Tumor necrosis factor and immune interferon synergistically increase transcription of HLA class I heavy- and light-chain genes in vascular endothelium

(cytokines/major histocompatibility complex/ β_2 -microglobulin/tumor necrosis factor receptors/interferon β)

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ABSTRACT Tumor necrosis factor and immune interferon synergistically increase cell-surface expression of class I major histocompatibility complex molecules in cultured human endothelial cells. We report that tumor necrosis factor and interferon γ each independently increase mRNA levels and together cause a greater-than-additive (i.e., synergistic) increase in steady-state mRNA levels and transcriptional rates of the class I heavy- and light-chain genes. HLA heavy-chain mRNA is equally stable in cytokine-treated and -untreated endothelial cells. Interferon γ does not increase tumor necrosis factor receptor number or affinity on human endothelial cells. We conclude that the synergistic increase in class I major histocompatibility complex cell-surface expression results principally from the synergistic increase in transcriptional rates. We propose that this increase is caused by the cooperative binding of independently activated transcription factors to the promoter/enhancer sequences of class I genes.

Most cells express class I major histocompatibility complex (MHC)-encoded molecules, termed HLA-A, -B, and -C in humans, on their surface (1). Each class I molecule consists of a polymorphic 45-kDa integral membrane heavy (α) chain, encoded by the MHC, that associates with a non-MHC-encoded, invariant 12-kDa light (β) chain, called β_2 -microglobulin. Class I MHC molecules bind foreign peptides, thereby forming a specific target structure that can be recognized by cytotoxic T lymphocytes (2, 3). Cells expressing higher densities of class I molecules on their surface are more stimulatory to and better targets of specific cytotoxic T lymphocytes (4, 5).

The level of class I surface expression on vascular endothelial cells (EC) is low relative to that on lymphocytes and macrophages. However, expression may be increased up to 10-fold by the cytokines tumor necrosis factor (TNF), lymphotoxin, or interferon both type I [leukocyte (IFN- α) and fibroblast (IFN- β)] and type II [immune (IFN- γ)] (6). Moreover, TNF combines with IFN- γ to yield a synergistic induction of class I surface expression (7). Such synergistic effects may well occur *in vivo* because combinations of cytokines are likely to be produced during immune responses. For example, IFN- γ and TNF are produced coordinately by the same T-cell clones (8), and IFN- β and TNF are induced coordinately by virus in human B cells and monocytes (9).

Synergy between cytokines has been seen in several other biological responses, but the molecular bases for cytokine interactions are not understood. We demonstrate here that synergy between TNF and IFN- γ is observed at the level of steady-state mRNA encoding both HLA α and β chain and in the rate of transcription for both chains. We propose that synergy is a result of positive interactions between transcrip-

tion factors activated by each cytokine, and we discuss elements in the *HLA-B27* promoter that are potential targets for the binding of cytokine-activated factors.

MATERIALS AND METHODS

Cells and Mediators. Human umbilical vein endothelial cells (EC) were isolated and cultured as described (10, 11). Cells used in these experiments were confluent and at passage levels 3 through 6. Recombinant human TNF (expressed in *Escherichia coli*, 2.5×10^7 units/mg, specific activity) was a gift of W. Fiers (State University of Ghent, Belgium). Recombinant human IFN- β (expressed in *E. coli*, 3×10^8 units/mg) and IFN- γ (expressed in *E. coli*, 2.5×10^7 units/mg) were obtained from Biogen. Cytokines, alone or in combinations, were added to the cultures in complete medium.

Fluorescence Flow Cytometry. Surface expression was determined by indirect immunofluorescent staining and quantitated on a fluorescence activated cell sorter (FACS) analyzer (Becton Dickinson). Staining was performed as described (12). Primary monoclonal antibodies (mAbs) used were (i) W6/32(IgG2a), which binds a monomorphic HLA-A,B,C determinant (13), (ii) E1/1.2(IgG2b), which is specific for a 96-kDa glycoprotein that is not regulated by the cytokines used in these experiments (14), and (iii) K16/16(IgG1 κ) as a control for nonspecific binding and autofluorescence (gift of D. Mendrick, Brigham and Women's Hospital, Boston). Secondary antibody was fluorescent rabbit IgG-anti-mouse-IgG (ICN).

mRNA Quantitation. mRNA was analyzed by S1 nuclease protection (15). RNA was prepared from EC by Nonidet P-40 lysis or by guanidinium lysis as noted (16). Equal amounts of RNA were hybridized to continuously labeled, single-stranded probes (see below) overnight at 43°C in 50% (vol/vol) formamide. After treatment with S1 nuclease at 400 units/ml (BRL) for 30 min at 37°C, samples were separated on 6% polyacrylamide gel containing 7.5 M urea (Sequagel, National Diagnostics, Manville, NJ), which was then dried and exposed to film (XAR-5, Kodak) with an intensifying screen (Biospeed, ABN, Hayward, CA) at -80°C.

Three specific probes were used in each experiment. HLA α -chain message was quantitated by hybridization to a 673-nucleotide *Pst*I-*Apa*I fragment of a genomic HLA-B7 clone, which encodes the highly conserved $\alpha 3$ domain. β_2 -Microglobulin was measured by hybridization to a 738-nucleotide *Eco*RI-*Sma*I fragment of the genomic clone $\beta 2M13$ (17).

Abbreviations: EC, endothelial cells; IFN- α , - β , and - γ , interferon α , β , and γ , respectively; ISRE, interferon-stimulated regulatory element; mAb, monoclonal antibody; MHC, major histocompatibility complex; TNF, tumor necrosis factor; TRE, 12-tetradecanoyl 13-acetyl phorbol-responsive element; FACS, fluorescence-activated cell sorter.

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This fragment includes the entire first exon, which is either 93 or 102 nucleotides long, depending upon the transcription start site used. A γ -actin probe was constructed with a 164-nucleotide *Ban* II–*Hind*III fragment from the 3'-untranslated region of the cDNA clone SP6 γ ACT (18). Quantitation of protected fragments was done by liquid scintillation counting or by densitometry with similar results.

Nuclear Run-Off Transcription Assay. Nuclei were prepared by Nonidet P-40 lysis and used immediately as described (19) with modifications (20). Labeled RNA was recovered by guanidinium lysis and centrifugation through CsCl (16); then it was hybridized to 5 μ g of plasmid DNA bound to nitrocellulose. The plasmids used were as follows: HLA-B7/pUC12, genomic clone; β 2M13, genomic clone of β ₂-microglobulin (17); SP6 γ ACT, γ -actin cDNA 3'-untranslated region (18); pGEM3 (Promega) and Bluescript (Stratagene) vectors, which were included as negative controls.

Determination of mRNA Stability. EC were given fresh complete medium with or without TNF (100 units/ml) and/or IFN- γ (250 units/ml) at time 0. Actinomycin D (5 g/ml, Sigma) was then added for 4 hr, 2 hr, 1 hr, or immediately before cytoplasmic RNA was harvested at 28 hr. Equal amounts of RNA were analyzed for HLA α -chain and γ -actin mRNA content by S1 nuclease protection, as described above. This concentration of actinomycin D was shown in run-off assays to block transcription in EC, and in these experiments longer times of actinomycin D treatment caused cell death.

TNF Receptor Quantitation. EC were treated for 18 hr with IFN- γ at 250 units/ml and harvested nonenzymatically. Cells (2.5×10^5) were incubated in 100 μ l of 1% bovine serum albumin (Sigma) in RPMI 1640 medium (GIBCO) with radio-labeled TNF (44.7 μ Ci/ μ g; 1 Ci = 37 GBq; New England Nuclear) with or without excess unlabeled TNF at 19°C for 150 min (by which time binding equilibrium is attained, data not shown). Samples were then layered over oil [0.2 ml, dibutyl phthalate/bis(2-ethylhexyl)phthalate 1.5:1.0 (vol/vol), Kodak] and centrifuged at $12,000 \times g$ for 15 sec. Cell pellets were recovered by cutting off the tube bottom; pellets and supernatant were counted in a γ counter (Beckman 5500B), and results were analyzed by the method of Scatchard.

RESULTS

TNF and IFN- β or IFN- γ Synergistically Induce HLA Class I Surface Expression and mRNA Accumulation. Effects of the cytokines TNF (100 units/ml), IFN- β (200 units/ml), and IFN- γ (250 units/ml) alone and in combination on HLA class I expression in cultured EC were examined by indirect immunofluorescence staining and FACS quantitation (Fig. 1, Table 1) and compared with mRNA levels determined by S1 nuclease protection (Fig. 2). As reported previously, class I MHC surface expression is uniformly increased by cytokine treatment (Fig. 1), and treatment with TNF together with either IFN- β or IFN- γ induces HLA class I expression that is significantly greater than the additive effects of each cytokine used singly (Table 1). Combined treatment with IFN- β and IFN- γ , in contrast, induces less than additive increases in HLA class I expression. These cytokine doses produce near-maximal induction of HLA class I molecules, although synergy between TNF and IFN- β or IFN- γ is also seen at lower cytokine doses (data not shown). Cytokine treatment did not alter the surface antigen detected by mAb E1/1.2 (Table 1, gp96).

Synergy between TNF and IFN- β or IFN- γ is also seen in the induction of steady-state mRNA levels for HLA class I α and β chains as measured in nuclease protection assays. As shown in Fig. 2 and Table 2, α and β class I mRNA are coordinately regulated by each of these cytokines, and transcripts using either start site for β -chain initiation are equally increased by cytokine treatment. In parallel with the observed induction of surface expression of class I molecules, TNF acts

FIG. 1. Cell number (y axis) vs. logarithm of fluorescence (x axis) of EC stained with an HLA class I-specific mouse mAb (W6/32) and mouse immunoglobulin-specific fluorescent antibodies. Expression was determined after 12 hr of treatment with TNF (100 units/ml) and IFN- γ (250 units/ml). —, Untreated cells; ···, TNF-treated cells; ---, IFN- γ -treated cells; -·-, cells treated with combined TNF and IFN- γ . Histograms were overlaid and smoothed on a FACS analyzer.

synergistically with both IFN- β and IFN- γ to increase the steady-state level of class I mRNAs. IFN- β and IFN- γ do not synergize in the induction of class I mRNA expression. IFN- β induces a greater accumulation of HLA class I mRNA than does IFN- γ under these conditions, although they appear to have induced similar levels of cell-surface expression. This discrepancy may arise because we are measuring the levels of mRNA and surface expression at the same time. Because increases in mRNA precede increases in surface expression, it is possible that at later times IFN- β -treated cells would show greater surface expression as well.

TNF and IFN- γ Synergistically Induce HLA Class I Transcription. Nuclear run-off experiments were performed to determine whether the accumulation of HLA class I mRNA in cells treated with TNF and IFN- γ is the result of an increased transcriptional rate. Equal numbers of counts incorporated into RNA were hybridized to DNA probes immobilized on nitrocellulose. RNA labeled by extension of nascent transcripts with radioactive rUTP can be detected after hybrid-

Table 1. Regulation of EC class I MHC surface molecules by TNF and IFNs

Treatment*	Corrected mean fluorescence [†]			
	Anti-HLA		Anti-gp96	
	W6/32	Normalized	E1/1.2	Normalized
None	6	(1)	73	(1)
TNF	16	2.7	78	1.1
IFN- γ	48	8.0	74	1.0
IFN- γ + TNF	137	22.8 [‡]	91	1.2
IFN- β	72	12.0	79	1.1
IFN- β + TNF	116	19.3 [‡]	90	1.2
IFN- β + IFN- γ	106	17.7	85	1.2

*Cells were given fresh medium and treated 22 hr with TNF (100 units/ml), IFN- γ (250 units/ml), and IFN- β (200 units/ml), stained with mAbs W6/32 (HLA class I specific), E1/1.2 (gp96 specific), or a control nonspecific antibody (K1616), and analyzed by FACS as described above; mean fluorescence of each sample was then determined.

[†]Mean fluorescence obtained with the nonspecific mAb K1616 was subtracted from values obtained with specific antibodies.

[‡]Synergy (value greater than the sum of values obtained with individual cytokine treatments).

FIG. 2. Cytokine-induced accumulation of HLA class I mRNA in EC. RNA was harvested from the same cultures of EC as those used in Table 1 as well as from a duplicate untreated culture. Duplicate samples of 5 μ g of cytoplasmic RNA were hybridized to continuously labeled, single-stranded HLA α -chain and β_2 -microglobulin probes or HLA α -chain and γ -actin probes (data not shown), treated with S1 nuclease, and electrophoresed on 6% acrylamide/7.5 M urea gel. Size standards were derived from *Msp* I-digested pBR322 DNA. Positions of the protected class I α - and β -chain fragments are indicated with labeled arrows. For normalization see Table 2. Gel depicted is representative of four similar experiments.

ization to HLA class I α or β DNA (Fig. 3, row 1). After treatment with TNF or IFN- γ more nascent transcripts are detected (rows 2 and 3, respectively), and the combination of these cytokines produces a synergistic increase in the transcription rate of HLA class I α and β genes (row 4).

HLA Class I mRNA Is Not Stabilized by IFN- γ and TNF. To investigate whether increased message stability could also contribute to the accumulation of HLA class I mRNA after

Table 2. Cytokine-regulated HLA class I mRNA expression

Treatment	HLA mRNA expression*	
	α	β
None	(1)	(1)
TNF	4.5	3.9
IFN- γ	5.7	3.7
IFN- γ + TNF	19.4 [†]	12.5 [†]
IFN- β	21.6	11.3
IFN- β + TNF	43.1 [†]	23.0 [†]
IFN- β + IFN- γ	21.6	15.3
None (duplicate)	0.9	0.8

*The gel shown in Fig. 1 was quantitated by densitometry. Duplicate RNA samples hybridized to γ -actin and *HLA-B7* probes were also analyzed by S1 nuclease protection. Values obtained by counting the protected γ -actin band were used to correct for unequal RNA amounts hybridized.

[†]Synergy.

FIG. 3. Effect of cytokine treatment on HLA class I α - and β -chain gene transcription. Nuclei were isolated from the same EC cultures used in the FACS experiment of Fig. 1. Nuclear run-off transcription assay and probes are described. Equal numbers of counts incorporated into RNA were hybridized to immobilized DNA (5 μ g per point). A 6-hr exposure of the autoradiograph is shown for *HLA-B7*; the rest is from a 12-hr exposure.

cytokine treatment, transcription was blocked in some cultures with actinomycin D 4 hr, 2 hr, and 1 hr before RNA harvest, and the rate of disappearance of HLA class I α -chain message was determined. Class I α -chain mRNA is equally stable in cytokine-treated and untreated EC (Fig. 4), indicating that TNF- and IFN- γ -induced accumulation of HLA mRNA is not the result of message stabilization.

IFN- γ Does Not Modulate Surface TNF Receptors on EC. IFN- γ induces a 2- to 3-fold increase of surface TNF receptors on the human cervical carcinoma cell line ME-180 (21), HeLa D98/AH2, and colon carcinoma HT-29 cells, but not SK-MEL-109 melanoma cells (22). To determine whether EC respond to IFN- γ with increased TNF-receptor expression, receptor-binding assays were done. Both IFN- γ -treated and -untreated EC populations bind an essentially equivalent amount of TNF at saturation. Scatchard analysis of the bound and free fractions (Fig. 5) revealed a K_d of 135 pM or 111 pM for the untreated and IFN- γ -treated populations, respectively, and 3850 or 3300 TNF receptors per cell. These values are similar to those reported for TNF receptors on human EC [K_d 105 pM, 1500 receptors per cell (23), K_d 43 pM, 664 receptors per cell (24)]; differences in receptor number may reflect variations in EC culture conditions. We conclude that TNF-receptor modulation is not the mechanism by which IFNs synergize with TNF.

DISCUSSION

Cytokine-regulated increase in HLA class I expression has been proposed as one mechanism by which EC at the site of

FIG. 4. Scatchard analysis of TNF receptors on untreated and IFN- γ -treated EC as described. $\square$, Data from untreated EC (K_d of 135 pM, 3850 receptors per cell) with broken-line fit by least-squares; $\blacksquare$, data from IFN- γ -treated EC (K_d of 111 pM, 3300 receptors per cell) with the solid line fitted. Data are from one of three experiments in which receptor numbers differed by <30% between control and IFN- γ -treated cells.

FIG. 5. HLA α -chain mRNA in EC treated with actinomycin D. Cells were treated with cytokine or left untreated for 24 hr. Actinomycin D was then added to some cultures 4 hr, 2 hr, 1 hr, or immediately before RNA harvest, and the expression of HLA α -chain mRNA and γ -actin mRNA was determined by nuclease protection. Data are from one of two similar experiments.

an immune reaction recruit and activate antigen-specific cytotoxic T lymphocytes from the circulation (25). Previous studies have shown that combined TNF and IFN- γ synergistically increase HLA class I molecule surface expression, but the basis of this synergistic interaction was not known (7). We demonstrate here that the synergy seen between TNF and IFN- γ in inducing HLA class I surface expression is also seen at the level of steady-state mRNA for HLA class I α and β chains and, furthermore, that TNF and IFN- γ synergize in the induction of elevated transcriptional rates of the genes encoding HLA class I α and β chains. Combined IFN- β and IFN- γ , on the other hand, produce no more than additive increases in cell-surface and steady-state mRNA expression.

Our observations suggest the following conclusions. (i) TNF acts to increase class I MHC antigen expression by increasing the transcriptional rate, as had been reported for IFN species (26). (ii) Synergy between TNF and IFN- γ results from an essentially multiplicative increase in transcriptional rate. In contrast, IFN- β is likely to act through the

same mechanisms as IFN- γ , leading to responses often less than additive when these cytokines are combined.

What mechanisms underlie the apparently independent effects on transcriptional rate produced by TNF and IFN- γ ? We find no evidence for IFN- γ induction of TNF receptors in endothelial cells, a mechanism proposed in other systems. Instead, we propose that TNF and IFN- γ each activate different, but possibly interacting, transcription factors (DNA-binding proteins) specific for different sites in the HLA class I genes. A similar mechanism has been demonstrated in the regulation of the human proenkephalin gene (27) and steroid-responsive genes (28, 29), except that in these cases an inducible transcription factor acts in combination with constitutive factors.

The DNA sequences responsible for IFN- γ -regulated expression of the human class I α -chain gene *HLA-B7* were shown by Weissman and colleagues (30) to be located between 660 base pairs (bp) 5' of the transcription start site to 2 kilobases (kb) downstream of the polyadenylation site. A sequence homologous to the 28-bp interferon consensus sequence, originally defined by comparing promoter sequences of genes regulated by IFN- α , has been identified in the 5' region of *HLA-A3* (26). A sequence homologous to the interferon consensus sequence has also been identified in the third intron of the human β_2 -microglobulin gene (17). Additionally, a 10-bp segment of the interferon consensus sequence (GTTTCACTTC) located 5' of the mouse MHC class I genes *H-2L^d* (31) and *H-2K^b* (32) has been shown to be required for binding nuclear factors induced by IFN- γ . Finally, this 10-bp sequence (inverted) is highly homologous to the 13-bp IFN-stimulated regulatory element (ISRE) that is sufficient to confer IFN- α and IFN- γ responsiveness upon a reporter gene and necessary for IFN responsiveness of the 9-27 gene (33). The region 5' of the *HLA-B27* gene contains this 10-bp sequence (Fig. 6a), as well as two elements partially homologous to the ISRE (Fig. 6b). It is likely, therefore, that the 10-bp element confers IFN responsiveness upon the HLA class I genes.

Two different families of promoter/enhancer elements have been shown to mediate gene regulation by TNF. Studies by Karin and colleagues (34) have demonstrated that TNF activates the transcription of *JUN* and *FOS*, which encode major components of the AP-1 transcription factor. AP-1, in turn, mediates the transcriptional activation of promoters containing the 12-tetradecanoyl 13-acetyl phorbol (TRE) (34, 35). Examination of the 5'-flanking region of *HLA-B27* reveals several sequences displaying partial homology with this element (Fig. 6).

A second mediator of TNF-regulated transcription is NF- κ B. It has been reported that TNF stimulates human interleukin 2 gene expression (36), interleukin 2-receptor α gene

A HLA-B27

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GAGCTCACTC TCTGGCATCA AGTTCCTCGT GATCAGTTTC CCTACCAAG
ATCCAAGAGG AGAGGTAAGG AGTGAGAGGC AGGGAGTCCA GTTCAGGGAC 100
AGGGATTCCA GGAGGAGAAG TGAAGGGGAA GCGGGTGGGC GCCATGGGG
GTCTCTCCCT GGTTCACACA GACAGATCCT TGTGCGGGAC TCAGGCAGAC 200
AGTGTGACAA AGAGGCTGGT GTAGGAGAAG AGGGATCAGG ACGAACGTCC
AAGGCCCCGG GCGCGGTCTC AGGGTCTCAG GCTCCGAGAG CCTGTCTGCG 300
ATTGGGAGG CGCAGAGTTG GGGATTCCCC ACTCCACCA GTTTCAGTTT
TTCTCCCAAC CTATGTCGGG TCCTTCTTCC AGGATACTCG TGACGCGTCC 400
CCATTTCCCA CTCCATTGG GTGTGGGGT TCTAGAGAAG CCAATCAGTG
TCGCCGGGGT CCCAGTTCTA AAGTCCCCAC GCACCCACCC GGACTCAGAA 500
TCTCTCTAGA CGCCGAGATG CGGGTCACGG CGCCCCGAAC CCTCTCTCTG

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B ISRE Homologies

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AGTTTCACTTC H-2Ld ICS (31)
HLA-B27 340-350
.....T..T 6-16 ISRE* (33)
.....TA..T 9-27 ISRE* (33)
.....CC.A. HLA-B27 35-45
G.....CACAG 161-171

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C TRE Homologies

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TGAGTCAG consensus TRE (44)
TGAG..... CRE region II (48)
GTGA.... HLA-B27 29-36
G..C.... 187-194
G.GA.... 232-239
G.TC.... 275-282
G.TC.... 283-290
A....G.. 11-4*
.....C. 193-186*
.....C. 498-490*

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FIG. 6. Analysis of the 5'-flanking region of *HLA-B27*. (A) Sequence is from GenBank, locus HUMMB27B, accession number M12967. CAAT box and variant TATA box (TCTAAA) are underlined. Partial and full ISRE homologies are boxed. Sequences homologous to the PRE are double underlined. The translation initiation codon at position 518 is indicated with an arrow (→). H2TF1/KBF1/NF- κ B binding-site homology is indicated by a dotted line above the sequence. (B) ISRE homologies are listed; identical nucleotides are indicated with a dot. (C) TRE homologies are listed; identical nucleotides are indicated with a dot.

expression (37), and expression regulated by the human immunodeficiency virus enhancer (38) by activating NF- κ B in T cells. The murine MHC class I *H-2K^b* gene promoter contains a palindromic sequence (TGGGGATTCCCCA) that is homologous with the NF- κ B-binding site and binds the constitutively expressed transcription factors KBF1 (39) and H2TF1 (40). Indeed, transcription factor NF- κ B competes with transcription factor H2TF1 for binding to this sequence (40, 41). This sequence is found in a similar position upstream of the *HLA-B27* gene (Fig. 6a). It was recently shown that an NF- κ B-like factor is activated in TNF-treated HeLa cells and that this factor can displace KBF1 from the *H-2K^b* promoter (42). In regulating expression of a reporter gene, however, a *Dde* I-*Hinf*I construct that deletes most of this palindrome responds even better to TNF than does a construct that retains this sequence (42).

The transcription factors implicated in TNF-induced gene expression, AP-1 and NF- κ B, differ in their sensitivity to the protein synthesis inhibitor cycloheximide. AP-1-mediated TNF activation of TRE-containing genes is blocked by cycloheximide, consistent with the observation that TNF acts by inducing *de novo* synthesis of *JUN* and *FOS* gene products (34, 43). In contrast, cycloheximide itself activates NF- κ B (44), which is associated with an inhibitor protein, I- κ B, in the cytoplasm of uninduced cells (40). Although cycloheximide increases transcription of HLA heavy-chain genes in neuroblastoma (26) and HeLa (20) cells, previous studies from our laboratory have demonstrated that cycloheximide blocks the TNF-induced accumulation of class I mRNA in EC (6). These data favor a role for transcription factors synthesized *de novo* in response to TNF, such as AP-1. Consistent with this proposal are the observations that AP-1 purified from HeLa cells binds to the several sites in the 5' upstream region of the murine class I gene *H-2D^d* (45) and that nuclear factors in murine tissue extracts also bind to one of these sites (46).

In conclusion, we hypothesize that in cultured EC, TNF and IFN- γ synergistically increase transcription of class I MHC genes by the following mechanism: (i) TNF activates nuclear factors that bind to AP-1 recognition elements, and (ii) this binding is augmented by cooperative interactions with interferon-activated transcription factors binding to the ISRE sequence.

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