Brief Definitive Report

A Model of Human Cytokine Regulation Based on Transfection of \( \gamma \) Interferon Gene Fragments Directly into Isolated Peripheral Blood T Lymphocytes

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Summary

An approach has been optimized permitting measurement of human cytokine reporter gene expression after transient transfection directly into purified human peripheral blood T lymphocytes. Comparing the expression of interleukin 2 (IL-2) CAT with a series of specially engineered \( \gamma \) interferon (IFN-\( \gamma \)) constructs, a fundamental difference in the molecular mechanisms regulating these two cytokines has been suggested. A potent, tissue-specific, constitutive-acting positive regulatory element was located between sequences -215 and -53 in the human IFN-\( \gamma \) gene. Deletion analyses suggested that sequences slightly upstream, between positions -251 to -215, exerted a powerful dominant suppressive influence over that positive element. Negative elements appear to play a major role in controlling the regulation of human IFN-\( \gamma \) gene expression. We thus propose a model of cytokine gene regulation in which selective derepression may be an important fundamental mechanism of induction and/or positive modulation.

Materials and Methods

Primary human T cells free of CD11\(^+\) cells were purified by double-panning and E-rosetting of normal, seronegative human PBMCs (10, 14). T cells cultured in 1.0 \( \mu \text{g/ml} \) PHA for 12-18 h at 3 \( \times \) 10\(^6\) cells/ml became transfection competent yet made no IL-2 and <3.0 U of IFN-\( \gamma \) per 10\(^6\) cells. 6 \( \times \) 10\(^6\) cells and indicated supercoiled plasmid DNAs at 50 \( \mu \text{g/ml} \) were electroporated in 0.3 ml of RPMI/10% FCS at 250 V, 980 \( \mu \text{F} \) using a Progenitor II (Hofer Scientific Instruments, San Francisco, CA) wired to an electroporation chamber (Bio-Rad Laboratories, Richmond, CA). Cells were activated 2 h later with 1.0 \( \mu \text{g/ml} \) of PHA and 10 ng/ml of PMA in fresh RPMI/10% FCS. 48 h after activation, cells were analyzed for production of IFN-\( \gamma \) by RIA (Centecor, Malvern, PA), IL-2 by CTLL analyses (1), and CAT as described (15) (except that CAT reactions were allowed to proceed overnight). IFN-\( \gamma \) CAT construct pIFN-697 was made by subcloning the 5' sequences, -697 to +14, from the human genomic clone pB9KB-1 (donated by Dr. Pat Gray, Genentech, So. San Francisco, CA), into an empty CAT vehicle (pBR CAT) made by deleting all RSV sequences from RSV CAT (16) with NdeI/HindIII (Fig. 1 C). Constructs -347, -251, -215, and -170 were made by 5' to 3' EXO III deletions of pIFN-697. Constructs -347, -251, -215, and -170, were similarly engineered using internal unidirectional EXO III digestion. Human IL-2 CAT (1) was generously donated by Dr. Jerald Crabtree, Stanford University, Stanford, CA. \( \beta \)-actin CAT was kindly provided by Drs. Art Sands and Bob Schwartz of Baylor College of Medicine. Unlike the traditional viral enhancer constructs RSV-CAT and SV\(_\gamma\)CAT, \( \beta \)-actin CAT was consistently unaffected by signals of T cell activation. Transfections were controlled for cell number, extract volume,
and quantity of plasmid DNA, and quantitated as molar equivalents of acetylated chloramphenicol produced by test plasmids relative to control plasmid pBR-CAT.

Results and Discussion

As shown in Fig. 1 A and Table 1, IL-2 CAT had no significant activity in primary resting (PHA only) T cells, yet was upregulated nearly 10-fold after PHA/PMA treatment of transfected cells. β-actin CAT had identical high activity in both resting and activated cells, while pBR-CAT had only baseline activity (Fig. 1 A).

Representative transfections of pIFN-697-derived constructs into primary human T cells are shown qualitatively in Fig. 1 B and quantitatively in Table 1. Resting peripheral blood T cells expressed considerable CAT activity (4-15-fold over background) after transient transfection with this construct. This phenomenon was recapitulated in resting Jurkat cells (data not shown), as well as in a resting murine T cell line transfected with an IFN-γ -550 CAT construct (17). No IL-2 CAT activity was noted in resting primary T cells and no substantial release of IL-2 or IFN-γ occurred. Transfected cells subsequently activated with PHA/PMA increased pIFN-697 CAT activity an additional two- to fivefold (Fig. 1 B and Table 1), upregulated IL-2 CAT activity about eightfold (Fig. 1 A and Table 1), and routinely produced 40-200 U of IFN-γ and 40-100 U of IL-2 per 10⁶ cells. Our data thus suggest that sequences in the proximal upstream region (-697 to +14) of the human IFN-γ gene, unlike those in IL-2, respond positively in trans to signals already present in minimally activated T cells. Presumably, additional negative regulatory sequences must prevent such baseline gene expression from occurring in the endogenous IFN-γ gene.

Deleting base pairs -697 to -348 (pIFN-347) dramatically diminished both resting transcription as well as upregulation by PHA/PMA (Fig. 1 B and Table 1). Further deletions to position -251 behaved similarly (Fig. 1 B and Table 1), suggesting positive cis-acting elements upstream of position -348. However, when 36 additional 5' bp were deleted (construct -215), high level constitutive CAT activity (5-60-fold over baseline) consistently reappeared in transfected primary T cells (Fig. 1 B and Table 1). The -215 construct also directed high level constitutive transcription in Jurkat cells (data not shown). In two experiments, the high baseline activity was even further upregulated by PHA/PMA (Table 1, Exps. 2, 3). Deleting to position -170 did not significantly

Table 1. Induction* of Human IFN-γ CAT Vectors Transiently Transfected into Human Peripheral T Cells

<table>
<thead>
<tr>
<th>PHA/PMA</th>
<th>Experiment</th>
</tr>
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<tbody>
<tr>
<td>-697</td>
<td>1²</td>
</tr>
<tr>
<td>-347</td>
<td>2²</td>
</tr>
<tr>
<td>-251</td>
<td>3²</td>
</tr>
<tr>
<td>-215</td>
<td>4²</td>
</tr>
<tr>
<td>-170</td>
<td>5²</td>
</tr>
<tr>
<td>-52</td>
<td>6²</td>
</tr>
<tr>
<td>-215 Δ(162-53)</td>
<td>7²</td>
</tr>
<tr>
<td>-215 Δ(110-53)</td>
<td>8²</td>
</tr>
<tr>
<td>pIL-2 CAT</td>
<td>9²</td>
</tr>
<tr>
<td>pβ-actin CAT</td>
<td>10²</td>
</tr>
<tr>
<td>pBR CAT</td>
<td>11²</td>
</tr>
</tbody>
</table>

* Fold induction compared with control plasmid pBR.

¹ Cells blasted 16-18 h with PHA.
² Cells blasted 16-18 h with both PHA and PMA.
³ (+) Stimulation with PHA/PMA after transfection.
⁴ (−) Incubation with media alone after transfection.

Figure 1. PHA-treated human PBLs were transfected with (A) control plasmids: pBR, a promoterless CAT construct; IL-2, IL-2 CAT; and β-actin CAT; (B) IFN-γ expression constructs: -697, pIFN-697 CAT; -347, pIFN-347 CAT; -251, pIFN-251 CAT; -215, IFN-215 CAT as shown in C. (+) Cells activated with PHA/PMA; (−) media only.

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Peripheral T cells (T) and Raji B cells (B) were transfected with the following DNAs as described: pBR CAT, pß-actin CAT, or pIFN-215 CAT. No secondary activating signals were used. Reducing this region's positive transcriptional influence (Table 1, Exps. 2, 4), but deleting to position −52 (IFN-γ CAAT/TATA promoter only) completely abolished all CAT activity (Table 1, Exps. 2, 4). By internally deleting sequences between positions −110 and −53 were shown to be required for that activity. The T cell specificity of the −215 construct was further demonstrated by its lack of transcriptional activity in either the B cell line, Raji (Fig. 2) or NIH 3T3 cells (data not shown). Of note, DNAse I footprinting studies in progress have now demonstrated a double strand, T cell-specific footprint over the sequence GTCTAAAGGAACTCTAACTACAACACC, which is within that region at positions −172 to −156.

Our preliminary findings of apparent positive and inducible elements upstream of position −348 in the IFN-γ promoter region corroborate our most recent observations (17) on that gene's induction. However, our higher resolution deletional analyses now point to additional constitutive enhancer-like elements which are proximally located (position −215 to −53), T cell specific, and apparently dominantly suppressed by more upstream regulatory elements presumably between positions −251 and −215. Perhaps related, that latter region contains a sequence AGAATCCCAC which has identity to the consensus sequence RRRRTTYCAY found in the 5′-flanking region of the human IL-2, GM-CSF, IL-4, and G-CSF genes (3). Thus, several separate lines of functional evidence strongly support the notion that negative elements control the regulation of human IFN-γ gene expression. Additionally, more recent studies in our laboratories have uncovered a T cell-specific, orientation-dependent silencer element within the −550 to −347 region of the IFN-γ gene (unpublished observations). Since only one report in the literature supports the notion of negative regulatory elements anywhere in the IL-2 gene (3), our composite data might be appropriately rectified by a slightly different model of cytokine inducibility (Fig. 3) than that envisioned for IL-2. It is hypothesized that transcription of the human IFN-γ gene is dominantly suppressed in mature resting T cells by several negative regulatory elements. Further, we propose that induction and modulation of that cytokine's gene expression may involve selective derepression of such suppressive influences along either contiguous or discontiguous regions within the human IFN-γ locus. This would facilitate constitutive upregulation of IFN-γ gene transcription via the potent constitutive 5′ upregulatory element described herein and/or the intronic enhancer elements we recently described (9, 11, 17, 18). It is noteworthy that such a model was recently proposed to explain the regulation of both IFN-α and β genes in human (19, 20). Further, removal of inhibitory influence by normal activating signals is thought to be the mechanism by which NF-κB responsive genes are activated (21). Understanding such reciprocal mechanisms will be an important first step in the development of epigenetic approaches to modulating such intercellular pathways.

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