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HLA Class I Heavy-Chain Gene Promoter Elements Mediating Synergy between Tumor Necrosis Factor and Interferons

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The cytokines tumor necrosis factor (TNF), beta interferon (IFN-β), and IFN-γ increase major histocompatibility complex class I molecule expression. A greater than additive (i.e., synergistic) induction of class I heavy-chain mRNA is observed in HeLa cells treated with TNF in combination with either type of IFN. To define the *cis*-acting elements mediating cytokine synergy, the promoter of a human major histocompatibility complex class I heavy-chain gene (HLA-B7) was placed in front of a reporter gene and transfected into HeLa cells. Deletion analysis mapped the elements required for synergy to a 40-bp region containing a κB-like element, which is necessary for the response to TNF, and an interferon consensus sequence (ICS), which is necessary for the responses to IFNs. When the orientation of these elements was reversed or their normal 20-bp spacing was reduced by 5 or 10 bp, i.e., one half or one full turn of the DNA helix, essentially equivalent responses were obtained, suggesting that these parameters are not critical. In electromobility shift assays, a p50-containing NF-κB nuclear factor from TNF-treated cells binds κB-containing probes, and ISGF-2 from IFN-γ-treated cells binds ICS-containing probes. A probe containing both the κB and ICS elements (κB-ICS) forms a novel complex with nuclear factors isolated from cells treated with both TNF and IFN-γ; this complex also forms when nuclear factors from individually cytokine-treated cells are mixed in vitro. The natural variant ICS found in HLA-A responds to IFN-γ and can mediate synergy with TNF. However, the variant κB found in HLA-C does not respond to TNF, nor can it mediate synergy between TNF and IFN-γ. These observations suggest that synergy between TNF and IFNs in the induction of HLA class I gene expression results from the sum of individual interactions of cytokine-activated enhancer-binding factors with the transcription initiation complex.

Major histocompatibility complex (MHC) class I molecules, called HLA-A, -B, and -C in humans, bind peptides derived from endogenously synthesized proteins, thereby forming a structure on the cell surface that is recognized by specific cytotoxic T lymphocytes (CTL). Class I-restricted CTL specific for viral or intracellular bacterial proteins constitute a major line of host defense against such infections (1). Nearly all cells synthesize and express class I molecules constitutively, and both synthesis and expression are strongly increased by the inflammatory cytokines tumor necrosis factor (TNF), beta interferon (IFN-β), and IFN-γ. Inducible MHC molecule synthesis at sites of infection is likely to be important because the ability of a cell to be recognized by CTL is a function of the rate of class I molecule synthesis and not the absolute level of class I molecule expression (10). This functional observation is consistent with the biochemical evidence that peptides bind to MHC class I molecules only during the folding and assembly of nascent class I heavy and light chains (β_2-microglobulin) in the endoplasmic reticulum (35, 52). TNF in combination with either type of IFN induces a greater than additive (i.e., synergistic) HLA class I surface and mRNA expression in endothelial cells (33), which results from the synergistic transcriptional activation of the structural HLA class I heavy- and light-chain genes by TNF and IFN-γ (29). The molecular basis of synergy is the subject of the present investigation.

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Cytokine-regulated transcription of many genes, including MHC genes, is mediated by the binding of proteins to DNA sequences located in the gene, often in the promoter region 5′ of the CAAT and TATA boxes (28). Sequences regulating transcription have been identified by measuring the response of transfected mutant genes, and several of the proteins binding these sequences have been characterized. The transcriptional response to TNF is mediated by two different DNA-binding factors, NF-κB and AP-1. TNF induces transcription of human immunodeficiency virus type 1 by activating NF-κB (15), perhaps by causing its dissociation from IκB, with which it constitutes a latent complex in the cytoplasm of nonlymphoid cells (19). Upon dissociation, free NF-κB moves to the nucleus, where it binds to the κB consensus sequence GGGRHTYYCC (34). TNF also induces the transcription of the genes encoding two principal subunits, c-Jun and c-Fos, of the transcription factor AP-1, which binds to the consensus sequence GTGAGTMA (5). Both the κB and AP-1 consensus sequences (when multimerized) can confer TNF responsiveness upon heterologous promoters (5, 28). Both elements are found in the HLA-B promoter (29). Repeated, degenerate NF-κB elements are present in the promoter of a murine MHC class I gene, *H-2K^b*, and have been implicated in the TNF response of this gene (28). However, only a single such element is clearly present in the human MHC class I gene promoter, and a single copy of the mouse κB element was not sufficient to mediate TNF-induced transcriptional activation (28).

The promoters of several IFN-α-responsive genes, including HLA-A2, were compared to determine the original, large (29-bp) interferon consensus sequence (ICS) (16). Subsequent studies have produced shorter consensus sequences, AGTTTCNNYTYY (48) and AGTTTC(N)NTTTCC (42).

IFN-α rapidly activates the transcription factor ISGF3 (44). Since IFN-α and IFN-β, called type I IFNs, bind to a common receptor (47), it is widely thought that the ICS also mediates the response to IFN-β. IFN-γ, called type II IFN, uses a distinct receptor (47) and slowly induces the synthesis of the ICS-binding transcription factor ISGF2 (17). A fragment of the HLA-B gene promoter that includes an ICS (. . .TCACTT. . .) mediates the response to both IFN-α and IFN-γ (23). A novel IFN-responsive element, called the IFN-γ activation site, has been recently described (11), but no homologous site has been identified in the HLA class I promoters. A second, canonical ICS located further upstream in the HLA-A2 promoter has been identified (2), and additional IFN-responsive enhancers have been mapped within the body of the HLA-B7 gene (18, 53), but their contribution to the IFN response of these genes has not been established.

The human MHC heavy-chain genes, HLA-A, -B, and -C, differ in their levels of constitutive expression in different tissues and in their regulation by cytokines (22). Although the promoters of these genes are homologous, it has been noted that the κB element differs between HLA-A and -B and HLA-C (51) and that the ICS element differs between HLA-A and HLA-B and -C (23). The difference in the κB elements has been suggested to mediate differential regulation by TNF (51), and the difference in the ICS has been reported to be the basis of differential regulation by IFNs (23).

The cytokine-responsive elements in the promoter of a human MHC class I gene, HLA-B7, are examined here. We find that synergistic induction of an HLA-B gene results from TNF acting through a κB element, which binds TNF-activated NF-κB, and IFNs acting through an ICS, which binds IFN-γ-induced ISGF2. The interaction of these elements in transfection assays or in vitro DNA-binding assays is independent of their orientation or spacing. Therefore, synergy appears to result from the summation of the individual effects of these cytokines on class I gene transcription. Finally, we show that a natural variant κB element found in the HLA-C locus does not bind NF-κB and is inactive in the context of the HLA-B7 promoter, whereas the variant ICS found in the HLA-A locus binds ISGF2 and can mediate a response to IFN.

MATERIALS AND METHODS

Cells and cytokines. HeLa cells (gift of R. Flavell, Yale University) were cultured in Dulbecco modified Eagle medium (GIBCO, Grand Island, N.Y.)–10% fetal calf serum–1 mM glutamine without antibiotics. Recombinant human TNF (expressed in *Escherichia coli*; 2.5×10^7 U/mg) was a gift of W. Fiers (State University of Ghent, Ghent, Belgium). Recombinant human IFN-β (expressed in *E. coli*; 3×10^8 U/mg) and IFN-γ (expressed in *E. coli*; 2.5×10^7 U/mg) were obtained from Biogen (Cambridge, Mass.). Treatments were for the times and at the doses noted in the figure legends.

S1 nuclease protection. Construction of the probes for γ-actin and the conserved third domain of HLA class I heavy chain has been described elsewhere (29). Cytoplasmic RNA was prepared by Nonidet P-40 lysis and phenol extraction (20). RNA (5 μg) was hybridized overnight to continuously labeled, single-stranded probes in 50% formamide at 43°C and digested with S1 nuclease (350 U/ml; Bethesda Research Laboratories, Gaithersburg, Md.), and the products were separated on a denaturing polyacrylamide gel. The gel was dried and exposed to film to produce an autoradiograph, which was quantitated by densitometry (Molecular Dynamics, Sunnyvale, Calif.). For quantitation, the specific value of the HLA class I band was determined by subtracting the background counts obtained from an area of equal size immediately above the class I band, and this specific value was divided by the specific value from the γ-actin band to correct for the minor loading differences.

Reporter gene construction. The promoter of the HLA-B7 gene (gift of T. Spies, Harvard University) was PCR subcloned into *Eco*RI- and *Sma*I-cut pUC19 with a 5′ primer that includes the endogenous *Eco*RI site at −670 bp (5′GGCTGCAGAATTCTAATCATTCAGGGA) and a 3′ primer that starts one nucleotide 5′ of the translation initiation codon (5′CTCGGCGTCTGAGGAGA) (49). Deletion mutants were made by the appropriate restriction enzyme digestions, Klenow filling, and ligation or by PCR between the M13/pUC sequencing primer and the following 5′ primers: A>, 5′CGTTGGGGATTCCCCACTCC; ICS>, 5′CCTGAGTTTCACTTCTTC; H>, 5′GACTCCCAACT TGTGTC; κB/C>B7, 5′CGTTGAGGATTCTCCACTCC; and κB/B-ICS/A>B7, 5′CGTTGGGGATTCCCCACTCC CCTGAGTTTCTTTTCTTCTCCC). Either promoter sequences were isolated in an *Nde*I-*Bam*HI fragment of pUC19 and placed in front of a promoterless human growth hormone (hGH) reporter gene (p0GH; Nichols Institute, San Juan Capistrano, Calif.) or PCR-generated deletion fragments were cut with *Bam*HI and ligated between a Klenow-filled *Hin*dIII site and the *Bam*HI site of p0GH. Oligonucleotides corresponding to the HLA-B7 κB and ICS elements, as well as the spacing mutants listed, were synthesized (Operon, Alameda, Calif.), annealed, and ligated into a *Hin*cII-digested H>B7.GH plasmid.

Transfection and hGH assay. HeLa cells were trypsinized, washed once in Hanks' buffered salt solution, and suspended in ice-cold electroporation buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.05], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose [8]) at approximately 10^7 cells per ml. Prior to transfection, the PCR-generated reporter gene constructs were digested with *Hin*dIII and *Eco*RI to separate the promoter-reporter gene fusion from the plasmid backbone. A small portion of the restriction-cut plasmids was analyzed on an agarose gel to confirm digestion and DNA concentration. Cell aliquots (0.4 ml) were mixed with 15 μg of reporter gene DNA and 5 μg of luciferase expression DNA (pGL2CON; Promega, Madison, Wis.) to control for transfection efficiency, placed in chilled electroporation cuvettes (0.2-cm gap; Bio-Rad, Melville, N.Y.), and electroporated at 750 V/cm and 960 μF (time constants ranged from 9 to 10 ms). Electroporated cells were mixed with medium (Dulbecco modified Eagle medium, 10% fetal calf serum, 1 mM glutamine) containing penicillin and streptomycin and divided into four wells of a 24-well plate. After 24 h, the medium was aspirated and replaced with cytokine-containing medium. Culture medium was harvested 18 to 20 h later, and 100-μl samples were assayed for hGH, using a solid-phase sandwich radioimmunoassay as described by the manufacturer (Nichols Institute). Radioactivity was measured in a gamma counter (model 5500B; Beckman, Palo Alto, Calif.). Cell lysates were assayed for luciferase by using a kit as described by the manufacturer (Promega) and read on a luminometer (Berthold LB9501).

Nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear proteins were extracted from approximately 5×10^6 HeLa cells by a miniprep modification of the procedure of Dignam et al. (13). Confluent cultures of HeLa

cells were placed on ice and rinsed three times with ice-cold Tris-buffered saline (0.15 M NaCl, 5 mM Tris [pH 7.8]). All of the following buffers were kept ice cold and supplemented with protease inhibitors (1 μg of leupeptin per ml, 5 μg of aprotinin per ml, 0.5 mM phenylmethylsulfonic acid [all from Sigma Chemical Co., St. Louis, Mo.], and 1 μM dithiothreitol (DTT; Bethesda Research Laboratories), and centrifugation was at 4°C ($10^4 \times g$). Cells were scrape harvested into 1.5 ml of Tris-buffered saline, pelleted, resuspended in 400 μl of hypotonic swell buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl), and left on ice for 15 m. Cells were then lysed by the addition of 100 μl of 2.5% Nonidet P-40 in the same buffer; the nuclei were pelleted for 30 s, resuspended in 50 μl of extraction buffer (20 mM HEPES [pH 7.9], 0.48 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol), and left on ice for 30 min. Nuclei were pelleted for 10 min, 50 μl of supernatant was withdrawn and mixed with 50 μl of freeze buffer (20 mM HEPES [pH 7.9], 0.1 M KCl, 0.2 mM EDTA, 20% glycerol), and aliquots were frozen in an ethanol bath at −70°C. Yields were quantitated by the Bradford assay (50) with a bovine serum albumin (BSA) standard and averaged 2 μg/μl. Annealed DNA oligonucleotides were labeled to approximately 5×10^3 cpm/fmol with polynucleotide kinase and [γ-^{32}P]ATP, phenol-chloroform extracted, passed through a Sephadex G-50 spun column, and quantitated by Cerenkov counting. The sequences of the oligonucleotides used are as follows (5′ to 3′; complement sequence not shown): κB, CGTTGGGGATTCCCCACTCC; ICS, CCA CAGTTTCACTTCTGCACCT; κBICS, CGTTGGGGATTC CCCACTCCCCTGAGTTTCACTTCTTCTCCC; κBΔ5ICS, CGTTGGGGATTCCCCACTGAGTTTCACTTCTTCTCCC; and κBΔ10ICS, CGTTGGGGATTCCCAGTTTCACTTCTT CTCCC. Nuclear extracts (4 μl) were added to 1 μl (1 μg) of poly(dI-dC) (Pharmacia, Piscataway, N.J.), and competitive inhibitors were added to 100-fold molar excess in 1 μl where indicated. Probe (1 fmol) was then added with 2 μl of binding buffer (0.16 M HEPES [pH 7.9], 0.3 M KCl, 11 mM EDTA, 2.5 mg of BSA per ml) for a total volume of 10 μl, and the incubation was continued for 20 min at room temperature. Samples (5 μl) were then withdrawn and electrophoresed at 15 V/cm through a native 4% polyacrylamide–2% glycerol gel (prerun for 30 min) in 0.25× Tris-borate-EDTA) electrode buffer at 20°C until the xylene cyanol tracking dye had migrated ~11 cm. The gel was dried and exposed to film or to storage phosphor screen for quantitation on a PhosphorImager (Molecular Dynamics).

Immunochemical identification of nuclear factors (antibody EMSA). Nuclear extracts were prepared as described above. Extracted protein (6 μg) was combined with supershift buffer (100 mM KCl, 25 mM Tris [pH 7.5], 1 mM DTT, 0.5 mM EDTA, 5% glycerol) for a total volume of 8 μl. Probe was then added in 10 μl with 1 μg of poly(dI-dC)–60 mM GTP–10 mg of BSA per ml–2 mM Tris (pH 7.6)–10 mM NaCl–0.2 mM DTT–0.2 mM EDTA–10% glycerol and incubated at room temperature for 20 min. Antibody (1 μl) was then added, and the mixture was incubated at 4°C overnight with gentle rocking. Antibodies used were anti-ISGF2, a polyclonal rabbit antiserum raised against purified ISGF2 (a generous gift of R. Pine, New York Public Health Research Institute) and anti-p50(NLS), anti-p65, and anti-c-Rel, all polyclonal antisera raised against peptides (Santa Cruz Biotech, Santa Cruz, Calif.). Samples (5 μl) were withdrawn and run on a 3.5% polyacrylamide–0.25× Tris-borate-EDTA gel at 4°C.

RESULTS

Endogenous HLA class I heavy-chain genes are induced synergistically by TNF and IFN in HeLa cells. HeLa cells were tested to determine whether they respond synergistically to TNF and IFN and so would be suitable recipients of mutant class I genes in a study of the promoter elements mediating synergy. Surface expression of HLA class I molecules, as measured by indirect immunofluorescence and flow cytometry, was increased on HeLa cells treated for 24 h with either TNF or IFNs, and a greater than additive increase was obtained following treatment with both TNF and IFN but not following treatment with both IFNs (data not shown). We next examined cytokine effects on HLA class I heavy-chain mRNA levels. HeLa cells were treated with the cytokines TNF, IFN-β, and IFN-γ, individually and in combinations, for 10, 24, or 56 h; they were then harvested, and mRNA levels were determined by S1 nuclease protection. A strikingly greater than additive (i.e., synergistic) induction of HLA heavy-chain mRNA is observed at the earlier time points (10 and 24 h; Fig. 1) in cells treated with TNF in combination with either type of IFN but not in cells treated with both types of IFN. This observation demonstrates that synergistic interactions between TNF and IFNs in regulating MHC gene expression are not restricted to endothelial cells and further suggested that the heavy-chain promoter elements mediating synergy could be defined by transfection studies in HeLa cells.

The κB and ICS elements of the HLA-B7 class I gene promoter are required for synergistic cytokine induction of a transfected reporter gene. A fragment of the HLA-B7 human class I promoter extending from approximately −680 to −20 bp (with +1 being the translation initiation site) was placed in front of a promoterless hGH reporter gene. Additional 5′ and internal promoter deletions were constructed by restriction enzyme digestions. These constructs were transfected into HeLa cells that were then treated with TNF and IFN-γ, and the response was determined by measuring hGH production. The full length −680 bp construct (B7.GH) and the 5′ deletion mutant to a *Tth*111I site at −240 bp (ΔET) both respond synergistically to TNF and IFN-γ (Fig. 2a). In contrast, constructs with 5′ deletions to the *Mlu*I site at −90 bp (ΔEM) or to the *Eco*RV site at −60 bp (ΔEE), as well as an internal deletion of the −240 to −90 region (ΔTM), are unresponsive to either cytokine individually or in combination. Results similar to those obtained with IFN-γ were obtained with IFN-β (data not shown). The responsive ΔET mutant was subject to a further deletion of a region that includes the previously defined enhancer B or region I element (31) (ΔETΔME) but does not interrupt a recently defined tissue-specific enhancer called the α site (12). This mutant retained a synergistic response although expression was substantially reduced compared with that of ΔET, suggesting that the enhancer B element is involved with increasing the efficiency of transcription initiation and not with mediating synergy directly. This role for the B element has also been suggested from studies of the murine class I promoter *H-2L*^{d} (14). These observations map the HLA-B7 promoter elements mediating the principal response to TNF or IFNs, and at least one of the elements required for synergy between these cytokines, to the 150-bp region between the *Tth*111I and *Mlu*I restriction endonuclease sites.

This 150-bp region contains two elements, the κB element and the ICS, previously implicated in the cytokine response of MHC genes. To focus on the roles of the κB and ICS

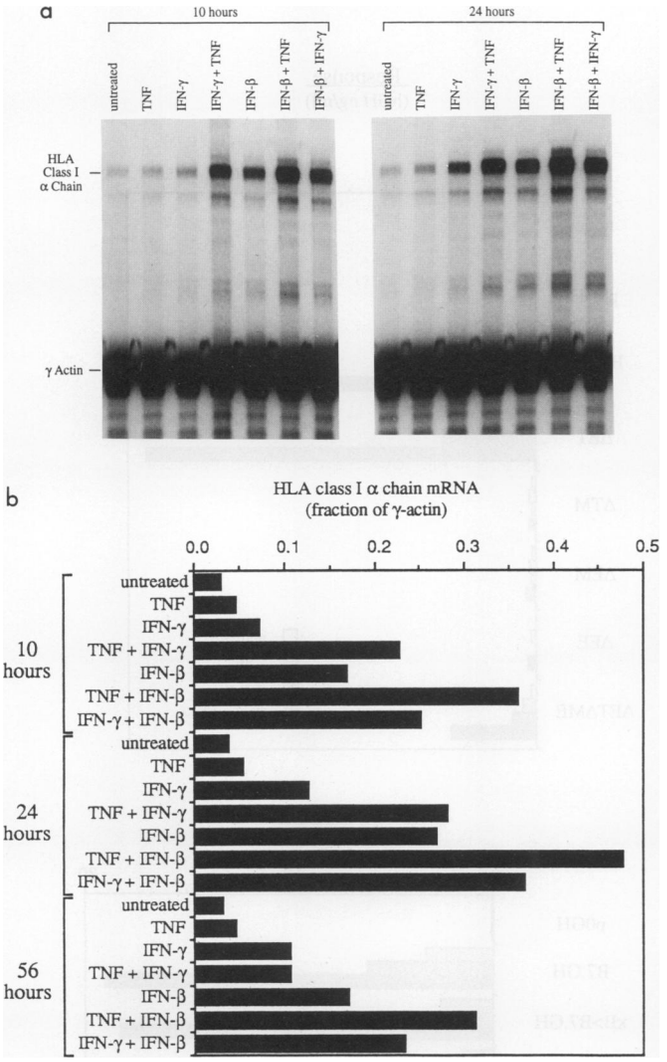

FIG. 1. TNF with either IFN-γ or IFN-β synergistically increases HLA class I heavy-chain mRNA in HeLa cells. (a) S1 nuclease protection assay of cytoplasmic mRNA encoding HLA class I heavy chain and γ-actin from HeLa cells treated with TNF (100 U/ml), IFN-γ (200 U/ml), or IFN-β (250 U/ml) for 10 or 24 h. (b) Quantitation of HLA class I heavy-chain expression. For each column, the HLA heavy-chain mRNA was quantitated by densitometry of the autoradiograph, and the resultant value was normalized by dividing by the value obtained for γ-actin in the same lane.

elements, we constructed a series of PCR-generated deletion mutants that began immediately 5′ of the κB element (κB>B7.GH), 5′ of the ICS (ICS>B7.GH), or immediately 3′ of both elements and created a *Hin*cII site (H>B7.GH). Promoter-reporter gene fragments were separated from the vector backbone by restriction enzyme digestion prior to transfection because transfection of the intact, undigested constructs by either electroporation or calcium phosphate precipitation resulted in cytokine responses that were independent of the κB or ICS element, perhaps resulting from cryptic, cytokine-responsive sequences in the vector. These deletion mutants were transfected into HeLa cells, and the hGH response was measured. The deletion mutant containing the κB element and downstream sequences responds to TNF and to IFN-γ and responds synergistically to combined TNF and IFN-γ (Fig. 2b, κB>B7.GH). The deletion mutant containing the ICS and downstream sequences, but excluding κB, responds to IFN-γ but not to TNF, and it does not respond synergistically to TNF with IFN-γ (ICS>B7.GH). The mutant containing only B7 promoter sequence 3′ of the ICS does not show a significant response to either IFN-γ or TNF (H>B7.GH). These results define the elements responsible for the principal responses to TNF and IFN-γ as the κB and ICS, respectively. A construct containing the κB element but not the ICS (κB.H>B7.GH) responds to TNF but is not induced synergistically with IFN-γ.

The κB and ICS elements are separated by approximately 10 bp, or about one turn of the DNA helix, suggesting that proteins bound to these elements could be positioned to interact with each other or with a third factor. To test the dependence of synergy upon element spacing and orientation, synthetic oligonucleotides matching the κB-ICS sequence and mutants with deletions of 5 or 10 bp between the κB and ICS elements were ligated into the *Hin*cII site of the unresponsive H>B7.GH construct in both the forward and reverse orientations. Insertion of the κB-ICS sequence in either orientation restored synergistic cytokine responsiveness to this construct (Fig. 2b, κB-ICS.H and ICS-κB.H). Similarly, oligonucleotides with deletions of 5 or 10 bp between the κB and ICS elements retained their ability to confer synergistic cytokine responsiveness, again in either orientation (Fig. 2b, κB-Δ5-ICS.H and κB-Δ10-ICS.H), albeit at somewhat lower levels. Similar results were obtained when constructs containing the entire B7 promoter (to −680) with deletions of 5 or 10 bp between the κB and ICS sequences were tested (data not shown).

To determine whether the ICS also mediated the response to IFN-β, HeLa cells were transfected with the same PCR-generated deletion mutants. The results obtained demonstrate that the ICS element also mediates both the response to IFN-β alone and is necessary for synergy between IFN-β and TNF (Fig. 3).

The κB and ICS elements bind nuclear factors isolated from cytokine-treated HeLa cells. Enhancers increase transcription by binding sequence-specific proteins that interact positively with the transcription initiation complex. The cytokine-responsive enhancer elements defined above, κB and ICS, were next tested in an EMSA for the ability to bind nuclear factors from TNF-, IFN-γ-, or IFN-β-treated HeLa cells.

Nuclear extracts from untreated cells and from cells treated with TNF for different times were incubated with double-stranded, radiolabeled κB oligonucleotide probes. Resultant DNA-protein complexes were resolved on a nondenaturing polyacrylamide gel and detected by PhosphorImager analysis and autoradiography. A weak complex is formed between the κB probe and nuclear factors from untreated HeLa cells (Fig. 4a, lane 2). TNF treatment for 4 h strongly activates κB-binding factors, which form a lower-mobility complex (lane 3). Both binding activities are specifically competed for by unlabeled κB but not by unlabeled ICS (lanes 6 and 7). No IFN-γ-induced κB-binding activity is observed (lane 4), nor does cotreatment with IFN-γ alter the TNF activation of κB-binding complexes (lane 5).

Three different complexes are formed between the ICS probe and nuclear proteins extracted from the nuclei of HeLa cells treated with IFN-γ for 4 h (Fig. 4b, lane 4). The upper and lower complexes are apparently induced de novo, whereas the factors forming the complex of intermediate mobility are present constitutively and are increased by IFN-γ. The binding of the IFN-γ-induced factors to the labeled probe is competitively inhibited by unlabeled ICS

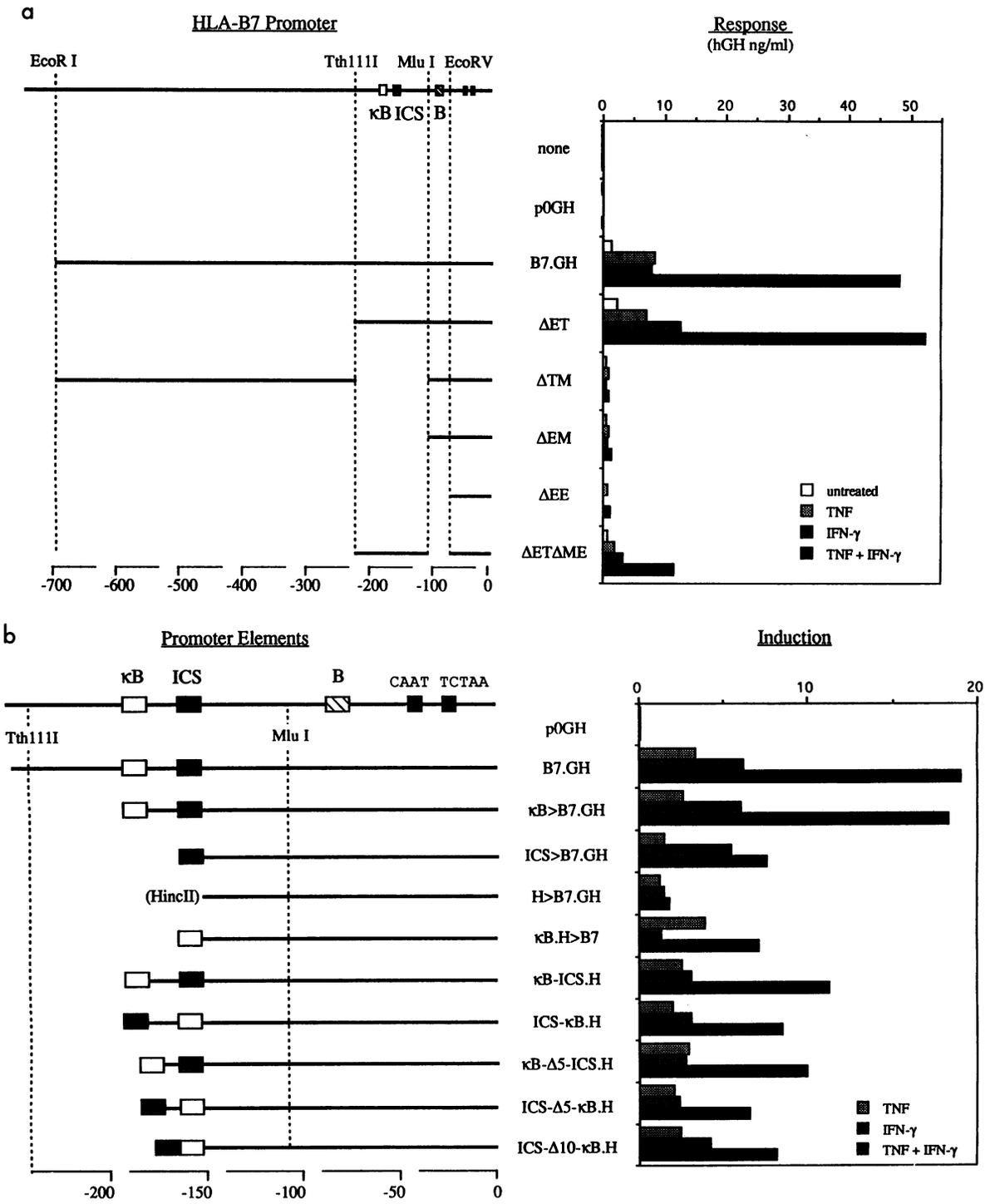

FIG. 2. Responses of transfected HLA-B7 promoter-hGH constructs to TNF and IFN-γ in HeLa cells. (a) The 150-bp *Tth*111I-*Mlu*I region of the HLA-B7 gene promoter is required for TNF and IFN-γ responses, as well as synergy between these cytokines, in transfected HeLa cells. HeLa cells were electroporated with 15 μg of experimental DNA and 5 μg of control luciferase DNA, divided equally into four cultures, and treated with cytokines (TNF, 100 U/ml; IFN-γ, 250 U/ml) 24 h later. The culture medium was assayed for hGH, and cell lysates were assayed for luciferase after an additional 24 h. (b) A single, intact κB element and the ICS are necessary for a synergistic response to TNF and IFN. HeLa cells were transfected with the constructs indicated and then cultured and treated as for panel a. Promoter-reporter gene fragments (experimental constructs) were digested with *Eco*RI and *Hin*dIII before transfection.

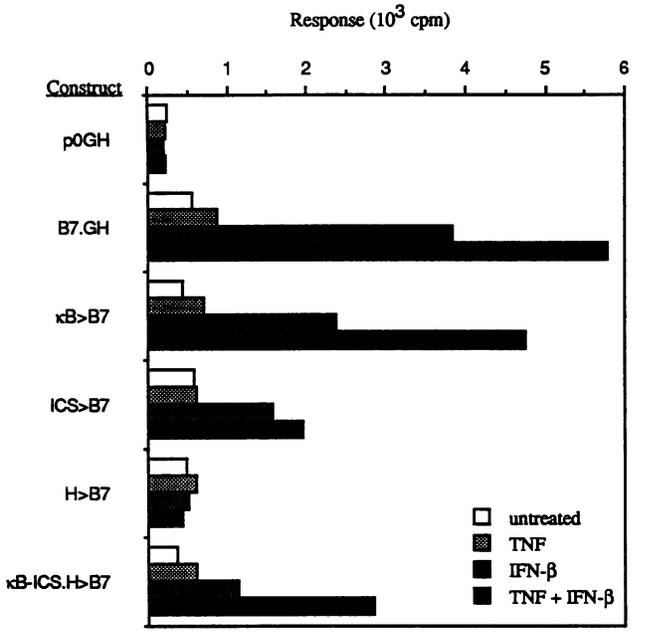

FIG. 3. Responses of HLA-B7 promoter mutants to TNF and IFN-β. HeLa cells were transfected with HLA-B7 promoter-hGH reporter gene constructs as described for Fig. 2.

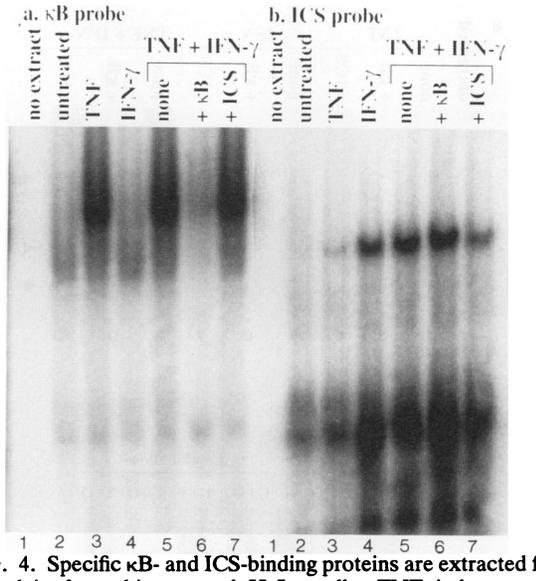

FIG. 4. Specific κB- and ICS-binding proteins are extracted from the nuclei of cytokine-treated HeLa cells. TNF induces a κB-binding nuclear factor, and IFN-γ induces an ICS-binding nuclear factor. HeLa cells were left untreated or treated with TNF (100 U/ml), IFN-γ (250 U/ml), or both TNF and IFN-γ for 4 h, and then nuclear extracts were prepared. Competitor DNA was added at 100-fold excess over labeled (probe) DNA.

but not κB oligonucleotides (Fig. 4b, lanes 7 and 6). Interestingly, TNF also induces the ICS-binding factors, albeit weakly (lane 3). ICS-binding factors from the nuclei of HeLa cells are detected after 1 h of IFN-γ treatment; this binding activity increases until 3 to 4 h and remains strong through 20 h. IFN-β only weakly induces ICS binding factors at 3 and 20 h (data not shown).

A probe containing both the κB and ICS sequences (κB-ICS) was tested in an EMSA with nuclear extracts from untreated cells and cells treated with TNF, IFN-γ, or both TNF and IFN-γ (Fig. 5a). TNF and IFN-γ each induce different κB-ICS-binding factors (lanes 2 and 5, single arrowheads), and combined treatment with both TNF and IFN-γ induces nuclear factors that produce a novel complex of yet lower mobility (lane 8, double arrowhead). This slower complex is disrupted when unlabeled κB competitor is added to the incubation, producing instead a complex that is identical to that formed with nuclear factors from cells treated with IFN-γ alone (lane 9). Unlabeled ICS competitor is less effective in disrupting the slower complex (lane 10) but does cause an increase in the complex that comigrates with the one induced by TNF alone (lane 2). This result suggests that the slower complex is formed by the binding of factors individually induced by TNF and IFN-γ and not by novel factors induced only in cells treated with both TNF and IFN-γ. To test this possibility, nuclear extracts from cells treated individually with TNF or IFN-γ were mixed in vitro. Individually treated cells contain nuclear factors that can combine with the κB-ICS probe in vitro to produce a novel complex of the same reduced mobility as that obtained with factors from cotreated cells (Fig. 5b, lanes 5 and 6). The transfection experiments described above demonstrated that reporter gene constructs with deletions of 5 or 10 bp between the κB and ICS elements still respond synergistically to TNF and IFN-γ. The binding of nuclear proteins to probes containing these deletions was tested in an EMSA. As shown in Fig. 5c, slower complexes are formed between nuclear proteins from TNF- and IFN-cotreated cells with the normal spacing, the Δ5 and Δ10 probes (lanes 5, 10, and 15, respectively), compared with treatments with either cytokine alone.

IFN-γ-induced ISGF2 binds to the ICS, and a TNF-activated p50- and p65-containing NF-κB binds to the κB element. The identities of the nuclear factors binding to the HLA class I κB and ICS elements were determined by supershift antibody EMSA. TNF-activated nuclear factors bound to the κB probe are recognized by antibodies specific for the p50 and p65 subunits of NF-κB (Fig. 6a). The IFN-γ-induced nuclear factor bound to the ICS probe is recognized by antibodies in the ISGF2-specific antiserum but not by the anti-p50 antiserum (Fig. 6b). These results suggest that the IFN-γ-induced nuclear factor is ISGF2 and that the TNF-activated nuclear factors contain the p50 and p65 subunits of NF-κB.

Functional differences are detected in the κB and ICS elements of the HLA-A, -B, and -C promoters. Although the HLA-A and -B and HLA-C promoters are very similar in the region from the κB site through the variant TATA (TCTA) box (Fig. 7a), sequence differences have been noted, especially in the ICS element of HLA-A and the κB element of HLA-C. To test the response of these variant elements, they were substituted into the HLA-B7 promoter-hGH reporter gene construct (κB/C-ICS/B>B7 and κB/B-ICS/A>B7), and the response of the transfectants to TNF and IFN-γ was measured. The construct with the κB element corresponding to HLA-C does not respond to either TNF alone or to TNF in combination with IFN-γ, although it responds normally to IFN-γ (Fig. 7b, κB/C versus κB/B). The construct with the ICS element corresponding to HLA-A responds normally to both TNF and IFN-γ individually, as well as synergistically to these cytokines in combination (Fig. 7b, ICS/A versus ICS/B).

The correlation between the enhancer function of the variant κB and ICS elements and their binding of TNF- and

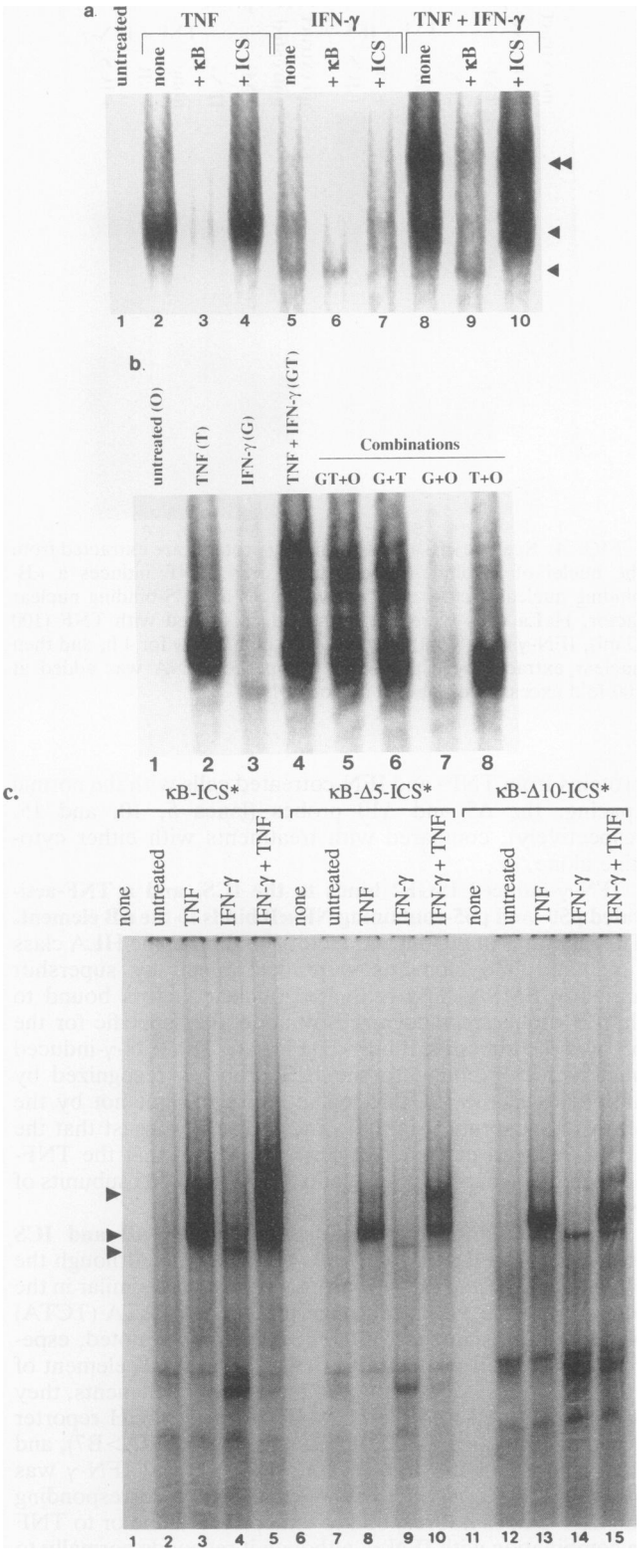

FIG. 5. Combined treatment with TNF and IFN-γ induces factors that produce a novel band of lower mobility with a κB-ICS probe. (a) TNF (100 U/ml), IFN-γ (250 U/ml) or both TNF and IFN-γ were added 4 h before nuclear extracts were prepared. The gel was run until the xylene cyanol marker dye had migrated approximately 15 cm. The double arrowhead marks a novel shifted band, the middle (single) arrowhead marks a TNF-induced nuclear factor complex, and the lower (single) arrowhead marks an IFN-γ-induced complex. (b) Nuclear factors from individually TNF- and IFN-γ-treated cells combine in vitro to produce a novel band of the same reduced mobility as that produced by factors from cell cotreated with TNF and IFN-γ. The same extracts were used here as were used in panel a. (c) TNF- and IFN-γ-induced factors bind to κB and ICS spacing mutant oligonucleotide probes. Probe sequence are listed in Materials and Methods. Treatments and EMSA conditions were as in panels a and b.

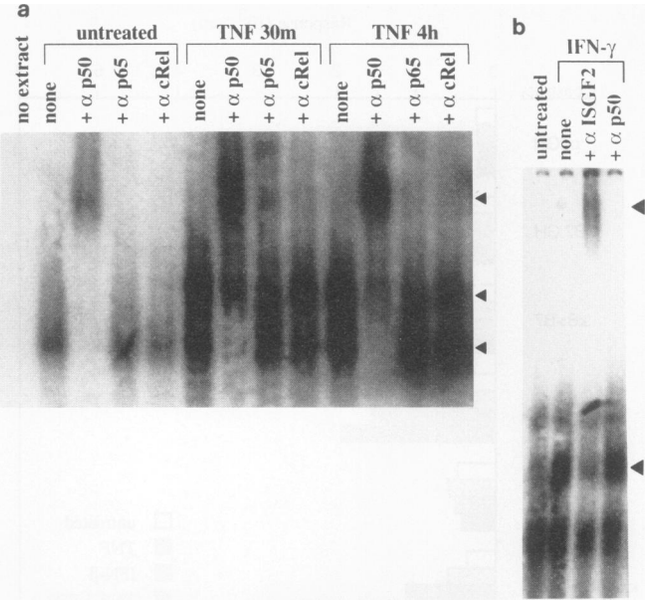

FIG. 6. Immunochemical identification of the TNF- or IFN-γ-induced nuclear factors binding to κB or ICS, respectively. (a) The TNF-induced κB-binding factor contains p50 and p65 subunits of NF-κB. HeLa cells were left untreated or treated with TNF (100 U/ml) for the times indicated, and nuclear extracts were prepared. The antibody EMSA was performed as described in Materials and Methods. (b) The IFN-γ-induced ICS-binding factor is ISGF2. HeLa cells were treated with IFN-γ (500 U/ml) for 4 h, and nuclear extracts were prepared.

IFN-γ-induced nuclear factors was tested in a competitive EMSA. Unlabeled κB/B competes effectively for the binding of TNF-activated nuclear factors to labeled κB/B, whereas unlabeled κB/C or ICS/B does not (Fig. 7c, lane 4 versus lane 5 or 6). Both unlabeled ICS/B and ICS/A compete, but κB/B does not compete, with labeled ICS/B for the binding of IFN-γ-induced nuclear factors, although ICS/B is more effective (Fig. 7d, lanes 4 and 5 versus lane 6). The relative strength of ICS/B versus ICS/A binding is observed at 1:100 and 1:500 probe/competitor ratios and is even more dramatic in EMSA at higher ionic strength (data not shown).

DISCUSSION

The term synergy has been widely used to describe greater than additive interactions between cytokines. The molecular basis of cytokine synergy has not been generally analyzed. Synergy between TNF and IFNs in the induction of HLA class I molecules was originally described in cultured vascular endothelial cells (33). Cytokine-regulated expression of MHC molecules in endothelium is particularly interesting because endothelial cells form the lining of blood vessels, in a position to recruit antigen-specific T cells from the circu-

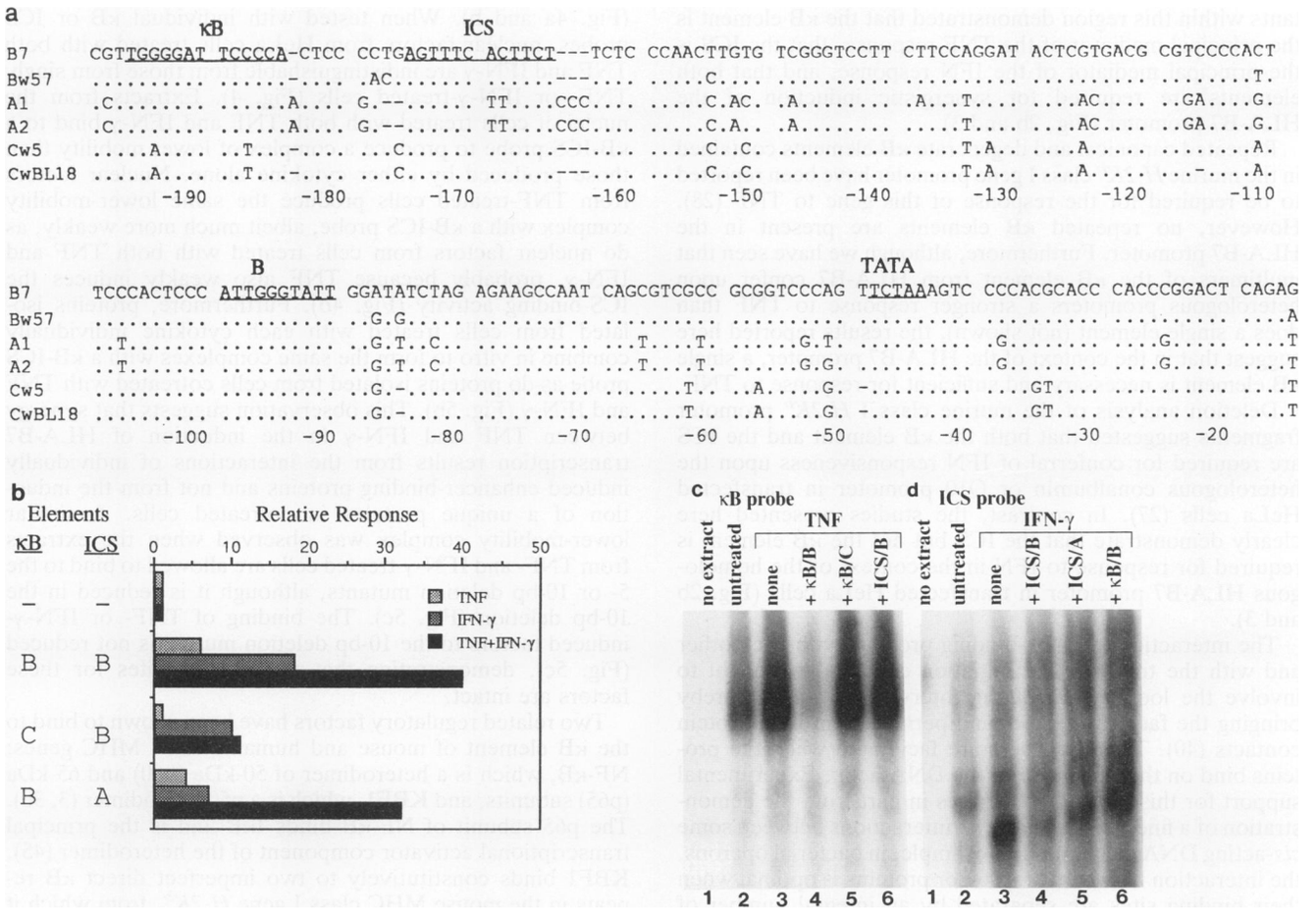

FIG. 7. The ICS element of HLA-A but not the κB element of HLA-C is able to mediate cytokine responses. (a) Sequence comparison of the promoters of HLA-A and -B and HLA-C. The HLA-B7 promoter was sequenced by us; additional sequences are from the literature: Bw57 (26), A1 (21), A2 (32), and Cw5 and CwBL18 (51). Sequences were aligned by inspection and numbered from the first nucleotide before the translation initiation codon of HLA-B7. Dots indicate nucleotide identity; dashes indicate gaps introduced to maximize homology. (b) Response to TNF and IFN-γ of the HLA-B7 promoter substituted with the natural HLA κB and ICS variants. Transfections and treatments were as in Fig. 2 and 3. Relative response was derived by dividing the values obtained from the treated cultures by the value obtained from the untreated culture. The constructs transfected and their untreated hGH responses (nanograms per milliliter) were as follows: H.B7>GH (−/−), 0.17; κB>B7.GH (B/B), 0.10; κB/C-ICS/B>B7 (C/B), 0.09; and κB/B-ICS/A>B7 (B/A), 0.06. The promoterless hGH gave no response over background regardless of treatment. Results shown are representative of three experiments. (c and d) HLA-C κB does not compete for TNF-induced NF-κB binding to HLA-B κB, and HLA-A ICS competes with HLA-B ICS for the binding of ISGF2. Unlabeled competitor oligonucleotides were added at 100-fold excess over the labeled probe, the incubation was continued for 20 min, and samples were separated on a nondenaturing polyacrylamide gel.

lating population into sites of immune reactions in the periphery. Cultured human endothelial cells are only inefficiently transfected, however; therefore, to pursue an investigation of the class I promoter elements mediating a synergistic cytokine response, HeLa cells were tested to see whether the class I genes of this efficiently transfected cell respond synergistically to TNF and IFNs. Flow cytometric analysis of HeLa cells showed that TNF, IFN-β, and IFN-γ each increase HLA class I surface expression and that a greater than additive induction is observed between TNF and either type of IFN (not shown). Analysis of HLA heavy-chain mRNA expression by S1 nuclease protection revealed a striking synergy between TNF and IFNs, particularly at early times (Fig. 1). For this reason, we chose to use HeLa cells to study the molecular basis of synergy. However, it should be noted that HeLa cells are not perfect models for endothelial cells. For example, although the HLA light chain (β_2-microglobulin) mRNA is also induced in HeLa cells by TNF or IFNs, synergistic induction of light-chain mRNA is not observed (not shown), in contrast to endothelial cells, in which the rate of light-chain gene transcription and mRNA levels are both synergistically increased by combined treatment with TNF and IFN-γ (29).

In this study of the class I promoter elements mediating synergistic induction by TNF and IFNs, HeLa cells were transfected with a reporter gene under control of the HLA-B7 human MHC class I gene promoter. Strong synergy was observed in a construct containing 680 bp of the promoter, and 5′ and internal deletions mapped the elements mediating TNF and IFN responses, as well as synergy between these cytokines, to a 150-bp fragment that contains a κB element (previously termed enhancer A [31]), which conforms to the NF-κB consensus binding sequence, and an ICS (Fig. 2a). Additional, PCR-generated 5′ deletion mu-

tants within this region demonstrated that the κB element is the principal mediator of the TNF response, that the ICS is the principal mediator of the IFN response, and that both elements are required for synergistic induction of the HLA-B7 promoter (Fig. 2b and 3).

Repeated canonical and degenerate κB elements contained in the murine *H-2K*^{b} class I gene promoter have been reported to be required for the response of this gene to TNF (28). However, no repeated κB elements are present in the HLA-B7 promoter. Furthermore, although we have seen that multimers of the κB element from HLA-B7 confer upon heterologous promoters a stronger response to TNF than does a single element (not shown), the results reported here suggest that in the context of the HLA-B7 promoter, a single κB element is necessary and sufficient for response to TNF.

Deletion analysis of the murine class I *H-2K*^{b} promoter fragments suggested that both the κB element and the ICS are required for conferral of IFN responsiveness upon the heterologous conalbumin or Q10 promoter in transfected HeLa cells (27). In contrast, the studies presented here clearly demonstrate that the ICS but not the κB element is required for response to IFN in the context of the homologous HLA-B7 promoter in transfected HeLa cells (Fig. 2b and 3).

The interaction of DNA-binding proteins with each other and with the transcription initiation complex is thought to involve the looping out of the intervening DNA, thereby bringing the factors together and permitting protein-protein contacts (40). These contacts are facilitated when the proteins bind on the same side of the DNA helix. Experimental support for this hypothesis derives in part from the demonstration of a fine spacing effect on interactions between some *cis*-acting DNA elements. For example, in bacterial operons, the interaction between λ repressor proteins is optimal when their binding sites are separated by an integral number of ~10-bp units, i.e., multiples of turns around the DNA helix (25). In addition, the normal spacing of several *cis*-acting elements in the avian skeletal α-actin promoter is not optimal for constitutive expression, suggesting that induction occurs by tissue-specific protein-induced torsional deformation that brings these elements into alignment (7). However, exceptions have also been reported. The interaction of two closely spaced, inducible enhancers in the human proenkephalin gene has been carefully investigated (9), and it was found that the effects of partial- or full-turn-of-the-helix deletions were measurable but not dramatic. Similarly, no clearly interpretable spacing effect was shown for interactions between the steroid receptor binding site and a second *cis*-acting element (46). The failure to demonstrate distance or phasing dependence of DNA-protein binding sites has been attributed to protein flexibility. To investigate the phasing dependence of cytokine synergy, the effect of altering the normal, approximately 10-bp spacing between the κB element and the ICS was tested. Essentially equivalent responses were obtained when the spacing between the κB element and the ICS was maintained or reduced by 5 or 10 bp, i.e. a half or a full turn of the DNA helix (Fig. 2b), suggesting that positive interactions between these inducible enhancers and the transcription initiation complex do not strictly depend on their binding to a particular face of the DNA helix.

Protein factors (*trans* acting) that bind *cis*-acting DNA elements regulate transcription by interacting with the transcription initiation complex. Proteins isolated from the nuclei of TNF- or IFN-γ-treated HeLa cells bind specifically to κB- or ICS-containing DNA probes, respectively, in EMSA (Fig. 4a and b). When tested with individual κB or ICS probes, nuclear factors from HeLa cells treated with both TNF and IFN-γ are indistinguishable from those from singly TNF- or IFN-γ-treated cells (Fig. 4). Extracts from the nuclei of cells treated with both TNF and IFN-γ bind to a κB-ICS probe to produce a complex of lower mobility than those produced by either cytokine alone. Nuclear factors from TNF-treated cells produce the same lower-mobility complex with a κB-ICS probe, albeit much more weakly, as do nuclear factors from cells treated with both TNF and IFN-γ, probably because TNF also weakly induces the ICS-binding activity (Fig. 4b). Furthermore, proteins isolated from cells treated with each cytokine individually combine in vitro to form the same complexes with a κB-ICS probe as do proteins isolated from cells cotreated with TNF and IFN-γ (Fig. 5b). This observation suggests that synergy between TNF and IFN-γ in the induction of HLA-B7 transcription results from the interactions of individually induced enhancer-binding proteins and not from the induction of a unique proteins in cotreated cells. A similar lower-mobility complex was observed when the extracts from TNF- and IFN-γ-treated cells are allowed to bind to the 5- or 10-bp deletion mutants, although it is reduced in the 10-bp deletion (Fig. 5c). The binding of TNF- or IFN-γ-induced factors to the 10-bp deletion mutant is not reduced (Fig. 5c), demonstrating that the binding sites for these factors are intact.

Two related regulatory factors have been shown to bind to the κB element of mouse and human class I MHC genes: NF-κB, which is a heterodimer of 50-kDa (p50) and 65-kDa (p65) subunits, and KBF1, which is a p50 homodimer (3, 30). The p65 subunit of NF-κB binds IκB and is the principal transcriptional activator component of the heterodimer (45). KBF1 binds constitutively to two imperfect direct κB repeats in the mouse MHC class I gene *H-2K*^{b}, from which it is displaced by the binding of an NF-κB-like factor (28). In some systems, p50 homodimers (KBF1) have been reported to be transcriptionally inactive and capable of suppressing p65-mediated transcriptional activation (45). However, KBF1 has also been implicated in the constitutive expression of HLA class I genes (3). A resolution of this apparent dilemma is suggested by a recent report demonstrating that transactivation by, but not the binding of, NF-κB is sensitive to seemingly minor sequence variations between the canonical κB and the MHC class I κB elements (38). Similarly, KBF1 may bind to the MHC class I κB sequence in such a way as to promote constitutive transcription. Alternatively, because the antibody used here was raised and purified against the p50 nuclear localization signal peptide that is almost identical to that found in p50B, the κB-binding complexes observed could contain either p50, p50B, or both. p50B is a distinct gene product that can form homodimers, which can associate with Bcl-3 to form a transcriptional activator (4).

Several nuclear factors that mediate IFN-regulated gene expression have been reported. The antibody EMSA gels shown here suggest that the IFN-γ-induced ICS-binding factor is ISGF2. ISGF2 (also known as IRF-1 and IBP-1 [39]) binds to AAGTGA repeats (36) and to the complementary TTCACTT-containing ICS of *H-2K*^{b} (2). IRF-2 binds to the same sequence and acts as a repressor (24), although an inducible, truncated form lacks repressor activity (37). This sequence is found in the ICS element contained on the coding strand of the ICS in HLA-B and -C, and the TTCTTTT-containing sequence of HLA-A also binds to ISGF2, although less strongly (Fig. 7c and d). ISGF2 is

reported to be induced by TNF in human fibroblasts (17) and induced by IFN-γ but not by type I IFN in HeLa cells (2), consistent with the results shown here. The role of ISGF2 in the transcriptional response to type I IFN is controversial (39). A recent test of the role of ISGF2 in IFN-β induction of HLA class I genes was reported in which transfectants with ISGF2 in the sense orientation expressed higher constitutive levels of HLA class I mRNA and were more IFN-β inducible, whereas antisense suppression of ISGF2 expression did not inhibit IFN-β induction of HLA class I mRNA (43). These results speak against a role for ISGF2 in transcriptional activation of HLA class I genes by IFN-β. Indeed, we have seen little, if any, induction of ISGF2 in HeLa cells by IFN-β. HLA class I induction by IFN-γ was not tested in these transfectants.

Type I IFN (IFN-α) rapidly activates the transcription factor ISGF3, which binds to a sequence termed the IFN-stimulated response element, a portion of which is homologous to the ICS. It is possible that IFN-β acts by inducing ISGF3. The ISGF3-ICS complex is reported to be unstable in the presence of higher salt concentrations (41), which may account for our inability to detect this activity in the high-salt extracts used here. The data presented here are most consistent with the conclusion that IFN-γ and IFN-β act by inducing different transcription factors that bind to the same *cis*-regulatory element. However, our results are not fully consistent with a role for ISGF3 as the mediator of IFN-β-induced class I expression. Since it has been proposed that IFN-γ increases one ISGF3 subunit (γ) and type I IFN activates the other (α), it might therefore be expected that type I and type II IFNs would synergize in their induction ISGF-3-regulated genes. As shown in Fig. 1, this combination of IFNs produces at best an additive increase in HLA class I heavy-chain mRNA. Similar lack of IFN interaction was found in endothelial cells (29). These results suggest that either ISGF3 is not the mediator of IFN-β-induced HLA class I gene expression or the model of ISGF3 activation through type I IFN versus type II IFN cannot be generalized to other cell types such as HeLa cells or endothelial cells.

The results presented here demonstrate the importance of the κB and ICS elements in the cytokine-regulated expression of HLA class I genes. These elements differ between HLA-A, -B, and -C (Fig. 7a). The difference in the ICS between HLA-A and HLA-B and -C has been reported to be the basis of the difference in the response of these genes to IFNs (23). However, IFN-γ responsiveness is retained when two nucleotides of the HLA-B7 ICS element are changed to match an HLA-A ICS (Fig. 7b). The fundamental difference between these studies is that the earlier study specifically changes the ICS of the HLA-A promoter to match the ICS of HLA-B, C, whereas the present study changes the ICS of HLA-B7 to match the ICS of HLA-A. Taken together, the results of these nearly reciprocal experiments suggest that additional sequences between the ICS and the transcription initiation site are important in the response of the HLA class I promoters to IFNs, a suggestion that has also arisen in the analysis of transgenic mice (6). The difference in the κB elements between HLA-C and HLA-A and -B has been proposed to underlie the difference in their responses to TNF (51). Indeed, we show here that the κB element of HLA-C (κB/C) is unresponsive to TNF alone or in combination with IFN-γ (Fig. 7b). The failure of the κB/C to mediate a TNF response correlates with the failure of κB/C to compete with κB/B for the binding of TNF-induced nuclear factors (Fig. 7c). In contrast, ICS/A competes with ICS/B, albeit relatively less effectively than ICS/B, for the binding of IFN-γ-induced nuclear factors (Fig. 7c).

In summary, the synergistic interactions between TNF and IFNs as regulators of HLA class I transcription can be fully explained by the independent effects of these two classes of cytokines on the class I promoter. Our spacing experiments have not revealed direct molecular interactions between TNF-induced factors binding to the κB element and IFN-induced factors binding to the ICS element. However, our identification of the relevant *cis* elements and the cytokine-induced proteins that bind to them will allow this question to be more precisely addressed in the future.

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