87 to −97) to the IL-2 receptor promoter (−80 to −70) (31) lie within this 275 bp IL-2 fragment. The latter homology is of interest because there is a similar sequence in a similar location in the IFN-γ gene, as shown in Table III A.

We further examined the 275 bp IL-2 fragment for internal homologies and compared the sequence to other inducible enhancer elements. A 15/18 bp homology is repeated twice within the 275 bp IL-2 fragment (Table III B). This sequence bears homology to the c-*fos* control element from −281 to −287 (on the minus strand) in the c-*fos* gene (32).

The c-*fos* gene is particularly interesting in relation to IL-2 gene regulation, because it falls into a class of genes that can be induced within 1 h after serum stimulation of NIH 3T3 cells (32) or PHA stimulation of human peripheral blood lymphocytes (15); both these inductions are likely to be, at least in part, dependent upon membrane protein-binding events. The functional role of the c-*fos* element remains to be shown, though its homology to viral core enhancer sequences has been noted (32). An understanding of the significance of these homologies awaits the discovery of both the relevant signal molecules and their specific target sequences, which will further define the mechanism by which membrane and cytosolic triggering events lead to the activation of T cell–specific genes.

Our results lead directly to the next logical step in the investigation of IL-2

gene regulation, namely, the isolation of the DNA binding molecule(s) that activate(s) the IL-2 gene after antigenic stimulation. Several assays have recently been developed that allow the detection and purification of DNA-binding proteins. These assays are most readily applied and interpreted once a small fragment of DNA with clear functional importance has been isolated, criteria met by the 275 bp fragment of IL-2 5' flanking sequence we have characterized.

Summary

Using a transient transfection assay, we have defined the sequences required for the activation of the IL-2 gene in response to signals from the T cell antigen receptor. To do so we have transfected the human T cell line Jurkat with hybrid DNA constructs in which fragments from the IL-2 gene are linked to an indicator gene. The indicator gene product, as well as IL-2 production from the endogenous IL-2 gene were assayed after activation of the transfected Jurkat cells by various stimuli. We have demonstrated that a 275 bp fragment stretching from 52 to 326 bp upstream of the IL-2 gene transcription initiation site is required for expression of the linked indicator gene. This IL-2 gene fragment has several of the characteristics of a transcriptional enhancer element, in that it functions in both orientations and will enhance the expression from the promoter of an unrelated gene. Such enhancement occurred only after activation of Jurkat cells through the T cell antigen receptor. More specifically, this 275 bp fragment activated the expression of a linked gene after binding of a monoclonal antibody to the Jurkat T cell antigen receptor in the presence of PMA. In addition, calcium ionophore, which circumvents antigen receptor binding in T cell activation, induced the expression of the linked gene through this 275 bp sequence, in the presence of PMA. Finally, in a mutant Jurkat cell line lacking T3/antigen receptor complexes at the cell surface, no expression due to the IL-2 5' flanking region was seen after exposure to antibody to the T cell antigen receptor plus PMA or to PHA plus PMA. In contrast, calcium ionophore plus PMA did induce the expression of a linked gene through the IL-2 5' flanking region in the mutant Jurkat cell line. The responsiveness of the transfected hybrid genes containing the IL-2 regulatory region paralleled the expression of the endogenous IL-2 gene, as determined by IL-2 bioassays. We conclude that the 275 bp IL-2 sequence (−326 to −52 bp) is a target for the signal pathway originating at the T cell antigen receptor. Definition of this 275 bp target sequence should now permit the isolation of the molecules that bind to and activate the IL-2 gene.

References

1. Reinherz, E. L., S. Meuer, K. A. Fitzgerald, R. E. Hussey, H. Levine, and S. F. Schlossman. 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. *Cell.* 30:735.
2. Weiss, A., and J. D. Stobo. 1984. Requirement for the co-expression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.* 160:1284.
3. Ohashi, P. S., T. W. Mak, P. Van den Elsen, Y. Yanagi, Y. Yoshikai, A. F. Calman,

C. Terhorst, J. D. Stobo, and A. Weiss. 1985. Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. *Nature (Lond.).* 316:606.

4. Meuer, S., R. E. Hussey, D. A. Cantrell, J. C. Hodgdon, S. F. Schlossman, K. A. Smith, and E. L. Reinherz. 1984. Triggering of the T3-Ti antigen-receptor complex results in clonal T-cell proliferation through an interleukin 2-dependent autocrine pathway. *Proc. Natl. Acad. Sci. USA.* 81:1509.

5. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.).* 308:693.

6. Weiss, A., R. L. Wiskocil, and J. D. Stobo. 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL-2 production reflects events occurring at a pre-translational level. *J. Immunol.* 133:123.

7. Wiskocil, R., A. Weiss, J. Imboden, R. Kamin-Lewis, and J. Stobo. 1985. Activation of a human T cell line: a two-stimulus requirement in the pretranslational events involved in the coordinate expression of interleukin 2 and gamma-interferon genes. *J. Immunol.* 134:1599.

8. Weiss, A., J. Imboden, D. Shoback, and J. Stobo. 1984. Role of T3 surface molecules in human T-cell activation: T3 dependent activation results in an increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. USA.* 81:4169.

9. Imboden, J. B., A. Weiss, and J. Stobo. 1985. The antigen receptor on a human T cell line initiates activation by increasing cytoplasmic free calcium. *J. Immunol.* 134:663.

10. Rasmussen, H. 1986. The calcium messenger system. Part I. *N. Engl. J. Med.* 314:1094.

11. Rasmussen, H. 1986. The calcium messenger system. Part II. *N. Engl. J. Med.* 314:1164.

12. Kronke, M., W. J. Leonard, J. M. Depper, and W. C. Green. 1985. Sequential expression of genes involved in human T lymphocyte growth and differentiation. *J. Exp. Med.* 161:1593.

13. Neefe, J. R., G. R. Curl, and J. N. Woody. 1981. Absolute requirement for adherent cells in the production of human interleukin 2 (IL-2). *Cell. Immunol.* 63:71.

14. Grabstein, K., S. Dower, S. Gillis, D. Urdal, and A. Larsen. 1986. Expression of interleukin 2, interferon-gamma and the IL-2 receptor by human peripheral blood lymphocytes. *J. Immunol.* 136:4503.

15. Reed, J. C., J. D. Alpers, P. C. Nowell, and R. G. Hoover. 1986. Sequential expression of proto-oncogenes during lectin-stimulated mitogenesis of normal human lymphocytes. *Proc. Natl. Acad. Sci. USA.* 83:3982.

16. Siebenlist, U., D. B. Durand, P. Bressler, N. J. Holbrook, C. A. Norris, M. Kamoun, J. A. Kant, and G. R. Crabtree. 1986. The promoter region of the IL-2 gene undergoes chromatin structure changes and confers inducibility on the CAT gene during activation of T cells. *Mol. Cell. Biol.* 6:3042.

17. Kant, J. A., and G. R. Crabtree. 1983. The rat fibrinogen genes. Linkage of the Aα and γ chain genes. *J. Biol. Chem.* 258:4666.

18. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044.

19. Gillis, S. M., M. Ferm, W. Ou, and K. A. Smith. 1978. T-cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.

20. McKnight, S. L., and R. Kingsbury. 1982. Transcriptional control signals of a eukaryotic protein-coding gene. *Science (Wash. DC).* 217:316.

21. Zinn, K., D. DiMaio, and T. Maniatis. 1983. Identification of two distinct regulatory regions adjacent to the human B-interferon gene. *Cell.* 34:865.

22. Dierks, P., A. van Ooyen, M. D. Cochran, C. Dobkin, J. Reiser, and C. Weissman.

1983. Three regions upstream from the Cap site are required for efficient and accurate transcription of the rabbit β-globin gene in mouse 3T6 cells. *Cell.* 32:695.

23. Walker, M. D., T. Edlund, A. M. Boulet, and W. J. Rutter. 1983. Cell-specific expression controlled by the 5'-flanking region of insulin and chymotrypsin genes. *Nature (Lond.).* 306:557.

24. Kronke, M., W. J. Leonard, J. M. Depper, S. K. Arya, F. Wong-Staal, R. C. Gallo, T. A. Waldmann, and W. C. Greene. 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proc. Natl. Acad. Sci. USA.* 81:5214.

25. Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard. 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eucaryotic cells by DNA mediated transfection. *Proc. Natl. Acad. Sci. USA.* 79:6777.

26. Ciliberto, G., L. Dente, and R. Cortese. 1985. Cell-specific expression of a transfected human alpha 1-antitrypsin gene. *Cell.* 41:531.

27. Emerson, B. M., and G. Felsenfeld. 1984. Specific factor conferring nuclease hypersensitivity at the 5' end of the chicken adult beta-globin gene. *Proc. Natl. Acad. Sci. USA.* 81:95.

28. Emerson, B. M., C. D. Lewis, and G. Felsenfeld. 1985. Interaction of specific nuclear factors with the nuclease-hypersensitive region of the chicken adult beta-globin gene: nature of the binding domain. *Cell.* 41:21.

29. Fujita, T., C. Takaoka, H. Matsui, and T. Taniguchi. 1983. Structure of the human interleukin 2 gene. *Proc. Natl. Acad. Sci. USA.* 80:7437.

30. Holbrook, N. J., K. A. Smith, A. J. Fornace, C. M. Comeau, R. L. Wiskocil, and G. R. Crabtree. 1984. T-cell growth factor: complete nucleotide sequence and organization of the gene in normal and malignant cells. *Proc. Natl. Acad. Sci. USA.* 81:1634.

31. Leonard, W. J., J. M. Depper, M. Kanehisa, M. Kronke, N. J. Peffer, P. B. Svetlik, M. Sullivan, and W. C. Green. 1985. Structure of the human interleukin 2 receptor gene. *Science (Wash. DC).* 230:633.

32. Treisman, R. 1985. Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. *Cell.* 42:889.