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Immunology

Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8

(C/EBP/protein–protein interaction/acute-phase reaction)

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ABSTRACT **Single binding sites for transcription factors NF-IL6 and NF-κB are present in the promoter of the interleukin (IL) 6 gene. Previous studies of internally deleted promoter mutants demonstrated that these two sites are important for the transcriptional regulation of this gene. In this report, we describe the synergistic activation of the IL-6 promoter by transcription factors NF-IL6 and NF-κB. Cotransfection of NF-IL6 with the NF-κB p65 subunit resulted in strong synergistic activation of an IL-6 promoter–reporter construct. Both the NF-IL6 and NF-κB binding sites in the IL-6 promoter were required for synergistic activation. Similar synergistic activation was observed in the IL-8 promoter, which also contains both NF-IL6 and NF-κB binding sites. Furthermore, we demonstrated that NF-IL6 and the NF-κB p65 subunit directly associated via the basic leucine-zipper domain of NF-IL6 and the Rel homology domain of p65. Since the promoters of many other genes involved in the inflammatory and acute-phase responses also contain binding sites for NF-IL6 and NF-κB, the cooperation between these two factors may have an important role in these responses. We also discuss the possible interplay between various viral gene products and these two factors in the process of viral infection and constitutive cytokine production.**

We have shown (1) that the transcriptional activation of the interleukin (IL) 6 gene by IL-1 was dependent on a 14-bp palindromic sequence located at position −150 and that an IL-1-inducible nuclear factor, termed NF-IL6, bound to this site. A cDNA encoding NF-IL6 was cloned and found to be a member of the C/EBP transcription factor family, which belongs to the larger family of basic leucine-zipper (b-Zip) transcription factors (2). This protein has recently been identified in several different species and designated AGP/EBP, LAP, IL-6DBP, rNFIL-6, C/EBPβ, CRP2, or NF-M (3–9). NF-IL6 not only activates the IL-6 gene but also is responsible for the regulation of genes encoding other inflammatory cytokines, acute-phase proteins, albumin, c-Fos, several adipocyte-specific proteins, and viral proteins.

On the other hand, several groups have reported that the NF-κB binding site located between bp −72 and −63 was important for the activation of the IL-6 gene by IL-1, tumor necrosis factor α (TNF-α) or lipopolysaccharide (LPS) (10–12). NF-κB was identified initially as a nuclear factor that binds to an enhancer element, called a κB motif, of the immunoglobulin κ light chain (13). NF-κB is thought to be involved in the expression of several viruses and many cellular inducible genes encoding cytokines, immunoregulatory receptors, and acute-phase proteins. The major form of NF-κB is a heterodimer composed of 50- and 65-kDa subunits (p50 and p65, respectively), both of which belong to the Rel family. NF-κB is constitutively present in the nuclei of mature B cells. However, in most other cells NF-κB is present in cytoplasm. Various stimuli, including mitogens, cytokines, viruses, and bacteria, cause NF-κB to translocate into the nucleus and bind to the specific binding site (for review, see refs. 14 and 15).

Because many immune response genes and acute-phase response genes contain both the NF-IL6 and the NF-κB sites, it is highly possible that cooperative interactions between NF-IL6 and NF-κB play an important role in the expression of these genes. In this report, we demonstrate the synergism between NF-IL6 and NF-κB in the transcription of the genes for the inflammatory cytokines, IL-6 and IL-8.

MATERIALS AND METHODS

Cell Culture. The human monocytic cell line U937 was cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. The murine embryonic carcinoma cell line P19 was cultured in minimal essential medium (α modification) supplemented with 10% fetal calf serum.

Plasmid Constructions. A cDNA clone encoding the human NF-κB p50 subunit was obtained by the PCR technique and cloned into BCMGneo. Three mutants of NF-IL6 [ΔSplNF-IL6, NF-IL6(S288A), and ΔSplΔSacNFIL6] were as described (16). These mutant NF-IL6 cDNAs and wild-type NF-IL6 cDNA were cloned into pEF-BOS. The IL-6 promoter–luciferase plasmid was as described (17). Two kinds of mutant IL-6 promoter–luciferase plasmids were constructed. In one the NF-κB site was disrupted by converting GGGATTTTCC to AATATTTTCC, and in the other NF-IL6 site was disrupted by converting ACATTGCACAATCT to ACACTACAAACTCT. The IL-8–luciferase gene was prepared by inserting the IL-8 gene from positions −94 to +44 into the luciferase reporter gene. As an internal control plasmid, elongation factor (EF)–β-galactosidase was used.

Electrophoretic Mobility Shift Assays (EMSAs) and Luciferase Assays. Double-stranded oligonucleotides spanning the NF-κB site of the IL-6 gene (GGGATTTTCC) were synthesized, annealed, and labeled. Nuclear extracts from U937 cells with or without LPS stimulation were prepared as described (16). EMSAs were carried out by the protocol of T. Fujita *et al.* (18). When recombinant protein was used, bovine

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Abbreviations: IL, interleukin; CMV, cytomegalovirus; LPS, lipopolysaccharide; TNF, tumor necrosis factor; b-ZIP, basic leucine-zipper; EMSA, electrophoretic mobility shift assay; MBP, maltose binding protein; EF, elongation factor.
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serum albumin was added to the reaction mixture at a final concentration of 0.5 mg/ml, and when cell extract was used, 2 μg of poly(dI-dC) was added. DNA transfection into P19 cells and luciferase assays were carried out as described (17).

Protein Crosslinking. Maltose binding protein (MBP) fusion proteins (MBP–NFIL6, MBP–ΔSplNFIL6, MBP–ΔSplΔSacNFIL6) and control MBP were bacterially expressed as described (16). CDM8 p65 was linearized at the *Xba* I site (for full-length p65), at the *Bsp*HI site, or at the *Sca* I site (for shorter forms of p65), respectively. Linearized DNAs were transcribed using the Megascript system (Ambion, Austin, TX). RNA was translated in the presence of [^{35}S]methionine (NEG 009T, DuPont), using rabbit reticulocyte lysate (Promega), and the ^{35}S-labeled proteins were purified on a Sephadex G-25 column. The crosslinking reaction was carried out as described (16).

RESULTS

NF-κB p50 and p65 Can Bind to the Putative NF-κB Site on the IL-6 Gene. Previous reports indicated that the putative NF-κB binding site located between positions −72 and −63 on the IL-6 gene is important for IL-6 induction (10–12), but it had not been demonstrated that the NF-κB p50–p65 heterodimer or the p65 homodimer could actually bind to this site. To test this hypothesis, we performed an EMSA using recombinant NF-κB p50 and p65 proteins made in a baculovirus expression system.

As shown in Fig. 1*A*, both p50 and p65 can bind avidly to the κB-like motif of the IL-6 gene. Incubation of the two proteins at 37°C for 60 min, which induces p50–p65 heterodimer formation, resulted in the appearance of complexes with an intermediate mobility. Addition of anti-p50 or anti-p65 antibody super-shifted these bands. These results indicate that the p50 homodimer, p65 homodimer, or p50–p65 heterodimer can bind to the NF-κB site in the IL-6 gene.

Next, to examine whether p50 and p65 are actually induced and bind to this NF-κB site, nuclear proteins were extracted

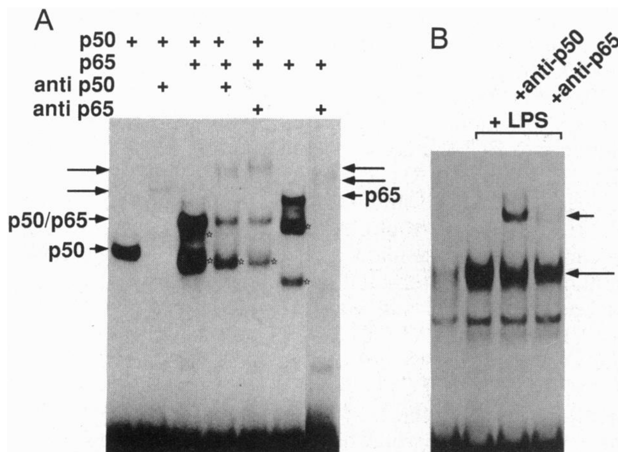

FIG. 1. NF-κB p50 and p65 subunits bind to the promoter region of the IL-6 gene. (*A*) EMSA using recombinant NF-κB p50 and p65 proteins expressed in baculovirus. The p50 proteins, the p65 proteins, or both were incubated with radiolabeled oligonucleotides containing a κB-like motif found in the IL-6 gene. Bands super-shifted by anti-p50 or anti-p65 antibodies are indicated by long arrows. The p65 proteins were easily degraded and the resulting degraded bands are indicated by stars. (*B*) EMSA using nuclear extracts from U937 cells. Nuclear extracts from U937 cells with or without stimulation of LPS were incubated with radiolabeled probe. The long arrow indicates specific complexes with the same mobility as the recombinant p50–p65 heterodimer. The short arrow indicates super-shifted bands by anti-p50 or anti-p65 antibodies. The fastest migrating bands are nonspecific.

from the human monocyte cell line U937 stimulated with LPS for 12 h and then analyzed by EMSA. As shown in Fig. 1*B*, LPS stimulation increased the retarded complex and the addition of either anti-p50 antibody or anti-p65 antibody induced a super-shifted band. These results indicate that p50 and p65 are induced by LPS and bind to the NF-κB site of the IL-6 gene in U937 cells.

Functional Cooperativity Between NF-κB and NF-IL6. To study the effects of NF-κB p50 and p65 and NF-IL6 on the transcriptional activation of the IL-6 gene, we performed transient cotransfection assays. When various combinations of the expression vectors for p50, p65, and NF-IL6 were cotransfected with the IL-6 promoter–luciferase reporter gene into murine embryonal carcinoma P19 cells, we did not detect endogenous p50, p65, or NF-IL6 in P19 cells by Western blot analysis or EMSA (data not shown).

Prior to experiments, we anticipated that one transcription factor might affect expression of the other. We found that NF-IL6 did not enhance the expression of the cytomegalovirus (CMV) enhancer/promoter–chloramphenicol acetyltransferase gene, and that p65 did not enhance the expression of EF-1α promoter/enhancer–β-galactosidase fusion gene (data not shown). Therefore, in this experiment we employed the CMV-p50 vector and the CMV-p65 vector, in which the p50 and the p65 cDNAs were placed under the control of the CMV enhancer/promoter, respectively, and the EF-NFIL6 vector, in which NF-IL6 cDNA was placed under the control of EF-1α enhancer/promoter. The efficiency of transfection and of NF-IL6 expression was monitored by cotransfecting EF–β-galactosidase control gene.

Cotransfection with various amounts of the p50 or p65 expression vector alone increased luciferase activity at most only 2-fold in our system (Fig. 2*A*). A combination of CMV-p50 and CMV-p65 increased luciferase activity to a similar extent (data not shown). When the expression vectors for p65 and NF-IL6 were cotransfected simultaneously, a drastic increase in luciferase activity was observed. Similar synergism was observed between p50 and NF-IL6, albeit to a lesser extent.

Since the major form of NF-κB consists of a p50–p65 heterodimer, we next studied the effect of cotransfecting various combination of p50 and p65. Various amounts of the p50 expression vector were cotransfected with a constant amount of the expression vectors for p65 and NF-IL6. In the absence of p50, the highest luciferase activity was observed. As the amount of the p50 expression vector was increased, less luciferase activity was observed, but even when high amounts (2 μg) of p50 were used luciferase activity never fell below that observed in cells transfected with NF-IL6 alone. These findings indicate that the p65 homodimers act most synergistically with NF-IL6, followed by p65/p50 heterodimers and then p50 homodimers.

Both the NF-IL6 and NF-κB Binding Sites in the IL-6 Gene Are Required for the Synergistic Activation. To examine whether both p65 and NF-IL6 need to bind DNA to act synergistically, we constructed two mutant reporter genes, in which either the NF-κB binding site or the NF-IL6 site was disrupted by multiple nucleotide sequence substitutions. As shown in Fig. 3, when the NF-κB binding site was disrupted, the synergistic effect between p65 and NF-IL6 was completely lost. When the NF-IL6 binding site was disrupted, the synergistic effect was dramatically impaired. We conclude that the binding of both p65 and NF-IL6 is required for synergistic transactivation.

Both Transcriptional Activity and DNA Binding Activity of NF-IL6 Are Required for the Synergistic Effect Between NF-IL6 and p65. To characterize the domains of NF-IL6 involved in the synergism, three mutants of NF-IL6 were used. ΔSplNFIL6 is a truncated mutant that can bind to DNA but lacks a transcriptional domain. ΔSplΔSacNFIL6 is a deriv-

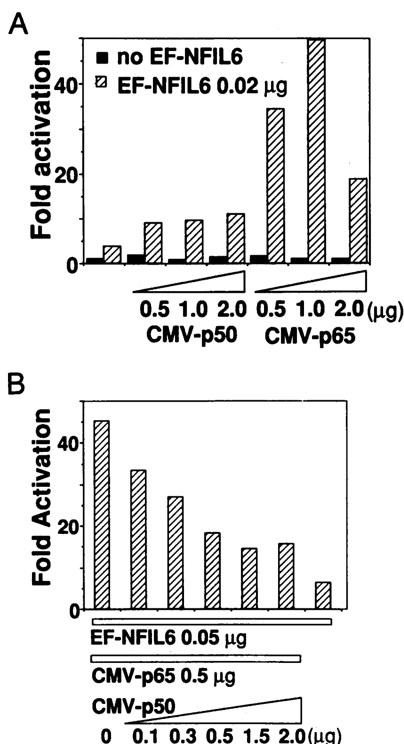

FIG. 2. Synergistic activation of the IL-6 gene by NF-IL6 and NF-κB. (*A*) P19 cells were cotransfected with 5 μg of the IL-6 promoter–luciferase reporter gene, 0.1 μg of the EF–β-galactosidase internal control plasmid, and the indicated amount of the expression vector. Various amounts of either CMV-p50 or CMV-p65 were cotransfected with or without 0.02 μg of EF-NFIL6. (*B*) An increasing amount of CMV-p50 was cotransfected with constant amounts of CMV-p65 (0.5 μg) and EF-NFIL6 (0.05 μg). Transcriptional activity was determined by assaying the luciferase activity of cellular extracts prepared 36 h after transfection. Transfection efficiencies were normalized by analyzing β-galactosidase activity. One unit of relative activity represents the luciferase activity obtained after transfection of the reporter gene only. The experiments were repeated three times, and similar results were obtained. The results of a representative experiment are shown.

ative of ΔSplNFIL6 that lacks both the transactivational domain and the N-terminal half of the leucine-zipper region. NFIL6(S288A) is a site-directed mutant that cannot bind to DNA due to the Ser-288 → Ala substitution. None of these mutants can act cooperatively with p65 (Fig. 4). These data indicate that both the DNA binding and transcriptional activation domains of NF-IL6 are required for synergistic activation with p65.

Direct Interaction Between NF-IL6 and NF-κB p65. The functional cooperative effect between NF-IL6 and NF-κB

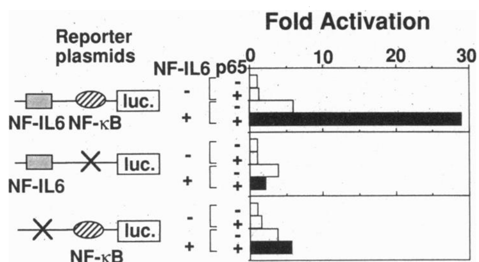

FIG. 3. Effects of disruption of either the NF-κB site or the NF-IL6 site on the synergy between NF-IL6 and p65. P19 cells were cotransfected with 5 μg of indicated reporter plasmid and 0.1 μg of EF–β-galactosidase, with or without 0.02 μg of EF-NFIL6, and with or without 0.5 μg of CMV-p50. Other conditions are the same as those described in Fig. 2.

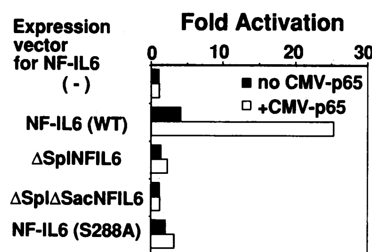

FIG. 4. NF-IL6 requires the transactivating domain and DNA binding ability for synergistic activation with p65. ΔSpl NFIL6 is a truncated mutant that deletes the *Spl* I fragment within the coding sequence. ΔSplΔSacNFIL6 is a derivative of ΔSplNFIL6 that is truncated in addition from Thr-307 to Leu-327 and, consequently, has lost its ability to dimerize. NFIL6 (S288A) was constructed by site-directed mutagenesis to replace Ser-288 of NF-IL6 with Ala, leading to a total loss of DNA binding. The indicated NF-IL6 expression construct at 0.02 μg was cotransfected into P19 cells with 5 μg of the IL-6 promoter–luciferase gene, 0.1 μg of EF–β-galactosidase, and with or without 0.5 μg of CMV-p65. Other conditions are the same as those described in Fig. 2.

p65 described above and a recent report that NF-IL6 associates with NF-κB p50 (19) prompted us to investigate the possibility that NF-IL6 may also interact directly with p65. Fusions between the MBP and full-length NF-IL6, ΔSplNFIL6, ΔSplΔSacNFIL6, and a control MBP protein alone were produced in bacteria. These four proteins were immobilized on amylose resin. Amylose resins containing equal amounts of each protein were incubated with radiolabeled full-length p65 or shorter forms of p65 synthesized by using a cell-free transcription–translation system and crosslinked with dithiobis(succinimidyl propionate). After washing 10 times, the crosslinked complexes were eluted with maltose and analyzed by SDS/PAGE under reducing conditions to cleave the crosslinkages.

Radiolabeled p65 bound to MBP-NFIL6 and to MBP-ΔSplNFIL6 (Fig. 5, lanes 3, 4, and 13) but not to MBP-ΔSplΔSacNFIL6 or to control MBP (lanes 1 and 2). The protein synthesized from the *Bsp*HI-cut template, truncated just outside the Rel homology domain, behaved similar to full-length p65 (lanes 5–8). However, the protein generated with the *Sca* I-cut template truncated within the Rel homology domain and did not associate with any of these fusion proteins (lanes 9–12). We conclude that the NF-IL6 and p65 proteins directly interact via the b-Zip domain and the Rel homology domain.

NF-IL6 and NF-κB Cooperatively Activate Transcription of the IL-8 Gene. We next examined whether the synergistic effect between NF-IL6 and NF-κB is also observed in the

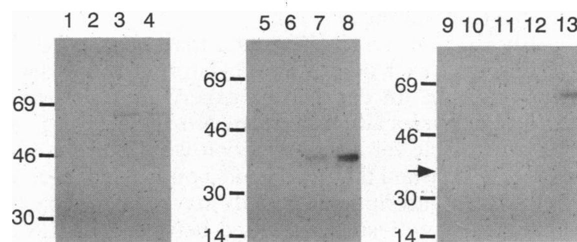

FIG. 5. Crosslinking of *in vitro*-synthesized NF-κB p65 and bacterially expressed NF-IL6 proteins. *In vitro*-translated full-length p65 (lanes 1–4 and 13) or the protein synthesized from the *Bsp*HI-cut template (lanes 5–8) or from the *Sca* I-cut template (lanes 9–12) was mixed with MBP-NFIL6 beads (lanes 4, 8, 12, and 13), MBP-ΔSplNFIL6 beads (lanes 3, 7, and 11), MBP-ΔSplΔSacNFIL6 beads (lanes 2, 6, and 10), or MBP beads (lanes 1, 5, and 9) and then crosslinked with dithiobis(succinimidyl propionate), washed, eluted, and analyzed by SDS/PAGE. The arrow indicates the predicted position of the protein generated from the *Sca* I-cut template.

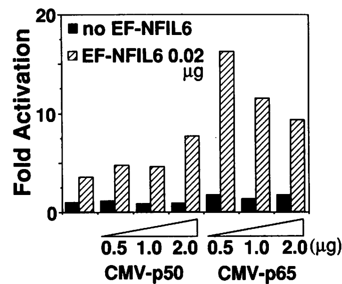

FIG. 6. Synergistic activation of the IL-8 gene by NF-IL6 and NF-κB. P19 cells were cotransfected with 5 μg of the IL-8 promoter–luciferase reporter gene, 0.1 μg of the EF-β-galactosidase internal control plasmid, and the indicated amount of the expression vector. Various amounts of either CMV-p50 or CMV-p65 were cotransfected with or without 0.02 μg of EF-NFIL6. Transcriptional activity was determined as described in Fig. 2.

expression of other genes that contain both NF-IL6 and NF-κB sites. We examined the IL-8 gene that contains adjoining NF-IL6 and NF-κB sites in its promoter and observed a weak synergistic effect between p50 and NF-IL6 and a strong synergistic effect between p65 and NF-IL6 (Fig. 6). This synergism required the integrity of both binding sites (data not shown). This indicates that the synergism between NF-IL6 and NF-κB is not restricted to the IL-6 promoter.

DISCUSSION

In this study we have shown that the NF-κB, especially the p65 subunit, and NF-IL6 cooperatively transactivate the expression of the IL-6 and IL-8 genes and that p65 and NF-IL6 directly associate with each other.

The various homodimeric and heterodimeric NF-κB/Rel-related proteins display preferential binding to slightly different DNA sequence elements. For the IL-6 gene, the κB-like motif GGGATTTCC was recognized by the p50 homodimers, the p50–p65 heterodimers, or the p65 homodimers, as shown in this study. However, in the absence of NF-IL6, NF-κB p50, p65, or any combination of p50 and p65 did not activate the IL-6 gene in our cotransfection systems. In the presence of NF-IL6, NF-κB p50 could enhance weakly, and p65 could enhance strongly, the transcription of the IL-6 gene. Previous reports indicate that the amount of p50 subunit exceeds that of the p65 subunit in resting cells and that the resulting p50 homodimer acts as an inhibitor of transcription (20). We obtained a similar but slightly different result; addition of p50 not only inhibited the cooperative effect of p65 and NF-IL6 but also acted cooperatively with NF-IL6 to stimulate IL-6 expression, although less effectively than p65.

Recently, LeClair *et al.* (19) found that p50 and NF-IL6 directly associate each other via the b-Zip domain and the Rel homology domain. In the present experiment, we demonstrated a direct association between NF-IL6 and p65. The regions responsible for this interaction were also the b-Zip domain of NF-IL6 and the Rel homology domain of p65. The requirement of transcriptional activity may explain in part the weak synergistic activation observed between NF-IL6 and p50 in spite of the strong protein–protein interaction observed between these two factors, as the transcriptional activity of p50 is much lower than that of p65.

During preparation of this manuscript, Stein *et al.* (21) demonstrate functional and physical associations between NF-κB and C/EBP family members. With regard to physical associations, their results are consistent with ours. However, they demonstrate synergistic stimulations of promoters with only C/EBP (NF-IL6) binding site, whereas we found that both binding sites were required for the synergism. This discrepancy may occur because they used a rather artificial reporter gene that is composed of a multimerized c-fos serum response element linked to a "TATA box."

There is accumulating evidence that both NF-IL6 and NF-κB binding sites are important for regulation of many immune response and acute-phase response genes including IL-8 (22), TNF-α (23, 24), IL-1β (25, 26), granulocyte colony-stimulating factor (24, 27), immunoglobulin κ light chain (13, 28), and serum amyloid A-1 (29), in addition to the IL-6 gene. In fact, we have demonstrated a cooperative effect between NF-IL6 and NF-κB proteins on the activation of the IL-8 gene by a transient cotransfection assay. The cooperation between these two factors may have an advantage in amplifying and ensuring the generation of inflammatory signals.

Although inflammatory cytokines are transiently induced during inflammation, constitutive expression of these cytokines is observed during some viral infections. The human T-lymphotrophic leukemia virus 1 constitutively activates IL-6 production via its transactivator protein Tax. The constitutive IL-6 production may be due in part to the activation of NF-κB by Tax as demonstrated in the IL-2 receptor system (30, 31). In addition, we have preliminary data showing that Tax synergistically enhances the activation of the IL-6 transcription by NF-IL6. Therefore, constitutive expression of IL-6 may be mediated by the activation of these two factors via Tax. A recent study has demonstrated that the X product of hepatitis B virus stimulates transcription of the IL-8 gene via both the NF-κB and NF-IL6 binding sites (32). Conversely, both NF-IL6 and NF-κB can bind to the regulatory regions of several viral genomes and, thereby, regulate viral expression. Therefore, if not properly regulated, the two transcription factors, viruses, and viral products may act to create a persistent and vicious cycle, resulting in persistent infection and inflammation. Furthermore, constitutive expression of inflammatory cytokines are observed in other pathological conditions of unknown etiology such as malignancies and autoimmune diseases (33). Therefore, it is highly possible that transcriptional factors controlling genes encoding inflammatory cytokines, including NF-κB and NF-IL6, are constitutively activated in these conditions. Little is known about the molecular mechanisms resulting in such deregulation of cytokine production, but investigation of these mechanisms may lead to a better understanding of the pathogenesis of these diseases.

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