MOLECULAR AND CELLULAR BIOLOGY, Aug. 1992, p. 3439–3448
0270-7306/92/083439-10$02.00/0

Vol. 12, No. 8

The Functional Importance of a Cap Site-Proximal Region of the Human Prointerleukin 1β Gene Is Defined by Viral Protein *trans*-Activation

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Received 9 March 1992/Accepted 14 May 1992

Prointerleukin 1β (IL-1β) is a cytokine that mediates a broad range of biological activities. Genomic sequences that regulate IL-1β transcription include both inducible regulatory elements located more than 2,700 bp upstream of the transcriptional start site (cap site) and proximal elements located near the TATA box of this gene. In this study, we focused on the identification and characterization of *trans*-acting nuclear regulatory proteins that bind to the cap site-proximal region of the human IL-1β gene. We identified a protein, termed NFIL-1βA (NFβA), that binds to a highly conserved 12-bp DNA sequence (−49 to −38) located upstream of the TATA box motif in both the human and murine IL-1β genes. The IL-1α gene, which lacks a TATA motif, does not possess an NFβA-binding sequence within the promoter region, suggesting that NFβA may selectively regulate IL-1β expression. Using electrophoretic mobility shift assays, we identified several distinct DNA-protein complexes that are expressed in a cell-type-specific manner. In monocytic cell lines, the relative abundance of these complexes varies rapidly following stimulation of the cells with phorbol esters or lipopolysaccharide. UV cross-linking analysis identified two distinct DNA-binding polypeptides that comprise distinct complexes. The functional role of NFβA was assessed in transient transfection assays. These data indicate that NFβA is required for both basal and inducible promoter activity in monocytic cells. Furthermore, the human cytomegalovirus immediate-early 1 gene product requires the presence of NFβA in order to *trans*-activate the proximal IL-1β promoter in a monocytic cell line. We propose that NFβA is a factor that mediates either direct or indirect activation by the immediate-early 1 gene product. The proximity of this essential factor to the TATA motif suggests a possible role in transcriptional initiation.

Cytokines are now recognized as major regulators of inflammation and immune system homeostasis. Prointerleukin 1 (IL-1) is a cytokine that mediates a wide variety of inflammatory, metabolic, and hematopoietic processes. Two distinct IL-1 polypeptides, termed IL-1α and IL-1β, have been identified and are the products of distinct genes (4, 34, 37). Both proteins can bind to the two known classes of high-affinity IL-1 receptors (11, 46). IL-1 is one of a group of proinflammatory cytokines with overlapping biological activities. This group also includes tumor necrosis factor α and interleukin 6 (IL-6). In combination, these cytokines can act synergistically on target cells (reviewed in reference 3). While the biological and clinical properties of IL-1 have been well characterized (reviewed in reference 18), the regulation of IL-1 production at the transcriptional and posttranscriptional levels (reviewed in reference 20) remains poorly understood.

The entire genomic sequences for human IL-1α (21), human IL-1β (14), and murine IL-1β (53) have been previously reported. Despite significant exon sequence divergence, the human IL-1α and IL-1β genes share a highly conserved overall intron-exon structure (14). Regions within the first intron of the human IL-1β gene appear to possess both positive (7) and negative (15) regulatory activities in transient transfection assays. Recently, the first intron of the human IL-1α gene was reported to contain sequences that mediate activation of this promoter by phorbol 12-myristate-13-acetate (PMA) (40). Moreover, Bensi et al. identified an enhancer element within the IL-1β gene that mediates induction of transcription by PMA (7). This enhancer is located between positions −2982 and −2795 upstream of the transcriptional start site. Sequence analysis of this enhancer region revealed the presence of DNA motifs similar to the AP-1 binding site of the collagenase gene (2) and the positive regulatory domain I binding site in the human beta interferon gene (22). Although these two DNA motifs may play a role in enhancer function, deletion of additional DNA upstream of these sequences dramatically reduces enhancer activity. The sequences required for induction of the human IL-1β gene by lipopolysaccharide (LPS) appear to overlap and extend upstream of this PMA-responsive element (45).

Transcriptional control of the IL-1 genes is ultimately mediated through the action of specific transcriptional regu-

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latory proteins that bind to DNA and receive stimulatory (or inhibitory) signals transmitted through one or more cellular second-messenger systems. These cellular signals may cause new transcriptional factors to be synthesized, recruit factors into the nucleus from the cytosol, or posttranslationally activate factors that were previously bound to the DNA. Eukaryotic genes clearly utilize a large number of transcriptional factors that mediate highly regulated cell-type and inducer-specific gene expression (reviewed in reference 39). While many transcriptional factors bind tightly to specific DNA sequences (e.g., AP-1, NF-κB, and SP-1), others bind weakly to DNA or not at all (e.g., herpes simplex virus VP16, adenovirus E1a, and the cytomegalovirus immediate-early 1 [IE1] gene product). At least one of these proteins, adenovirus E1a, probably mediates its effects indirectly by modulating the activity of other factors, such as E2F (6). Some transcriptional factors may directly interact with components of the basic transcriptional machinery, such as the TATA-box-binding protein TFIID, although they may require additional proteins in order to initiate transcription (31, 43). These additional proteins are generally referred to as adaptors or coactivators (reviewed in reference 32).

Here we report the first detailed molecular analysis of the human IL-1β promoter, including the identification of nuclear regulatory proteins that appear to play critical roles in the transcriptional expression of this important cytokine. We identified and characterized a factor, termed NFIL-1βA (NFβA), that binds to a 12-bp highly conserved sequence within the cap site-proximal (CSP) promoter regions of the human and murine IL-1β genes. Multiple specific DNA-protein complexes were observed by electrophoretic mobility shift assay (EMSA) analysis using a double-stranded oligonucleotide containing only the NFβA-binding sequence as a probe, suggesting that there may be several distinct NFβA polypeptides, that a single NFβA protein can be modified at the posttranslational level to generate the multiple observed complexes, or both. Two distinct DNA-binding polypeptides have been identified, supporting the possibility that there are multiple NFβA gene products. The relative abundance of these complexes in crude nuclear extracts prepared from monocytic cells is affected by the presence of phosphatase inhibitors (PIs) and varies rapidly following activation with PMA or LPS. In addition, transient transfection experiments indicate that NFβA is required for both basal IL-1β promoter activity and inducible activity mediated by the human cytomegalovirus (HCMV) IE1 gene product.

MATERIALS AND METHODS

Cell culture and nuclear extracts. THP-1, U937, and RAW264.7 monocytic cells (American Type Culture Collection) were grown in RPMI 1640 culture medium (Fisher Mediatech) containing 10% heat-inactivated fetal bovine serum (HyClone), 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (complete medium). Jurkat and Raji cells were grown as indicated above, using a final concentration of 5% fetal bovine serum in the culture medium. All medium components were assayed for endotoxin levels, and only reagents containing less than 15 pg of endotoxin per ml (final concentration) were used. Normal human peripheral blood monocytes (PBMs) were kindly provided by Raymond Donnelly (Division of Cytokine Biology, U.S. Food and Drug Administration). Normal human neutrophils were kindly provided by Elizabeth Simons (Department of Biochemistry, Boston University School of Medicine). Stimulation of THP-1 and U937 cells was performed by incubating the cells in the presence of 20 ng of PMA (Sigma) per ml for various times as indicated. Nuclear extracts were prepared from nonquiescent cell cultures by the method of Dignam et al. (17), with slight modifications. Specifically, nuclei were obtained following hypotonic lysis in the presence of 10 mM KCl, 0.3 M sucrose, 5 mM $MgCl_2$, 10 mM β-glycerol phosphate, 0.2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg each of aprotinin, antipain, chymostatin, leupeptin, and pepstatin A per ml. Nuclear proteins were extracted by incubating the nuclei for 30 min on ice using 320 mM KCl. Crude extracts were recovered and dialyzed to a final KCl concentration of 100 mM in the presence of 15% glycerol. Extracts were stored in aliquots at −80°C.

DNA constructs and probes. DNA probes for EMSA analysis were prepared by using subclones of the human IL-1β genomic clone λBDC-454 (14) as described in the text. Custom-synthesized double-stranded oligonucleotide probes (Oligo's Etc.) were obtained for the native (designated M1.2 [ACTTCTGCTTTT]) and mutant (designated M5.6 [ACT TCTGGTTTT]) NFβA-binding sequences. DNA fragments were endlabelled at 5′ overhangs by the addition of α-^{32}P-labelled deoxynucleotide triphosphates (DuPont-NEN), using *Escherichia coli* DNA polymerase Klenow fragment (U.S. Biochemical) as recommended by the manufacturer. Unincorporated nucleotides were removed by using Sephadex G-25 or G-50 columns (5 Prime-3 Prime Inc.).

EMSA. Radiolabelled probe DNA (0.1 ng) was incubated with 2 to 4 μg of crude nuclear extract in the presence of 2 μg of poly(dI-dC) (PL-Pharmacia) in a final volume of 20 μl as previously described (47). In competition experiments, unlabelled DNA was added just prior to the addition of the extract at a molar excess indicated in the text. Binding reactions were performed at room temperature for 30 min, and then a portion of the mixture (typically 6 μl) was electrophoresed on 4% nondenaturing low-ionic-strength mobility shift polyacrylamide gels, using an electrophoresis buffer containing 22 mM Tris-HCl (pH 8.0), 22 mM borate, and 0.5 mM EDTA (0.25× TBE). Gels were dried and visualized by autoradiography.

Methylation interference and DNase I footprinting. Methylation interference footprinting was performed as described previously (13). DNA methylation and single-end radiolabelling of DNA probes were performed as previously described (38). Partially methylated probe DNA (2×10^5 cpm) was incubated in a 10-fold scale-up EMSA binding reaction mixture, using 50 to 100 μg of crude THP-1 nuclear extract. The mixture was then fractionated on a preparative-scale 4% mobility shift polyacrylamide gel. Wet gels were visualized by brief autoradiography, and the bands containing the bound and unbound DNA were excised. Radiolabelled DNA was recovered from the gel slices by electroelution, cleaved with piperidine, denatured, and electrophoresed on an 8% denaturing polyacrylamide sequencing gel. The gel was then dried without fixation and visualized by autoradiography. DNase I footprinting was performed exactly as previously described (5).

UV cross-linking. Custom-synthesized oligonucleotides (Oligo's Etc.) were obtained for use as template and primer strands. The template oligonucleotide was an antisense-strand sequence consisting of residues −59 to −20 (relative to the transcriptional start site). The primer oligonucleotide was a sense-strand sequence consisting of residues −59 to −51. Template and primer DNA were annealed at a molar ratio of 1:2, and second-strand synthesis was performed by

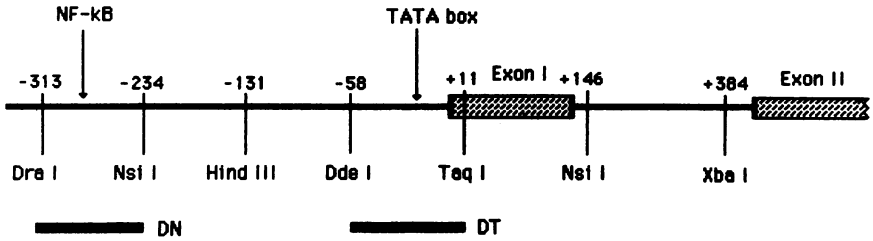

FIG. 1. Restriction map of the human IL-1β promoter (14) and genomic subclones used for EMSA analysis. Restriction enzyme site locations are numbered relative to the initiation of transcription (+1). Genomic subclones (dark horizontal bars) are denoted by two-letter codes.

using *E. coli* DNA polymerase Klenow fragment (U.S. Biochemical) in the presence of 50 μCi of [α-^{32}P]dATP (DuPont-NEN) and 0.8 mM 5-bromo-dUTP (Sigma). This probe (2 × 10^5 cpm) was used in a 10-fold scale-up EMSA binding reaction mixture containing 50 μg of THP-1 or Raji nuclear extract. Following fractionation of the reaction mixture on a mobility shift polyacrylamide gel, the entire gel was exposed to UV light (312 nm), using an inverted UV light source (IBI) at a distance of 5 cm from the light source for 30 min on ice. Wet gels were visualized by brief autoradiography, and the bands containing the bound and unbound DNA were excised. DNA-protein complexes were eluted from the gel slices overnight at 4°C in Laemmli sample buffer (125 mM Tris-HCl [pH 6.8]), 2% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 10% glycerol, 0.5 μg of bromphenol blue per ml), then denatured by boiling, and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide gel). Prestained molecular weight protein standards (Amersham) were electrophoresed in adjoining lanes. Gels were subsequently dried and visualized by autoradiography.

Transfections and CAT assays. The vector used for these studies was pBLCAT3, a plasmid which carries a promoterless chloramphenicol acetyltransferase (CAT) gene downstream of a multiple cloning site (35). A portion of the human IL-1β promoter (−131 to +11) was inserted into pBLCAT3 upstream of the CAT gene. To delete the NFβA-binding sequence, a portion of the native genomic DNA (−58 to +11) was replaced by an identical double-stranded oligonucleotide fragment containing seven specific nucleotide substitutions creating unique *Bgl*II and *Bam*HI restriction sites which flank the NFβA-binding sequence (GagatCTACTTC TGCTTTTGgAtcC; lowercase letters denote the modified sequence) to generate plasmid 7mHT. A deletion mutant (7mHTΔβA) was generated by removing the *Bgl*II-*Bam*HI fragment containing the NFβA-binding sequence from 7mHT. Sequence modifications were confirmed by DNA sequencing. Expression constructs which contain the coding sequences for the HCMV IE1 (pCC), IE2 (pCSdlAcc), and IE1 plus IE2 (pCS) genes have previously been described (25, 36, 51, 52).

Transient transfection of THP-1 cells was performed as we have previously described, using the DEAE-dextran method (27). Cells were exposed to 20 μg of total plasmid DNA (10 μg of each plasmid) in a 100-μg/ml solution of DEAE-dextran (PL-Pharmacia) in serum-free RPMI 1640 for 60 min at 37°C, washed once with RPMI 1640 containing 1.5 U of heparin per ml, and then washed once in RPMI 1640 without heparin. Cells were cultured overnight in 100-mm-diameter dishes at a density of 10^6 cells per ml in complete medium. LPS (10 μg/ml; serotype O26:B6) was added to the cultures, and cells were harvested 24 h later. CAT assays were performed as described previously (23). Acetylated derivatives were separated from the nonacetylated chloramphenicol by ascending thin-layer chromatography, using chloroform-methanol (95:5). The plates were visualized by autoradiography and quantitated by using a thin-layer chromatograph scanner (Radiomatic Instrument & Chemical Co.).

RESULTS

Identification of novel DNA-binding activities. Portions of the human IL-1β proximal promoter region were subcloned into the plasmid vector pUC19 as shown in Fig. 1 and used as probes to identify sequence-specific protein-binding activities, using EMSA analysis. We had previously shown that both the *Dra-Nsi* (DN) and *Dde-Taq* (DT) restriction fragments could generate specific DNA-protein complexes, the latter in a highly tissue-specific manner (15, 19). The DT fragment (−58 to +11) was subsequently assayed by EMSA, using nuclear extracts prepared from various cell types (Fig. 2A). This DNA fragment is of particular interest since it contains the TATA box motif and other sequences that are

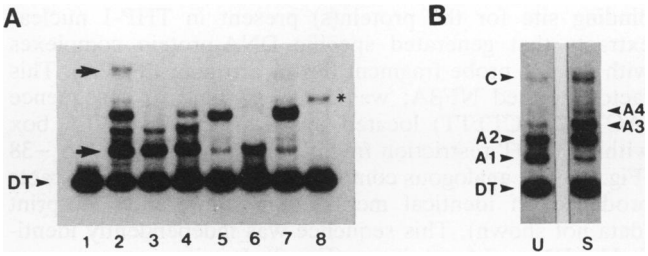

FIG. 2. (A) EMSA analysis using the DT probe fragment and nuclear extracts prepared from various cell types. Lanes: 1, no extract; 2, THP-1 cells; 3, U937 cells; 4, normal human PBMs; 5, murine RAW264.7 cells; 6, normal human neutrophils; 7, Raji human B-lymphoblastoid cells; 8, Jurkat human T-lymphoblastoid cells. The upper arrow denotes the distinct DNA-binding activity of NFβC (see text); the lower arrow denotes the NFβA complex that was subsequently evaluated by DNA footprinting. The asterisk denotes a nonspecific band generated by several of the nuclear extracts. Unbound DT probe DNA migrates at the bottom of the gel. (B) Designation of distinct NFβA complexes. EMSA analysis was performed with the DT probe and nuclear extracts prepared from unstimulated U937 cells (lane U) or cells stimulated with PMA (20 ng/ml) for 1 h (lane S). Shown are the locations of the specific NFβC and NFβA complexes. Cells were stimulated with PMA to induce βA4 expression. The βA4 complex is specifically induced in stimulated cells but comigrates with a nonspecific band observed in some experiments (see asterisk in panel A).

highly conserved between the human and murine IL-1β genes (14, 15, 53). EMSA data generated by using the DT probe fragment showed that extracts prepared from several cell types generated multiple DNA-protein complexes. These complexes were found to be specific, as shown below. Both human (THP-1, U937, and PBMs) and murine (RAW264.7) monocytic cells expressed multiple DNA-binding activities. The relative abundance of these complexes differed among the cell types tested, as well as among extracts prepared from cultures of THP-1 or U937 cells obtained from different sources (data not shown). Also, there was no clear correlation between the relative abundance of these complexes in resting cells and the levels of IL-1β mRNA expressed by these cells following activation. Furthermore, not all immune cells express these DNA-binding activities, since nuclear extracts prepared from Jurkat T-lymphoblastoid cells did not generate specific complexes with the DT probe.

During the course of these experiments, we found that consistent patterns of DNA-protein complexes were obtained only when a cocktail of protease and PIs was included in the buffers used to prepare the nuclear extracts. In addition, we found that nuclear extracts prepared from living cells generated a different pattern of complexes compared with extracts prepared from cell pellets that had been previously frozen and stored at −80°C prior to preparation of the extracts, presumably as a result of proteolysis resulting from the freeze-thaw lysis of lysozomes. We conclude that these methodological differences explain the discrepancies between the EMSA data presented in Fig. 2A and previously reported data (19). Subsequent experiments (described below) demonstrated that the higher-mobility DNA-protein complexes shown in Fig. 2A were generated by proteins that bound to a single common sequence. For clarity, these complexes are designated βA1 to βA4 (Fig. 2B). The lowest-mobility complex (upper arrow) was generated by proteins binding to a distinct sequence (data not shown). This factor, termed NFβC, will be described elsewhere (39a).

Identification of the NFβA binding site. Methylation interference footprinting was then used to identify the precise binding site for the protein(s) present in THP-1 nuclear extracts that generated specific DNA-protein complexes with the DT probe fragment (lower arrow in Fig. 2A). This factor, termed NFβA, was found to bind to a sequence (ACTTCTGCTTTT) located upstream of the TATA box within the DT restriction fragment at positions −49 to −38 (Fig. 3). The analogous complex generated by PBM extracts produced an identical methylation interference footprint (data not shown). This sequence was independently identified by DNase I footprinting (Fig. 3). It is interesting to note that the footprinted region observed by using DNase I is not significantly larger than that observed by using methylation interference and does not extend into the TATA box motif. This finding suggests that the TATA box may be fully accessible for binding by TFIID even in the presence of bound NFβA. The NFβA binding site is absolutely conserved between the human and murine IL-1β genes (100% identity over 12 bp), as is the location of this sequence relative to the TATA box of each gene. The latter observation is consistent with the possibility that NFβA directly interacts with TFIID. A search of the GenBank nucleic acid sequence data base revealed that the NFβA-binding sequence does not resemble the binding site for any previously described nuclear regulatory proteins. Thus, NFβA is the first regulatory protein to be identified that binds to a novel sequence within the human and murine IL-1β genes. The

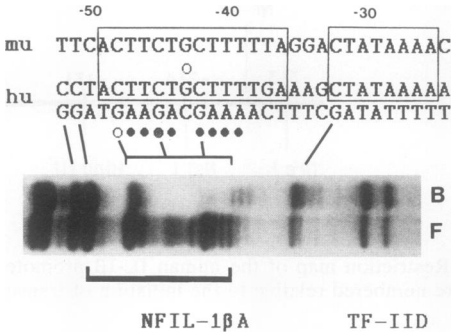

FIG. 3. Identification of the NFβA-binding site by methylation interference DNA footprint analysis. The DT probe was incubated with nuclear extracts prepared from unstimulated THP-1 cells. Specific DNA-protein complexes were fractionated on preparative nondenaturing polyacrylamide gels, eluted from the gel (see lower arrow in Fig. 2A), and subsequently cleaved with piperidine, and the products were fractionated on denaturing polyacrylamide gels as described previously (13). Shown are the strong (filled circles) and weak (open circles) DNA-protein contact points. Also shown are the conserved sequences from the human (hu) and murine (mu) IL-1β genes which contain the binding sites for NFβA and the TATA-box-binding factor TFIID. Protected sequences observed by using DNase I footprinting are also shown (dark horizontal bar). The locations of these sequences are denoted relative to the start of transcription (designated +1). B, bound DNA; F, free DNA.

human IL-1α gene, which does not possess a canonical TATA box, lacks promoter sequences that are homologous to the NFβA binding site (21), although a highly similar sequence is located within 3′ untranslated region of this gene. The murine IL-1α gene does not possess a similar sequence. Additional homologous sequences have also been found in the promoters of the IL-6 and IL-1ra genes (Table 1), although it is not known whether these sequences can bind NFβA.

The specificity of DNA-protein complexes shown in Fig. 2A was confirmed by competition experiments using various unlabelled DNA fragments and double-stranded oligonucleotides (oligonucleotide sequences are shown in Materials and Methods). As shown in Fig. 4, both the DT fragment and a 19-bp oligonucleotide containing the native binding sequence for NFβA (M1.2) competed for binding, whereas the DN fragment and a 19-bp mutant oligonucleotide (M5.6) did not compete with the DT probe. EMSA analysis of the nuclear extracts was next performed by using the M1.2 oligonucleotide probe, which contains the native binding sequence for NFβA. The results shown in Fig. 5 revealed a pattern of binding similar to that observed by using the DT probe fragment (Fig. 2A) and confirmed that multiple DNA-

TABLE 1. Comparison of sequences present in heterologous genes that are homologous to the NFβA-binding sequence[a]

Gene	Sequence	Location	Reference
Human IL-1β	ACTTCTGCTTT	Promoter	14
Murine IL-1β	ACTTCTGCTTT	Promoter	53
Human IL-1α	CCTTCTGCTTT	3′ untranslated region	21
Human IL-6	ACTTCAGCTTT	Promoter	44
Human IL-1ra	ACTATTTCTTT	Promoter	48

[a] Homologous sequences were identified by using the PC/Gene (IntelliGenetics) FASTSCAN program, which utilizes the Lipman-Pearson FASTA algorithm (42).

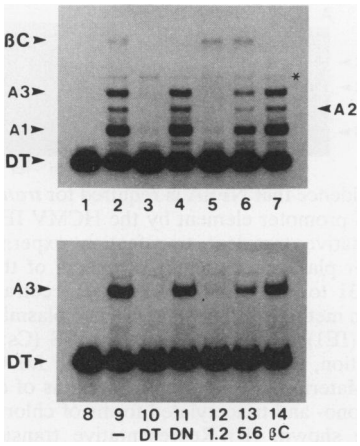

FIG. 4. Sequence specificity of the observed DNA-proteins complexes. EMSA analysis was performed with the DT probe and nuclear extracts prepared from either THP-1 (lanes 2 to 7) or Raji (lanes 9 to 14) cells. Lanes 1 and 8 represent unbound DT probe. The locations of specific NFβA and NFβC complexes are shown. Unlabelled competitor DNA (200-fold molar excess) was added to several binding reaction mixtures as follows: lanes 3 and 10, DT; lanes 4 and 11, DN; lanes 5 and 12, M1.2 (1.2); lanes 6 and 13, M5.6 (5.6) lanes 7 and 14, an oligonucleotide containing the NFβC-binding sequence (βC). The asterisk denotes a nonspecific band generated by the THP-1 nuclear extract.

protein complexes could be generated by using a probe that contained only the NFβA-binding sequence. This result suggested that either distinct nuclear proteins could specifically bind to the same DNA sequence, that a single protein could exist in several posttranslationally modified forms such that each form generates a distinct complex, or both. Precedents for each possibility have been reported (12, 50). Another formal possibility is that the various complexes are generated by proteolytic fragments of a single NFβA protein which are capable of sequence-specific DNA binding (30), although the use of a broad spectrum of protease inhibitors during extract preparation minimizes this possibility.

Identification of two distinct DNA-binding polypeptides. UV cross-linking analysis of the βA1 and βA3 complexes was performed to determine whether these complexes contained a common DNA-binding subunit. EMSA binding reactions were performed by using nuclear extracts prepared from THP-1 or Raji cells and a radiolabelled oligonucleotide probe containing the photosensitive nucleotide 5-bromodeoxyuridine. The specific DNA-protein complexes were resolved on mobility shift gels, and covalent cross-linking was performed by directly exposing the entire gel to UV light. The specific βA1 and βA3 complexes were then excised from the mobility shift gel and analyzed by SDS-PAGE. As shown in Fig. 6, the βA1 complex contained a single cross-linked DNA-protein band corresponding to the cross-linked polypeptide and oligonucleotide probe (lanes 2 and 3), whereas the βA3 complex from both THP-1 (lanes 4 and 5) and Raji extracts (lanes 6 and 7) contained a single band of distinct size. By subtracting the apparent molecular weight of the probe, the sizes of these DNA-binding polypeptides are estimated to be 28 and 39 kDa for βA1 and βA3, respectively. A single 28-kDa protein was also found to be a component of the βA1 complex generated by nuclear extracts from normal human PBMs and neutrophils (data not shown), although the possibility that these proteins are identical remains to be established. Similarly, we do not know whether the 39-kDa proteins that comprise the βA3 complex in THP-1 and Raji cells are identical. Also, it should be noted that the 28- and 39-kDa proteins observed here may not represent the sole components of the βA1 and βA3 complexes, respectively, since additional polypeptides that do not directly contact the DNA would not be cross-linked by this method.

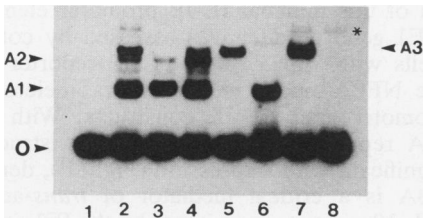

FIG. 5. Evidence that an oligonucleotide probe containing only the NFβA-binding sequence can generate multiple DNA-protein complexes. EMSA analysis was performed with the 19-bp M1.2 oligonucleotide probe (O) and nuclear extracts prepared from various cell types. Lanes: 1, no extract; 2, THP-1 cells; 3, U937 cells; 4, PBMs; 5, RAW264.7 cells; 6, neutrophils; 7, Raji B cells; 8, Jurkat T cells. The asterisk denotes a nonspecific band generated by several of the extracts.

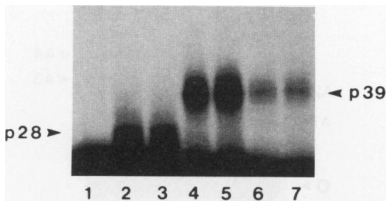

FIG. 6. Identification of two distinct DNA-binding polypeptides by UV cross-linking. A radiolabelled double-stranded DNA oligonucleotide probe containing 5-bromodeoxyuridine was incubated with THP-1 or Raji nuclear extracts, and the resultant DNA-protein complexes were then resolved on a mobility shift gel. The gel was exposed to UV light, and specific bands containing the βA1 and βA3 complexes were excised. Cross-linked protein-oligonucleotide complexes were eluted from the gel slices and fractionated by SDS-PAGE (10% polyacrylamide gel). Positions of migration of the specific cross-linked complexes are indicated by arrowheads, and the molecular sizes of the distinct polypeptide components are shown. Lanes: 1, free probe; 2 and 3, βA1 complexes from THP-1 extracts; 4 and 5, βA3 complexes from THP-1 extracts; 6 and 7, βA3 complexes from Raji extracts.

Evidence for posttranslational modification of NFβA. Subsequent experiments addressed the possibility that at least some of the NFβA-specific DNA-protein complexes observed by using EMSA analysis resulted from posttranslational modification of the NFβA polypeptides. One type of posttranslational modification that has been shown to affect transcriptional regulatory protein function is phosphorylation (8, 29, 41, 49). Nuclear extracts were prepared from unstimulated or PMA-stimulated U937 cells in the presence or absence of the PI β-glycerol phosphate (protease inhibitors were included in both cases) and evaluated by EMSA analysis. We found that the relative expression of the distinct NFβA activities in both resting and activated cells was markedly affected by the presence of the PI, suggesting that NFβA can be modified by phosphorylation. Specifically, in extracts prepared from unstimulated cells in the absence of the PI, the βA1 form was predominantly expressed. In contrast, βA1 activity was diminished and βA2

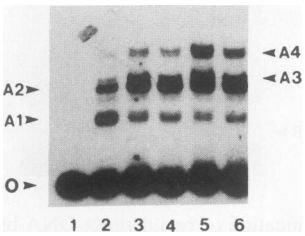

FIG. 7. Rapid changes in the relative abundance of NFβA complexes following stimulation. EMSA analysis was performed with the M1.2 probe (O) and nuclear extracts prepared from U937 cells. Cells were stimulated with PMA (20 ng/ml) for various times, the cells were then harvested, and nuclear extracts were prepared by using buffers containing β-glycerol phosphate (10 mM). The locations of specific NFβA complexes are shown. Lanes: 1, no extract; 2, no stimulation; 3, 15-min stimulation; 4, 30-min stimulation; 5, 1-h stimulation; 6, 3-h stimulation.

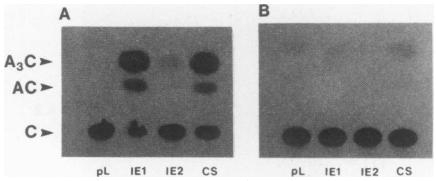

FIG. 8. Evidence that NFβA is required for *trans*-activation of a minimal IL-1β promoter element by the HCMV IE1 gene product. (A) Representative transient transfection experiment using the 7mHT reporter plasmid containing a portion of the human IL-1β promoter (−131 to +11). THP-1 cells were cotransfected by the DEAE-dextran method with both 7mHT and plasmid pLink760 (pL; vector), pCC (IE1), pCSdlAcc (IE2), or pCS (Cs; IE1 plus IE2). After transfection, cell lysates were assayed for CAT activity as described in Materials and Methods. Positions of chloramphenicol (C) and the mono- and triacetylated forms of chloramphenicol (AC and A_3C) are shown. (B) Representative transient transfection experiment using the 7mHTΔβA reporter plasmid, which lacks the NFβA-binding sequence.

activity was enhanced in extracts prepared from unstimulated cells in the presence of the PI. Extracts prepared from PMA-stimulated cells showed a more dramatic dependence on the PI. As reported below, PMA induces a rapid change in the relative expression of the NFβA complexes. In extracts prepared in the absence of the PI, the βA1 and βA2 forms predominated, although a small amount of βA3 activity was also observed. In the presence of the PI, the level of βA1 activity was greatly reduced, whereas the level of βA3 activity was enhanced. In addition, the βA4 complex was observed only in extracts prepared in the presence of the PI from PMA-stimulated cells. Thus, the presence of a general protein PI has a marked effect on the relative levels of NFβA DNA-binding activities observed in crude nuclear extracts. We did not consistently observe changes which suggested that phosphorylation or dephosphorylation of any particular complex resulted in conversion to a second complex, supporting the possibility that phosphorylation instead differentially affects the affinity of individual NFβA polypeptides for the DNA.

The observation that the relative expression of the various NFβA activities was dramatically altered after PMA stimulation was explored in greater detail by preparing nuclear extracts from U937 cells (using PIs) at various times after PMA stimulation and then assessing the NFβA-binding activities by EMSA analysis. We found that expression of the individual NFβA complexes could be independently up- or down-regulated within 15 min following PMA stimulation (Fig. 7). Specifically, βA1 levels decreased rapidly after stimulation and remained low, whereas the levels of βA2 and βA3 rapidly increased and remained high throughout the times examined. While βA4 was not expressed by resting cells, this activity was transiently expressed following activation, although with distinct kinetics. Similar changes in the relative highs of βA1 to βA3 activities were also observed following LPS stimulation, although little βA4 activity was expressed in these extracts (data not shown).

NFβA is required for promoter activation by the HCMV IE1 protein. We have previously shown that the IE gene products of HCMV markedly up-regulate expression of the human IL-1β gene in monocytic cell lines (27). We used transient transfection analysis to determine whether NFβA plays a role in the activation of the IL-1 promoter by the IE1 protein. In these studies, THP-1 cells were transfected with both a CAT reporter construct (7mHT) and expression plasmids containing the HCMV IE1 and/or IE2 genes. The 7mHT CAT construct contains a portion of the human IL-1β promoter (−131 to +11) and differs from the native promoter sequence in that seven nucleotide substitutions which flank the NFβA-binding sequence were introduced to allow subsequent deletion of this site (see Materials and Methods for sequences). These mutations did not affect the ability of this promoter fragment to be *trans*-activated by IE1 gene products, which we had previously reported (16). As shown in Fig. 8A, the IE1 gene product was capable of markedly *trans*-activating the 7mHT reporter construct. While we had previously shown that THP-1 cells cotransfected with an IE1 expression plasmid could *trans*-activate CAT constructs containing large portions of IL-1β upstream sequences (−1097 to +11 [27]), these data show that as little as 131 bp of upstream sequences is sufficient to induce significant CAT expression (approximately 83% conversion of chloramphenicol to its acetylated derivatives) in cells cotransfected with the IE1 construct (pCC). Cotransfection of cells with the IE2 construct (pCSdlAcc) provides only minimal *trans*-activation of the reporter plasmids (approximately 6% conversion). Cotransfection of cells with constructs containing both IE1 and IE2 (pCS) sequences induced a moderate level of *trans*-activation (approximately 62% conversion). Cells that were cotransfected with an expression vector which lacked IE gene sequences (pLink760) did not induce CAT expression. In parallel experiments, promoterless CAT reporter plasmids were not *trans*-activated when cotransfected with the IE expression plasmids.

Subsequently, the requirement for NFβA in the *trans*-activation of this minimal IL-1β promoter element by the HCMV IE1 gene product was assessed by cotransfecting THP-1 cells with 7mHTΔβA, a CAT reporter plasmid in which the NFβA-binding sequence was deleted from the IL-1β promoter, and the IE constructs. With use of the 7mHTΔβA reporter plasmid, the IE1 construct failed to induce significant CAT expression (Fig. 8B), demonstrating that NFβA is a critical mediator of *trans*-activation of minimal IL-1β promoter sequences by the IE1 gene product. The potential for an NFβA-binding sequence to confer IE1 responsiveness to a heterologous promoter has not yet been tested. Additional studies using reporter plasmids containing larger upstream regions of the IL-1β promoter (specifically, −1097 to +11 or −1755 to +11) have shown that deletion of the NFβA-binding sequence does not abolish the ability of these reporter plasmids to be *trans*-activated by the IE1

TABLE 2. Effect of LPS on reporter plasmid expression

Expt.	% conversion of chloramphenicol to its acetylated derivatives with reporter:							
	7mHT				7mHTΔβA			
	Control	LPS	IE1 + IE2	IE1 + IE2 + LPS	Control	LPS	IE1 + IE2	IE1 + IE2 + LPS
1	0.5	1.6	9.1	40.7	0.2	0.4	0.4	0.9
2	0.4	2.1	13.0	96.4	1.0	0.2	2.4	11.5
3	0.4	1.5	40.4	98.9	0.3	3.8	3.6	5.8
4	1.8	16.8	62.0	98.8	2.2	0.1	3.2	6.2
5	0.8	4.7	92.7	98.1	0.9	0.2	11.7	22.7
Stimulation index[a]		5.5	59.4	145.4		2.9	6.1	14.6

[a] Average fold stimulation over control values.

construct (data not shown). This finding suggests that the IE1 gene product may also activate the IL-1β promoter via several distinct upstream factors.

To further assess the role of NFβA in the *trans*-activation of the proximal human IL-1β promoter, the effect of an additional stimulus (LPS) was examined. We had previously shown that LPS-stimulated THP-1 cells exhibited approximately 30-fold-higher CAT activity (using a −1097 to +11 promoter fragment to direct CAT expression) when cotransfected with pCS (IE1 plus IE2) than did the same cells in the absence of LPS stimulation (27). Furthermore, we had found that LPS increased CAT activity only twofold in the absence of the IE gene products. In the present studies, we used minimal promoter constructs in order to eliminate the contribution of upstream factors to transcriptional activation of the promoter. THP-1 cells were cotransfected with the 7mHT or 7mHTΔβA reporter plasmid and the pCS (IE1 plus IE2) construct as described above. The cells then either were stimulated with LPS or remained unstimulated for 24 h prior to performance of the CAT assays. As shown in Table 2, LPS did not substantially induce CAT expression from the 7mHT reporter plasmid in the absence of the IE proteins. In contrast, cells cotransfected with pCS in the absence of LPS stimulation exhibited approximately 60-fold-higher CAT activity. LPS-stimulated cells transfected with both 7mHT and pCS exhibited a 145-fold-higher level of CAT expression. In contrast, cells cotransfected with pCS and the 7mHTΔβA reporter plasmid exhibited approximately 10-fold-lower CAT activity in the presence or absence of LPS relative to cells transfected with pCS and 7mHT. While transfection of THP-1 cells with 7mHT alone generates only extremely low levels of CAT activity, no CAT activity was observed in cells transfected with 7mHTΔβA. Taken together, these data suggest that NFβA is required for basal transcriptional activity and the activation of the minimal promoter element by the IE1 protein but does not appear to directly or solely mediate the activation of the IL-1β gene by LPS. The latter possibility is supported by the recent identification of an enhancer element located more than 3 kbp upstream of the cap site which can confer LPS responsiveness on a heterologous promoter (45).

DISCUSSION

While expression of IL-1 genes has been extensively studied in a variety of cell types, the genomic regulatory elements that control transcription of these genes are far less well understood. Transient transfection approaches have recently been used to identify both phorbol ester (7)- and LPS (45)-inducible enhancer elements within the human IL-1β gene which lie far upstream of the transcriptional start site. In this study, we used EMSA analysis and DNA footprinting to provide the first description of nuclear proteins that bind to a common novel sequence within the CSP human IL-1β promoter. These studies revealed an NFβA family of proteins that consist of at least two distinct polypeptides which recognize a conserved DNA sequence located upstream of the TATA motif of the human and murine IL-1β genes. The presence of this NFβA-binding sequence appears to be required for both basal and HCMV IE1-inducible transcription directed by the CSP promoter element in monocytic cell lines.

In the absence of additional upstream elements, the CSP promoter region of the human IL-1β gene can direct only low levels of transcription of a reporter gene (15) (Table 2). This minimal transcription can be greatly augmented by the presence of the IL-1β enhancer (7, 15, 45), suggesting that NFβA is not acting solely as an enhancer factor. In vivo, the cellular IL-1β gene is transcribed only in specific cell types, such as monocytic cells, following stimulation. Since our transient transfection data showed that the activity of the CSP promoter element used in these studies (−131 to +11) was not markedly augmented by LPS stimulation alone (Table 2), this finding suggests that NFβA alone is not solely responsible for the LPS inducibility of the IL-1β gene. Furthermore, activation of monocytic cells with PMA (Fig. 7) or LPS (data not shown) rapidly and transiently affects the relative levels of the various NFβA activities, suggesting that NFβA may still indirectly participate in the inducible response of this gene. Experiments are under way to determine whether NFβA is a phosphoprotein and whether the observed changes in NFβA activities in stimulated cells are due, at least in part, to posttranslational modification of distinct NFβA polypeptides.

The highly conserved location and binding sequence for NFβA in the human and murine IL-1β genes (Fig. 3) suggest that NFβA plays an important role in the selective expression of this cytokine. This sequence is not present at a comparable location within the IL-1α gene, which lacks a canonical TATA motif, although a similar sequence is located over 9,400 bp downstream of the cap site (53) (Table 1). The absence of a homologous sequence within the murine IL-1α gene suggests that NFβA does not participate in the regulation of IL-1α gene expression and provides a possible mechanism by which the IL-1β gene could be regulated independently of the IL-1α gene within the same cell type. It is not currently known whether cell types that express predominately IL-1α, such as keratinocytes (28), also express lower levels of NFβA. However, this possibility is consistent with the lack of NFβA expression by Jurkat T

cells (Fig. 2A), since T cells have been reported to express predominantly IL-1α (1), and with the observation that WI38 fibroblasts and HeLa cells express only very low levels of NFβA activity (data not shown). The existence of two distinct DNA-binding polypeptides that comprise the βA1 and βA3 complexes suggests that they may perform different functions in vivo. This possibility is underscored by the observation that the relative levels of these activities changes rapidly and inversely following stimulation (Fig. 7).

Transient transfection studies to determine the functional role of NFβA revealed that the HCMV IE1 protein was capable of *trans*-activating a minimal IL-1β promoter element (−131 to +11) in an NFβA-dependent fashion (Fig. 8). This finding extends our previous observations showing that HCMV IE gene products can markedly enhance IL-1β gene expression in LPS-stimulated THP-1 cells (27). Although reporter constructs containing IL-1β promoter sequences could be induced with IE1 alone, the endogenous gene was previously shown to not be similarly activated. This finding may be due to negative regulatory elements within intron 1 of the IL-1β gene that may prevent activation of the endogenous gene by IE1 in the absence of LPS (15). The mechanism by which IE1 can *trans*-activate a promoter remains unclear, since IE1 itself does not appear to specifically bind to DNA (unpublished data). In the case of the HCMV IE promoter itself, several studies have suggested that IE1 activates this promoter via interaction with NF-κB which is bound to specific sequences within the 18-bp repeats of this promoter (10, 36). The significance of this observation is not clear, however, since IE1 does not activate all promoters which contain these 18-bp sequences, including the human immunodeficiency virus long terminal repeat (unpublished data). Although we found that NFβA was required for activation of the IL-1β CSP promoter element (−131 to +11) by IE1, it was not required for the activation of sequences with 5′ termini at −1097 or −1795 (data not shown). It is possible that these longer sequences contain the binding sites for additional transcriptional activating factors that may also be affected by IE1.

Taken together, these data suggest that NFβA itself is not a potent *trans*-activator but is required for activation of the IL-1β promoter by IE1. These findings are similar to the actions of ATF2, a mammalian transcriptional factor that binds to DNA in a sequence-specific manner and mediates the activation of several genes by the adenovirus E1a protein (33). Since E1a does not bind to DNA directly, interaction with ATF2 provides a means by which E1a can be recruited to a promoter. Furthermore, ATF can directly interact with TFIID (26). TFIID is regarded as a commitment factor whose binding to the TATA box is a prerequisite for assembly of the basal transcriptional apparatus (9), and thus it may serve as a target for activation by upstream factors. Functional interaction between upstream factors and the basal apparatus may also require additional components known as a coactivators or adaptors (reviewed in reference 32). While the term "adaptor" has most often been applied to as yet uncharacterized proteins that do not bind to DNA, the adaptor concept applies equally as well to DNA-binding proteins such as ATF. One possible model for NFβA action is that it functions as an adaptor factor, although any direct interaction between NFβA and TFIID remains to be demonstrated. It is also possible that NFβA acts to recruit TFIID to the promoter in a manner similar to that of ATF (24, 26). The conserved location of the NFβA-binding sequence near the TATA box, as well as the observation that the DNase I footprint of NFβA (Fig. 3) does not extend into the TATA motif, implies that NFβA and TFIID may be capable of binding to the promoter simultaneously. The effect of the HCMV IE1 proteins may be to facilitate this interaction.

An alternative model for the *trans*-activation of the IL-1β promoter by IE1 is based on the recent finding that E1a can release transcriptional factors from complexes with cellular inhibitor proteins (6). In one model, the transcriptional factor E2F is maintained in an inactive complex via direct interaction with the retinoblastoma gene product (Rb). This model further predicts that E1a dissociates E2F from inactive complexes by binding to Rb. Thus, E1a may activate transcription by binding to inhibitory proteins and releasing necessary transcriptional factors (reviewed in reference 54). By analogy, NFβA may function as a transcriptional activator but may be bound to an inhibitory factor in unstimulated cells. The IE1 protein may activate the CSP IL-1β promoter by binding to this putative inhibitor protein and dissociating it from NFβA. Our current data are consistent with both of these models.

Future studies will be directed toward understanding the precise nature of the potential interactions between elements of the CSP IL-1β promoter and the upstream factors that bind to the IL-1β enhancer. We will also need to determine how these factors may be affected by posttranslational modifications which are induced in a stimulus-specific manner by distinct cellular second-messenger systems. Together, these processes represent key steps in the tightly regulated expression of the IL-1β gene.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants AI-29088 to M.J.F., HL-37121 and HL-07638 to G.W.H., AI-13562 to M.F.S., and AI-27850 to P.E.A.

We thank Jeffrey Kosiba and Bridget Martell for assistance with the DNA footprinting analysis. Jon Buras and Gyorgy Frendl are acknowledged for continued assistance and helpful discussions. We also thank S. Alex Mitsialis for critically reviewing the manuscript prior to submission and for generously providing assistance with the computer homology analysis and data base searches.

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