cis-ACTING SEQUENCES REQUIRED FOR CLASS II GENE REGULATION BY INTERFERON γ AND TUMOR NECROSIS FACTOR α IN A MURINE MACROPHAGE CELL LINE

BY YVONNE R. FREUND,† RUSSELL L. DEDRICK,† AND PATRICIA P. JONES†

From the *Department of Immunology and Infectious Diseases, Research Institute, Palo Alto Medical Foundation, Palo Alto, California 94301; and the †Department of Biological Sciences, Stanford University, Stanford, California 94305

The class II genes of the mouse MHC encode four polypeptide chains that are expressed as two heterodimers, the AαAg and the EαEγ molecules, on a restricted number of cell types. On thymic epithelial and dendritic cells, expression of the class II (Ia) molecules is necessary for normal T cell maturation, and in the periphery, cells expressing these molecules play a crucial role in the initiation of antigen-specific immune responses.

Ia-expressing cells in the periphery include B cells, macrophages, dendritic cells, and some epithelial and endothelial cells. Exogenous antigens must be processed by these cells and presented in the context of the Ia molecules in order to be recognized by the Th cell receptor. In light of evidence that APC, in particular macrophages, internalize and process self, as well as foreign antigens (1), the requirement for strict regulation of Ia expression may be critical to avoid presentation of self molecules, and triggering of autoimmune disease. Thus, on mouse macrophages, expression of the Ia molecules can be modulated by various cytokines and mediators of inflammation.

The principal mediator of Ia induction is IFN-γ, and the molecular mechanisms by which this cytokine exerts its effect have been a subject of great interest (reviewed in references 2 and 3). In murine and human cell lines, IFN-γ has been shown to increase the expression of class II molecules in a coordinate manner (4, 5) and to function primarily by increasing rates of transcription of these genes (6, 7). The structural basis for this coordinate regulation of transcription is likely to be due to the sequence similarities in the 5' flanking regions of the class II genes; and these cis-acting elements, with their corresponding DNA-binding proteins, are currently being intensively studied (8–17). Two conserved sequences, which have been designated the X and Y boxes, are found 5' of the transcription initiation site in all class II genes and have been shown to be required for promoter activity (8, 9). Several investigators have proposed that the coordinate responses of the class II genes to various

This work was supported by National Institutes of Health grant AI-19964 to P. P. Jones and Biomedical Research Support grant 507 RR-05513-26 to Y. R. Freund. Y. R. Freund and R. L. Dedrick were supported by fellowships from the Arthritis Foundation.

Address correspondence to Patricia P. Jones, Department of Biological Sciences, Stanford University, Stanford, CA 94305.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/90/04/1283/17 $2.00
lymphokines may be mediated by the X and Y consensus regions (8, 10). 5' of the X box is another conserved sequence called the heptamer sequence or the H box (13, 14). The H box is included in a region that has been called the Z box in the HLA-DRα gene (11) and the W box in the HLA-DQα gene (12). The Z box sequence has been implicated in tissue-specific and IFN-γ-inducible responsiveness (11), and the H box, in IFN-γ-specific induction (11, 15, 16). In a recent study (17), we demonstrated that the H, X, and Y box elements of the Aα gene promoter are all required for constitutive expression in B lymphoma cells and for IFN-γ-inducible expression in a myelomonocytic cell line.

TNF-α, a product of activated macrophages, has been shown to induce Ia surface expression (18), as well as expression of other immunoregulatory molecules such as IL-1 (19), IL-6 (20), and the IL-2-R (21). TNF-α frequently enhances the activity of IFN-γ, and the two cytokines act synergistically in the induction of Ia expression, antiviral activity, and 2'-5' oligoadenylate synthetase activity (22). TNF-α increases Ia expression by increasing levels of mRNA for the Ia molecules (18); however, it is not known whether it increases transcription or stabilizes the RNA. In this study, we investigated the effect of TNF-α on Ia gene expression in the murine myelomonocytic cell line WEHI-3, in the presence or absence of IFN-γ, and compared its mode of action with that of IFN-γ. We found that both cytokines increase total mRNA for the Aα gene and increase the activity of the Aα promoter. Mutational analysis of this promoter has revealed that TNF-α-mediated induction requires all of the sequence elements that have been shown to be necessary for IFN-γ-mediated induction. Furthermore, an additional sequence element was found to affect induction by TNF-α, but not by IFN-γ.

Materials and Methods

Cell Culture. WEHI-3, a myelomonocytic cell line derived from a BALB/c mouse (23), was used in these studies. Cells were cultured in RPMI 1640 with 10% FCS (HyClone Laboratories, Logan, UT), 5 × 10^-5 M β-mercaptoethanol, and penicillin-streptomycin (50 U/ml and 50 μg/ml), and were screened monthly for mycoplasma infection.

Reagents. All cytokines used in this study were generously provided by Dr. M. Shepard, Genentech, Inc. (South San Francisco, CA). Recombinant murine IFN-γ, produced in *Escherichia coli*, had a specific activity of 1.03 or 1.74 × 10^5 antiviral U/mg protein. Recombinant murine TNF-α had a specific activity of 2.9 × 10^-7 U/mg protein and <0.125 EU/ml of endotoxin, as assayed by the limulus amoebocyte lysate assay. 14C-chloramphenicol, [dichloroacetyl-1,2-14C] with a specific activity of 60.0 mCi/mmol was obtained from New England Nuclear (Boston, MA). Acetyl coenzyme A (sodium salt) was obtained from Sigma Chemical Co. (St. Louis, MO); DEAE dextran, from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ) and p-Luciferin from Analytical Luminescence Laboratory (San Diego, CA).

mAbs. The following FITC-conjugated mAbs were used in this study: MKD6 (anti-Aβ) (24), 10-3.6 (anti-Aβ) (25), 14-4-4 (anti-Eα) (26), and 34-1-2 (anti-H-2Kβ, Dα) (27).

Measurement of Cell Surface Ia Expression. WEHI-3 cells were seeded at 2.5 × 10^4 cells per 60-mm tissue culture dish and treated for 48 h with IFN-γ, TNF-α, or both cytokines. Maximal induction was observed using 10 or 20 U/ml IFN-γ and 1 nM TNF-α. Cells were removed from dishes by pipetting, then pelleted, resuspended in optimal amounts of FITC-conjugated mAbs (1 μg antibody per sample), and incubated for 30 min at room temperature. Medium used for the labeling was RPMI 1640 + 5% FCS + 0.02% sodium azide. Cells were washed, resuspended in medium containing propidium iodide at a final concentration of 1 μg/ml to stain dead cells, and analyzed on an EPICS 753 flow cytometer (Coulter Electronics Inc., Hialeah, FL). The analyses in this study were gated to eliminate dead, propidium iodide-pos-
itive cells, and scatter-gated to eliminate large, aggregated cells and debris. Staining with 10-3.6 served as a control for nonspecific binding of antibody to Fc receptors.

**Northern Blot Analysis.** RNA was isolated from WEHI-3 cells treated for 48 h with IFN-γ (10 U/ml), TNF-α (1 nM), or both cytokines at these concentrations, according to the method of Chirgwin et al. (28). 10 μg of total RNA was subjected to electrophoresis in a 1.5% agarose- formaldehyde gel, transferred onto Nytran nylon membrane (Schleicher & Schuell, Inc., Keene, NH), and hybridized to an Aα probe, as previously described (4). The probe was a 790-bp Pst I cDNA fragment originally obtained from M. Davis (Stanford, CA), which was labeled with 32P-dCTP by the hexamer priming method (29). Kodak X-AR5 x-ray film was preflashed so that exposure of the film would be linearly related to radioactivity on the filter, and films were exposed using Cronex Lightening Plus intensifying screens. Films were analyzed using an Ultra-scan XL laser densitometer (LKB Instruments, Inc., Gaithersburg, MD) to quantitate increases in total mRNA.

**Plasmid Construction.** The pAα plasmid containing the genomic Aα gene and ~5 kb of 5' flanking sequence was generously provided by Dr. Leroy Hood (Pasadena, CA), and the promoter region was identified and sequenced as described elsewhere (17). The plasmid pAα CAT 1, containing the Aα promoter region fused to the chloramphenicol acetyl transferase (CAT) reporter gene, was constructed by blunt-end ligating 2.5 kb of the Aα promoter, using the Bam HI site at position −1 and filling in with Klenow enzyme, to pA10CAT2 (30) (generously provided by Dr. Jane Parnes, Stanford, CA), which had been digested with Hind III and made blunt by filling in. The pAα CAT-276 plasmid was constructed using an Sst I–Bam HI fragment of pAα CAT 1 that extended from position −276 of the Aα promoter through to the SV40 poly(A) site of the CAT reporter gene. This fragment was blunt-end ligated into the Eco R1 site of pUC18 so that the polylinker of pUC 18 was at the 5' end of the Aα sequence. The luciferase plasmid, pSV2ALΔ 5', was generously provided by Dr. Suresh Subramani (La Jolla, CA).

**Construction of 5' Deletion Mutants.** Mutants of the 5' region of the Aα promoter were constructed using Bal31 exonuclease digestion of the 5' region of the pAα CAT-276 plasmid, as previously described (17). A fragment of the Eα gene was cloned into the Sma I site of the pAα CAT-276 plasmid to protect the Xba I restriction site in the pUC18 polylinker from bidirectional digestion by Bal 31, and aliquots of the reaction were terminated at various times. The remaining Eα DNA was removed by Bam HI digestion and the plasmid ends were made blunt with Klenow enzyme and ligated so that an intact Xba I site bordered the deletion end-point. Constructs were characterized by sequencing using the method of Sanger et al. (31).

**Construction of Linker-Scanner Mutants.** The linker-scanner mutants were constructed as a set of recombinant plasmids all containing the CAT gene and −276 bp of the Aα promoter, starting from the site of transcription initiation. In each construct, a 12-bp piece of the promoter region was replaced by linker sequences. These constructs were created by generating paired 3' and 5' deletions and ligating pairs to create the mutation of interest. Generation of the deletions that were used as the 3' end was described above. To generate the 5' ends, the same principles were followed, except that mung bean nuclease, rather than Klenow enzyme, was used to blunt the ends of the deleted DNA before ligating. Fragments were ligated, bacteria were transformed, and clones were screened for the amount of deleted DNA and characterized by sequencing. 5' and 3' deletions were matched to generate sequential 12-bp inserts spanning the first 173 bp 5' from the Aα initiation site. This procedure has been detailed elsewhere (17). The resulting mutants were designated according to the most 5' and most 3' nucleotides altered relative to the initiation site.

**Transfection.** WEHI-3 cells were transfected with 12.5 μg of the pAα CAT DNA and 2.5 μg of luciferase DNA using DEAE dextran (32). Briefly, WEHI-3 cells were seeded at 3 × 10⁶ cells/100-mm plate, 16–18 h before transfection. The medium was removed, and 3 ml of RPMI containing 10 μg/ml of DEAE dextran and DNA was added to each plate. Cells were incubated for 4 h at 37°C, shocked for 2 min with 10% DMSO in RPMI, washed, pooled, and replated so that promoter activity could be assayed in cells treated with: (a) no

---

1 Abbreviation used in this paper: CAT, chloramphenicol acetyl transferase.
cytokines; (b) 20 U/ml IFN-γ; (c) 1 nM TNF-α; or (d) both cytokines at these concentrations.
Transfection with the pUC18 plasmid was used as a control for endogenous CAT activity.
In this case, no luciferase gene was cotransfected, and these extracts were also used to measure background activity in the luciferase assay.

**Luciferase and CAT Enzyme Assays.** Luciferase activity was assayed on 20 μl of cell extract from transfected cells that had not been treated with cytokines, according to the method of deWet et al. (33), using a Monolight 2001 luminometer. Cell extracts were then heated to 60°C for 7 min (34), and CAT activity was measured according to Gorman et al. (35), using 14C-choramphenicol. Acetylated products were separated by TLC and visualized by autoradiography. Quantitative measurements were obtained by cutting out the acetylated spots and measuring radioactivity in a scintillation counter. Promoter activity was determined by subtracting the values obtained from the pUC18 control transfectants from each sample's 14C counts and luciferase activity; then dividing counts by luciferase activity to normalize for the amount of DNA that was transfected into each sample. When data from several experiments were compared, the induction of CAT activity using the mutant constructs was expressed as a percent of the induction using the full-length construct.

**DNA Sequence Comparisons.** The IFIND and ALIGN programs from IntelliGenetics (Mountain View, CA) were used to search the Genbank/EMBL Data Bank for sequences with homology to unique elements in the Aβ promoter. These programs are based on the algorithm developed by Wilbur and Lipman (36).

**Results**

**IFN-γ and TNF-α Increase Cell Surface Expression of the I-A and the I-E Proteins.** WEHI-3 cells were cultured for 48 h with either 20 U/ml IFN-γ, 1 nM TNF-α, or a combination of the two cytokines, and surface expression of the I-A or I-E proteins was analyzed (Fig. 1). Uninduced WEHI-3 cells expressed essentially no surface I-A or I-E proteins.
molecules. Both IFN-γ and TNF-α induced class II antigen expression on the majority of cells after a 48-h exposure. TNF-α induction of both I-A and I-E molecules was bimodal, indicating that there were two distinct populations of cells that could be induced to express either higher or lower levels of these molecules. This bimodal induction was not a function of the dose of TNF-α administered, since cells were treated with TNF-α in a dose range between 0.5 and 50 nM for 48 h, and no further shift in the population was observed at doses >1 nM (data not shown). In addition, when cells cultured for 24 h were compared with cells cultured for 48 h, an almost identical pattern of bimodality was observed. As shown in the lower panels of Fig. 1, when WEHI-3 was treated for 48 h with both IFN-γ and TNF-α, all of the cells strongly expressed I-A and I-E antigens, indicating that the addition of the two cytokines together was able to convert more cells to the highly positive phenotype than addition of TNF-α alone.

The Induction of I-A and I-E Cell Surface Expression Occurs through an Increase in mRNA. To determine the effect of IFN-γ on Aα mRNA levels, RNA was isolated from treated cells and analyzed by Northern blot hybridization (Fig. 2). There was no detectable message for the Aα gene product in uninduced cells. Treatment with IFN-γ induced Aα mRNA, and treatment with TNF-α induced Aα mRNA to even higher levels. Densitometric analysis of the autoradiograms indicated that when cells were induced with both IFN-γ and TNF-α, levels of Aα message were three- to fourfold greater than when cells were induced with either cytokine alone. These results demonstrated that Ia surface expression is accompanied by an increase in levels of mRNA.

Both IFN-γ and TNF-α Increase Activity of the Aα Promoter. We had previously de-
determined, using nuclear run-on experiments, that the increase in $\alpha$ mRNA after IFN-γ treatment of WEHI-3 cells was due to an increase in the rate of transcription of the $\alpha$ gene (7). To determine the effect of IFN-γ and TNF-α on the activity of a class II promoter, we fused the $\alpha$ promoter to the bacterial CAT reporter gene. Promoter activity was measured by assaying CAT activity. When the CAT gene was fused to the $\alpha$ promoter with 2.5 kb of 5' flanking DNA, CAT activity in WEHI-3 cells was induced by both IFN-γ and TNF-α (Fig. 3, lanes 3-6). Mock transfected cells (no DNA; Fig. 3, lanes 1 and 2) and cells transfected with the CAT construct in the reverse orientation (lanes 7 and 8) showed no CAT activity. A similar pattern of induction was obtained using pA$\alpha$CAT-276, which contained only 276 bp of the 5' flanking sequence. This indicated that -276 bp of 5' flanking sequence was sufficient to confer inducibility, although we have not ruled out the possibility that additional regulatory elements exist between -276 and -2.5 kb. We have also shown that when pA$\alpha$CAT-276 was transfected into L cells, which do not express Ia, or into constitutively expressing B cell lymphomas, the relative CAT activity reflected the Ia phenotype of the recipient cell (17).

105 bp of 5' Sequence Are Required for $\alpha$ Promoter Inducibility by IFN-γ or TNF-α. To determine how much of the 276-bp 5' sequence was critical for induction by TNF-α, we used a series of 5' deletion mutants. The 5' ends of these deletions are illustrated by the circled numbers in Fig. 4. These mutants were transfected into WEHI-3 cells, and the effects of IFN-γ and TNF-α on CAT expression were determined (Fig. 5). Data were corrected for the efficiency of transfection, using luciferase activity as previously described in Materials and Methods, and results were expressed as relative CAT activity. As was observed with IFN-γ-mediated induction, response to TNF-α alone was eliminated when the promoter region was deleted to <105 bp 5' of the initiation site.
Figure 4. Nucleotide sequence of the promoter region of the murine \(\alpha\) gene from positions -169 to -1 (17). The circled, bold-faced numbers below the sequence indicate the 5' endpoints of the deletion mutations. The Z, H, X, and Y box homologies are indicated by boxed areas in the \(\alpha\) sequence. Stippled boxes indicate the \(\beta\)-like enhancer region residues and the T box residues. The arrow represents the position of the transcription start sites. The linker-scanner mutations are indicated by boxes below the sequence that span the regions altered by each mutation. Linker-scanner mutations replaced the regions between the mutation endpoints with the following linker sequence: GATCCCTCTAGAG.

Three Sequences in the Region between -126 and the Initiation Site Are Required for Induction of CAT Activity by Both IFN-\(\gamma\) and TNF-\(\alpha\). To define regulatory sequence elements in more detail, we created a series of nested linker-scanner mutations covering the region of -173 to -5, altering sequential 12-bp portions of the promoter. The boxes in Fig. 4 illustrate the regions of the \(\alpha\) promoter altered by the various linker-

Figure 5. Effect of 5' deletions of the \(\alpha\) promoter on CAT expression in WEHI-3 cells by IFN-\(\gamma\), TNF-\(\alpha\), or both cytokines. Data are averages from four experiments, expressed as relative CAT activity ± SEM and corrected for transfection efficiency by assaying for luciferase activity (see Materials and Methods). Deletions without SEM were assayed once.
SEQUENCES REQUIRED FOR CLASS II GENE REGULATION

Figure 6. Data from a representative transfection experiment, showing results of the CAT assay on extracts from WEHI-3 cells transfected with selected linker-scanner constructs. Data are expressed as relative CAT activity, indicating that they have been corrected for transfection efficiency by assaying for luciferase activity.

Scanner mutants, and variation from the wild-type sequence is described in the figure legend. Fig. 6 shows results from one such experiment, using selected constructs that showed the most pronounced effects on IFN-γ and TNF-α induction. The data show that two linker-scanner mutations resulted in nearly total loss of promoter activity in the presence of cytokines: LS[-55,-42], which alters the Y box, and LS[-75,-62], which covers the 3' end of the X box (see Fig. 4). Several other linker-scanner mutations also reduce induction by the cytokines. Fig. 7 summarizes data from six experiments. The results have been normalized to the amount of induction seen with the wild-type plasmid in each experiment. This was done to facilitate averaging of results from several experiments, since induced levels of Ia and CAT expression varied from one experiment to the next. The vertical line at 1.0 represents CAT activity when cells were transfected using the wild-type pA\text{\textsubscript{276}} CAT-276 and treated with a particular cytokine. The analysis shows that three mutant constructs consistently decreased promoter inducibility to 25% or less of that obtained with the full-length construct. Two of these are the constructs that alter the Y box (LS[-55,-42]) and the 3' end of the X box (LS[-75,-62]). The third alters the 5' end of the X box (LS[-86,-73]). Two other mutations also consistently decreased the induction by both IFN-γ and TNF-α. These were LS[-112,-99], which encompasses the H box described by Servenius et al. (13) and Thanos et al. (14), and LS[-126,-115], which covers a region homologous to the kB enhancer of the Ig genes (37). Each of these mutations had similar effects on induction by IFN-γ, TNF-α, or by both cytokines. Finally, the only sequence found to be uniquely important for induction by one cytokine and not by the other is the sequence altered by LS[-31,-18]. This alteration decreases, but does not eliminate, TNF-α responsiveness; however, it has no effect on induction by IFN-γ. (see Figs. 6 and 7). Alteration of this sequence also reduces CAT transcription in cells treated with both cytokines.

In summary, linker-scanner analysis has revealed three sequences that are critical, and one that appears to be important, for optimal induction by both IFN-γ
and TNF-α. These are the X, Y, and H boxes, and the kB enhancer. In addition, a sequence between -31 and -18 appears to be important for optimal induction by TNF-α but not by IFN-γ. No sequences appear to be uniquely required for IFN-γ induction.
The murine class II MHC molecules, I-A and I-E, can be induced on the surface of the WEHI-3 macrophage cell line by both IFN-$\gamma$ and TNF-$\alpha$. We have demonstrated that IFN-$\gamma$ induces I-A and I-E expression on virtually all cells, and TNF-$\alpha$ induces a bimodal increase in the expression of both molecules. When IFN-$\gamma$ and TNF-$\alpha$ are administered together, all of the cells become strongly I-A and I-E positive. The basis for this synergy is not known, but could reflect effects of IFN-$\gamma$ on TNF-$\alpha$ receptors, as has been demonstrated in human cells (38-40). Both IFN-$\gamma$ and TNF-$\alpha$ exert their effect on class II expression by increasing levels of mRNA. Northern blot analysis of mRNA from cells treated with both IFN-$\gamma$ and TNF-$\alpha$ demonstrated that the combination of the two cytokines was twice as stimulatory as if the effects of each cytokine had been added together. This synergy has also been observed by Chang and Lee (18). The increase in mRNA levels occurs primarily via an increase in transcription of the $\alpha$ gene, since nuclear run on assays (7; and our unpublished results) detected no transcription of the class II genes without induction by IFN-$\gamma$ or TNF-$\alpha$.

We have examined sequences 5' of the $\alpha$ transcription initiation site to determine whether IFN-$\gamma$ and TNF-$\alpha$ require identical or distinct sequences to activate the $\alpha$ promoter. These results confirm and extend our recent findings on $\alpha$ promoter expression in B lymphoma cells and in IFN-$\gamma$-induced WEHI-3 cells (17). Mutational analysis demonstrated that sequences 3' of residue -105 were essential for induction by IFN-$\gamma$ and TNF-$\alpha$. Induction by each individual cytokine dropped significantly when the promoter was deleted from -115 to -105, removing part of the $H$ box, and was completely abolished on deletion to -89, completely removing the $H$ box. Although induction by each individual cytokine was lost on deletion to -105, induction by both cytokines together was still evident when the promoter was deleted to -78. It appears that residues between -105 and -78 allow some synergistic induction but are not sufficient for induction by the individual cytokines. A better understanding of this observation will require analysis of proteins binding to this DNA region after induction or activation by the two cytokines individually and in combination.

Analysis using linker-scanner mutations indicated that the conserved class II promoter elements are required for induction by both cytokines. Mutation of the $Y$ box (LS[-55,-42]) and the $X$ box (LS[-75,-62] and LS[-86,-73]) caused decreases of 75% or more in induction by IFN-$\gamma$ or TNF-$\alpha$, when compared with induction using the full-length (-276) construct. Mutation of the $H$ box (LS[-112,-99]) also resulted in a 75% decrease in IFN-$\gamma$ induction and a slightly smaller decrease in TNF-$\alpha$ induction. The $Y$ box is an inverted CCAAT box between residues -48 and -39, with the sequence CTGAT'TGGTT, which is a perfect match with the consensus $Y$ box sequence (5) and is critical for $\alpha$ promoter activity. Binding of nuclear proteins to the $Y$ box has been extensively investigated in human and mouse class II genes (8, 9, 12, 17, 41-43), and it is currently believed that only one protein, a ubiquitous CCAAT box binding protein, NFY, binds to this box (9). No unique $Y$ box binding protein is induced by IFN-$\gamma$, since a comparison of IFN-$\gamma$-induced and -uninduced macrophages revealed no differences in $Y$ box binding factors (8, 17, 43).
LS[−75,−62] and LS[−86,−73]) alter the 3' and 5' ends of the X box, respectively, and result in a major loss in promoter activity. The X box of the Aα promoter diverges more substantially from the consensus X box sequence than X boxes found in other class II genes (17, 43), and it appears that a variety of X box binding proteins may exist that are specific for the various Ia genes (43). Binding of proteins to the X box of Aα has been shown to be independent of cell type or lymphokine stimulation (17, 43); therefore, although the X box is critical for promoter activity, it may mediate IFN-γ or TNF-α-specific induction only in the context of other, more specific cis-acting sequences.

Linker-scanner mutation LS[−112,−99] spans an H box element contained within residues −106 to −100, which matches the consensus H box sequence of RRAYCTT (13). Alteration of this sequence causes a loss in IFN-γ-induced CAT expression and, to a lesser extent, in CAT expression induced by TNF-α or by IFN-γ and TNF-α in combination. The H box is conserved in all of the Ia genes, as well as in several human genes (13, 15). In the human DRα (15) and DQβ (16) genes, sequences in the area of the H box have been shown to be necessary, but not sufficient for IFN-γ induction. We have demonstrated that this sequence is not uniquely required for IFN-γ inducibility, since it also appears to contribute to induction by TNF-α. In addition, the H box has been shown to be important for expression of the Aα promoter in constitutively Ia-positive B cell lines (17).

In addition to the X, Y, and H elements, linker scanner analysis has implicated two sequences, whose alteration decreased promoter activity by ∼50–60%. LS[−126, −115] resulted in decreases in both IFN-γ and TNF-α induction. The region between residues −129 and −118 shows homology with the κB core enhancer sequence found in the Ig κ L chain. The consensus sequence is GGGANTC (44). κB-like enhancer elements are also found in the H-2Kb, β2-microglobulin, HIV-1 and -2, human IL-2, and the IL-2-R α chain genes (45). The IL-2-R α chain and HIV-1 κB-like enhancers have been the subject of intense interest recently, in part because both are transcriptionally regulated by TNF-α (44, 46, 47). The κB-like enhancers from both of these genes are capable of conferring TNF-α inducibility to a heterologous promoter, and selective deletion of this sequence abrogates TNF-α inducibility in primary human T cells as well as in Jurkat cells (44). The most recent DNA binding studies revealed two proteins binding to the enhancer in the IL-2-R after stimulation with TNF-α: an 86-kD protein that corresponds to the HIVEN86A protein, (a nuclear protein that binds the HIV-1 LTR, [48]), and a 51-kD protein corresponding to NF-κB. Induction by TNF-α also requires binding of several constitutively expressed factors to one upstream and two downstream sequences (44). These additional sequences are not present in the Aα promoter; however, this pattern of induction may be a model for the function of TNF-α in the Aα promoter, in that binding to the κB-like enhancer is important but not sufficient for induction, without the concomitant binding of constitutively expressed factors to other sequences. While the κB element of the Aα promoter seems to influence induction by IFN-γ and TNF-α, it is intriguing that some induction still occurs when this element had been removed by 5′ deletions.

A final sequence element appears to play a role in transcription induced by TNF-α, but not by IFN-γ. We have tentatively called this sequence, which spans residues from −31 to −17, the T box, and have indicated its location in Figs. 4 and 8. The
boundaries of this element were assigned on the basis of homologies with three mammalian sequences discovered, in part, by a search of the Genbank/EMBL Data Bank. These sequences have been aligned in Fig. 8. A perfect homology to the T box sequence is found in the HLA-DQα gene and a 14/15-bp match is found in the rat RT1.Bα gene. These genes are the human and rat homologues of the murine Aα chain (49, 50). When the three genes are aligned so that there is maximum nucleotide identity in the Y box and T boxes, an 11-bp insert is observed at the 3' end of the HLA-DQα Y box. In the rat Bα gene, the 3' portion of the T box includes a TGTAA (TATA) box that is present in approximately the same position in both the Eα gene and in the human homologue, HLA-DRα, but not present in the Aα or HLA-DQα genes.

The full T box sequence is not found in the Eα or the HLA-DRα genes. However, as illustrated in Fig. 8 A, mouse Eα and human DRα share with Aα the TGNTTTTGG sequence in the center of the T box. It is possible that this 7-bp sequence may be sufficient to mediate the effect of TNF-α, since it is homologous to

---

**Figure 8.** (A) Alignment of a portion of the 5' upstream sequence of the Aα gene with sequences from human HLA-DQα, rat RT1.Bα, murine Eα, and HLA-DRα. Sequence of HLA-DQα are from Jonsson et al. (49), RT1.Bα from Barran and McMaster (50), Eα from Mathis et al. (62), and HLA-DRα from Das et al. (63). Boxes identify the Y box and the T box. Dots indicate identity when compared with the corresponding base in the murine Aα sequence. Nucleotide sequences in each gene corresponding to the consensus core enhancer elements are shown in bold type. (B) Alignment of the 5' upstream regions of the Aα gene with the rat c-myc gene and the core transcription enhancer elements found in SV40 virus, the HLA-DRα first intron, and Ig H chain. The T box sequences in Aα and c-myc are indicated, and the core consensus described by Weiher (51) is shown. Sequences of rat c-myc are from Hayashi et al. (56) and of μ H chain are from Gillies et al. (53).
the core transcription enhancer described by Weiher (51) and observed in SV40, polyoma virus, Ig, and the HLA-DRα genes (3, 51–53). The consensus sequence for this enhancer is TGG\textsuperscript{TTT}AAA\textsubscript{G}, where the first G has been shown to be absolutely required for enhancer activity (51). We have indicated the core transcription enhancer sequence in bold letters in each of the sequences in Fig. 8. In all genes, the critical first G is present in the sequence. It is of interest to note that there are no sequences corresponding to the T box, or to the SV40 core enhancer, in the promoter regions of either the mouse Aβ and Eβ genes (see sequences in references 54 and 55), indicating that there may potentially be differential regulation of α and β chains of the class II genes by TNF-α.

A search of the Genbank/EMBL Data Bank revealed strong homology of the T box sequence to a 15-bp region in the second intron of the rat, mouse, and human c-myc genes (56–58). Regulation of class II genes, as well as the c-myc gene by TNF-α, could be mediated either via the core transcription element or via this larger region of homology. TNF-α has been found to downregulate transcription of the c-myc gene in the human promyelocytic leukemia cell line, HL-60 (59), and in HeLa cells (60). Sequences responsible for this regulation have not been located, however, it is likely that they are not in the 5' flanking region (61). It is intriguing to speculate that this regulation involves the T box-like element in the second intron of the c-myc gene.

Evaluation of the significance of the T box sequence in the Aα gene and its role in TNF-α responsiveness will await determination of whether TNF-α responsiveness can be transferred to a heterologous promoter by this element, and whether proteins in extracts from TNF-α-treated cells bind the T box and other core elements.

In conclusion, this analysis of cis-acting sequences has led to the following model for the transcriptional activation of the Aα gene in WEHI-3 cells. At least four 5' sequences are necessary for optimal induction by both IFN-γ and TNF-α: the X, Y, and H boxes and the kB-like enhancer. In addition, a sequence between -31 and -17, which we have designated the T box and which contains homology to the core enhancer sequence, appears to be important for induction by TNF-α. Having delineated the cis-acting sequences required for activation by IFN-γ and TNF-α, it appears that to understand transcriptional activation of the Aα gene, and of other class II genes, it will be necessary to identify the proteins binding to all of the critical cis-acting sequences and to determine whether binding to one region influences binding to another.

Summary

In this report, we have demonstrated that IFN-γ and TNF-α increase expression of both the I-A and I-E region gene products on the surface of the myelomonocytic cell line WEHI-3, and that they mediate this increase via an increase in Aα transcription. Constructs containing 5' deletion mutations of the Aα promoter attached to the bacterial chloramphenicol acetyl transferase gene were used to delineate the minimum 5' flanking sequences required for promoter activity, and for inducibility by IFN-γ and TNF-α. Approximately 115 bp of 5' sequences are required for minimum induction by IFN-γ or TNF-α when the cytokines are present separately. This includes the three conserved promoter elements, the X, Y, and H boxes. Nested linker-scanner mutations demonstrated that additional regions were also critical for optimal induction by IFN-γ or TNF-α. These include the kB-like enhancer and a
SEQUENCES REQUIRED FOR CLASS II GENE REGULATION

TNF-α-specific sequence that we have tentatively called the T box. The T box sequence was also found in the promoter regions of the human HLA-DQα and rat RT1.Bα genes. Although the entire T box sequence element was not found in the other mouse class II genes, all class II α genes contained the SV40 core enhancer element in the regions included by the T box. Mouse class II β genes appear to contain neither the T box nor the core enhancer element in this region, suggesting differential regulation of class II α and β genes by TNF-α.

We thank M. Ryan for technical assistance; T. Zimmer and J. Feder for critically reading the manuscript; and S. Subramani, L. Hood, and M. Davis for generously providing plasmid DNA.

Received for publication 16 October 1989 and in revised form 21 December 1989.

References

1. Marrack, P., and J. Kappler. 1988. T cells can distinguish between allogeneic major histocompatibility complex products on different cell types. Nature (Lond.). 332:840.
2. Giacomini, P., P. B. Fisher, G. J. Duigou, R. Gambari, and P. G. Natali. 1988. Regulation of class II MHC gene expression by interferons: insights into mechanisms of action of interferon (Review). Anticancer Res. 8:1153.
3. Sullivan, K. E., A. F. Calman, M. Nakanishi, S. Y. Tsang, Y. Wang, and B. M. Peterlin. 1987. A model for the transcriptional regulation of MHC class II genes. Immunol. Today. 8:289.
4. Paulnock-King, D. M., K. Sizer, Y. R. Freund, P. P. Jones, and J. Parnes. 1985. Coordinate induction of Ia α, β and I m mRNA in a macrophage cell line. J. Immunol. 135:632.
5. Flavell, R. A., H. Allen, L. C. Burkly, D. H. Sherman, G. L. Waneck, and G. Widera. 1986. The molecular biology of the H-2 histocompatibility complex. Science (Wash. DC). 233:437.
6. Rosa, F. M., and M. Fellous. 1988. Regulation of HLA-DR gene by IFN-γ, transcriptional and post-transcriptional control. J. Immunol. 140:1660.
7. Jones, P., R. A. Aiyer, Y. R. Freund, G. K. Pavlath, and T. Zimmer. 1986. Interferon-gamma: receptors and effects on MHC antigen expression. Prog. Immunol. 6:458.
8. Dorn, A., B. Durand, C. Marfing, M. Le Meur, C. Benoist, and D. Mathis. 1987. Conserved major histocompatibility complex class II boxes- X and Y are transcriptional control elements and specifically bind nuclear proteins. Proc. Natl. Acad. Sci. USA. 84:6249.
9. Dorn, A., J. Bollekens, A. Staub, C. Benoist, and D. Mathis. 1987. A multiplicity of CCAAT box-binding proteins. Cell. 50:863.
10. Liou, H.-C., M. Boothby, and L. H. Glimcher. 1988. Distinct cloned class II MHC DNA binding proteins recognize the X box transcription (%) element. Science (Wash. DC). 242:69.
11. Tsang, S. Y., M. Nakanishi, and B. M. Peterlin. 1988. B-cell-specific and interferon-γ-inducible regulation of the HLA-DR α gene. Proc. Natl. Acad. Sci. USA. 85:8598.
12. Miwa, K., C. Doyle, and J. L. Strominger. 1987. Sequence-specific interactions of nuclear factors with conserved sequences of human class II major histocompatibility complex genes. Proc. Natl. Acad. Sci. USA. 84:4939.
13. Servenius, B., L. Rask, and P. A. Peterson. 1987. Class II genes of the human major histocompatibility complex. The DO4 gene is a divergent member of the class II β gene family. J. Biol. Chem. 262:8759.
14. Thanos, D., G. Mavrothalassitis, and J. Papamatheakis. 1988. Multiple regulatory regions on the 5' side of the mouse Eα gene. Proc. Natl. Acad. Sci. USA. 85:3075.
15. Basta, P. V., P. A. Sherman, and J. P.-Y. Ting. 1988. Detailed delineation of an inter-
feron-γ-responsive element important in human HLA-DRA gene expression in a glioblastoma multiform line. Proc. Natl. Acad. Sci. USA. 85:8618.

16. Boss, J. M., and J. L. Strominger. 1986. Regulation of a transfected human class II major histocompatibility complex gene in human fibroblasts. Proc. Natl. Acad. Sci. USA. 83:9139.

17. Dedrick, R. L., and P. P. Jones. 1990. Sequence elements required for activity of a murine major histocompatibility complex class II promoter bind common and cell-type-specific nuclear factors. Mol. Cell. Biol. 10:593.

18. Chang, R. J., and S. H. Lee. 1986. Effects of interferon-γ and tumor necrosis factor-α on the expression of an Ia antigen on a murine macrophage cell line. J. Immunol. 137:2853.

19. Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, Jr., and J. V. O'Connor. 1986. Tumor necrosis factor ( cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. 163:1433.

20. Katz, Y., and R. C. Strunk. 1989. IL-1 and tumor necrosis factor. Similarities and differences in stimulation of expression of alternative pathway of complement and IFN-β/IL-6 genes in human fibroblasts. J. Immunol. 142:3862.

21. Lowenthal, J. W., D. W. Ballard, H. Bogerd, E. Bohnlein, and W. C. Greene. 1989. Tumor necrosis factor-α activation of the IL-2 receptor-α gene involves the induction of xB-specific DNA binding proteins. J. Immunol. 142:3121.

22. Wong, G. H. W., and D. V. Goeddel. 1988. Biological activities and production of TNF-α. In Monokines and Other Non-Lymphocytic Cytokines. M. C. Rowanda, J. J. Oppenheim, M. J. Kluger, and C. A. Dinarello, editors. Alan R. Liss, Inc., New York. 251-260.

23. Warner, N. C., M. A. S. Moore, and D. J. Metcalf. 1969. A transplantable myelomonocytic leukemia in BALB/c mice: cytology, karyotype and muramidase content. J. Natl. Cancer Inst. 43:963.

24. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin producing T cell hybridomas. J. Exp. Med. 153:1198.

25. Oi, V., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. Curr. Top. Microbiol. Immunol. 81:115.

26. Ozato, K., N. M. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. Cancer Res. 12:5707.

27. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463.

28. Lopta, M. A., D. W. Cleveland, and B. Sollner-Webb. 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. 12:5707.

29. deWet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. 7:725.

30. Mercola, M., J. Goverman, C. Mirell, and K. Calame. 1985. Immunoglobulin heavy-
chain enhancer requires one or more tissue-specific factors. Science (Wash. DC). 227:366.
35. Gorman, C. M., L. Moffat, and B. Howard. 1982. Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol. Cell. Biol. 2:1044.
36. Wilbur, W. J., and D. J. Lipman. 1983. Rapid similarity searches of nucleic acid and protein data banks. Proc. Natl. Acad. Sci. USA. 80:726.
37. Sen, R., and D. Baltimore. 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell. 46:705.
38. Ruggiero, V., J. Tavernier, W. Fiers, and C. Baglioni. 1986. Induction of the synthesis of tumor necrosis factor receptors by interferon-γ. J. Immunol. 136:2445.
39. Tsujimoto, M., Y. K. Yip, and J. Vilcek. 1986. Interferon-γ enhances expression of cellular receptors for human tumour necrosis factor. J. Immunol. 136:2441.
40. Aggarwal, B. B., T. E. Eessalu, and P. E. Hass. 1985. Characterization of receptors for tumour necrosis factor and their regulation by γ-interferon. Nature (Lond.). 318:665.
41. Celada, A., M. Shiga, M. Imagawa, J. Kop, and R. A. Maki. 1988. Identification of a nuclear factor that binds to a conserved sequence of the I-Ag gene. J. Immunol. 140:3995.
42. Sherman, P. A., P. V. Basta, T. L. Moore, A. M. Brown, and J. P.-Y. Ting. 1989. Class II box consensus sequences in the HLA-DRα gene: transcriptional function and interaction with nuclear proteins. Mol. Cell. Biol. 9:50.
43. Boothby, M., H.-C. Liou, and L. H. Glimcher. 1989. Differences in DNA sequence specificity among MHC class II X box binding proteins. J. Immunol. 142:1005.
44. Greene, W. C., E. Bohnlein, and D. W. Ballard. 1989. HIV-1, HTLV-I and normal T-cell growth: transcriptional strategies and surprises. Immunol. Today. 10:272.
45. Cross, S. L., N. F. Halden, M. J. Lenardo, and W. J. Leonard. 1989. Functionally distinct NF-κB binding sites in the immunoglobulin κ and IL-2 receptor α chain genes. Science (Wash. DC). 244:466.
46. Lowenthal, J. W., E. Bohnlein, D. W. Ballard, and W. C. Greene. 1988. Regulation of interleukin 2 receptor α subunit (Tac or CD25 antigen) gene expression: binding of inducible nuclear proteins to discrete promoter sequences correlates with transcriptional activation. Proc. Natl. Acad. Sci. USA. 85:4468.
47. Lowenthal, J. W., D. W. Ballard, E. Bohnlein, and W. C. Greene. 1989. Tumor necrosis factor α induces proteins that bind specifically to κB-like enhancer elements and regulate interleukin 2 receptor α-chain gene expression in primary human T lymphocytes. Proc. Natl. Acad. Sci. USA. 86:2331.
48. Franza, R. B. Jr., S. F. Josephs, M. Z. Gilman, W. Ryan, and B. Clarkson. 1987. Characterization of cellular proteins recognizing the HIV enhancer using a microscale DNA-affinity precipitation assay. Nature (Lond.). 330:391.
49. Jonsson, A.-K., J.-J. Hyldig-Nielsen, B. Servenius, D. Larhammer, G. Andersson, F. Jorgensen, P. A. Peterson, and L. Rask. 1987. Class II genes of the human major histocompatibility complex. Comparisons of the DQ and DX α and β genes. J. Biol. Chem. 262:8767.
50. Barran, P. A., and W. R. McMaster. 1987. DNA sequence analysis of the rat RT1.Bα gene. Immunogenetics. 26:56.
51. Weiher, H., M. König, and P. Gruss. 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science (Wash. DC). 219:626.
52. Wang, Y., A. S. Larsen, and B. M. Peterlin. 1987. A tissue-specific transcriptional enhancer is found in the body of the HLA-DR α gene. J. Exp. Med. 166:625.
53. Gillies, S. D., S. L. Morrison, V. T. Oi, and S. Tonegawa. 1983. A tissue-specific transcriptional enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell. 33:717.
54. Larhammer, D., U. Hammerling, M. Denaro, T. Lund, R. Flavell, L. Rask, and P. Peterson. 1983. Structure of the murine immune response I-Ag locus: sequence of the I-Aβ gene and an adjacent B chain second domain exon. Cell. 34:179.
55. Saito, H., R. A. Maki, L. K. Clayton, and S. Tonegawa. 1983. Complete primary structures of the $\text{Eg}$ chain and gene of the mouse major histocompatibility complex. Proc. Natl. Acad. Sci. USA. 80:5520.
56. Hayashi, K., R. Makino, H. Kawamura, A. Arisawa, and Y. K. Yoneda. 1987. Characterization of rat $c$-$\text{myc}$ and adjacent regions. Nucleic Acids Res. 15:6419.
57. Gazin, C., S. Dupont De Dinechin, A. Hampe, J.-M. Masson, P. Martin; D. Stehelin, and F. Galibert. 1984. Nucleotide sequence of the human $c$-$\text{myc}$ locus: provocative open reading frame within the first exon. EMBO (Eur. Mol. Biol. Organ.) J. 3:383.
58. Bernard, O., S. Cory, S. Gerondakis, E. Webb, and J. M. Adams. 1983. Sequence of the murine and human cellular $\text{myc}$ oncogenes and two modes of $\text{myc}$ transcription resulting from chromosome translocation in B lymphoid tumours. EMBO (Eur. Mol. Biol. Organ.) J. 2:2375.
59. Kronke, M., C. Schluter, and K. Pfizenmaier. 1987. Tumor necrosis factor inhibits MYC expression in HL-60 cells at the level of mRNA transcription. Proc. Natl. Acad. Sci. USA. 84:469.
60. Yarden, A., and A. Kimchi. 1986. Tumor necrosis factor reduces $c$-$\text{myc}$ expression and cooperates with interferon-γ in HeLa cells. Science (Wash. DC). 234:1419.
61. Seliger, B., G. Stark, and K. Pfizenmaier. 1988. Tumor necrosis factor-α affects LTR-controlled oncogene expression in transformed mouse fibroblasts at the post-transcriptional level. J. Immunol. 141:2138.
62. Mathis, D. J., C. D. Benoist, V. E. Williams II, M. R. Kanter, and H. O. McDevitt. 1983. The murine $E_{\text{nu}}$ immune response gene. Cell. 32:745.
63. Das, H., S. Lawrance, and S. Weisman. 1983. Structure and nucleotide sequence of the heavy chain gene of HLA-DR. Proc. Natl. Acad. Sci. USA. 80:3543.