Identification of Cytokines That Might Enhance the Promoter Activity of HLA-B27

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ABSTRACT ***Objective.*** Although the mechanism by which HLA-B27 induces ankylosing spondylitis is unclear, a minimum threshold of transcription is essential, a process controlled at the promoter. Our aim was to scan the effect of a panel of cytokines on the promoter of the HLA-B27 gene over serial timepoints.
Methods. The promoter region of B*2705 gene was cloned into a luciferase reporter, stably transfected into HeLa cells, and used to monitor the serial effect of 25 cytokines. Results of HLA-B27 promoter-reporter assays were compared to those of real-time polymerase chain reactions.
Results. After an initial delay, significant activation of the HLA-B27 promoter was observed with tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IFN-ß. While early response of the HLA-B27 promoter was highest with TNF-α and IFN-γ, ultimately the highest activity was observed with IFN-ß.
Conclusion. The only promoter of HLA-B alleles studied in the past was that of HLA-B7, and always at only a single fixed timepoint of culture. This is the first study to show that activation of HLA-B27 promoter is a sequential event, and that TNF-α and IFN-ß are major participants at different timepoints.
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Key Indexing Terms:
HLA-B27 PROMOTER TUMOR NECROSIS FACTOR-α
INTERFERONS ANKYLOSING SPONDYLITIS

HLA-B27, a gene associated with ankylosing spondylitis (AS), is an allele of the HLA-B locus of the class I major histocompatibility complex (MHC). The protein is a complex between the $ß_2$-microglobulin ($ß_2$m) monomorphic light chain and the HLA-B27-specific heavy chain. How HLA-B27 contributes to arthritis is unclear. The 3 current hypotheses are that HLA-B27 causes arthritis by presenting "arthritogenic peptides," or by engaging natural killer receptors, or by inducing "endoplasmic reticulum unfolded protein response" (ER UPR)[1]. These hypotheses are based on the activities of the HLA-B27 protein, and hence would require the HLA-B27 gene to be transcribed.

The first stage of transcription is largely controlled at the promoter region. This has never been studied in HLA-B27. In homologous HLA-A2 and HLA-B7 genes, the enhancers have been mapped into 2 major domains, containing, respectively, a nuclear factor-κB (NF-κB) and an interferon-simulated response element (ISRE) consensus motif (Figure 1)[2]. These 2 domains bind to transcription factors activated by cytokines. The only cytokines that have been studied for HLA-A2 and HLA-B7 are tumor necrosis factor-α (TNF-α) and the interferons (IFN)[3-6].

To study the HLA-B27 promoter, we cloned the 5' untranslated region (UTR) of B*2705, and inserted it into a vector upstream of a luciferase reporter gene. A stable transfectant was generated in the HeLa, a cell line used in all previous promoter-reporter studies on HLA-A and HLA-B alleles. In our experiments, we tested a total of 25 different cytokines.

The purpose of these experiments was not to find out how HLA-B27 contributes to arthritis, but simply to identify the factors that might be responsible for its transcription. This is important because several observations support the hypothesis that factors that modulate HLA-B27 transcription play a role in the pathogenesis of AS. First, in rats that carry the HLA-B27 transgene, arthritis would develop only if the expression of HLA-B27 exceeds a critical threshold[7]. Second, in the same rat model, the expression of HLA-B27 in the bone marrow-derived macrophages would be upregulated when the cells were cultured with certain cytokines[8]. Third, the surface expression of HLA-B27 is greater in patients with AS compared to HLA-B27-positive subjects without arthritis[9].

MATERIALS AND METHODS

Cell lines. HeLa, HeLa-pNF-kB-luc cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and Panomics (Fremont, CA,

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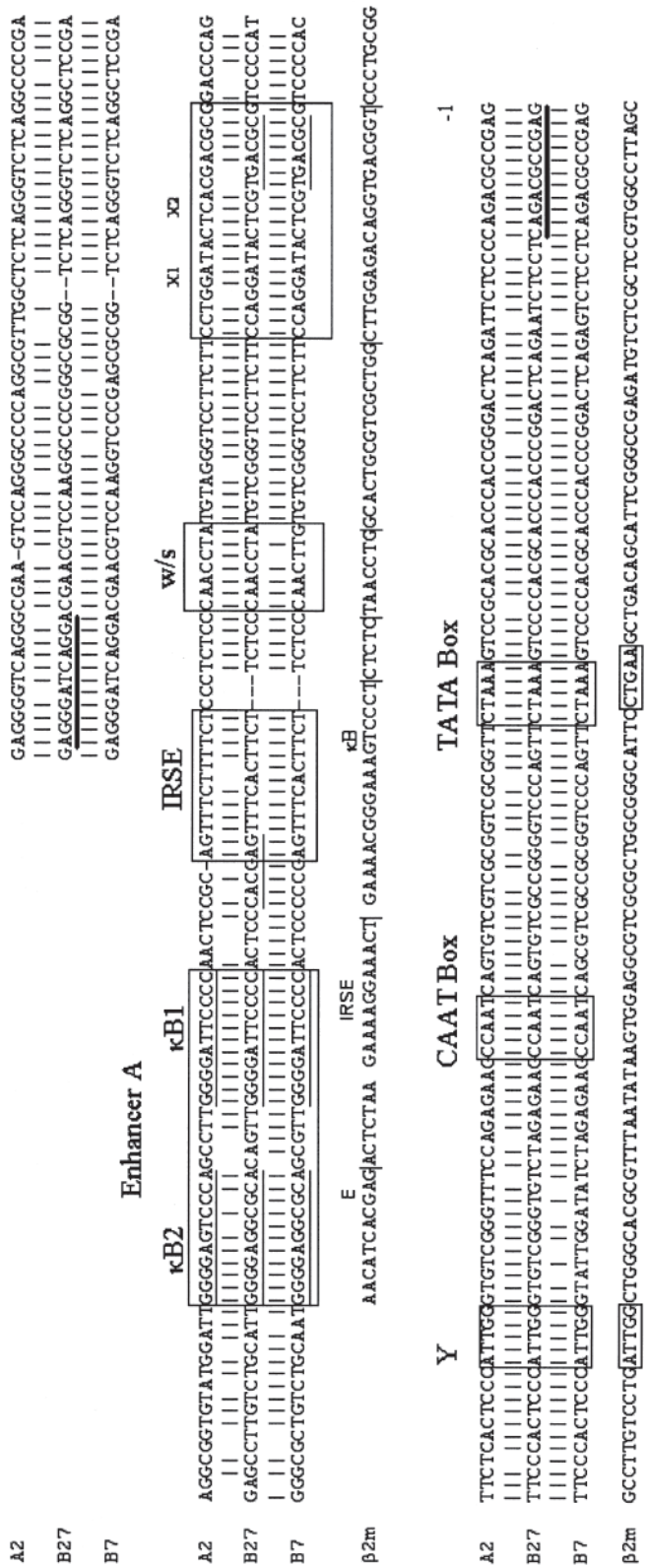

Figure 1. Alignment of the 5'UTR of HLA-A2, HLA-B27, HLA-B7, and $ß_2$m. Enhancer A contains κB2 and κB1 sites. The lightly underlined CACGAG 5' of ISRE is the E box, the lightly underlined TGACGC in the X2 box is site α. Heavily underlined sequences correspond to PCR primers for cloning the B27 5'UTR. Labels for boxes specific for HLA class I follow those published by van den Elsen, *et al*[2]. Definition of 5'UTR follows that of the IMGT/HLA database, with the first nucleotide 5' of the ATG start codon being –1.

USA), respectively[10]. The CCL6 cells (Henle-407, HLA-A68, and HLA-B72) and CCL6 cells stably transfected with the genomic DNA of B*2705 (CCL-6-B27 cells) have been described previously[11]. CCL6 is allegedly an immortalized human intestinal epithelial cell line. The cultures of these cells have been described[12].

Reagents. Escherichia coli-competent cells, T4 DNA ligase, Taq DNA polymerase, restriction enzymes were purchased from Invitrogen; pGL4.14-luc2 and pGL3 control vector from Promega; Qiaquick Gel Extraction Kit, Plasmid mini kit, and Plasmid Maxi kit from Qiagen (Valencia, CA, USA); Britelite Reporter Gene Assay system from Perkin Elmer (Shelton, CT, USA); Fugene 6 from Roche (Indianapolis, IN, USA); and pISRE-luc, a 5 repeat of the ISRE of ISG54, from Stratagene (La Jolla, CA, USA). Plasmid pE.1-B27 containing the genomic clone of B*2705 was a gift from J. Taurog (University of Texas Southwestern Medical Center, Dallas, Texas)[13].

Recombinant human interleukin 1α (IL-1α), IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-15, IL-17, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-α, and IFN-γ were purchased from eBioscience (San Diego, CA, USA); recombinant human IL-12-p70, IP-10, MCP-1, RANTES, MIP-1α, IFN-ß, and transforming growth factor-ß (TGF-ß) from Cell Science (Canton, MA, USA); recombinant human IL-18 and IL-22 from R&D Systems (Minneapolis, MN, USA); and a panel of recombinant type I interferons from PBL (Piscataway, NJ, USA).

Construction of promoter-reporter. pGL4-B27 contained a 432 bp portion of the HLA-B27 5'UTR, which was amplified from pE.1-B27 by polymerase chain reaction (PCR) primers 5'-GGC GAG CTC AGG ATT CAG AA-3' and 5'-CGA AGC TTC TCG GCG TCT G-3', and inserted into pGL4.14-luc2 (Figure 1). Sequencing validated the identity with that in GenBank accession M12967. A mutant of pGL4-B27 was purchased from Bio S&T (Lachine, Quebec, Canada) in which the κB1 region of HLA-B27 was replaced by that of HLA-E (5'-GAG GAT TCT C-3'). This plasmid was named pGL4-mB27.

Generation of transfectants. For transient transfection, 5000 cells were plated into each well of a 96-well flat-bottom plate (Matrix Technology, Hudson, NH, USA), cultured 8 to 12 hours, and then transfected with plasmids using Fugene 6 (Roche, Palo Alto, CA, USA). Cells were then cultured another 12 hours before adding other reagents. To normalize variations in transfection efficiency, all transient transfection also included a ß-galactosidase plasmid (Invitrogen).

To generate the stable transfectants HeLa-pGL4-B27, aliquots of 3×10^5 cells were plated into 60-mm diameter plates and transfected with plasmids using Fugene 6. Cells were subcultured 24 hours later into medium containing 100 µg/ml hygromycin B. After 2 weeks, hygromycin-resistant colonies were separately isolated and expanded. The one clone most responsive to TNF-α plus IFN-γ was used in all subsequent studies. This clone is referred to here as the B27 promoter-reporter HeLa cells.

Luciferase, viability, and NF-κB colorimetric assays. All studies were carried out in triplicates. To assay the amount of luciferase activity in stable transfectants, cells were lysed with Britelite substrate (Perkin Elmer) and luminescence measured with a microplate luminometer (Berthold, Oak Ridge, TN, USA). Cell viability was assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). In all the experiments viability was near 90% and did not vary with the reagents added. When assaying for luciferase activity in transient transfection, to allow for compatibility with the ß-galactosidase enzyme assay system, we used the Luciferase Assay System (Promega). ß-galactosidase activity was measured colorimetrically (Promega). The luciferase results were normalized according to the ß-galactosidase readings. Results of luciferase assays were expressed as -fold changes calculated by reference to samples cultured with no activation reagents.

To assay for relative amounts of 5 different NF-κB components in cell nuclei, nuclear proteins were extracted using a nuclear extract kit (Active Motif, Carlsbad, CA, USA). Ten micrograms of nuclear protein were added to each well of a TransAm NFB family kit, and tested following the instructions from Active Motif.

Measurement of HLA-B27 transcript by real-time PCR. CCL6-B27 cells were cultured with the concentration of TNF-α and IFN-ß that was tested to be optimal for the HeLa cells. Total RNA was isolated using Trizol reagent, contaminating DNA was digested with DNase I (Invitrogen), and transcripts were reverse-transcribed using the Omniscript RT kit (Qiagen). Primers for HLA-B27 were as follows: forward primer 5'-GTC CAC CGT CCC CAT CG-3'; reverse primer 5'-ACG CAG CCT GAG AGT AGC-3'. Real-time PCR with Sybr Green was assayed in a 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Samples were done in triplicates and normalized to the corresponding GAPDH values.

Statistical analysis. Assessments of statistical results were done by paired t or Student t tests. Error bars in graphs represent standard deviations.

RESULTS

Alignment of 5'UTR of HLA-B27 with HLA-B7, HLA-A2, and $ß_2m$. We retrieved the 5'UTR sequences of all 54 HLA-B alleles available in the IMGT/HLA Database (http://www.ebi.ac.uk/imgt/hla), and compared them with HLA B*2705. Only 6 alleles carry 5'UTR sequences identical with B*2705; these are B*2706, B*2732, B*180101, B*1803, B*1817N, and B*370101.

Next, we aligned the first 300 bp in the 5'UTR of HLA-B27 with HLA-B7 (Figure 1). The proximal promoter regions of these 2 genes contain 3 major regulatory elements: an "enhancer A" comprising NF-κB response elements κB1 and κB2, an IFN-stimulated response element (ISRE), and the S-X-Y modules. Within these particular elements, HLA-B27 differs from HLA-B7 in one nucleotide each in the "enhancer A" subregion and in the S module. There are differences in 8 nucleotides outside these elements. Differences between HLA-B27 and HLA-A2 and $ß_2$m are more numerous (Figure 1).

Effect of cytokines on HLA-B27 promoter-reporter. The B27 promoter-reporter HeLa HeLa-pGL4-B27 cells were cultured up to 96 hours with 25 different cytokines: IL-1α, IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-22, TNF-α, GM-CSF, IFN-α, IFN-ß, IFN-γ, IP-10, MCP-1, RANTES, MIP-1α, and TGF-ß. Significant induction of the HLA-B27 promoter was observed at 48 hours with TNF-α and the 3 interferons, the highest being with TNF-α and IFN-γ ($p = 0.002$ and $p = 0.007$ comparing TNF-α to IFN-α and IFN-ß, respectively). Beginning at 72 hours, there was a surprising switch of hierarchy from TNF-α to IFN-ß. By 96 hours, the effect of IFN-ß was very significantly higher than TNF-α, IFN-α, and IFN-γ ($p = 0.008$, $p = 0.007$, $p = 0.007$, respectively), the effect of IFN-ß being actually twice that of TNF-α. No effect was observed throughout this period of culture with the other cytokines in the 25-cytokine panel. Effect of all 25 cytokines on cells transfected with control luciferase vector was negligible. The positive results are shown as average of 3 separate experiments in Figure 2 (upper panel, arrow indicates results with TNF-α). Results of IFN-α are shown in Figure 2 (lower panel) and Figure 3. In additional experiments, instead of using the same stable transfectant, HeLa cells were transiently transfected with the pGL4-B27, cultured separately with TNF-α, IFN-α, IFN-ß, IFN-γ, and assayed separately at 24, 48, and 72 hours. The results confirmed that the strongest inducer at 72 hours was IFN-ß (results not shown).

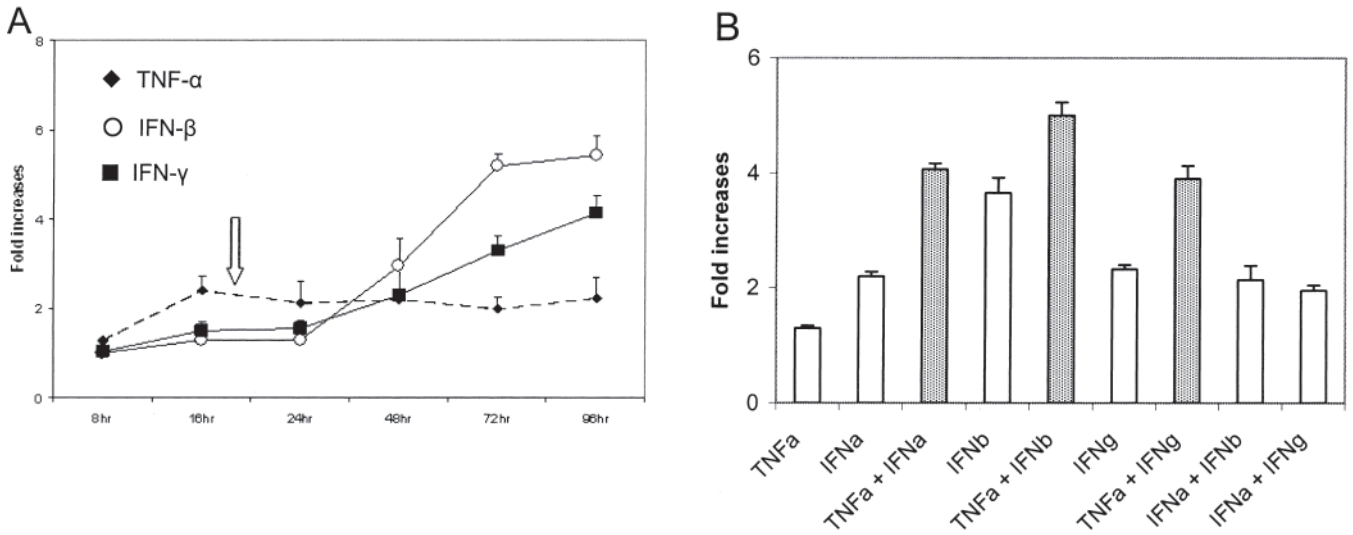

Figure 2. Fold increases in luciferase activity induced by culture of the B27 promoter-reporter HeLa Hela-pGL4-B27 cells with various cytokines. In Figures 2–4, fold changes were calculated by reference to samples cultured with no cytokines. A. Arrow indicates result with TNF-α. At 48 hours, compared to control samples, Bonferroni-corrected p values for TNF-α, IFN-α, IFN-ß, and IFN-γ were 0.05, 0.02, 0.0006, and 0.02, respectively. B. 48-hour culture with various combinations of cytokines.

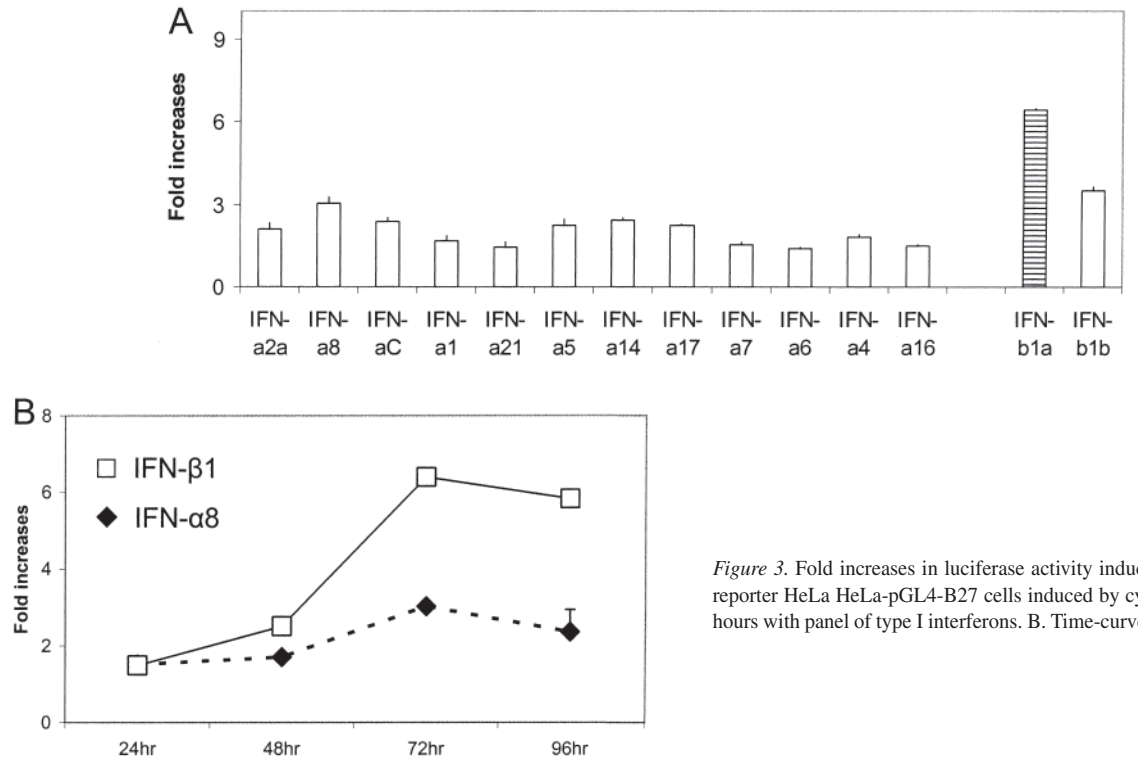

Figure 3. Fold increases in luciferase activity induced in the B27 promoter-reporter HeLa HeLa-pGL4-B27 cells induced by cytokines. A. Results at 72 hours with panel of type I interferons. B. Time-curve for IFN-ß1 and IFN-α8.

Dose-response experiments were evaluated for 10 of the cytokines: TNF-α, IFN-α, IFN-ß, IFN-γ, IL-1α, IL-1ß, IL-4, IL-12, IL-18, and TGF-ß1. Experiments as described above were carried out with the optimum concentrations: 20 ng/ml for TNF-α and 1000 units/ml for all interferons. For the other cytokines, concentrations used were those recommended by the manufacturers.

Next, we tested whether there would be more luciferase activities if either TNF-α or the 3 interferons were each cultured in combination with each of the cytokines in the 25-cytokine panel. Results with the B27 promoter-reporter HeLa HeLa-pGL4-B27 cell line indicated no enhancing or suppressive effect by adding to TNF-α or IFN-α or IFN-ß or IFN-κ in combination with any of the following: IL-1α, IL-1ß, IL-2,

IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12-p70, IL-13, IL-15, IL-17, IL-18, M-CSF, GM-CSF, IP-10, MCP-1, RANTES, MIP-1α, or TGF-ß1 (data not shown). However, when TNF-α was combined with IFN-α or IFN-ß or IFN-κ, the effects were additive ($p < 0.0001$ comparing combination to individuals; Figure 2 lower panel is representative of 2 experiments). In contrast, there was no additive effect when the IFN subtypes were combined with each other ($p = 0.1$, results not shown).

Testing 12 subtypes of IFN-α. Both IFN-α and IFN-ß are type I interferons. IFN-α has multiple subtypes; the one tested in the previous experiments was IFN-α1. Next, we compared IFN-ß with the following subtypes of IFN-α: IFN-α2a, IFN-α8, IFN-αC, IFN-α1, IFN-α21, IFN-α5, IFN-α14, IFN-α17, IFN-α7, IFN-α6, IFN-α4, and IFN-α16. We also included IFN-ß1b, a laboratory-created mutant of the natural IFN-ß1a (representations of 2 experiments shown in Figure 3). The effect of IFN-ß1a exceeded the effect of the highest IFN-α subtype at 48, 72, and 96 hours of culture ($p = 0.004$, $p = 0.0004$, $p = 0.0004$, respectively). This hierarchy was also observed with the therapeutic reagent IFN-ß1b.

Testing the NF-κB and the ISRE domains separately. For HLA-A2 and HLA-B7, the κB1 and the ISRE domains of the promoter are separately responsible for binding to TNF-α and the type I interferon-induced transcription factors. To verify this in HLA-B27, we tested an ISRE mutant of the HLA-B27 promoter-reporter. HeLa cells transfected with this mutant indeed responded to TNF-α, but not to the 3 interferons (results not shown).

Next, we tested if the delay in response to IFN-ß was in general peculiar to the ISRE element. HeLa cells were transfected with either a NF-κB consensus reporter or an ISRE consensus reporter, and cultured separately in our panel of 25 cytokines. The NF-κB reporter responded only to TNF-α but not to interferons (Figure 4, upper panel), and vice versa for the ISRE reporter (Figure 4, lower panel; arrow indicates results with TNF-α). The difference from the HLA-B27 promoter-reporter was that both reached a peak of response at 4–8 hours, subsiding to baseline by 24 hours. The other unexpected finding was that the NF-κB promoter-reporter responded very strongly to both IL-1α and IL-1ß, although both cytokines did not affect the B27 promoter-reporter.

To investigate why IL-1α and IL-1ß were strong activators of the HeLa-pNF-κB–luc but weak activators of HeLa-pGL4-B27, we cultured HeLa cells for 6 hours with TNF-α, IL-1α, and IL-1ß, extracted their nuclei, and compared the amounts of 5 different components of the NF-κB complex (average of triplicate samples shown in Figure 4, lowest panel). Much higher amounts of the 2 major NF-κB components p50 and p65 were observed with TNF-α compared to IL-1α and IL-1ß. Apparently the NF-κB luciferase reporter was not sensitive enough to discriminate the differences between TNF-α and IL-1.

Validating the hierarchy of TNF-α and IFN-ß and the time sequence of response using real-time PCR in a completely different cell line. CCL6-B27 cells were cultured for 24, 48, and 96 hours with TNF-α and IFN-ß, and the relative amounts of HLA-B27 transcripts were measured serially by real-time PCR. Both TNF-α and IFN-ß induced increases in HLA-B27 transcripts at 24 hours of culture ($p = 0.02$, $p = 0.003$, respectively). Parallel to the promoter-reporter experiments, the most effective cytokine early in culture was TNF-α, but by

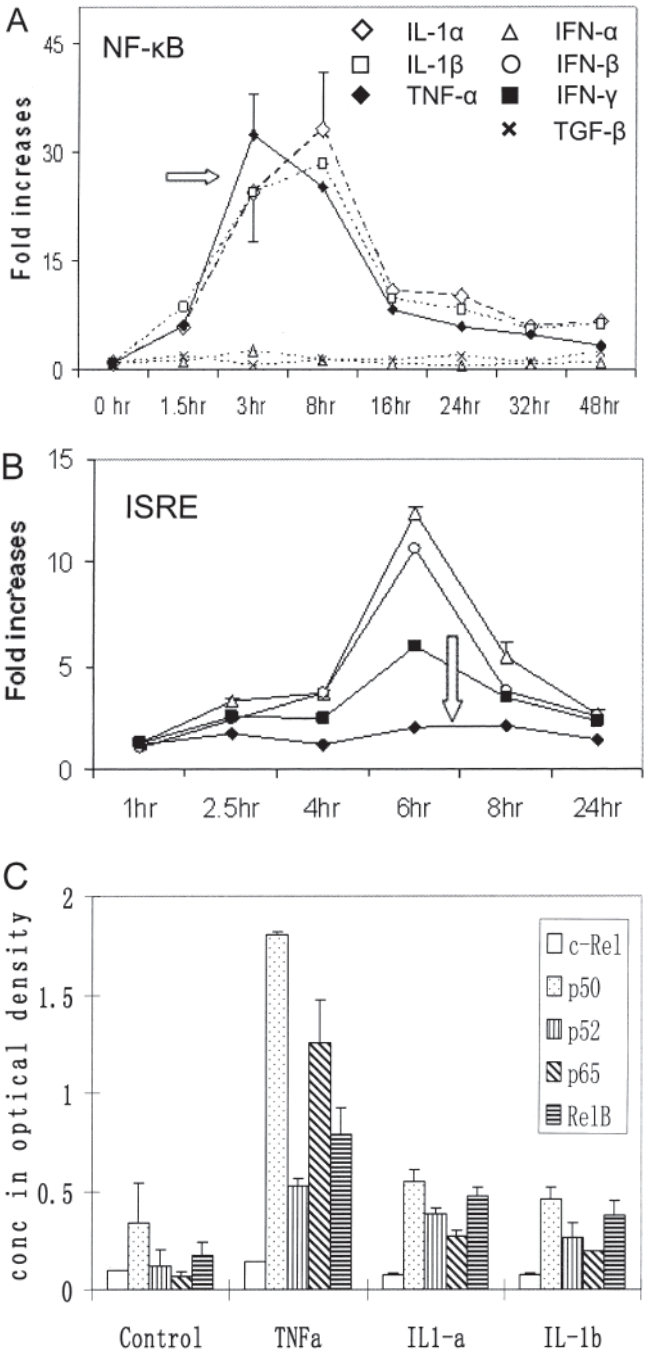

Figure 4. Fold increases in luciferase activity induced by culture of Hela cells with various cytokines. Promoters for luciferase gene are (A) NF-κB consensus and (B) ISRE. Arrow indicates result for TNF–α. C. Relative amount of NF-κB components in HeLa cells activated by 3 different cytokines.

48–96 hours, the effect of IFN-ß was much higher (p = 0.01 for IFN-ß at 96 hours). TNF-α showed no enhancement at 48 and 96 hours of culture (Figure 5; arrows indicate results with TNF-α). No transcript was observed with the parent CCL6 cells, validating the specificity for HLA-B27 for this pair of CCL6 cell lines.

DISCUSSION

Transcription of HLA class I genes is largely controlled by elements in the 300 bp of the 5'UTR. Since the sequence of the proximal promoter of HLA-B27 is identical with only 4 other HLA alleles, there is no guarantee that control of the HLA-B27 promoter activity is identical to that of HLA-B7, the only HLA-B allele promoter studied to date[2]. To our knowledge, our study is the first to use a promoter-reporter to investigate the HLA-B27 gene, to test a large panel of cytokines, and, importantly, to construct a time-curve of response to individual cytokines. Because the 5'UTR sequences of the arthritis-causing B*2705 and reportedly non-arthritis-causing B*2706 are identical, experiments were carried out only with a promoter-reporter cloned from the B*2705 gene. In agreement with reports concerning the promoters of HLA-A2 and HLA-B7, we discovered that for HLA-B27, the active cytokines are also TNF-α and IFN-γ[3-6].

The major finding of our study is the identification of IFN-ß as a late but strong activator of HLA-B27, especially because it has not been studied before with HLA-A2 and HLA-B7. Until recently, most studies of interferons in the pathogenesis of spondyloarthropathies (SpA) have focused on the role of IFN-γ. Because of a bias against the Th1 response, the expression of IFN-γ is actually lower in SpA than Th2 cytokines[14]. IFN-ß, on the other hand, is a member of the type I interferons, and is independent of Th1 or Th2 lymphocytes. That IFN-ß is a key participant in SpA is in agreement with observations from a study[8] of the possibility that SpA in rats is caused by HLA-B27-induced endoplasmic reticulum unfolded protein response (ER UPR). The study showed that in HLA-B27-transgenic rats, the cytokine that by itself induced the highest expression of HLA-B27 and ER UPR was IFN-ß[8].

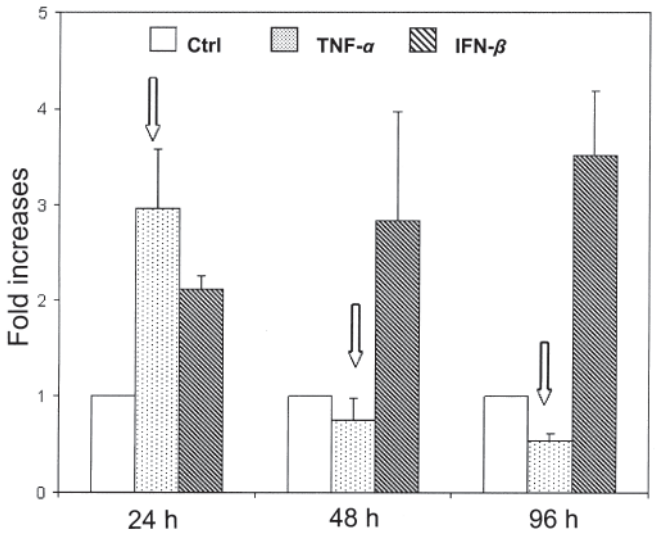

Figure 5. Fold increases in real-time PCR values of HLA-B27 in CCL6-B27 cells after 24, 48, and 96 hour cultures with no cytokines (Ctrl), TNF-α, and IFN-ß. Fold increases are with reference to samples cultured for the same period of time without cytokines. Arrows indicate TNF-α results. Greatest increases were observed with IFN-ß at 48 and 96 hours.

In our promoter-reporter experiments, the enhancement induced by TNF-α and interferons was only 2 to 5-fold that of control. Because of lack of an anti-HLA-B27-specific heavy-chain antibody, the changes in HLA-B27 protein could not be measured. Most studies to date on induction of HLA-B27 heavy chains by cytokines have been carried out with immortalized cell lines or cells derived from transgenic animals. To our knowledge, the degree of increase of HLA-B27 protein induced by cytokines in primary cells derived from patients with AS has been reported only once. Using flow cytometry, it was demonstrated that the surface expression of HLA-B27 was increased to 2 to 5-fold by IFN-α and IFN-γ[15]. This range is very similar to that observed in our study.

There are several limitations to extending our observations as general principles of HLA-B27 regulation. First, in our experiments we used only the HeLa cells for promoter-reporter experiments, although to a certain extent the positive cytokines were verified by the CCL6-B27 cell line. It has been shown in HLA-B27-transgenic rats that upregulation of HLA-B27 by IFN-γ is much greater in bone marrow-derived macrophages compared to splenocytes[8]. Thus, we can conclude from our HeLa cell experiments that the HLA-B27 promoter is modulated by TNF-α and the interferons, but we cannot conclude that the other cytokines, which are inactive with HeLa cells, might not be active with other cell types. The second limitation in interpretation is that in our experiments we used a promoter-reporter, so that we could actually test the effect of mutating certain domains; but because of that, we cannot confirm that the conclusions can be extended to endogenous HLA-B27. Finally, by use of the 342 bp of the 5'UTR region alone, these experiments do not address possible control of HLA-B27 transcription by elements beyond that region or in the introns or 3' regions.

The complexity of HLA-B27 promoter activity is illustrated by 2 findings we observed with reporters for NF-κB binding and the ISRE sequences. First, these reporters responded rapidly to cytokines, whereas there was a comparatively long delay with the HLA-B27 promoter-reporter containing these sequences. Second, the NF-κB binding-promoter responded strongly to IL-1α and IL-1ß, whereas there was no response with the HLA-B27 promoter-reporter. This might be partly explained by our finding that IL-1 did not induce as much NF-κB inside the nuclei compared to TNF-α. In any case, these findings illustrate that simply having certain transcription binding sequences does not guarantee a promoter to equivalent responses to the corresponding transcription factor acti-

vators. Access to transcription factors also depends on the overall sequences of the promoters, and on the chromatin structure. The chromatin structure, on the other hand, is dependent upon epigenetic controls, which would vary with cells and their environments. The experimental system described here will allow investigators to explore these complex issues with the HLA-B27.

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