The Proximal Regulatory Element of the Interferon-γ Promoter Mediates Selective Expression in T Cells*

(Received for publication, July 30, 1996, and in revised form, September 26, 1996)

Laurie A. Penix‡§, Marianne T. Sweetser¶, William M. Weaver‡, James P. Hoeffler‡, Tom K. Kerppola**, and Christopher B. Wilson††‡‡‡§§

From the ‡Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06520-8064, the departments of §Pediatrics and ¶Immunology, University of Washington School of Medicine, Seattle, Washington 98195, ¶¶Invitrogen Corporation, San Diego, California 92121, and the **Howard Hughes Medical Institute and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0650

Interferon-γ (IFN-γ) is produced by natural killer cells and certain subsets of T cells, but the basis for its selective expression is unknown. Within the region between −108 and −40 base pairs of the IFN-γ promoter are two conserved and essential regulatory elements, which confer activation-specific expression in T cells. This report describes studies indicating that the most proximal of these two regulatory elements is an important determinant of its restricted expression. The proximal element is a composite site that binds members of the CREB/ATF, AP-1, and octamer families of transcription factors. Jun is essential for activation-induced transcription and binds preferentially as a heterodimer with ATF-2. In contrast, CREB appears to dampen transcription and binds preferentially to a different site. This element, the CpG dinucleotide in this element is selectively methylated in Th2 T cells and other cells that do not express IFN-γ, and methylation markedly reduces transcription factor binding. As a target for DNA methylation and for binding of transcription factors that mediate or impede transcription, this element appears to play a central role in controlling IFN-γ expression.

Differential expression of lymphokines by individual T cells is central to the proper regulation of the immune response and in resistance to infection. For example, production of interferon-γ (IFN-γ)1 facilitates host defense to intracellular pathogens. In contrast, IL-4 appears to antagonize host defense to such agents while contributing to protection from intestinal helminths and facilitating the development of allergic responses (1, 2). Consequently, abnormal regulation of lymphokine production contributes to the pathogenesis of certain infectious, allergic, and autoimmune diseases. Since the production of these and other cytokines by T cells in response to activation is predominantly regulated at the level of transcription (3–5), it follows that differential transcription of lymphokine genes is essential to a properly regulated immune response.

Activation of T cells leads to an increase in intracellular Ca2+, which activates calcineurin, and protein kinase C and c-Jun amino-terminal/stress-activated protein kinase isozymes are triggered. Recent work has established the mechanism by which these events lead to the activation of IL-2 and IL-4 transcription. Both promoters contain regulatory elements that engage one or more NFAT (nuclear factor of activated T cells) proteins (6–11). Preformed NFAT resides in the cytosol in resting T cells and translocates to the nucleus on activation; this translocation is regulated by calcineurin, the action of which is inhibited by cyclosporin (7, 9, 12, 13). Calcineurin also acts in concert with protein kinase C to activate c-Jun amino-terminal kinases, which phosphorylate Jun and ATF-2 (activating transcription factor) (14). Jun and Fos (AP-1 (activator protein)) proteins bind in concert with NFAT to multiple sites in the IL-2 (10, 15, 16) and IL-4 (9, 17) promoters and thereby contribute to transcriptional activation. However, the transcription factors binding to these elements are common to cell types other than T cells, and the distal NFAT site and the PK NFAT site can be exchanged between the IL-2 and IL-4 promoters without affecting specificity of promoter function for Th1 (T helper lymphocytes) and Th2 T cell clones, respectively (9, 18). Thus, these elements are sufficient to account for activation-specific, cyclosporin-sensitive promoter function, but not for specificity of their promoters.

Like IL-2, IFN-γ expression is restricted to the Th1 subset of primed CD4+ T cells, whereas IL-4 is expressed by Th2 cells. IFN-γ expression does not completely parallel that of IL-2 since IFN-γ is expressed by primed but not naive T cells and, among primed T cells, also is highly expressed by the CD8− subset, whereas IL-2 is not (2, 4, 19, 20). Despite its importance in regulation of the immune response, compared with IL-2 and IL-4, little information exists regarding the regulation of IFN-γ expression. We recently reported that the proximal 108 bp of 5′-flanking sequence from the IFN-γ gene are necessary and sufficient to confer T cell and activation-specific, cyclosporin-sensitive expression and that the activity of this region is associated with two essential and evolutionarily conserved elements (21). The proximal element (−73 to −48 bp) shares homology with the NFIL-2A element of the IL-2 promoter (21) and is a target for methylation at its central CpG dinucleotide (22). Methylation at this site correlates strongly and inversely with the capacity of primary T cell populations and Th1 and Th2 T cell clones to express IFN-γ (22, 23). This suggests that the IFN-γ proximal element is likely to play an important role in the regulation and perhaps the specificity of this lymphokine’s expression.

This work indicates that the IFN-γ proximal element, like
Control of IFN-γ Expression

13165

FIG. 1. Activation requirements of the proximal element of the IFN-γ promoter. Constructs containing no promoter (pE3Q), the basal (pIFN-39) or proximal (pIFN-538) IFN-γ promoter, or the proximal element dimer were transfected into Jurkat T cells, which were unstimulated (uns) or stimulated with 1 μM ionomycin (iono), I) and/or 25 ng/ml PMA (P) in the absence or presence of 500 ng/ml cyclosporin A (CSA). Values are the means ± S.E. of two experiments. CPR, chlorophenol red production.

elements defined in the IL-2 and IL-4 promoters, is a composite binding site for a complex array of transcription factors including ATF-2, c-Jun and other Jun proteins, CREB (cAMP response element-binding protein), ATF-1, and Oct-1 (octamer). c-Jun engages this element, preferentially as a heterodimer with ATF-2, and activates transcription in response to T cell activation. In contrast, CREB and ATF-1, which are cAMP- and calcium/calmodulin-responsive transcription factors (24), also bind to this element with high affinity, and CREB impedes transcription. In addition, methylation of the central CpG dinucleotide of this element diminishes transcription factor binding, which is consistent with the inverse relationship between methylation of this element and expression of IFN-γ by T cell subsets. These observations suggest that the proximal element of the IFN-γ promoter, through competitive engagement of transcription factors and differential methylation, is likely to play an important role in the selective expression of IFN-γ in T cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The line of Jurkat T cells (which expresses IFN-γ following activation), the human monocyte-macrophage cell line U937 (American Type Culture Collection, Rockville, MD), and the Epstein-Barr virus-transformed B cell line 816 (D. Pious, University of Washington, Seattle) were maintained as described previously (21), except that the 816 cells were grown in medium supplemented with 15% fetal calf serum (Hyclone Laboratories, Logan, UT). L929 murine fibroblasts were grown as described previously (25).

The following antibodies to transcription factors were used. For the CREB/ATF family, anti-CREB-1 (C-21), anti-ATF-1 (C41-51), anti-CREB-1/ATF-1/CREM (25C10D0), and anti-ATF-4 (25) were mouse monoclonal antibodies, and anti-ATF-3 (C-19) was a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal (F2BR1) and rabbit polyclonal (F2poly) antibodies to human ATF-2 were provided by J. P. Hoeffer (Invitrogen, San Diego, CA). For the AP-1 and Octamer families, rabbit polyclonal anti-Jun (c-Jun/AP-1(1D)), anti-c-Jun-specific (c-Jun/AP-1(1N)), anti-Fos (K-25), and anti-Oct-1 were obtained from Santa Cruz Biotechnology.

Plasmid Constructs—The pIFN-538, pIFN-108, pIFN-39, and pILS-508-β-galactosidase reporter and β-actin-CAT constructs were described previously (21). Plasmids containing head-to-tail (5' to 3') dimers of the IFN-γ proximal element (71 to 44 bp from the transcription start site) were generated by subcloning ligated oligomers synthesized with terminal BglII (5') and BamHI (3') restriction sites into the pIFN-39 basal promoter construct. Proximal dimers with mutations M1–M4 (see Fig. 5A) were produced in a similar manner. The full-length promoter (pIFN-538) containing the M5 (C to T) mutation was prepared by polymerase chain reaction site-directed mutagenesis and splicing by overlap-extension (26) to generate a 240-bp fragment that was subcloned into pIFN-538A–177–109 (21). The sequence of each construct was confirmed with dideoxynucleotide chain termination sequencing.

Plasmids containing the cDNAs for CREB and CREB containing a mutation in the DNA-binding domain that allows dimerization but blocks binding to target DNA (KCREB) (27, 28) were obtained from R. H. Goodman (Vollum Institute, Oregon Health Sciences University, Portland, OR). Plasmids containing cDNAs for murine ATF-2 (CREBP2.1) and murine ATF-2 with a mutation abrogating DNA binding (CREBP1mu) were obtained from K. Georgopoulos (Massachusetts General Hospital, Charlestown, MA) (29). Plasmids containing wild-type human ATF-2 (2-ATF2P) and a truncated form lacking the transcriptional activation domain (ATF-2BR) (30) and wild-type c-Jun (31, 32) have been described; the plasmid containing c-Jun with a deletion of residues 260–266 in the DNA-binding domain (c-JunΔ266) was analogous to that described in Ref. 33. These cDNAs were received or cloned into the pRcRSV vector (Inveritrogen).

Transient Transfection Assays—Jurkat, U937, and 816 cells were transiently transfected with both 30 μg of IFN-γ β-galactosidase plasmid and 16 μg of control β-actin-CAT plasmid in 0.5 ml of RPMI 1640 medium by electroporation (ECM600, BTX Inc., San Diego, CA) at 850 V, 186 ohms, and 1900, 1500, or 1050 microfarads, respectively. In cotransfection experiments, the indicated amounts of expression plasmids containing transcription factor cDNAs under the control of the RSV (Rous sarcoma virus) promoter were also included. The total amount of RSV vector per cuvette was kept constant by the addition of control pRcRSV plasmid lacking a cDNA insert. After electroporation, the cells were unstimulated or stimulated with 1 μM ionomycin and phorbol 12-myristate 13-acetate (PMA) as described previously (21). Where indicated, cells were pretreated for 20 min with cyclosporin A (500 ng/ml). Cell lysates were analyzed for β-galactosidase activity using chlorophenol red as substrate (34) and corrected for transfection efficiency by normalizing to CAT content as described previously (21). L929 cells were transiently transfected with 5 μg of IFN-γ β-galactosidase plasmid and 2.5 μg of control β-actin-CAT plasmid using 2000 μg/ml DEAE-dextran for 3 h and then shocked with 10% dimethyl sulfoxide in phosphate-buffered saline for 1–2 min. The cells were stimulated, and cell lysates were prepared as described above.

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from Jurkat T cells that were untreated or stimulated for 2 h as described (21, 35, 36). CREB-1 (the DNA-binding domain, amino acids 254–327), from Santa Cruz Biotechnology, as well as recombinant c-Jun (the DNA-binding domain, amino acids 199–334) and c-Fos (the DNA-binding domain, amino acids 139–200), provided by T. K. Kerpola (32), were purified by nickel chelate affinity chromatography of bacterial lysates (37). Recombinant ATF-1 (full-length) and ATF-2BR (the DNA-binding domain, amino acids 350–505) were obtained from bovine lens preparations of bacteria without further purification and are ~90% pure (30).

 Sequences of oligonucleotides used with their consensus sequences underlined are as follows: the IFN-γ proximal element (−71 to −47 bp), 5′-AAAACTTGTTGAAAAATCTGAACTCTTG-3′; the NFIL-2A element (36), 5′-GAAAAATATGTTAATATGTTAAACAT-3′; the consensus so- matostatin CRE (cAMP response element) (38), 5′-GATCCGGTGACCTATCAGGCATCTACGACGTTGGG-3′; the consensus metallothionein IIa AP-1 element (39), 5′-GAGCCGCGGATGCTATCGGCGGGG-3′; and the consensus DRA octamer (40), 5′-CTGACCATTAAAGCTACATACACTCTCCCTGGG-3′. The GATA element of the T cell receptor-α enhancer (41), 5′-GTTAGAGATATGGCCCGCCA-3′, was used as a nonspecific competitor. Oligonucleotides were labeled, and the binding reactions were performed as described previously (21), except that reactions containing recombinant proteins were incubated in buffer containing 10 mM HEPES, pH 7.5, 125 mM NaCl, 10% glycerol, 2 mM dithiothreitol, 0.1% Nonidet P-40, 5 μg/ml bovine serum albumin, 2 mM phenylmethylsulfonyl fluoride, and 2 μg/ml peptide A.

In experiments using antisense or monoclonal antibodies to specific transcription factors, these were routinely preincubated with nuclear extracts for 2 h on ice before the addition of probe. Bound complexes were analyzed on 6% DNA polyacrylamide (30%) gels at room temperature in 0.4 X Tris borate/EDTA buffer, and then the gels were dried and autoradiographed overnight.

DNA Binding Assays—The DNA template for the footprinting experiment was made by end labeling a human (−350 to +50 bp) IFN-γ promoter fragment at the downstream end. The binding reactions and DNase I digestions were carried out as described previously (9). The
RESULTS

The IFN-γ Proximal Element Directs Activation-specific, Cyclosporin-sensitive Expression in T Cells—Using transient transfection of reporter constructs containing 10-bp scanning internal deletions, our laboratory identified two highly conserved, regulatory elements in the immediate 5'-flanking sequence of the human IFN-γ gene, which were necessary for activation-specific and cyclosporin-sensitive expression in Jurkat T cells (21). To determine if the most proximal of these transcriptional regulatory elements, like the intact IFN-γ promoter, is sufficient to confer activation-specific and cyclosporin-sensitive expression, two tandem copies of this element were placed upstream of the basal IFN-γ promoter driving expression of a β-galactosidase reporter gene and transfected into Jurkat T cells (Fig. 1). Expression from the proximal dimer construct was stimulated to the same extent as that from the full-length promoter (pIFN-538) by ionomycin and PMA and was blocked by cyclosporin (>85%). Likewise, expression of both these constructs was minimal in unstimulated cells and in cells stimulated with ionomycin or PMA alone. Thus, a dimer of the proximal element was sufficient for properly regulated expression in Jurkat T cells.

In contrast to the robust expression observed in Jurkat T cells, expression from the proximal dimer and the full-length promoter constructs was similar to that from a promotorless construct (pEQ3) and lower than that from the basal promoter construct (pIFN-39) in unstimulated or stimulated U937 monocyte-macrophages or L929 fibroblasts (data not shown). Very low levels of expression were observed in the 816 B cell line, which is consistent with data indicating that transformed B cell lines express IFN-γ in low amounts (42, 43). Thus, like the full-length promoter, the proximal dimer appears sufficient to confer activation- and cell lineage-specific expression.

The IFN-γ Proximal Element Binds Members of the CREB/ATF, AP-1, and Octamer Families—The proximal element of the IFN-γ promoter has partial homology to the NFIL-2A element of the IL-2 gene, which binds octamer, AP-1 (including c-Jun, JunD, and Fos proteins), and NFAT proteins (10, 36, 44–46). The binding sites of AP-1 and CREB/ATF transcription factors are closely related, and certain members of these families can form cross-family heterodimers (47). Accordingly, binding of nuclear proteins to the IFN-γ proximal element probe was compared with that of probes containing NFIL-2A, octamer, AP-1, and CRE elements by electrophoretic mobility shift assay (EMSA) (Fig. 2A). In previous studies, EMSA of the proximal IFN-γ element showed two intense complexes, A and B (21), which are now resolved into doublets, and a previously undetected intermediate-mobility complex. Also noted in response to activation, the A1 complex is barely detectable, and A2 is the predominant complex (Fig. 2A, lanes 4 and 5). This difference was seen in five independently prepared Jurkat nuclear extracts (data not shown).

When used as unlabeled competitors in EMSA (Fig. 2A), the CRE oligonucleotide markedly reduced all the complexes generated by the IFN-γ proximal element probe, with the exception of the intermediate-mobility complex. The NFIL-2A competitor completely blocked formation of the intermediate-mobility complex.

binding reactions with the Jurkat nuclear extracts utilized 3 μg of protein.
The proteins present in Jurkat T cell extracts shown in the other figures. The complexes in these EMSAs cannot be compared directly with that of binding domains rather than the full-length proteins, the mobility of the recombinant proteins used were, with the exception of ATF-1, the DNA-binding domains of the CREB/ATF-1 family (Fig. 2). This complex was inhibited by the IFN-γ proximal element complexes (data not shown). An antisem recognizing all Jun proteins completely blocked the A2 complex in both stimulated and unstimulated extracts (Fig. 2B, lanes 4 and 11), and a c-Jun-specific antisem produced a supershifted complex in stimulated Jurkat extracts (Fig. 2C, lane 6). In contrast, antibodies to the Fos family of proteins or to c-Fos had no effect on complexes formed with the proximal element probe, although each antibody blocked and/or supershifted complexes formed with an AP-1 probe (Fig. 2C, lanes 7–12).

Several observations suggest that the intermediate complex is generated by binding of Oct-1 to the IFN-γ proximal element (Fig. 2A). 1) The intermediate-mobility complex comigrated with the predominant complex generated by the NFIL-2A probe. 2) This complex was inhibited by the IFN-γ proximal element, NFIL-2A, and consensus octamer oligonucleotides, but not by CRE- or AP-1-containing oligonucleotides. 3) Oct-1-specific antisem blocked formation of this complex and the corresponding NFIL-2A complex, but had little or no effect on the mobility of complexes present in Jurkat T cell extracts shown in the other figures.

Fig. 3. IFN-γ proximal element is capable of binding recombinant ATF-1, CREB, Jun, and Jun/ATF-2 heterodimers. A. EMSA using CRE (lanes 1–4), the proximal IFN-γ element (lanes 5–8), AP-1 (lanes 9–12), and NFIL-2A (lanes 13–16) as probes with recombinant proteins in the presence of 25 ng of poly(dI-dC). For each probe, a 10 nm concentration of the indicated recombinant protein was added: CREB (amino acids 254–327), ATF-1 (full-length), CREB plus ATF-1, or ATF-2 (amino acids 350–505). B, EMSA using the same probes with recombinant c-Jun (amino acids 199–334), c-Jun plus ATF-2, ATF-2, or c-Jun plus c-Fos (amino acids 139–200). For each probe, c-Jun and c-Fos were added at 100 nm and ATF-2 at 10 nm. Note that since the recombinant proteins used were, with the exception of ATF-1, the DNA-binding domains rather than the full-length proteins, the mobility of the complexes in these EMSAs cannot be compared directly with that of the proteins present in Jurkat T cell extracts shown in the other figures.
In contrast, the AP-1 and NFIL-2A probes bound ATF-2 and c-Jun/ATF-2 heterodimers poorly or not at all compared with c-Jun or c-Jun/c-Fos.

To better define the regions of interaction of these proteins with the proximal element, DNase I footprint analysis was performed. Recombinant ATF-2 protected a large region of the proximal element spanning from $-63$ to $-43$ bp, whereas AP-1 (recombinant c-Jun and Fra-1) protected an overlapping but smaller region ($-63$ to $-53$ bp) (Fig. 4 and data not shown). NFATp, either alone or in conjunction with AP-1 or ATF-2, did not bind to this region. The region protected by recombinant ATF-2 corresponded to that protected by the Jurkat nuclear extracts. No differences were seen in the footprints generated with unstimulated or stimulated nuclear extracts or when AP-1 and ATF-2 were added together (data not shown).

In summary, these results suggest that the IFN-$\gamma$ proximal element binds CREB, ATF-1, ATF-2, and c-Jun under conditions similar to those required by elements containing these consensus motifs. CREB/ATF-1 and c-Jun/ATF-2 bind readily as heterodimers. Upon stimulation, c-Jun is induced and binds as a homodimer or preferentially as a heterodimer with ATF-2. Although not observed, the presence of Jun/Fos heterodimers in low abundance cannot be excluded. In contrast to the NFIL-2A element, NFATp does not bind to this site.

**Mutations within the Proximal Element Identify Bases Critical for Binding and Markedly Reduce Reporter Gene Expression**

The central ACGT sequence of the IFN-$\gamma$ proximal element is highly conserved (21), lies within the sequence with the best homology to a consensus CRE motif, and is within the region protected by ATF-2 and AP-1 in the footprint analysis. Consistent with this, these were identified as the most critical bases for binding in EMSA (Fig. 5). A probe containing the M3 mutation, which disrupts this sequence, failed to bind any of the specific complexes usually seen with the wild-type probe, with the exception of a more rapidly migrating complex (Fig. 5B, lanes 7–8). The M5 mutation, which consists of a single base change from C to T within the CRE motif, bound only the Oct-1 complex (Fig. 5B, lanes 11 and 12). The retention of Oct-1 binding may be due to reconstitution of the sequence AATAT-GTAA found in the NFIL-2A element, which overlaps the putative octamer motif (36), and was confirmed using Oct-1 antisera (data not shown). In contrast, oligonucleotides with the M1, M2, and M4 mutations had minimal effects. The M1 and M4 mutations produced complexes that were identical to the wild-type probe. Although the M1 mutation appears to bind complexes more intensely, this was not a consistent finding. Similar results were seen with the M2 mutation, except it did not form the Oct-1 complex (Fig. 5B, lanes 5 and 6). The bases in the center of the CRE homology were also critical for binding of recombinant proteins. In Fig. 5C, the results with heterodimers of CREB/ATF-1 and Jun/ATF-2 are shown. Results with the individual recombinant factors were similar (data not shown). When used as competitors, the M1, M2, and M4 oligonucleotides competed nearly as well as the wild type, but the M3 and M5 mutations failed to alter binding of complexes to the wild-type IFN-$\gamma$ element to any appreciable extent.

The effects of these mutations on expression were tested by transient transfection of reporter constructs containing head-to-tail dimers of the proximal element with the M1–M4 mutations or with the full-length IFN-$\gamma$ promoter (pIFN-538) containing the C to T mutation (M5). As shown in Fig. 6,
expression from the M3 and M5 reporter constructs, in which the mutations disrupted the central ACGT sequence, was reduced to the low level of the basal promoter (pIFN-39). In contrast, the reporter constructs with the M1 and M2 mutations, which minimally affected binding by EMSA but which are within the region footprinted both by ATF-2 and by AP-1, showed markedly decreased expression. In contrast, the reporter construct with the M4 mutation, which is located at the 3'-end of the proximal element in a region footprinted only by ATF-2, was expressed at levels comparable to those of the wild-type proximal element dimer and the full-length IFN-γ promoter (pIFN-538).

Role of Jun, ATF-2, and CREB in Regulation of Expression via the IFN-γ Proximal Element—The data described above suggested that members of the CREB/ATF-1, ATF-2, and Jun families play an important role in the function of the proximal element. To assess this, wild-type and dominant-negative mutated genes were tested in cotransfection assays. Wild-type CREB, which as a homodimer or heterodimer with ATF-1 was the most abundant complex observed by EMSA, had an inhibitory effect on expression from the proximal dimer (Fig. 7B); a similar trend, although not statistically significant, was observed with the full-length promoter (Fig. 7A). These effects were not observed with the DNA-binding mutant KCREB. The DNA-binding mutant of c-Jun (c-Jun3BR) consistently and markedly inhibited expression from both the full-length promoter and proximal dimer constructs even at low concentrations (Fig. 7, C and D). Wild-type c-Jun had little effect on the proximal dimer, but at higher concentrations inhibited the full-length promoter. In contrast to the results with cotransfection of CREB, c-Jun, and their mutants, cotransfection of wild-type human ATF-2 or of a dominant-negative ATF-2 mutant containing only the DNA-binding domain (ATF-2BR) did not consistently affect expression (Fig. 7, E and F). Likewise, cotransfection of murine ATF-2 (CRE-BP2.1) or of a mutant containing a mutation in the DNA-binding domain (CREBP1mu) had little effect (n = 3) (data not shown). These results suggest that CREB and c-Jun have opposing effects on the IFN-γ proximal element and promoter, whereas a role for ATF-2 was not demonstrated by these cotransfection experiments.

Methylation of the CpG Dinucleotide Impedes Binding of Factors to the IFN-γ Proximal Element—Previous studies indicated that the degree of methylation of the CpG dinucleotide within the IFN-γ proximal element correlates inversely with the capacity of T cell populations or T cell clones to produce this lymphokine (22, 23). In CD4+ Th1 clones, this CpG is fully demethylated, whereas in Th2 clones, it is fully methylated; this difference is selective in that there is not a general hypomethylation of all CpG dinucleotides in the IFN-γ gene in Th1 as compared with Th2 T cell clones (22).

To determine the effect of methylation on binding of transcription factors to this element, EMSA was performed with oligonucleotide probes containing 5'-methylcytosine in the CpG of each strand. Compared with non-methylated IFN-γ proximal element and CRE probes, the characteristic complexes formed with stimulated Jurkat nuclear extracts were markedly reduced when methylated probes were used (Fig. 8A). In addition, novel faster migrating complexes were observed with the methylated IFN-γ proximal element probe. The lower apparent binding affinity of the methylated oligonucleotides for CREB, ATF-2, and Jun proteins present in nuclear extracts was confirmed using non-methylated probes and methylated oligonucleotides as unlabeled competitors (data not shown). Consistent with this, a methylated IFN-γ proximal element oligonucleotide competed much less effectively than the non-methylated oligonucleotide for binding of recombinant CREB, ATF-2, and c-Jun (Fig. 8B).

FIG. 6. Transient transfection of Jurkat T cells confirms that mutations that alter binding are critical for activation-induced expression. A, β-galactosidase expression observed with the promoterless pE3 construct, the basal (pIFN-39) and full-length (pIFN-538) IFN-γ promoter constructs, and constructs containing dimers of the wild-type or mutated M1–M4 sequences shown in Fig. 5A. Results are the means ± S.E. of two experiments. B, β-galactosidase expression observed with the full-length IFN-γ promoter containing the wild type (pIFN-538) or M5 (C to T) mutation. Results are the means ± S.E. of three experiments. *P, ionomycin and PMA; uns, unstimulated; CPR, chlorphenol red production.

DISCUSSION

The current results indicate that the proximal element of the IFN-γ promoter plays an important role in gene regulation and may contribute to the differential expression of this lymphokine. As shown, the proximal element contains sufficient information to mimic with a high degree of fidelity the properties of the endogenous gene and of the full-length promoter with respect to activation requirements and cell lineage-specific expression. Furthermore, in transiently transfected primary human T cells, the IFN-γ proximal element dimer directs activation-specific expression, which is 30-fold greater than that of the basal promoter (pIFN-39b) and T cells from transgenic mice in which a dimer of this element directs expression of a luciferase reporter gene produce luciferase in an activa-

L. A. Penix, unpublished data.
tion-specific, cyclosporin-sensitive manner.3

The IFN-γ proximal element displayed a complex binding pattern, engaging Oct-1, CREB/ATF, and Jun proteins, while lacking a consensus element for any of these transcription factors. Previous studies have demonstrated that members of the CREB/ATF and AP-1 families display binding affinities that vary with dimer composition and that depend on the sequence of the binding site (47, 49, 50). As seen in Fig. 3, the proximal element more closely resembles a CRE-like site and displayed a pattern of binding similar to, but distinct from, that of the CRE, AP-1, and NFIL-2A elements. The results obtained by EMSA also are supported by preliminary fluorescence anisotropy data (51): ATF-2 bound to the IFN-γ proximal element with higher affinity than did CREB or GST/c-Jun and bound this element with an affinity similar to that of a consensus CRE, while CREB and GST/c-Jun bound the consensus CRE with greater affinity than the proximal element (data not shown). These findings suggest that multiple factors bind to the IFN-γ proximal element, but binding by ATF-2-containing complexes may be favored.

Jun or Jun/ATF-2 Facilitate IFN-γ Promoter-mediated Transcription—The simplest interpretation of the EMSA, foot-printing, and cotransfection experiments is that activation-dependent transcription through the IFN-γ promoter and its proximal element follows in part from the induction of c-Jun expression and binding of c-Jun to this element either as a homodimer or a heterodimer with ATF-2 or other Jun proteins (Fig. 9). T cell activation results in the calcineurin-dependent activation of c-Jun amino-terminal/stress-activated protein kinases, which phosphorylate c-Jun and ATF-2 (14, 52), and would allow these proteins to participate in transcription initiation. Supporting this interpretation is the inhibitory effect of the c-Jun DNA-binding domain mutant, which blocked transcription from the full-length IFN-γ promoter and the proximal element dimer. At high concentrations, Jun partially inhibited expression from the full-length promoter, suggesting that c-Jun, or transcription factors with which it interacts, may act on regions within the IFN-γ promoter in addition to the proximal element. The lack of an inhibitory effect of full-length c-Jun on the proximal element may reflect its ability to function in this context as a homodimer, whereas it may interact more efficiently as a heterodimer at other regions in the IFN-γ promoter. Preliminary results suggest that the distal element of this promoter is one such region (data not shown). Additional evidence supporting a role for Jun or Jun heterodimers comes from a recent report by Young and co-workers (53), who found that cotransfection of c-Jun plasmids containing activation do-

3 T. Aune, L. A. Penix, M. Rincon, and R. Flavell, submitted for publication.
main mutations inhibited IFN-γ promoter function, although the locus of the inhibitory effect was not defined. The current studies indicate that c-Jun contributes to activation through the proximal element of the IFN-γ promoter, but do not exclude a role for other Jun proteins in this process. Functional overlap among different Jun family proteins has been suggested in the IL-2 and IL-4 promoters (9, 15, 16, 54).

In contrast to the results observed with c-Jun constructs, no consistent effect on transcription was observed when murine or human wild-type ATF-2 or two different types of dominant-negative ATF-2 were cotransfected along with the IFN-γ promoter or proximal element constructs. Similarly, no effect of ATF-2 was observed when it was cotransfected in combination with c-Jun (data not shown). This was not anticipated from the EMSA studies, which suggested that Jun/ATF-2 heterodimers would bind preferentially over other Jun-containing complexes in stimulated extracts. However, this is consistent with the results of the footprinting and transfection experiments, which demonstrated that mutations within the region footprinted jointly by AP-1 and ATF-2 inhibited expression; by contrast, the M4 mutation, which is within the region footprinted by ATF-2 but not by AP-1, had no effect. These results support the notion that Jun-containing dimers are necessary for transcriptional activation, but the other constituent of Jun-containing dimers may not be critical. It is also possible that ATF-2 is not limiting in Jurkat T cells or that the effects observed in cotransfection assays were in part indirect since ATF-2 and c-Jun and c-Jun/ATF-2 are thought to be phosphorylated by c-Jun amino-terminal/stress-activated protein kinases when T cells are activated. The center of this element contains a CpG dinucleotide, the methylation of which impedes transcription factor binding and is observed in types of T cells (e.g., Th2 T cells) that do not transcribe IFN-γ.

In addition to the potential interactions of both positively and negatively acting transcription factors at this site, a CpG dinucleotide within the proximal element of the IFN-γ promoter serves as a target for methylation. Since phosphorylation of CREB in T cells may enhance its binding to DNA (56), elevation of cAMP could contribute to this dampening effect; this is consistent with the observation that agents that elevate intracellular cAMP inhibit IFN-γ production (57). Interestingly, IL-4 transcription is not impeded by cAMP (58, 59), and differences in sensitivity to or content of cAMP have been proposed to contribute to differential lymphokine production or proliferation by Th1 and Th2 CD4+ T cells (60).

Potential Role of the Proximal Element in Selective IFN-γ Expression—In addition to the potential interactions of both positively and negatively acting transcription factors at this site, a CpG dinucleotide within the proximal element of the IFN-γ promoter serves as a target for methylation. Since methylation impeded binding of Jun or Jun/ATF-2 proteins to this element (Fig. 8), methylation may raise the effective threshold concentrations of phosphorylated Jun or Jun/ATF-2 needed to activate transcription above that generally present in activated T cells in situ. Consistent with this, methylation of the IFN-γ

---

* L. H. Glimcher, personal communication.
proximal element correlates strongly and inversely with the capacity of primary T cell populations and T cell clones to produce IFN-γ (22, 23). In addition to impairing the binding of transcription factors, methylation of DNA at CpG dinucleotides may impair promoter function by other mechanisms. Methylation of CpG engages specific proteins (61, 62) that may block transcription either through the competitive binding of transcription factors having minimal agonistic activity (CREB/ATF-1) or through methylation of the central CpG dinucleotide of the element (Fig. 9). This parsimonious use of a single transcription factor containing proximal element may provide a locus at which cells can markedly limit, if not block, the capacity of the endogenous IFN-γ gene to be transcribed.

In sum, the current results indicate that the IFN-γ proximal element, like the IFN-γ promoter, is selectively active in T cells. This selectivity does not appear to reflect the binding of transcription factors that are unique to cells that express this lymphokine. Rather, the IFN-γ promoter contains within its proximal element the potential to engage proteins that mediate transcription either through the competitive binding of transcription factors having minimal agonistic activity (CREB/ATF-1) or through methylation of the central CpG dinucleotide of this element (Fig. 9). This parsimonious use of a single transcriptional regulatory element may be important in allowing T cells to rigorously control the expression of this potent immunoregulatory lymphokine.

Acknowledgments—We thank Timothy Hoey and Ya-Lin Sun (Tu-lairk, Inc.) for performing the DNA footprint analysis, Richard Good- man for suggestions and assistance with the fluorescence anisotropy experiments, Katia Georgopoulos for antibodies and murine ATF-3 cDNAs, and Laurie Glimcher (Harvard School of Public Health) for permission to cite unpublished data.

REFERENCES
1. Locksley, R. M., and Wilson, C. B. (1995) in Principles and Practice of Infectious Diseases (Mandell, G. L., Bennett, J. E., and Dolin, R., eds) Vol. 1, 4th Ed. pp. 102–149, Churchill-Livingstone, Inc., New York.
2. Seder, R. A., and Paul, W. E. (1994) Annu. Rev. Immunol. 12, 635–763.
3. Ullman, K. S., Northrop, J. P., Verweij, C. L., and Crabtree, G. R. (1990) Annu. Rev. Immunol. 8, 421–452.
4. Liu, B., Yu, C. C., Meyer, J., English, B. K., Kahn, S. J., and Wilson, C. B. (1991) J. Clin. Invest. 87, 194–202.
5. Rao, A. (1994) Immunol. Today 15, 274–281.
6. Chuvpilo, S., Schomberg, C., Gerwig, R., Heinfling, A., Reeves, R., Grummt, F., and Heinfling, A. (1993) J. Biol. Chem. 268, 3747–3752.
7. Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerson, L. A., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994) Nature 369, 497–502.
8. Rooney, J. W. Hedge, M. R., McCaffrey, P. G., Rao, A., and Glimcher, L. H. (1994) Mol. Cell. Biol. 15, 6299–6310.
11. Tsuruta, L., Lee, H.-J., Masuda, E. S., Koyano-Nakagawa, N., Arai, N., Arai, K., and Yokata, T. (1995) J. Immunol. 154, 5255–5264.
12. Rooney, J. W., Hedge, M. R., McCaffrey, P. G., Rao, A., and Glimcher, L. H. (1994) EMBO J. 13, 625–633.
13. Schreiber, S. L., and Crabtree, G. R. (1992) Immunol. Today 13, 136–142.
14. Saiki, R., Joung, E., Hnilo, M., Kallunki, T., Kan, M., and Ben-Neriah, Y. (1994) Cell 77, 727–736.
15. Boise, L. H., Petryniak, B., Mao, X., June, C. H., Wang, C. Y., Lindsten, T., Bravo, R., Kovary, K., Leiden, J. M., and Thompson, C. (1993) Mol. Cell. Biol. 13, 1911–1919.
16. Jain, I., McCaffrey, P. G., Valge-Archer, V. E., and Rao, A. (1992) Nature 356, 801–804.
17. Stahbo, S. J., Gold, J. S., Murphy, T. L., and Murphy, K. M. (1993) Mol. Cell. Biol. 13, 4793–4805.
18. Lederer, J. A., Liou, H. C., Mefferd, L., and Lichtman, A. H. (1994) J. Immunol. 153, 77–86.
19. Lewis, D. B., Prickett, K. S., Larsen, A., Grabstein, K., Weaver, M., and Wilson, C. B. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 9743–9747.
20. Sow, S. L., Bradley, I. M., Craft, M., Tinkham, S., Atkins, G., Weinberg, A. D., Duncan, D. D., Hedrick, S. M., Dutton, R. W., and Huston, G. (1991).