

# Regulatory anatomy of the murine interleukin-2 gene

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## ABSTRACT

We have cloned the mouse IL2 gene and sequenced 2800 bp of 5' flanking DNA. Comparison to the previously reported human sequence revealed extensive identity (~86%) between the two genes from +1 to -580 with additional small islands of homology further upstream. Proximal sites which have been shown to be important in regulation of the human IL2 gene are well conserved in sequence and location. Transfection experiments using hybrid gene constructs containing varying lengths of the mouse 5' flanking DNA linked to a CAT reporter gene have demonstrated the presence of several novel positive and negative regulatory elements. One negative regulatory region lying between -750 and -1000 consists primarily of alternating purines and pyrimidines and is absent from the human gene. The conserved region from -321 and -578, an upstream segment from -1219 to -1332, and another region of ~450 bp from -1449 to -1890, which contained a well-conserved sequence of 60 bp, were each associated with enhanced levels of expression. We found no evidence for intragenic or downstream enhancer elements in this gene. All the elements identified affect only the magnitude of the inducible response, for no region when deleted had the effect of altering either the need for induction, the kinetics of stimulation, or the cell-type specificity of expression. Deletion studies suggest a strong requirement for NFAT binding even in the presence of extensive 5' flanking sequence. Therefore we conclude that IL2 gene expression is controlled primarily through a central T<sub>H</sub>1-specific signaling pathway, which acts through proximal elements, while distal cis-elements exert a secondary modulating effect.

## INTRODUCTION

Perturbation of the antigen receptor/CD3 complex on resting T cells initiates a variety of biochemical responses whose proximal results include phospholipid hydrolysis, production of intracellular second messengers, transmembrane calcium fluxes and activation of protein kinase C<sup>1</sup>. These common, activation-specific events result in the expression of a preprogrammed set of genes by the

responding cells, leading ultimately to proliferation and/or the elaboration of effector function<sup>2</sup>.

The mechanisms by which cell-type-specific patterns of gene expression are allocated to discrete subsets of cells are obviously a fundamental aspect of T-cell development. To investigate the nature of these mechanisms, we have focused on the regulation of the murine IL2 gene. IL2 is subject to particularly stringent control, both in cell-type specificity and in its requirements for particular activating stimuli. The extreme transience of the kinetics of IL2 expression, and the refractoriness of proliferating normal T cells to IL2 induction, suggest that this gene is negatively as well as positively regulated. In addition, various T-cell subsets differ in the precise activation signals they require for IL2 induction<sup>3,4</sup>. These considerations indicate that the minimal mechanisms involved in activating IL2 transcription in tumor cells may not fully account for the sophisticated physiological control of this gene.

In this work, we have therefore characterized the sequences and potential regulatory elements in the mouse IL2 gene in regions extending considerably beyond the minimal 300 bp flanking sequences previously shown to be required for expression. The murine and human sequences appear to be strikingly well conserved over a discrete region extending about 600 bp upstream of the transcriptional start site. Additional islands of high sequence identity appear up to at least 1700 bp upstream. This high degree of conservation implies a functional role in regulation, which we have tested here.

## MATERIALS AND METHODS

### Cloning the mouse IL2 gene

A BALB/c genomic library in the vector  $\lambda$  Charon 4A was kindly provided by Dr. L. Hood. The insert DNA consisted of equal masses of sperm DNA that had been partially digested with Eco RI or partially digested with a combination of Hae III and Alu I followed by Eco RI linker addition<sup>5</sup>. The library was probed with a nick-translated 470 bp Bgl II-Acc I mouse IL2 cDNA fragment from pCD-mIL2<sup>6</sup> (generously provided by Dr. K.-I. Arai, Institute of Molecular and Cellular Biology, Palo Alto, CA). Positive plaques were isolated and the resulting phage purified through two additional rounds of infection. Phage DNA was prepared by standard plate lysate procedures<sup>7</sup> and the Eco RI

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restriction fragment pattern was compared to the previously published mouse IL2 map by DNA gel blot analysis using the entire IL2 cDNA insert from pCD-mIL2 as a probe. Two clones,  $\lambda$ IL2-9.1 and  $\lambda$ IL2-2.1, which contain the entire coding region and flanking DNA were selected for further analysis.

### Sequencing the IL2 5' flanking region

A 618 bp Rsa I-Pst I fragment extending from -578 to +40 relative to the start site of transcription was subcloned into the Sma I-Pst I sites of pSP65 (Promega Biotec) and the sequence determined according to Maxam and Gilbert<sup>8</sup>. A 2700 bp Eco RI-Acc I fragment from  $\lambda$ IL2-9.1 extending from -2800 to -100 was subcloned into the Eco RI-Sma I sites of pGem7Zf(+) (Promega) and the sequence determined by the dideoxy chain termination method using the Sequenase<sup>TM</sup> 2.0 kit as directed by the manufacturer (U.S. Biochemicals).

### Plasmid construction

A series of IL2-CAT hybrid genes was constructed in which expression of the bacterial gene for chloramphenicol acetyltransferase (CAT) is under the control of increasing amounts of IL2 5' flanking DNA. These plasmids are designated as pIL2 (-X) where X indicates the 5' terminal nucleotide, relative to the transcriptional start site, that is included in the construct. pIL2 (-2800) was constructed by first subcloning a 2.8 kb Eco RI-Pst I (-2800 to +40) fragment from  $\lambda$ IL2-9.1 into the corresponding unique sites of pSP65, followed by insertion of a 2.25 kb Pst I fragment from pTK-CAT (gift of C. Gorman and B. Howard, NIH<sup>9</sup>). This Pst I fragment contains 76 bp of HSV TK gene 5' untranslated DNA, the bacterial gene for CAT, and SV40 downstream processing signals. pIL2 (-1890) was constructed in a similar manner but starting with the 1.9 kb Eco RI-Pst I fragment from  $\lambda$ IL2-2.1 instead of the 2.8 kb fragment. pIL2 (-321) was made from pIL2 (-1890) by first digesting with Bgl I followed by partial Xmn I digestion and isolation of the largest fragment on a low-melt agarose gel. This fragment was recircularized by ligation to an Sma I-Bgl I fragment from pSP64. Plasmid pIL2 (-103) was generated from pIL2 (-321) by digesting completely with Acc I, filling in with Klenow polymerase and religating. pIL2 (-578) was made by cloning a 618 bp Rsa I-Pst I fragment, extending from -578 to +40, into the Sma I-Pst I site of pSP65, followed by digestion with Pst I and insertion of the 2.25 kb Pst I CAT cassette described above. pIL2 (-232) and pIL2 (-753) were made in the identical manner beginning with, respectively, a 272 bp Dde I-Pst I fragment (-232 to +40) and a 793 bp Ssp I-Pst I fragment (-753 to +40). Plasmids pIL2 (-1449), pIL2 (-1332), pIL2 (-1219), and pIL2 (-351) are deletions made by digesting pIL2 (-2800) with Stu I (-2205) followed by addition of Bal 31 nuclease. Aliquots were removed at 5, 10, and 15 min, treated briefly with the Klenow fragment of E. coli polymerase I to generate flush ends, and digested to completion with Bgl I. DNA in the desired size range was isolated on a low-melt agarose gel and recircularized by ligation to a Bgl I-Sma I fragment from pSP65. The exact extent of the deletions was determined by sequencing.

pIL2 (-1890;  $\Delta$ NFAT-1) was derived from pIL2 (-1890) and pIL2 (-321). Both plasmids were digested to completion with Xmn I and the following fragments were isolated on a low-melt agarose gel: 1) a 4.5 kb fragment from pIL2 (-321) which contained IL2 promoter sequences from -261 to +45, the CAT gene and plasmid sequences; and 2) a 2.4 kb fragment from pIL2 (-1890) which contained the remainder of the plasmid and IL2

sequences from -1890 to -322. These were ligated together and the loss of the 60 bp Xmn I fragment confirmed by dideoxy sequencing. Sequencing also revealed the loss of a C residue at position -261. Thus, the  $\Delta$ NFAT-1 deletion extends from -321 to -261, inclusive.

Plasmids containing intragenic or 3' flanking DNA were constructed as follows. Genomic fragments (see Fig. 1A) designated A (350 bp Pvu II-Eco RI), B (2.2 kb Eco RI-Hind III), C (1.8 kb Hind III), D (0.9 kb Hind III), E (1.1 kb Eco RI-Asp 718), F (1.8 kb Asp 718-Eco RV), and G (2.0 kb Eco RV-Eco RI) were cloned in both orientations into the unique Hind III site in pIL2 (-1890), either by blunt-end ligation (fragments A, B, E, F, and G) or cohesive-end ligation (fragments C and D). These were designated pIL2 (-1890) Y (+) or pIL2 (-1890) Y (-), where Y denotes the inserted fragment (A-G) and +/- indicates, respectively, the sense and anti-sense orientation of the inserted fragment with respect to the IL2-CAT gene.

All plasmids were purified on CsCl-ethidium bromide density gradients before use.

### Drugs and reagents

The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma) was dissolved in Me<sub>2</sub>SO at a concentration of 10  $\mu$ g per ml and the calcium ionophore A23187 (Calbiochem) was made up in Me<sub>2</sub>SO to a final concentration of 0.37 mg per ml; both were stored in small aliquots at -20°C.

### Cell lines and DNA transfections

The human T-cell leukemia line Jurkat (kindly provided by Dr. G. Crabtree, Stanford University) and the murine lines EL4.E1, NS-1, P388D1, and BW5147 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 50  $\mu$ M 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. The IL2-dependent lines HT-2, MTL2.8.2, and CTLL-2 were grown in the same medium supplemented with 5% TPA-stimulated EL4.E1 cell supernatant as a source of growth factor. The 32D cl 5 line was grown in Dulbecco's Modified Eagle Medium (DMEM), which contained 10% FBS, 2 mM L-glutamine, and 20% WEHI-3B conditioned medium as a source of IL3. Ltk<sup>-</sup> cells were grown in DMEM without IL3. D10.G4.1 were passaged every 10 days with antigen and antigen presenting cells as described<sup>10</sup>, except that we used 5% EL4.E1 conditioned medium instead of rat Con A supernatant as a source of growth factors. Cells were maintained in logarithmic growth prior to being transfected.

Transfection of hematopoietic cells with plasmid DNA was by DEAE-dextran facilitation. Cells to be transfected were washed in growth medium and counted. They were then rewashed in serum-free DMEM containing 10 mM HEPES, pH 7 (hereafter DME/H). Finally, the washed cells were resuspended to a density of  $1 \times 10^7$  per ml in a transfection cocktail consisting of DME/H, 0.1 mM chloroquine, 250  $\mu$ g per ml DEAE-dextran ( $2 \times 10^6$  M.W.), and 10  $\mu$ g per ml supercoiled plasmid DNA. The cells were put into loosely capped 17  $\times$  100 mm round-bottom polypropylene tubes (Falcon 2059) and placed in a 37°C/7% CO<sub>2</sub> incubator for 30 min (EL4.E1) or 60 min (Jurkat). The tubes were swirled gently every 10 min to prevent extensive cell clumping. Generally,  $1-2 \times 10^7$  cells were transfected with each plasmid in an experiment. After incubation in the transfection cocktail, 6 ml of DME/H were added to each tube and the cells were pelleted at  $500 \times g$  in a room temperature centrifuge. Jurkat

cells were then plated into two 60 mm tissue culture plates. EL4.E1 cells were washed an additional time in 3 ml DME/H containing 150 units per ml heparin (sodium salt; Sigma) to reduce clumping before being plated. Approximately 24 hr post-transfection, one plate of each pair received TPA and A23187 to final concentrations of 10 ng per ml and 37 ng per ml, respectively. After an additional 18 hr of incubation, cells were harvested for assay of CAT activity as described previously<sup>9</sup> except that extracts were incubated for 5 hr.

All comparisons were based on multiple transfections using at least two independent DNA preparations of each plasmid. Transfections included pBR322 as a negative control and pRSV-CAT as a positive standard for transfection efficiency. Relative CAT activity was calculated by setting the pBR322 sample to background and subtracting its cpm from the other samples. The percent acetylation per  $10^6$  cell equivalents was then calculated for each plasmid and normalized with respect to pIL2(-321), which was arbitrarily set to 1.0. Results are presented as mean  $\pm$  S.E.M. While an internal standard for transfection efficiency was not available during much of this work, the comparisons presented in Figure 7 were based on a large number of repeated experiments, in each of which all plasmids were transfected in parallel, using the same transfection cocktail and target cells. In each case, pRSV-CAT yielded 1–3% chloramphenicol acetylation (in 5 hr) per  $10^6$  cells.

Ltk<sup>-</sup> cells were transfected by calcium phosphate precipitation as described<sup>11</sup>. Cells ( $8 \times 10^5$ ) were seeded in 100 mm tissue culture plates one day prior to transfection. Each plate received 25  $\mu$ g of plasmid without added carrier DNA.

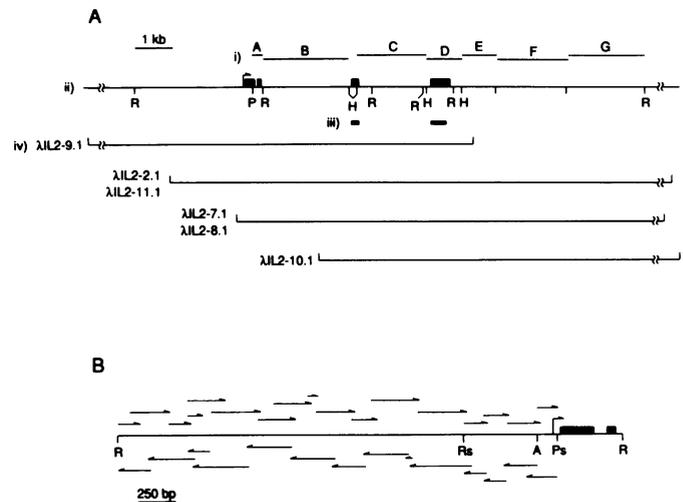
### RNA extraction and gel blot analysis

Cytoplasmic RNA was extracted after 5 hr of stimulation with TPA and A23187 as described, in the presence of 10 mM vanadyl ribonucleoside complexes<sup>12</sup>. Approximately equalized masses of RNA, usually 5–10  $\mu$ g, were electrophoresed on denaturing 1% agarose/formaldehyde gels and stained with acridine orange to visualize the rRNA. The presence of residual vanadyl ribonucleoside complexes led to some variation in the actual amounts of RNA loaded. The RNA was then blotted onto nylon membranes (Nytran, Schleicher and Schuell) according to the manufacturer's instructions. After baking at 80°C for 60 min to fix the RNA, the filters were probed with random primed cDNAs specific for human IL2<sup>13</sup> and mouse IL2<sup>6</sup> and IL4<sup>14</sup>. All filters were also probed with a mouse  $\alpha$  skeletal actin cDNA (N. Davidson, Caltech, unpublished) to verify the integrity of the RNA and to allow the signal to be normalized to the amounts of RNA present in each lane. Hybridizations were for 20 hr at 42°C in 5 $\times$ SSPE, 50% formamide, 0.2% SDS, 5 $\times$ Denhardt's and 10% dextran sulfate. Filters were washed three times for 1 min each at room temperature in 2 $\times$ SSC, 0.2% SDS and 0.05% NaPP<sub>i</sub>, followed by two 30 min washes at 68°C in 0.2 $\times$ SSC, 0.1% SDS, 0.05% NaPP<sub>i</sub>. They were then exposed to film at -70°C with an intensifying screen.

## RESULTS

### Isolation of the mouse IL2 gene

We isolated phage containing the murine IL2 gene from a BALB/c genomic library in  $\lambda$  Charon 4A, using a 470 bp Bgl II-Acc I nick-translated cDNA probe to identify six positive clones representing four different genomic inserts (Fig. 1A). The identities of the resulting clones were verified by comparison with



**Figure 1.** Cloning the mouse IL2 gene and sequencing of its 5' flanking region. (A) (i) Top lines (A-G) show the intragenic and 3' fragments that were tested for the presence of transcriptional regulators. (ii) The organization of the mouse IL2 gene is shown. Thin line denotes chromosome; thick boxes represent exons. Arrow marks start site and direction of transcription. Letters under chromosome represent restriction enzyme sites relevant to the constructions. R = EcoRI, P = PvuII, H = HindIII, As = Asp718 and RV = EcoRV. (iii) Thick lines below exons 3 and 4 indicate the sequences included in the 3' cDNA probe used in the library screen. (iv) Regions represented in genomic clones are diagrammed. (B) Expanded view of exons 1 and 2 and 5' flanking DNA showing the sequencing strategy employed. Letters designate restriction enzyme sites. R = EcoRI, Rs = RsaI, A = AccI and Ps = PstI.

predictions from the genome organization and partial sequence previously reported by Fuse *et al.*<sup>15</sup>. These authors had shown that, in the mouse, the IL2 gene resides on four contiguous EcoRI fragments spanning 8.3 kb of DNA, as shown in Fig. 1A. We identified three genomic clones that contain at least 2 kb of 5' flanking DNA in addition to the entire coding region. One of the clones,  $\lambda$ IL2-9.1, contains the intact 3.3 kb genomic Eco RI fragment upon which exons 1 and 2 reside, with at least 2.8 kb of upstream sequence. Clones  $\lambda$ IL2-2.1 and  $\lambda$ IL2-11.1 appear identical and possess a 2.4 kb Eco RI fragment that hybridizes to a 5' IL2 cDNA probe (not shown). Their 5' termini are at a HaeIII site, modified with Eco RI linkers, 1.9 kb upstream of the transcriptional start site. All of the clones isolated except  $\lambda$ IL2-9.1 also contain >7 kb of 3' flanking DNA.

We determined the sequence of the 2.8 kb of 5' flanking sequence present on the 3.3 kb genomic Eco RI fragment, as described in Materials and Methods. Figure 1B shows the sequencing strategy used. The major features of our murine sequence (Fig. 2), in comparison to the human IL2 sequence, are discussed in the following.

### Comparison between murine and human sequences

A dot matrix comparison between the human<sup>16</sup> and mouse IL2 5' flanking sequences is shown in Fig. 3. There is extensive sequence identity extending from -1 to -616, where it ends abruptly, and also some scattered islands of similarity further upstream. A direct sequence comparison of the mouse and human 5' flanking regions up to -580 (-585 in the human) is shown in Fig. 4. The identity throughout this region is 86%, which is similar to the degree of conservation between the proximal upstream regions of other mouse and human lymphokine genes. However, the length of the conserved upstream region is longer

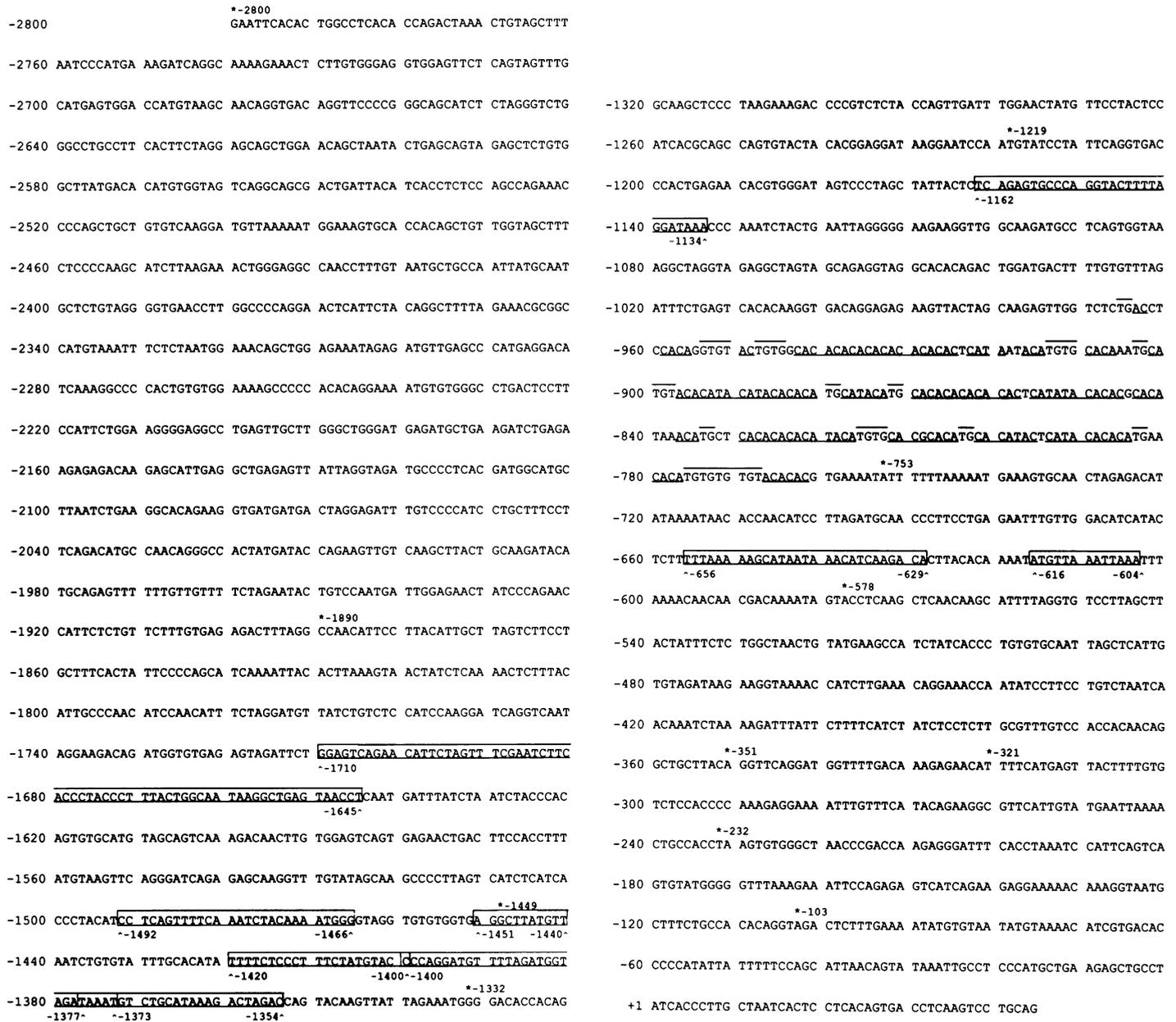


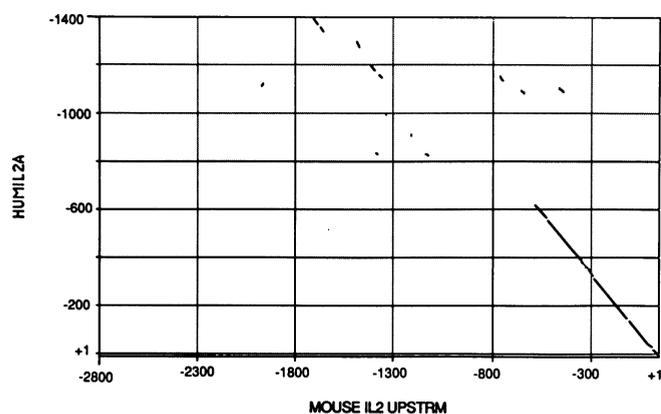
Figure 2. Sequence of the mouse IL2 gene 5' flanking region from -2800 to +45. Sequence was determined by both chemical degradation and dideoxy chain termination methods. Regions upstream of -580 that show homology to the human IL2 gene are boxed (see Fig. 4). Underlined and overlined regions show stretches of poly d(CA) and poly d(GT), respectively. Asterisks indicate the 5' termini of the IL2 sequences in the constructs diagrammed in Fig. 6.

in the IL2 gene than in IL3, IL6, and GM-CSF<sup>17-19</sup>. As for other lymphokine genes, this degree of flanking sequence conservation is considerably greater than that of the coding regions of the two genes. Such strong evidence of evolutionary pressure against sequence divergence suggests that sequences throughout this region play a role in DNA-protein interactions required for IL2 gene regulation.

The sequence similarity terminates abruptly about 100 bp from the border of a striking feature of the murine sequence, namely the block of alternating purines and pyrimidines between -759 and -960. There is nothing in the human gene which corresponds to this murine sequence. On the coding strand, poly(dC-dA) predominates with interspersed islands of poly(dG-dT), in runs

of up to 48 residues (Fig. 2). The alternating Pu-Py tracts may have been introduced into the murine gene, or deleted from the human gene, en bloc, because where mouse-human sequence similarities resume further upstream, they are out of register. The murine sequence is usually present at a position about 250 bp further from the RNA cap site than its human counterpart (see Figs. 3 and 5). Of interest is the presence of two contiguous 7 bp direct repeats of the sequence 5'-ACACATA-3' between -746 and -733 of the human IL2 gene, suggesting the possible loss of a longer alternating Pu-Py stretch by recombinational excision.

Figure 5 lists the additional promoter-distal regions of sequence similarity identified by the dot-matrix comparison of the murine



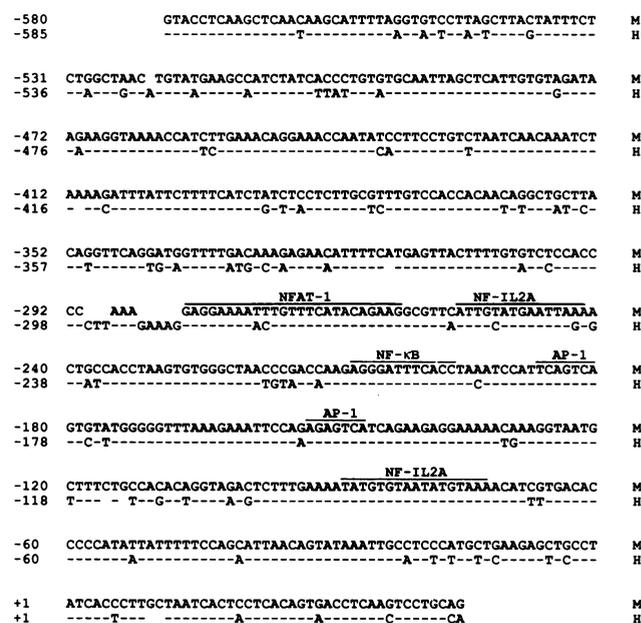
**Figure 3.** Dot matrix comparison of mouse IL2 upstream region from  $-2800$  to  $+45$  and human sequence from  $-1370$  to  $+45$  using the Pustell program from the Mac-Vector DNA analysis software package (IBI). The hash level was 6 and the window size was 25 and the minimum positive score was set at 65%. Human sequence was from Holbrook<sup>16</sup>, with the start site of transcription moved 8 bp to the 3' direction in accord with Taniguchi<sup>42</sup> and Devos *et al.*<sup>43</sup>.

and human genes. Some of these matches are impressive; 28/30 identical base pairs with one gap (between  $-1645$  and  $-1674$  in the murine gene); 15/16 identical ( $-1695$  to  $-1710$ ); 24/27 identical (between  $-1466$  and  $-1492$ ); and 19 identical in a contiguous run of 21 (between  $-1400$  and  $-1420$ ). These do not represent obvious repetitions of motifs found in the more proximal regions, nor do the conserved sequences include recognizable binding sites for the DNA-binding proteins considered in the next section. Their functional significance will be discussed in a later section.

#### Candidate binding sites for transcription factors

Several DNA binding proteins have been shown to footprint over the region from  $-1$  to  $-279$  in the human IL2 gene<sup>20,21</sup> and these protected regions have been shown by genetic analysis to play a role in its inducible expression<sup>20,22,23</sup>. The regions protected by two of these proteins, NF-IL2A (Oct-1) and NFAT-1, are indicated in Fig. 4. The proximal NF-IL2A site is perfectly conserved between the two genes, while the distal sites differ at 7 out of 39 nucleotides. The binding site for NFAT-1, a protein restricted to activated T cells<sup>24</sup> whose appearance precedes IL2 gene transcription, is also conserved well, though not perfectly, between mouse and human. This is not surprising in view of the fact that nuclear extracts from activated EL4.E1 cells contain a protein which has similar properties to NFAT-1 in a gel retardation assay<sup>24</sup>. Binding sites for NF- $\kappa$ B and AP-1-like factors in the human gene<sup>25-27</sup> are conserved in both sequence and position in the mouse 5' flanking region.

A computer-assisted search of the IL2 5' region identified several potential binding sites for known regulatory proteins in the promoter-distal flanking regions of the murine IL-2 gene. These identifications are provisional because they are based solely upon homology to published consensus sequences. We could not locate consensus motifs for T-cell receptor decamer (AGTGA<sup>T</sup><sub>CA</sub>TCA, at a level of 80%)<sup>28</sup>, Sp1 (GGGCGG, at a level of 85%) or the cAMP response element CREB (TGACGTCA, at a level of  $>75\%$  matching). The failure to identify consensus motifs of course does not exclude other binding



**Figure 4.** Comparison of mouse and human IL2 immediate upstream regions from  $-580$  to  $+45$ . Dashes indicate identity, and gaps were added by visual inspection to maximize matches. Indicated on the figure are binding sites for NFAT-1, NF- $\kappa$ B, NF-IL2A and AP-1 (see text). Other proteins footprint over this region but have not been identified<sup>25</sup>. Overlines indicate binding sites for proteins demonstrated to interact with the mouse or human IL2 promoter. The extent of each binding site shown is from published consensus sites for AP-1 and NF- $\kappa$ B<sup>26,44</sup>, from deletion analysis for NFAT-1 and the proximal NF-IL2A site<sup>20</sup>, and from footprint analysis for the distal NF-IL2A site<sup>20</sup>. For sites that were determined by several methods, the minimum size of the site is shown.

sites for any of these factors. However, we did locate four glucocorticoid response element (GRE) core motifs (TGTTCT)<sup>29</sup> at  $-1913$ ,  $-1704$ ,  $-1194$ , and  $-327$ . The GRE at  $-1704$  falls within an extended region of similarity between the mouse and human IL2 genes (Figs. 3 and 4B). Normally, the signals inducing IL2 transcription can be mimicked by the combination of calcium ionophore and TPA<sup>30</sup>. The proteins AP-1 and AP-3 are involved in TPA-inducible gene responses, and consensus binding sites have been proposed for both. In addition to the probable AP-1 binding sites at  $-153$  and at  $-185$ <sup>25,27</sup>, there are two more potential sites further upstream at  $-1515$  and  $-1015$ . Three possible AP-3 sites are present at  $-2708$ ,  $-2492$ , and  $-2264$ .

#### Far upstream elements modulate IL2 expression in T<sub>H</sub>1-type lymphomas

Extensive analysis of the human IL2 gene promoter has revealed numerous regions between  $-60$  and  $-361$  which, when deleted, reduce the level of inducible expression of a linked reporter gene following transfection into the Jurkat and EL4 T-cell lines<sup>20,22,23,26</sup>. At least five regions have been implicated thus in activation. There is no evidence, however, that this catalogue of positive elements is complete. Furthermore, there has been no test of the functional roles of negative regulatory sequences in the maintenance of strict activation and/or tissue specificity of this gene. Because the upstream conservation between mouse and human IL2 genes suggests a functional role for sequences lying 5' to  $-300$ , we constructed a series of IL2-CAT plasmids in which the bacterial gene for chloramphenicol acetyltransferase



**Figure 5.** Comparison of nine mouse and human similarity regions located upstream of  $-580$ . Top line is mouse sequence. Gaps have been introduced by inspection to maximize similarity. Numbers above and below the sequences indicate their position relative to the transcriptional start sites of the respective genes.

is under the control of the mouse IL2 gene promoter. To minimize non-IL2 regulatory sequences that might elevate basal levels of expression nonspecifically, no heterologous promoter or enhancer sequences were included. The structures of these chimeric molecules, containing between 103 bp and 2800 bp of 5' flanking IL2 DNA, are diagrammed in Fig. 6.

The smallest construct, pIL2 ( $-103$ ) CAT, contains only the promoter-proximal NF-IL2A enhancer element. The pIL2 ( $-232$ ) construct includes, in addition, three putative sites for inducible activation factors: NF- $\kappa$ B (at  $-206$ ) and two proposed AP-1 sites (at  $-185$  and  $-153$ ). All sites known to be 'essential' are included in the pIL2 ( $-321$ ) and longer constructs<sup>2</sup>.

To test for a role of promoter-distal elements in controlling the magnitude of inducible IL2 expression, this series of plasmids was introduced into Jurkat and EL4.E1 cells, and the resulting CAT activity determined after 18 hr of stimulation with A23187 and TPA (Fig. 6). In all cases, the plasmids were transfected into the same cell population in parallel, with our shortest expressible construct [pIL2( $-321$ )] serving as a normalization standard for the resulting levels of CAT activity. The combined results of a series of analyses are presented schematically in Fig. 7.

With the exception of pIL2 ( $-103$ ) and pIL2 ( $-232$ ), all the IL2-CAT plasmids yielded easily detectable amounts of CAT

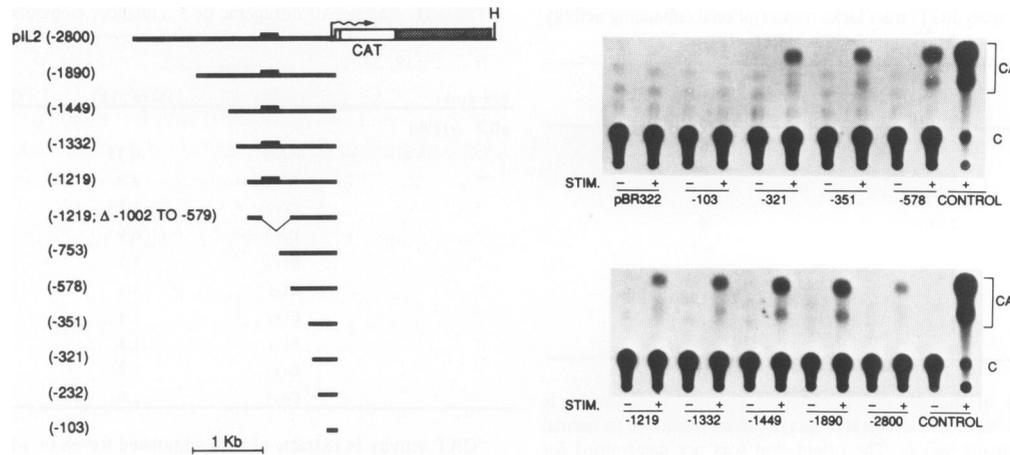
activity in extracts from transfected cells following stimulation (Fig. 7). There was no detectable CAT activity in any case unless the cells were stimulated, in accordance with the fact that transcription of the IL2 gene is linked tightly to cell activation<sup>2,31</sup>. The failure of pIL2 ( $-103$ ) to be expressed under any conditions indicates that this fragment lacks intrinsic promoter function in the absence of enhancing elements between  $-103$  and  $-321$ . Expression was detectable with pIL2( $-232$ ), but this was at least an order of magnitude less than from pIL2( $-321$ ). Thus, the NF- $\kappa$ B, Oct-1 and AP-1 candidate sites are not sufficient for efficient induction in these cells.

There were several effects from increasing the amount of IL2 flanking DNA beyond  $-321$ . While both positive and negative effects were observed, in general they were small in magnitude when assayed in the EL4.E1 or Jurkat tumor cells. In these constructs, the upstream elements were not separated from powerful proximal enhancer elements which may have obscured their function. The description that follows is provided nevertheless, in view of the possibility that some of these sites could play a stronger role in normal cells with more stringent regulation of IL2.

First, the increase in 5' flanking DNA from  $-321$  to  $-578$  was correlated with a reproducible increase in CAT activity, in both cell lines. This suggests that additional positive regulatory elements are located between  $-321$  and  $-578$  in the murine gene, in agreement with the evidence for stringent sequence conservation. The effects of adding these sequences had not been noted previously in studies with the human gene<sup>22</sup>.

Second, there appears to be a weak negative regulatory region lying somewhere between  $-578$  and  $-1219$ . In both cell lines examined, CAT activity was reproducibly about two fold lower with pIL2 ( $-1219$ ) than with pIL2 ( $-578$ ). The pIL2 ( $-1219$ ) plasmid encompasses the block of alternating purine-pyrimidine residues that is not found in the human 5' flanking region. To test whether the poly d(CA) tract or other sequences in this region act as negative regulators, we cloned a 260 bp *Hin*FI-*Ssp*I fragment containing the poly d(CA) region upstream of the IL2 promoter sequences in pIL2 ( $-103$ ) and pIL2 ( $-321$ ). These constructs were then transfected into EL4.E1 cells in parallel with the unmodified parental plasmids. That this region lacks intrinsic enhancing activity in EL4.E1 is shown in Table I. The presence of one or two copies of the d(CA) tract was unable to impart transcriptional activity to pIL2 ( $-103$ ). Furthermore, when one to three copies of this element are inserted upstream of the expressible ( $-321$ ) construct, the resulting plasmids showed a lower level of CAT activity than did the unmodified parental plasmids. Thus, rather than possessing positive enhancing activity, the poly d(CA) region could account for the lower level of expression seen with pIL2 ( $-1219$ ). To test alternative sites for the negative element(s) in this region, we examined two additional constructs, pIL2 ( $-753$ ) and pIL2 ( $-1219$ ;  $\Delta$ -1002 to  $-579$ ). Results (not shown) revealed that both pIL2 ( $-753$ ) and pIL2 ( $-1219$ ;  $\Delta$ -1002 to  $-579$ ) were expressed as well as pIL2 ( $-578$ ), thus implicating the poly d(CA) tract as most likely to account for the negative effect on IL2 promoter function.

Third, there was evidence for two novel positive regulatory elements further upstream. Their effects were detected more clearly in the conspecific EL4.E1 line than in the human Jurkat cells, which tend to express the longer constructs poorly in general (see below). The negative effect of sequences between  $-1219$  and  $-578$  was reproducibly found to be reversed by including additional 5' IL2 sequences as little as 113 bp further



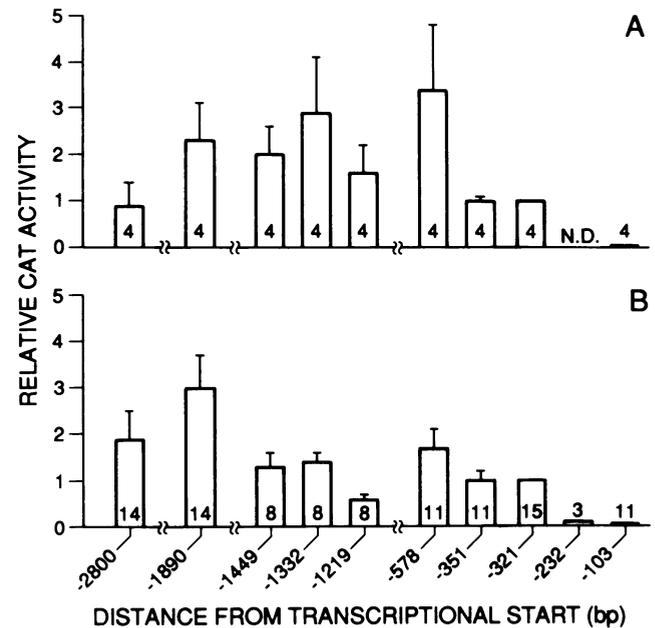
**Figure 6.** (Left) pIL2-CAT constructs. The number in parentheses refers to the 5' terminal nucleotide located in the plasmid. All IL2 sequences terminate downstream at +45 in the 5' untranslated region. Thick line, IL2 5' sequence; black box, poly(dC-dA) tract; open box, CAT gene; stippled box, SV40 sequences containing small t intron and polyadenylation sites. Vector is pSP65 (not shown). Arrow indicates start site of transcription. H indicates location of unique HindIII site in pIL2 (-1890) used in construction of plasmids with intragenic and 3' genomic sequences. (Right) Representative CAT assay of extracts from Jurkat cells transfected with each of the indicated constructs. Unacetylated chloramphenicol and its monoacetylated derivatives are indicated with (C) and (CA), respectively. - = uninduced; + = 18 hr stimulation with TPA + A23187.

upstream, suggesting the presence of a positive element in this interval. Both pIL2 (-1332) and pIL2 (-1449) showed higher levels of expression than pIL2 (-1219) (Fig. 7). At least one other possible positive regulator of IL2 gene expression lies further upstream. Inclusion of the sequences between -1449 and -1890 reproducibly resulted in the highest level of IL2-promoted CAT activity achieved in EL4.E1 cells. Note that this region includes the highly conserved sequences between -1466 and -1492, between -1645 and -1673, and between -1694 and -1710, as described above. An additional increase to -2800 had a modest negative effect in EL4.E1, which was more substantial in human Jurkat cells.

In general, the positive or negative effects on expression of each increment of 5' flanking sequence were observed both in murine EL4.E1 cells and in human Jurkat cells. However, as shown in Fig. 7, the two lines differ in their relative strengths of reporter gene expression from the IL2(-578)-CAT and IL2(-1890)-CAT constructs, with EL4.E1 showing considerably higher expression from the longer constructs. Whether this reflects a cell-type-specific (or species-specific) positive regulatory element, or alternatively a systematic difference in the utilization of longer fragments of exogenous DNA, is not presently resolved.

#### Absence of 3' and intragenic enhancers

Transcriptional enhancers have been discovered 3' to the mouse T-cell receptor *Cβ2* locus<sup>32</sup> and *CD3δ* gene<sup>33</sup> and the human *CD2* gene<sup>34</sup>. We therefore tested the possibility that additional transcriptional regulatory sequences reside within or 3' to the IL2 gene by subcloning different fragments of genomic DNA (Fig. 1A) into pIL2 (-1890) as described in Methods. In these constructs, the genomic fragments were inserted 0.6 kb downstream of the two SV40 derived polyadenylation sites, and therefore were not included in the IL2/CAT transcription unit. In this way, 5.0 kb of 3' flanking DNA and all but 100 bp of intragenic DNA were tested. All of these constructs were expressed at a lower level than the parent plasmid from which they were derived (Table II). Clearly there are no positive regulatory elements in these regions that can act in a position-



**Figure 7.** Pooled CAT assay data from transfected Jurkat (A) and EL4.E1 (B) cells. Relative CAT activity calculated as in Materials and Methods. Numbers in bars are the number of times that a given plasmid was tested in parallel with pIL2 (-321) CAT as a reference standard. Only results from 18 hr stimulated cells are shown. Results are presented as mean  $\pm$  S.E.M.

independent way. These results do not, however, identify sequence-specific negative regulatory regions, because the degree of inhibition seemed primarily related to the length of the inserted DNA. While the relative reductions seen in CAT activity greatly exceed the decreases in the molar concentration of plasmid DNA in the transfection protocol, it remains possible that nonspecific mechanisms were responsible for limiting expression. The more dramatic negative effect of B (second intron) may, however, indicate that some sequences limiting IL2 expression are indeed located outside of the 5' flanking region.

**Table I.** The polyd(CA) · Ipolyd(GT) tract lacks transcriptional enhancing activity in EL4.E1 cells

Plasmid	Insert <sup>a</sup>	Relative CAT activity <sup>b</sup>
pIL2 (-103)	-	0
"	1 x CA (+)	0
"	1 x CA (-)	0
"	2 x CA	0
pIL2 (-321)	-	1.0
"	1 x CA (+)	1.0±0.3
"	1 x CA (-)	0.78±0.01
"	2 x CA	0.48±0.08
"	3 x CA	0.42±0.01

<sup>a</sup>The number of copies of the 260 bp *Hin*FI-*Ssp*I polyd(CA) fragment is indicated. (+) or (-) indicates the orientation of this fragment relative to its normal location in the IL2 upstream region. The orientation was not determined for plasmids containing multiple copies.

<sup>b</sup>Plasmids were transfected into EL4.E1 cells along with pBR322 as a negative control. Relative CAT activity was determined as indicated in Table III after 18 hr stimulation with 10 ng/ml TPA + 37 ng/ml A23187. Results are the mean ± range of two experiments.

### Control of tissue-specific IL2 expression is mediated through the 5' flanking DNA by a T<sub>H</sub>1-specific signalling pathway

Normally, IL2 gene transcription appears to be restricted to activated T<sub>H</sub>1 cells, even though these cells share a common precursor with all hematopoietic cells. To test the roles of IL2 regulatory sequences in maintaining cell-type specificity, the series of pIL2-CAT plasmids was transfected into a variety of hematopoietic and non-hematopoietic cell lines by DEAE-dextran facilitation, as shown in Table III. The panel included other T-cell types, such as T<sub>H</sub>2 cells and CTLs, in addition to non-T cells. To ensure delivery of an activation signal we used agonists of the universal phosphoinositide pathway, namely the calcium ionophore A23187 and the phorbol ester TPA. After an 18 hr stimulation with TPA + A23187, the cells were harvested and assayed for the presence of CAT.

None of the plasmids was expressed in any cell line tested except EL4.E1 and Jurkat, although only the results for pIL2 (-1890) are shown here. This lack of expression by other cell lines is not due to their refractivity to transfection or to an inability to respond to TPA + A23187. All the cells listed express easily detectable amounts of CAT protein when transfected with a control plasmid, pRSV-CAT, and several of them (32D cl5, S49.1, our subline of BW5147, and others) possess an activation pathway capable of further elevating expression from the RSV LTR upon stimulation. The successful stimulation of the HT-2, 32D cl 5, and D10.G4.1 lines was demonstrated by their synthesis of endogenous IL4 RNA under these conditions (Fig. 8 and data not shown). Table III shows that even the combination of transfectability and inducibility in these cell lines is not sufficient to permit expression from any IL2 promoter fragment, even when introduced as naked DNA. Thus, cell-type specific expression of IL2 appears to depend upon a signalling response specific to T<sub>H</sub>1-type lymphomas.

### Subordination of distal modulator elements to a common activation pathway

In general the different extents of 5' flanking sequence affected the magnitude but not the kinetics of CAT expression from the IL2-CAT constructs after induction. Comparison of the relative levels of CAT activity expressed at 6 hr, 9 hr, and 24 hr of

**Table II.** Absence of intragenic or 3' enhancer elements in the mouse IL2 gene

Plasmid	Insert	Size (kb)	Relative CAT activity <sup>a</sup>
pIL2 (-1890)			1.0
"	A(+)	0.35	0.54±0.12
"	B(+)	2.3	0.23±0.08
"	C(+)	1.8	0.40±0.17
"	D(+)	0.9	0.50±0.11
"	E(+)	1.1	0.43±0.08
"	E(-)	1.1	0.53±0.12
"	F(+)	1.8	0.71±0.27
"	F(-)	1.8	0.49±0.16
"	G(+)	2.0	0.48±0.18
"	G(-)	2.0	0.53±0.20

<sup>a</sup>CAT activity in extracts of cells stimulated for 18 hr with TPA + A23187 was determined as in Table III except that values were normalized with respect to pIL2 (-1890). Results are given as mean ± range of two experiments for fragments A-D and three experiments for fragments E-G.

induction showed indistinguishable kinetics with any of the plasmids (data not shown). Thus, the positive and negative effects on expression appear to be kinetically subordinate to a common time course of stimulation.

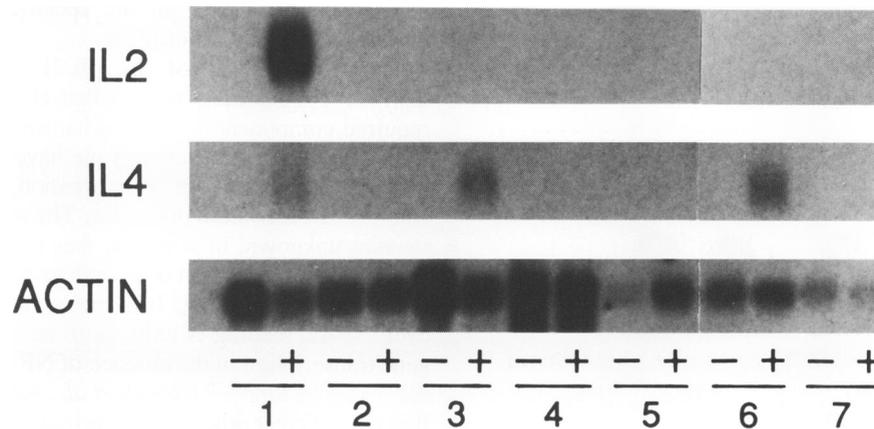
To test whether the stimulatory effects of the upstream elements could obviate the requirement for the NFAT-1 site, we constructed a derivative of pIL2(-1890) from which the NFAT-1 site was specifically excised. This construct, pIL2(-1890; ΔNFAT-1), was transfected into EL4.E1 cells in parallel with the parental pIL2(-1890) and the pIL2(-321) constructs. As shown in Fig. 9, upon stimulation the pIL2(-1890; ΔNFAT-1) construct was expressed at severely reduced levels even in comparison with the minimal pIL2(-321) construct. Thus, no signaling mediators generated in these tumor cells were capable of activating the upstream sequences independently of NFAT-1. The particular severity of this reduction of activity, in comparison with the milder effects of NFAT-1 site deletions in shorter human IL2 constructs<sup>20,23</sup> may further indicate a necessary role for the conserved sequence from -301 to -322 (Fig. 4), which is also removed from the ΔNFAT construct.

## DISCUSSION

### Structure of the murine IL2 5' flanking region

In order to understand the molecular basis for the restricted expression of the murine IL2 gene we have cloned the gene and determined the nucleotide sequence of the 5' flanking region. Our upstream sequence extends to -2800 relative to the start site of transcription; this is 2300 bp further upstream than sequences previously reported<sup>15</sup>. This region showed no extended homology to any other murine lymphokine gene. Comparison of this sequence to that previously determined for the human IL2 gene<sup>16</sup> revealed extensive similarity from -1 to -616 with the regions between -1 and -580 displaying 86% identity throughout their length. Thus, the evolutionarily conserved sequence in the IL2 5' flanking region extends ~350 bp beyond the sequences previously reported to be required for maximal induction of the human IL2 promoter<sup>20,22,23</sup>, with patches of similarity resuming further upstream. The extensive conservation suggests a role for multiple regulatory sequence elements in the relatively complex control of IL2 gene expression.

The sequence conservation between -1 and -616 is



**Figure 8.** Gel blot analysis of cytoplasmic RNA extracted from induced and noninduced hematopoietic cell lines. Total cytoplasmic RNA was fractionated on denaturing agarose/formaldehyde gels, blotted, and hybridized with the indicated probes as described in Methods. Data presented are from sequential hybridizations of the different probes with the same filter. - = uninduced; + = induction with TPA + A23187. Cell lines are (1) EL4.E1, (2) BW5147, (3) HT-2, (4) P388D1, (5) WEH1-3B, (6) 32 D cl 5, (7) NS-1. Note that our subline of BW5147 is not inducible for IL2, in contrast to others in the literature<sup>45</sup>.

remarkable less for its degree than for its length. In general, comparisons of the immediate upstream regions of other lymphoid-specific genes show regions of similar conservation between murine and human. The extent of these homologous regions, however, tends to be on the order of 300 bp<sup>17-19</sup>. For example, the mouse and human IL2R $\alpha$  gene upstream regions contain only 42 mismatches in the first 300 bp (86% similarity), but contain 51 mismatches in the next 92 bp (45% similarity; data and numbering scheme from ref. 35).

In addition to overall sequence conservation, the human and mouse IL2 promoters also contain binding sites for identified trans-acting factors located in the same order and in the same place. It is likely that other factors, as yet unidentified, interact with sequences upstream of -300 since there must be selective pressure to keep these sequences so similar. The high degree of homology in the regions between known sites suggests that some factors may bind very close to one another or even overlap in their DNA recognition specificity. The possible inclusion of negative regulatory sites could explain some of the internal deletion results of Williams *et al.*<sup>23</sup>, in which a smaller internal deletion from -361 to -335 in the human IL2 promoter reduced expression much more severely than a larger deletion from -361 to -292. Postulating contiguous and/or overlapping sites can also explain why the identified protein binding sites have not moved around relative to one another since the divergence of mice and humans as they have in other genes (e.g., metallothionein-I<sup>36</sup>).

Deletion analysis of the mouse IL2 upstream region suggests a net positive role for the conserved sequences between -351 and -578, although these are not required for expression of the murine or the human<sup>20,22,23</sup> gene. Transfection experiments utilizing IL2 promoter-CAT constructs demonstrated that an increase in 5' DNA from -351 to -578 was correlated with an increase in inducible CAT activity. This is in accord with the results of Williams *et al.*<sup>23</sup> showing that certain deletions in this region could dramatically reduce expression from the IL2 promoter even when the binding sites for NFAT-1, NF- $\kappa$ B, and Oct-1 and AP-1 were intact. That sequences upstream of -321 could play a role in regulating IL2 gene expression is further supported by experiments of Nabel *et al.*<sup>37</sup> using various upstream regions of the human IL2 gene as probes in gel mobility shift assays.

**Table III.** pIL2-CAT expression is cell-type specific and dependent on both cell-type and gene-specific inductive signals

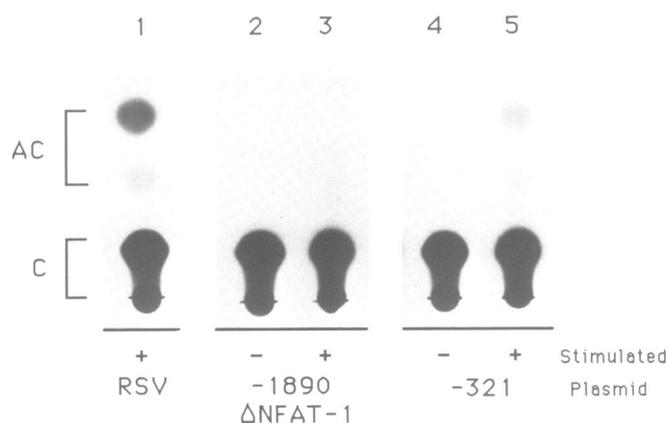
Cell line	Cell type	Endogenous mRNA <sup>a</sup>		pRSV-CAT activity <sup>b</sup>		Fold induction	pIL2(-1890) CAT activity <sup>b</sup>	
		IL2	IL4	-	+		-	+
NS-1	B-cell	-	-	0.33	0.33	1.0	0	0
32D cl 5	Pre-mast cell	-	+	3.8	14.3	3.8	0	0
P388D1	Macrophage	-	-	0	0.1	$\infty$	0	0
HT-2	Th2	-	+	0.55	3.3	6.0	0	0
D10.G4.1	Th2	-	+	N.D. <sup>c</sup>	0.28	N.D.	0	0
BW5147	Thymoma	-	-	0.7	4.2	6.0	0	0
MTL2.8.2	CTL	-	-	9.9	9.3	0.9	0	0
CTLL-2	CTL	-	-	9.7	19.3	2.0	0	0
Ltk <sup>-</sup>	Fibroblast	-	-	17	22	1.3	0	0
EL4.E1	Thymoma	+	+	0.35	3.4	10	0	0.42
Jurkat	Th1	+	N.D.	8.7	12.8	1.5	0	4.1

<sup>a</sup>Cytoplasmic RNA was extracted after 5 hr stimulation with 10 ng/ml TPA + 37 ng/ml A23187 and analyzed as described in Materials and Methods.

<sup>b</sup>CAT activity is presented as % acetylation per  $1 \times 10^6$  cell equivalents in a 5 hr assay, except for Ltk<sup>-</sup> extracts, which were assayed for 30 min. - = unstimulated; + = 18 hr stimulation with 10 ng/ml TPA + 37 ng/ml A23187, except for D10.G4.1 cells, which were cultured at  $2 \times 10^5$  per ml for 45 h with  $6.7 \times 10^5$  mitomycin C-treated spleen cells and 100 mg conalbumin per  $\mu$ l. 0 = < 0.05.

<sup>c</sup>Not done.

Further upstream, the murine and human sequences diverge, but regulatory effects of specific sequences are still seen. A potential positive element or elements can be correlated with a region where several islands of excellent mouse-human similarity reappear (between -1440 and -1890). Another likely positive element occurs in the region between -1219 and -1332. On the other hand, a striking aspect of the mouse IL2 upstream region is the poly d(CA) region from -1000 to -750, apparently associated with a negative regulatory effect. Stretches of alternating purines and pyrimidines are found upstream of a number of murine lymphokine genes (e.g., IL6, GM-CSF)<sup>17,18</sup> and in their large introns (e.g., IL4, IL6)<sup>17,38</sup>, but apparently not in their human counterparts. The regulatory implications of this difference are not clear. Poly d(GT)·Id(CA) is able to assume



**Figure 9.** Defective expression of pIL2 (-1890; $\Delta$ NFAT). The figure shows a representative CAT assay of EL4.E1 cells transfected with the indicated constructs as described in Materials and Methods. Similar results were obtained in three separate experiments. - = uninduced; + = induction with TPA + A23187. In this experiment stimulation was for 5 hr. In another experiment (not shown), pIL2 (-1890;  $\Delta$ NFAT) gave 4% as much activity as pIL2 (-1890) at 18 hr of stimulation.

a Z-DNA configuration<sup>39</sup>, and d(GT) $\cdot$ Id(AC)<sub>30-130</sub> has been reported to show weak transcriptional enhancing activity in fibroblasts in conjunction with a truncated SV40 early promoter<sup>40</sup>. Because the poly d(CA) tract is correlated with lower levels of expression from the IL2 promoter, it is possible that such tracts have either enhancing or silencing effects, depending on the promoter.

#### Role of regulatory sequences in control of IL2 gene expression

The critical question is the relationship between the regulatory properties of these novel upstream elements and those of the promoter-proximal elements described before<sup>2</sup>. There are three general possibilities. First, the upstream elements may be enhancers (and partial silencers) in their own right, normally cooperating with downstream inducible elements but capable of autonomous action. Second, the upstream elements may represent inducible enhancers and silencers, interdependent with the proximal elements but conferring altered sensitivity to particular inductive stimuli or altered cell-type specificity. Third, the upstream elements may be subordinate to the proximal elements, capable of modulating levels of expression but not of activating expression autonomously. Our results argue against the first two models and support the third.

An important result of our investigations was the inability to segregate any of the determinants of induction-dependence or pharmacological sensitivity (in Novak, Chen, and Rothenberg, and Novak and Rothenberg, mss. submitted for publication), from expression *per se*. By contrast, Serfling *et al.*<sup>25</sup> have suggested that when IL2 upstream sequences are combined with a heterologous promoter, the resulting expression is somewhat less induction-dependent when shorter (~300 bp) IL2 sequences are used than with longer (~2000 bp) ones, possibly due to artefactual read-through transcription by RNA polymerases<sup>41</sup>. In our experiments using the natural IL2 promoter with its regulatory sequences, it is striking that all constructs from pIL2 (-321) to pIL2 (-2800) were induced or inhibited in parallel, with indistinguishable kinetics, although to different maximal levels. These results strongly suggest that the overall progress of IL2 promoter induction is governed by a single rate-limiting

mechanism, to which all the specific positive and negative upstream sites are subordinate.

The poor activity of the pIL2(-232) and pIL2(-1890;  $\Delta$ NFAT-1) constructs suggest that NFAT binding may be one required component of this mechanism in EL4.E1 cells. Thus, the distal regulatory elements we have described appear to be quantitative modulators of expression, rather than targets of autonomous activation processes. The mechanism of their action remains unknown. In principle, they may act as sites for specific DNA protein interaction or as regions which alter DNA topology or nucleosome phasing. It is important to note, however, that even NFAT binding is unlikely to be sufficient to activate IL2 gene transcription in the absence of NF- $\kappa$ B-like, Oct-1-like, and AP-1-like factors<sup>20,26</sup> (Novak *et al.*, submitted for publication). It is not yet clear whether the upstream sequences might be able to compensate for a deficit in one of these other positive regulatory factors.

A final conclusion is that the activation pathway responsible for IL2 gene induction appears to be confined to T<sub>H</sub>1-type cells. The results in Table III are clearly inconsistent with a single universal set of phosphoinositide pathway response factors in T cells, since many T-cell lines could not express any IL2 promoter construct, even when introduced as naked DNA. The nonexpressors included cell lines capable of expressing IL4 RNA in response to stimulation, showing that the difference between 'T<sub>H</sub>1' and 'T<sub>H</sub>2' cell types does not reside exclusively in the methylation status or accessibility of their endogenous IL2 genes in chromatin. Elsewhere, we show that even within the same clonal cell population, the two lymphokine RNAs show differential susceptibility to cAMP (Novak and Rothenberg, submitted for publication). The implication is that IL2 and IL4, which has a broader cell-type distribution than IL2, are induced through distinguishable sets of transcriptional regulators. The identification of key differences between these sets will allow the developmental relationship between T<sub>H</sub>1 and T<sub>H</sub>2 cells to be explicated in molecular terms.

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