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A Lymphoid Cell-Specific Nuclear Factor Containing c-Rel-Like Proteins Preferentially Interacts with Interleukin-6 κB-Related Motifs Whose Activities Are Repressed in Lymphoid Cells

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The proto-oncoprotein c-Rel is a member of the nuclear factor κB transcription factor family, which includes the p50 and p65 subunits of nuclear factor κB. We show here that c-Rel binds to κB sites as homodimers as well as heterodimers with p50. These homodimers and heterodimers show distinct DNA-binding specificities and affinities for various κB motifs. In particular, the c-Rel homodimer has a high affinity for interleukin-6 (IL-6) and beta interferon κB sites. In spite of its association with p50 in vitro, however, we found a lymphoid cell-specific nuclear factor in vivo that contains c-Rel but not p50 epitopes; this factor, termed IL-6κB binding factor II, appears to contain the c-Rel homodimer and preferentially recognizes several IL-6κB-related κB motifs. Although it has been previously shown that the IL-6κB motif functions as a potent IL-1/tumor necrosis factor-responsive element in nonlymphoid cells, its activity was found to be repressed in lymphoid cells such as a Jurkat T-cell line. We also present evidence that IL-6κB binding factor II functions as a repressor specific for IL-6κB-related κB motifs in lymphoid cells.

Nuclear factor κB (NF-κB) was originally identified as a B-cell-specific nuclear factor binding to an enhancer element called κB of the κ immunoglobulin light-chain gene (58). It is now clear, however, that NF-κB is present in many different types of cells and plays an important role in the inducible expression of diverse cellular genes through binding to various κB sequences. These genes mostly include those involved in host defense, such as genes for various cytokines, interleukin-2 receptor α chains, cell adhesion molecules, major histocompatibility complex (MHC) class I and II antigens, and acute-phase proteins; NF-κB, in addition, binds to κB sites in several viral enhancers such as those of human immunodeficiency virus (HIV), cytomegalovirus, and simian virus 40 (SV40) and is thought to be involved in viral activation (2, 38). Although NF-κB is constitutively present in the nucleus of B cells, NF-κB is an inducible nuclear factor in other types of cells (59). Subsequent studies have shown that NF-κB preexists in the cytoplasm in the inactive form complexed with an inhibitory protein termed IκB; it has been suggested that stimulation of cells by a variety of agents such as phorbol ester, bacterial lipopolysaccharide, virus, double-stranded RNA, interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and human T-cell leukemia virus type 1 (HTLV-1) Tax transactivator results in the dissociation of the IκB/NF-κB complex and subsequent translocation of NF-κB to the nucleus (3, 4). Although the activation of protein kinase A or C and subsequent phosphorylation of IκB has been proposed to be a major activation pathway for NF-κB (19, 61), other studies suggest the involvement of other signal transduction pathways as well (27, 48).

NF-κB contains two proteins of 50 and 65 kDa termed p50 and p65, respectively (5). Although earlier studies showed that only p50 subunits have DNA-binding properties and therefore suggested that the DNA-binding activity of NF-κB is mediated through its p50 portion (5, 32), more recent studies have shown that p65 can also bind DNA apparently as homodimers (53, 56, 64). Thus, these proteins bind to κB sequences primarily as heterodimers (p50/p65) but also bind as homodimers. In particular, the p50 homodimer shows a high affinity for symmetrical κB motifs such as κB sequences of MHC class I gene promoters (6, 34). By contrast, asymmetric prototype κB motifs found in the enhancers of the κ immunoglobulin gene and in the SV40 and HIV viral enhancers are weak binding sites for the p50 homodimer but have a high affinity for the p50/p65 heterodimer (NF-κB) (6, 63). Since p50 has been shown to be identical to a previously characterized constitutive nuclear factor that specifically binds to the MHC κB sequence (34), the p50 homodimer appears to be physiologically present in vivo (6, 34). However, it is not known whether p50 or p65 homodimers have any physiological roles in vivo.

Recent cloning and sequencing of cDNA clones encoding p65 and a 110-kDa precursor of p50 have revealed an extensive sequence homology between these proteins in their N-terminal portions. Surprisingly, the predicted sequences of these proteins are also highly homologous to those of the c-*rel* proto-oncogene and of the *Drosophila* maternal morphogen dorsal gene; in particular, p65 is related to c-Rel more closely than to p50 (9, 20, 21, 47, 53, 56). c-*rel* is the cellular cognate of v-*rel*, the transforming gene of highly oncogenic reticuloendotheliosis virus strain T that induces an acute fatal lymphoma in young birds. Interestingly, the products of the c-*rel* and dorsal genes, in addition to their sequence similarity to p50 and p65, show unique subcellular partitioning, as NF-κB does (21). Furthermore, it has been shown more recently that the c-Rel and v-Rel proteins can recognize some κB sequences (8, 28, 34). Thus, these proteins constitute a distinct family of transcription factors recognizing κB elements and showing unique subcellular partitioning. However, it remains to be established that

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various complexes of the members of this family have different DNA-binding specificities or affinities and transcriptional potentials or are present physiologically in vivo.

In this report, we show that c-Rel homodimers and p50/c-Rel heterodimers show distinct DNA-binding specificities and affinities for various κB motifs. Particularly, the c-Rel homodimer has a strong affinity for a κB motif found in the IL-6 gene promoter, and the p50/c-Rel heterodimer cannot bind to the IL-6κB motif in the presence of the c-Rel homodimer. We also show the presence of a lymphoid cell-specific nuclear factor that is distinct from c-Rel homodimers or p50/c-Rel heterodimers but contains c-Rel epitopes and has an IL-6κB-specific DNA-binding property. Evidence is also presented that this factor might function as a transcriptional repressor specific for the IL-6κB-related motifs in lymphoid cells. IL-6 is a pleiotropic cytokine involved in the regulation of the immune response, hematopoiesis, and inflammation (35). We and others have previously shown that the IL-6κB motif is an important promoter element in the regulation of IL-6 gene expression (42, 60, 68). Thus, the results presented in this paper suggest that c-Rel-related factors play a role in the host defense through the regulation of the IL-6 gene and possibly also of some other cytokine genes having IL-6κB-related promoter elements.

MATERIALS AND METHODS

Cloning of cDNAs encoding human p50 and c-Rel by using the polymerase chain reaction. The following polymerase chain reaction primers were synthesized on the basis of the sequences of human p50 and c-*rel* cDNAs (11, 34): p50 5′ primer, 5′-AATCTAGACTCGCCACCCGGCTTCAGA-3′ (nucleotides 152 to 173 in the 5′ noncoding region; this primer contains an *Xba*I site [underlined]); p50 3′ primer, 5′-TCTCTAGAAAGAGGTTATCCT-3′ (nucleotides 1665 to 1686); c-Rel 5′ primer, 5′-TACATATGGCCTCCGGTGCGT ATAAC-3′ (nucleotides 178 to 199; this primer contains an *Nde*I site [underlined] in the methionine start codon); and c-Rel 3′ primer, 5′-TCGGATCCAAGTTATACTTGAAAA AATT-3′ (nucleotides 2021 to 2043).

Two micrograms of poly(A)$^+$ RNA isolated from HeLa cells was reverse transcribed; 1/10 of the reverse transcripts was run for each reaction as described previously (37), using 35 cycles of 1 min at 90°C, 2 min at 55°C, and 3 min at 70°C. Then 1.5-kb p50 cDNA (nucleotides 152 to 1686, amino acids 1 to 502) and 1.8-kb c-*rel* cDNA (nucleotides 178 to 2043, amino acids 1 to 587) were amplified, gel purified on a 0.7% agarose gel, and cloned into the *Sma*I or *Hin*cII site of the Bluescript pKSII$^+$ vector. In contrast to the previously reported clone isolated from a Daudi cDNA library (11), our c-*rel* cDNA clone did not contain an in-frame 96-bp *Alu* fragment.

Synthesis of human p50 and c-Rel proteins in bacteria and antibody preparation. p50 cDNA cloned into pKSII$^+$ was cut out by *Bam*HI and recloned into the *Bam*HI site of the pET-3b expression vector (55); this p50 expression vector encodes the 63-kDa p50 protein (amino acids 18 to 504 of the p50 precursor), which is larger than the physiological p50 subunit of NF-κB (5). c-*rel* cDNA was cut out by *Nde*I and cloned into the *Nde*I site of the pET-3a expression vector; this plasmid codes for the near-full-length 79-kDa c-Rel protein (amino acids 1 to 582) (11). These expression vectors were introduced into *Escherichia coli* BL21. The transformed bacteria were grown and then induced with 1 mM isopropylthiogalactopyranoside (IPTG) for 2 to 3 h. Most of the p50 protein remained soluble after extraction of harvested bacteria and was purified by DEAE and phosphocellulose column chromatography. The c-Rel protein which was largely in the inclusion body fraction after centrifugation was purified by DEAE-Sephacel column chromatography in the presence of 8 M urea and subsequently renatured as described previously (25). Rabbit antisera were prepared by six subcutaneous injections of 100 μg of proteins with complete Freund's adjuvant at weekly intervals. The specificities of these antisera were established by Ouchterlony and Western immunoblot analysis.

In vitro transcription, translation, and immunoprecipitation. Two micrograms of pBluescript (pKSII$^+$) containing either p50 cDNA (amino acids 1 to 504) or c-*rel* cDNA (amino acids 1 to 587) was linearized with *Eco*RI which recognized *Eco*RI sites in the polylinker of pKSII$^+$. RNAs were synthesized in a 20-μl reaction mixture, using T7 RNA polymerase as recommended by the manufacturer (Promega). Two-microliter samples of RNAs were used for in vitro translation, using a rabbit reticulocyte lysate in a 30-μl reaction mixture containing 25 μCi of ^{35}S-labeled methionine as recommended by manufacturer (Promega). Immunoprecipitation was carried out as described previously (51) in a 100-μl volume containing 1.2 μl of antisera, 40 μl of Immsorbin (Wako Co. Ltd.), and 3 μl of ^{35}S-labeled p50 translated products, 1 μl of c-*rel* products, or 3 μl of cotranslated products of p50 and c-*rel*. The immune complexes were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE).

Analysis of protein-DNA interaction by EMSA. The probes used for the electrophoretic mobility shift assay (EMSA) were prepared from plasmids containing various κB sequences cloned in the multiple cloning site of pBLCAT3 by cutting at the 5′-end *Xba*I and 3′-end *Hin*fI sites and were 3′ end labeled as described previously (60). Nuclear and cytosolic extracts were prepared from unstimulated cells or cells stimulated with IL-1α or TNF-α for 30 min by the methods of Dignam et al. (17) and Baeuerle and Baltimore (3), respectively. IL-6κB binding factor II (BFII) was partially fractioned from Jurkat nuclear extracts by gel filtration on Sephacryl S-400. NF-κB was partially purified from nuclear extracts of TNF-stimulated HeLa cells by DEAE-cellulose, phosphocellulose (P11), and DNA-cellulose column chromatography. EMSA was performed essentially as described previously (60) except that polyvinyl alcohol was omitted. For heterodimerization, recombinant p50 proteins were incubated with recombinant c-Rel proteins at room temperature for 10 min in 20 μl of binding buffer before addition of DNA probes. In the experiments using antibodies, 1 μl of antisera was incubated for 1 h at room temperature with nuclear extracts, purified fractions, or recombinant proteins in the binding buffer prior to addition of DNA probes.

DNA transfection and analysis of gene expression. The chloramphenicol acetyltransferase (CAT) reporter genes containing various κB motifs were constructed by inserting synthetic κB motifs at the 5′ end of the IL-6 TATA element as described before (60). For the expression of Tax proteins, pHTLV-1 Tax, which contains the Tax coding region of HTLV-1 under the control of the HTLV-1 long terminal repeat, was used (52). Mouse L-TK$^-$, HeLa, and Hep3B cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. The human T-cell line Jurkat was maintained in RPMI 1640 medium containing 10% fetal calf serum. Cells were transfected with 2 μg of CAT reporter genes and 1 μg of HTLV-1 Tax expression vectors per plate by the DEAE-dextran method as described

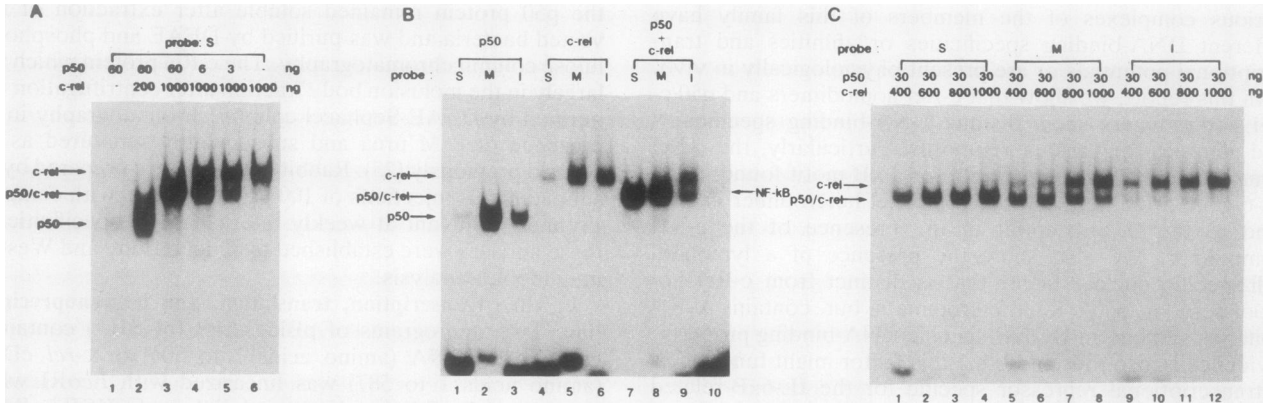

FIG. 1. c-Rel binds various κB motifs as homodimers as well as heterodimers with p50. (A) Formation of c-Rel homodimers and p50/c-Rel heterodimers. Recombinant p50 and c-Rel proteins were incubated with SV40κB probes and analyzed by EMSA. Lanes: 1, p50 (60 ng); 2, p50 (60 ng) and c-Rel (200 ng); 3, p50 (60 ng) and c-Rel (1000 ng); 4, p50 (6 ng) and c-Rel (1,000 ng); 5, p50 (3 ng) and c-Rel (1,000 ng); 6, c-Rel (1,000 ng). (B) Binding specificities of p50 homodimers, p50/c-Rel heterodimers, and c-Rel homodimers for SV40 (S), MHC (M), and IL-6 (I) κB probes. Lanes: 1 to 3, p50 (60 ng); 4 to 6, c-Rel (600 ng); 7 to 9, p50 (60 ng) and c-Rel (600 ng); 10, purified NF-κB from HeLa cells. (C) Differential affinities of p50/c-Rel heterodimers and c-Rel homodimers for SV40 (lanes 1 to 4), MHC (lanes 5 to 8), and IL-6 (lanes 9 to 12) κB probes. Lanes: 1, 5, and 9, p50 (30 ng) and c-Rel (400 ng); 2, 6, and 10, p50 (30 ng) and c-Rel (600 ng); 3, 7, and 11, p50 (30 ng) and c-Rel (800 ng); 4, 8, and 12, p50 (30 ng) and c-Rel (1,000 ng).

previously (44). For TNF stimulation, cells were incubated with recombinant TNF-α (34 ng/ml) for 24 h before being harvested for determination of CAT activity (23).

RESULTS

c-Rel homodimers and p50/c-Rel heterodimers show distinct DNA-binding specificities and affinities for various κB motifs. The human p50 protein containing the N-terminal DNA-binding portion (amino acids 18 to 504) and near-full-length c-Rel protein (amino acids 1 to 582) were synthesized in bacteria by using the pET3 expression system. The purified recombinant p50 and c-Rel proteins had apparent sizes of 63 and 79 kDa, respectively, on SDS-PAGE. The recombinant p50 is larger than the physiological p50 subunit (5), since the p50 precursor is presumably processed at sites more N terminal to amino acid 504 of p50. To examine whether c-Rel recognizes κB motifs as a homodimer as well as a heterodimer with p50, we used the prototype κB sequence (SV40κB, GGGGACTTTCCAC) found in the κ immunoglobulin, HIV, and SV40 enhancers as a probe for EMSA. As shown in Fig. 1A (lane 6), the c-Rel protein formed a DNA/protein complex, the mobility of which was considerably slower than that of the p50/DNA complex (lane 1), being consistent with the larger size of the c-Rel protein. Surprisingly, without combined renaturation, mixing at room temperature for 10 min of p50 and c-Rel proteins with κB probes resulted in the formation of the DNA/protein complex, the mobility of which was intermediate between those of p50 and c-*rel* DNA/protein complexes (lane 2 to 5) and was comparable with that of HeLa-derived NF-κB (Fig. 1B, lane 10). These results indicate that c-Rel proteins can bind DNA as a homodimer but also as a heterodimer with p50. In addition, the results of competition experiments fully confirmed DNA-binding specificities of the c-Rel-mediated nucleoprotein complex formation (data not shown). Figure 1A also shows that the relative amounts of the p50/c-Rel heterodimers formed were dependent on the relative amounts of p50 and c-Rel proteins present in the incubation mixture: 10 times more c-Rel proteins were required for equivalence in p50/c-Rel heterodimer formation (600 to 1,000 ng of c-Rel versus 60 ng of p50) (Fig. 1A, lanes 2 to 5). This result can be explained by the fact that bacterially synthesized c-Rel proteins are not completely renatured after resolubilization; bacterial p50 proteins, by contrast, remain soluble, and the purification of p50 does not require a solubilization step. Alternatively, the relative inefficiency of c-Rel proteins may be an intrinsic property.

We then tested whether c-Rel homodimers and c-Rel/p50 heterodimers show different DNA-binding specificities or affinities for different κB sequences. We used MHCκB (GGGGATTCCCC) and IL-6κB (GGGATTTTCCC) motifs in addition to SV40κB as probes and used fixed amounts of p50 (60 ng) and c-Rel (600 ng). Figure 1B shows representative EMSA results. As previously shown (34, 63), the p50 homodimer had a higher affinity for symmetrical κB motifs such as the MHCκB motif than for the asymmetrical SV40κB motif; the affinity of p50 for IL-6κB was intermediate between them (lanes 1 to 3). The c-Rel homodimer also showed only weak binding to the asymmetrical SV40κB motif (lane 4). However, the p50/c-Rel heterodimer showed comparable binding to both symmetrical (MHCκB) and asymmetrical (SV40κB) motifs (lanes 7 and 8). Interestingly, while the p50/c-Rel heterodimer/DNA complexes were predominantly formed with MHC and SV40 κB probes (lanes 7 and 8), significant amounts of the c-Rel homodimer/DNA complex in addition to the p50/c-Rel heterodimer/DNA complex were formed with an IL-6κB probe under the same conditions (lane 9). These results were also confirmed by cross-competition experiments using recombinant p50 and c-Rel proteins and SV40, MHC, and IL-6κB sites (data not shown). Thus, the c-Rel homodimer appears to recognize the IL-6κB motif with a higher affinity than does the p50/c-Rel heterodimer. In addition, the κB motif found in the beta interferon promoter (IFκB, GGGAAATTCC) (39) was also found to have a higher affinity for c-Rel homodimers than for p50 homodimers or p50/c-Rel heterodimers (data not shown). To pursue this issue further, the fixed amount of p50 (30 ng) and increasing amounts of c-Rel (400 to 1,000 ng) were incubated with various κB probes. As shown in Fig. 1C, c-Rel homodimer/DNA complexes were not readily visible with the SV40κB probe even at the input of 1,000 ng

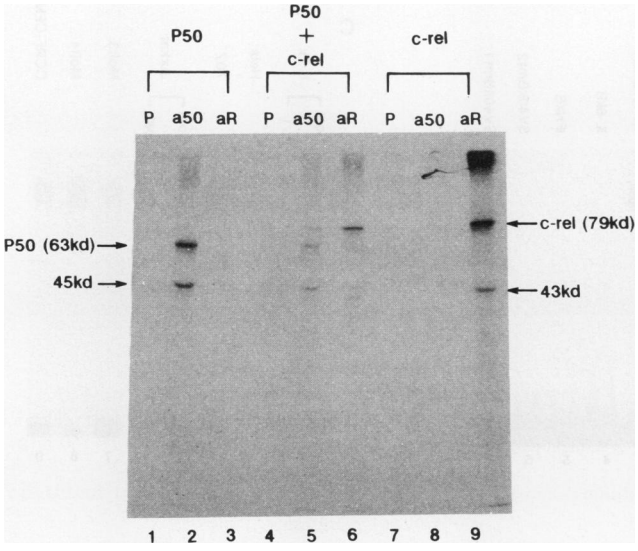

FIG. 2. Association of c-Rel with p50 in the absence of DNA-binding sites. The *c-rel* and p50 cDNAs were translated alone or cotranslated together in reticulocyte lysates that contained [^{35}S]methionine. The in vitro-translated p50 (lanes 1 to 3), p50 and c-Rel (lanes 4 to 6), and c-Rel (lanes 7 to 9) were subjected to immunoprecipitation with preimmune sera (P; lanes 1, 4, and 7), anti-p50 antisera (a50; lanes 2, 5, and 8), or anti-c-Rel antisera (aR; lanes 3, 6, and 9). Immune complexes were analyzed on SDS–10% polyacrylamide gels.

of c-Rel (lane 4). By contrast, even at the input of 400 ng of c-Rel, significant p50/c-Rel heterodimer/DNA complexes were not seen with the IL-6κB probe (lane 9). The MHCκB probe appeared to have comparable affinities for the p50/c-Rel heterodimer and c-Rel homodimer (lanes 5 to 8). Thus, the results of these experiments indicate that the c-Rel homodimer preferentially binds to some κB motifs such as IL-6κB and IFκB.

We also examined whether p50 and c-Rel proteins can associate in the absence of DNA-binding sites. After in vitro transcription and translation, association was evaluated by coimmunoprecipitation using anti-p50 or anti-c-Rel antisera. As shown in Fig. 2, p50 proteins (63 kDa) translated in vitro were specifically recognized by anti-p50 (lane 2) but not by anti-c-Rel (lane 3) antisera; a minor component (45 kDa) which was also recognized by anti-p50 antisera (lane 2) is probably a prematurely terminated product. Anti-c-Rel antisera also specifically reacted with in vitro-translated c-Rel proteins (79 kDa) and a 43-kDa prematurely terminated product (lane 9). Thus, antisera prepared against recombinant p50 and c-Rel proteins were specific and did not cross-react each other despite considerable homology in their N-terminal portions. However, both antisera coimmunoprecipitated p50 and c-Rel proteins from the reaction mixture containing p50 and c-Rel (lanes 5 and 6), indicating the formation of p50/c-Rel heterodimers in the absence of DNA. Interestingly, anti-p50 antisera coimmunoprecipitated the 43-kDa c-Rel product in the presence of p50 and anti-c-Rel antisera coimmunoprecipitated the 45-kDa p50 product in the presence of c-Rel (lanes 5 and 6), suggesting that these smaller p50 or c-Rel proteins can heterodimerize. The formation of heterodimers and homodimers in the absence of DNA was also confirmed by glutaraldehyde cross-linking experiments (data not shown).

A lymphoid cell-specific nuclear factor specifically recognizing IL-6κB-related motifs contains c-Rel epitopes. Since expression of the c-*rel* gene has been shown to be much higher in lymphoid cells than in nonlymphoid cells (10, 12) and since the c-Rel homodimer shows a high affinity for the IL-6κB site (Fig. 1), we examined whether c-Rel homodimers could be detected in nuclear extracts from lymphoid cells by using IL-6κB probes. We prepared nuclear extracts from various lymphoid and nonlymphoid cells and analyzed them by EMSA. A representative EMSA pattern seen with nuclear extracts from Jurkat helper T cells is shown in Fig. 3A. As shown previously (45, 54), stimulation of Jurkat cells with TNF-α resulted in the induction of κB-binding factors (termed NF-κB) which had the mobility comparable to that of HeLa-derived NF-κB; the results of epitope analysis indicated that Jurkat NF-κB is mostly a p50/p65 heterodimer (see Fig. 5B); Jurkat NF-κB recognized IL-6 and SV40 κB motifs with comparable affinities (Fig. 3A, lanes 1 to 4). The IL-6κB probe, in addition, formed the DNA/protein complex, the mobility of which was considerably slower than those of nucleoprotein complexes mediated by NF-κB (lanes 1 and 2) or by the c-Rel homodimer (data not shown). This factor is present constitutively and tentatively termed IL-6κB BFII. Since IL-6κB BFII has a higher affinity for multimerized IL-6κB sites than for a single site (lanes 5 and 6), we used multimerized (three copies) IL-6κB probes in the subsequent experiments. Figure 3B shows the results of competition analysis. The DNA/protein complex formed by IL-6κB BFII and IL-6κB was specifically abolished by IL-6κB (lane 4) and IFκB (lane 5) but not significantly by SV40κB (lane 2) and MHCκB (data not shown). As shown in Table 1, IL-6 and IFκB sequences both have 5-bp AT-rich sequences in the middle of their κB motifs, but SV40κB has a cytosine residue in these AT-rich sequences. We therefore constructed several mutants of the SV40κB motif (Table 1) and tested them in competition experiments. Interestingly, the SV40κB mutants having mutated central AT-rich sequences (mt2 and mt7) competed for IL-6κB BFII-mediated complex formation (Fig. 3B, lane 6, and unpublished data), indicating that central AT-rich sequences are important for the interaction of IL-6κB BFII with DNA. We also analyzed the IL-6κB BFII activity in various types of cells. The results (Fig. 3C and 4) showed that the presence of IL-6κB BFII is specific for lymphoid cells. However, some deviations from this conclusion were noted: nuclear extracts from several human (KM-1, KM-2, and AMO-1) and mouse (MPC-11) myeloma cell lines as well as from phagocytic cell lines (HL-60 and U937) showed reduced IL-6κB BFII activity (Fig. 4).

The results that IL-6κB BFII recognizes specifically IL-6κB and is present only in lymphoid cells (Fig. 3 and 4) suggest that IL-6κB BFII is related to c-Rel. To further clarify this point, we tested the reactivity of IL-6κB BFII with anti-c-Rel antisera by EMSA. As expected from the results of immunoprecipitation analysis with anti-p50 and anti-c-Rel antisera (Fig. 2), anti-p50 reacted only with p50 and supershifted the recombinant p50 homodimer/DNA complex (some inhibition of p50 binding to DNA was also observed) (Fig. 5A, lanes 2 and 8); anti-c-Rel antisera specifically supershifted the recombinant c-Rel homodimer/DNA complex (lanes 3 and 9), and both anti-c-Rel and anti-p50 antisera supershifted the recombinant p50/c-Rel heterodimer/DNA complexes (lanes 5 and 6). In addition, anti-p50 antisera strongly reacted with purified HeLa NF-κB (Fig. 5B, lane 2) and NF-κB from IL-1-stimulated Hep3B cells (lane 5) or from TNF-stimulated Jurkat cells (lane 8).

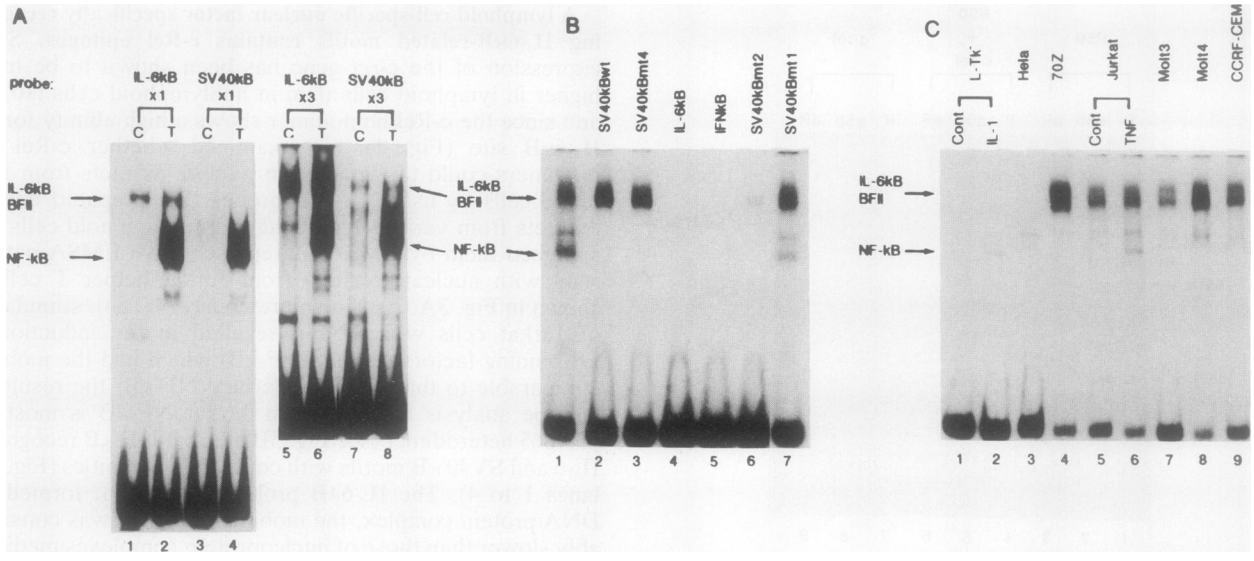

FIG. 3. Detection of an IL-6κB-specific nuclear factor (IL-6κB BFII) in lymphoid nuclear extracts. (A) EMSA of Jurkat nuclear extracts from unstimulated (C) and TNF-stimulated (T) cells with IL-6κB or SV40κB probes (×1 and ×3 indicate probes containing one and three copies of κB motifs, respectively). DNA/protein complexes mediated by IL-6κB BFII and NF-κB are indicated by arrows. (B) Competition binding analysis. The IL-6κB ×3 probe, nuclear extracts from TNF-α-stimulated Jurkat cells, and the following fragments (100 ng) containing various multimerized (×3) κB sequences were used in competition analysis: lane 1, none; lane 2, wild-type SV40κB; lane 3, SV40κB mt4; lane 4, IL-6κB; lane 5, IFκB; lane 6, SV40κB mt2; lane 7, SV40κB mt1 (see Table 1 for sequences). (C) Analysis of IL-6κB BFII activity in a variety of lymphoid and nonlymphoid cells. Nuclear extracts from various cells were analyzed by EMSA by using multimerized IL-6κB probes. In some analyses, nuclear extracts from unstimulated (Cont) as well as IL-1- or TNF-stimulated cells were used.

By contrast, anti-c-Rel antisera barely reacted with HeLa or Hep3B NF-κB (lanes 3 and 6) and showed only minimal reactivity with Jurkat NF-κB (lane 9). The results of epitope analysis on IL-6κB BFII with these antisera are shown in Fig. 5C. Only anti-c-Rel antisera blocked significantly (60 to 80% inhibition) the formation of the IL-6κB BFII/DNA complex in repeated analyses, indicating that IL-6κB BFII contains c-Rel but not p50 epitopes.

Differential responsiveness of the IL-6κB-related motifs to cytokines and HTLV-1 Tax in lymphoid and nonlymphoid cells. We have previously shown that the IL-6κB motif can function as a potent IL-1–TNF-responsive promoter element in nonlymphoid cells such as L-TK$^-$ and HeLa cells when inserted upstream of TATA elements (60). Since the foregoing results show that a c-Rel-related factor (IL-6κB BFII) specifically recognizing IL-6κB is present in lymphoid cells (Fig. 3 to 5), we were prompted to examine the in vivo activity of IL-6κB in lymphoid cells. Thus, a series of CAT reporter constructs in which multimerized (three copies) SV40, MHC, and IL-6 κB sequences were inserted at the 5′ end of the IL-6 TATA element were transfected into either nonlymphoid L-TK$^-$ or Jurkat T cells, and response to

TABLE 1. Summary of sequence comparison and of DNA-binding and in vivo activities of various κB motifs[a]

κB motif	Sequence	DNA binding			In vivo response	
		c-Rel[b]	IL-6κB BFII	NF-κB	L-TK$^-$	Jurkat
SV40κB						
Wild type	TGGGGACTTTCCAC	±	−	++	++	++
mt4	TGGGGACTTTCC**ca**		−	++	++	++
mt5	**ga**GGGACTTTCCAC		−	++	++	++
mt1	TG**tt**GACTTTCCAC	−	−	−	−	−
mt3	TGGGGACTTT**gg**AC		−	−	−	−
mt6	TGGG**at**CTTTCCAC		−	−	−	−
mt2	TGGGGA**t**TTTCCAC	+	+	+	++	−
mt7	TGGGGA**aa**TTCCAC		+	+	+	−
IL-6κB	TGGGATTTTCCCA	+++	++	++	++	−
IFκB	TGGGAAATTCCTC	+++	++	+	+	−
MHCκB	TGGGGATTCCCCA	+	±	++	++	++
Other IL-6κB related[c]						
IL-1β (15)	GGGAAAATCC					
IL-8 (50)	TGGAATTTCC					
IP-10 (46)	GGGAAATTCC					

[a] The results shown in Fig. 1, 3, 4, 5, and 6 and unpublished data are summarized.
[b] The ability of each sequence to bind to c-Rel homodimers in the presence of p50/c-Rel heterodimers (Fig. 1) is indicated by ± to +++.
[c] References are given in parentheses.

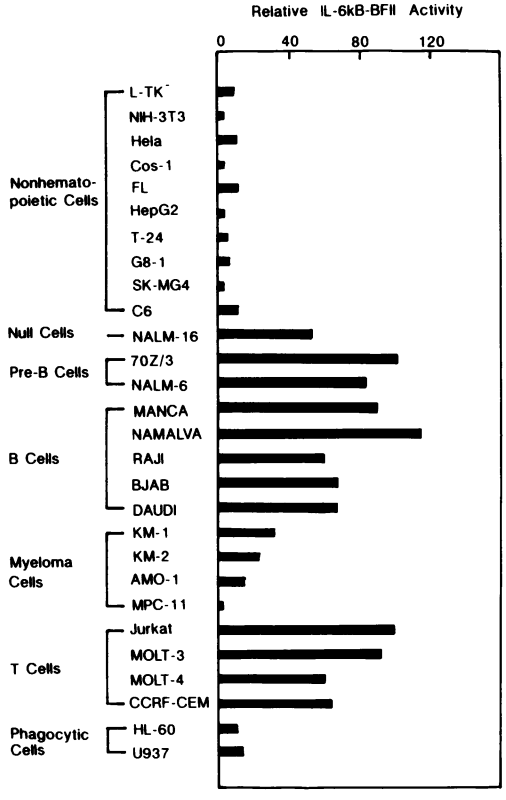

FIG. 4. Summary of results of extensive analyses on IL-6κB BFII in various cells. IL-6κB BFII activities were expressed relative to the IL-6κB BFII activity in Jurkat cells, which was set at a value of 100.

TNF-α stimulation or to cotransfection of the HTLV-1 Tax expression vector was determined by measuring CAT activity. As shown previously (60), all of the IL-6CAT constructs containing IL-6, SV40 (Fig. 6B), or MHC (not shown) κB sequences showed comparable responses to TNF-α and Tax (Fig. 6B) or to IL-1α (not shown) in L-TK⁻ and HeLa cells (data not shown). Surprisingly, however, while the CAT reporter genes containing SV40 or MHCκB motifs responded effectively to TNF-α as well as to Tax in Jurkat cells, the CAT reporter gene containing IL-6κB was virtually unresponsive to these stimuli in Jurkat cells: weak but significant induction by Tax of the IL-6κB CAT reporter gene was noted (Fig. 6A). To determine sequence specificities involved in the lymphoid cell-specific unresponsiveness, we constructed CAT reporter genes containing mutated SV40κB sequences (Table 1) and tested these constructs for their in vivo responsiveness to TNF-α in L-TK⁻ and Jurkat cells. The results (Fig. 7; summarized in Table 1) showed that (i) the constructs containing mutated SV40κB sequences which are defective in NF-κB binding (mt1, mt3, and mt6) (Fig. 3B, lane 7) were totally inactive both in L-TK⁻ and Jurkat cells; (ii) SV40κB mt4 and mt5, the binding specificities of which are similar to those of the wild-type SV40κB (Fig. 3B, lanes 2 and 3), responded to TNF-α in both L-TK⁻ and Jurkat cells, though the sequence (TGGGGACTTTC CCA) of mt4 closely resembles of that of IL-6κB (TGG GATTTTCCCA); and (iii) the mutants having substituted middle AT-rich sequences (mt2 and mt7) recognized IL-6κB BFII (Fig. 3B, lanes 6) and responded to stimuli in L-TK⁻ cells but not in Jurkat cells. Thus, the in vivo unresponsiveness of several κB sequences to TNF-α and Tax in Jurkat cells appears to be correlated to their ability to bind IL-6κB BFII.

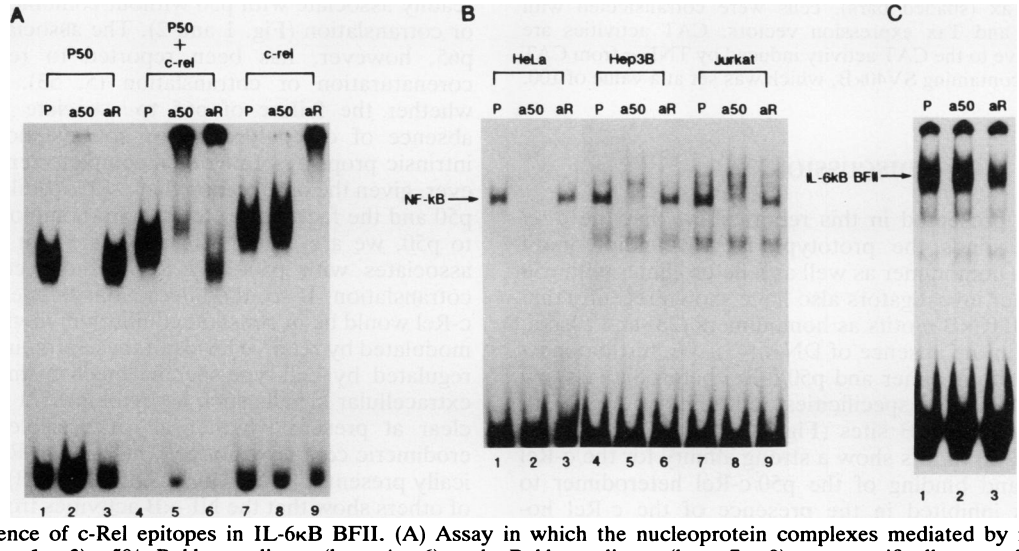

FIG. 5. Presence of c-Rel epitopes in IL-6κB BFII. (A) Assay in which the nucleoprotein complexes mediated by recombinant p50 homodimers (lanes 1 to 3), p50/c-Rel heterodimers (lanes 4 to 6), and c-Rel homodimers (lanes 7 to 9) were specifically supershifted by anti-p50 (a50; lanes 2, 5, and 8) or anti-c-Rel (aR; lanes 3, 6, and 9) antisera. As a control, preimmune sera (P; lanes 1, 4, and 7) were used. (B) NF-κB activities from various sources reacted with anti-p50 antisera. Either partially purified HeLa NF-κB (lanes 1 to 3) or nuclear extracts from IL-1-stimulated Hep3B cells (lanes 4 to 6) and from TNF-stimulated Jurkat cells (lanes 7 to 9) were incubated at room temperature for 30 min with 1 μl of preimmune sera, anti-p50 antisera, or anti-c-Rel antisera prior to addition of probes. The SV40κB (×1) probe was used in the experiments shown in panels A and B. (C) Inhibition of IL-6κB BFII activity by anti-c-Rel but not anti-p50 antisera. IL-6κB BFII partially fractioned from Jurkat nuclear extracts was incubated with 1 μl of preimmune sera (lane 1), anti-p50 antisera (lane 2), or anti-c-Rel antisera (lane 3) prior to addition of IL-6κB (×3) probes. Preimmune sera from rabbits immunized with c-Rel or p50 were tested, and the results for preimmune sera from the rabbits immunized with c-Rel are shown.

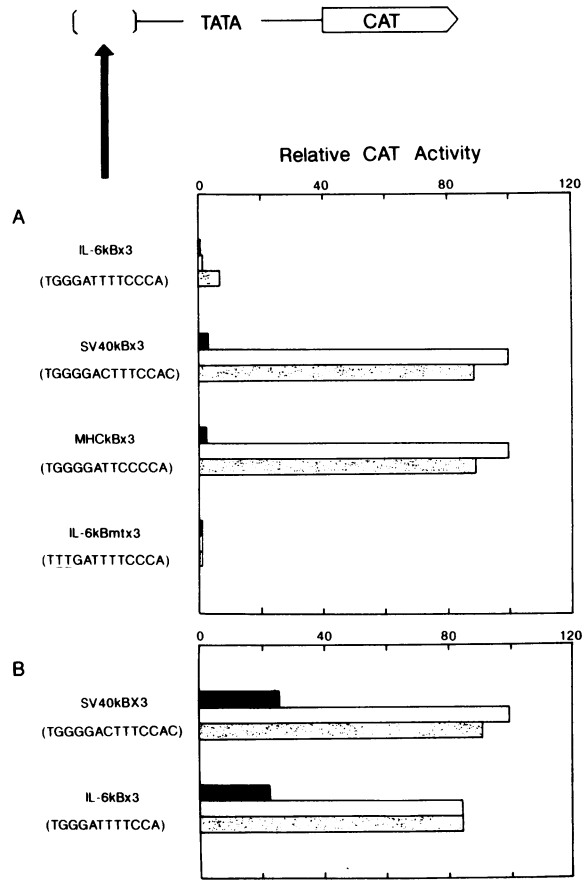

FIG. 6. Differential responsiveness of the IL-6κB motif to TNF-α and HTLV-1 Tax in Jurkat (A) and L-TK⁻ (B) cells. After transfection of various reporter genes, cells were stimulated with TNF-α for 24 h (open bars) or were unstimulated (closed bars); for induction by Tax (shaded bars), cells were cotransfected with reporter genes and Tax expression vectors. CAT activities are expressed relative to the CAT activity induced by TNF-α from CAT reporter genes containing SV40κB, which was set at a value of 100.

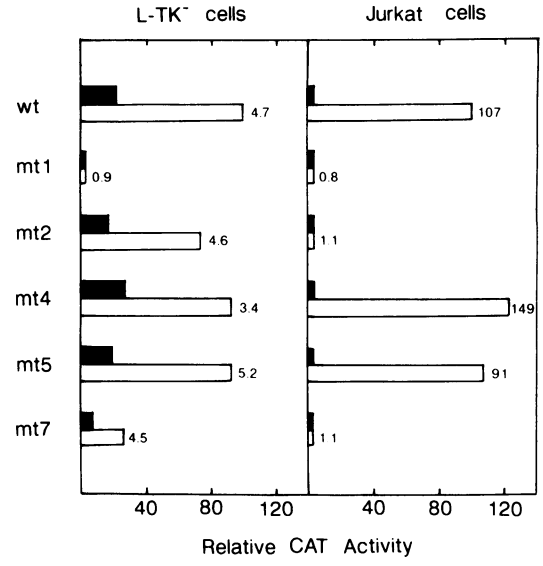

FIG. 7. Responsiveness of various SV40κB mutants to TNF-α in Jurkat and L-TK⁻ cells. CAT reporter genes containing two copies of various SV40κB mutant sequences (see Table 1) were transfected into Jurkat or L-TK⁻ cells. The degrees of TNF-α activation (fold induction) are indicated by numbers at the right of the bars.

DISCUSSION

The results presented in this report show that the c-*rel* gene product binds the prototype NF-κB-binding motif (SV40κB) as a homodimer as well as a heterodimer with p50 (Fig. 1A). Other investigators also have shown recently that c-Rel binds MHCκB motifs as homodimers (28) and associates with p50 in the absence of DNA (43). We further show that the c-Rel homodimer and p50/c-Rel heterodimer show different DNA-binding specificities and affinities for SV40, MHC, IL-6, and IF κB sites (Fig. 1B). In particular, the IL-6κB and IFκB motifs show a strong affinity for the c-Rel homodimer, and binding of the p50/c-Rel heterodimer to these sites is inhibited in the presence of the c-Rel homodimer (Fig. 1C). By contrast, the SV40κB motif is a weak binding site for p50 and c-Rel homodimers but binds effectively to the p50/c-Rel heterodimer. Although the p50 homodimer shows a very strong affinity for MHCκB, the extensive binding of p50 homodimer to MHCκB was not observed in the presence of c-Rel (Fig. 1). The previous studies also showed that the p50 homodimer and p50/p65 heterodimer (NF-κB) have distinct DNA-binding specificities and affinities for various κB motifs (63). Thus, the association of p50, p65, and c-Rel in various combinations theoretically should provide the complexes with diverse but distinct DNA-binding specificities and affinities for various κB motifs and possibly with different transcriptional activation potentials. It is not surprising, therefore, that the in vivo activities of various κB elements are differentially regulated in different types of cells, through variations in relative nuclear levels of p50, p65, and c-Rel in the cells.

One of the surprising findings in this study is that c-Rel can readily associate with p50 without combined corenaturation or cotranslation (Fig. 1 and 2). The association of p50 with p65, however, has been reported to require combined corenaturation or cotranslation (5, 53). It is not clear whether the failure of p65 to associate with p50 in the absence of corenaturation or cotranslation is due to an intrinsic property of p65 or incomplete renaturation. However, given the observation that c-Rel readily associates with p50 and the fact that c-Rel is more homologous to p65 than to p50, we are tempted to speculate that p65 also readily associates with p50 or c-Rel without corenaturation or cotranslation. If so, the interactions between p50, p65, and c-Rel would be in constant equilibrium in vivo and would be modulated by relative levels of these proteins, which may be regulated by cell-type-specific mechanisms as well as by extracellular signals such as cytokines. It is not, however, clear at present whether all of possible homo- or heterodimeric complexes of p50, p65, and c-Rel are physiologically present in vivo. Our results (Fig. 5B) as well as those of others show that the NF-κB activities from HeLa, Jurkat, Hep3B, and U937 cells are largely the complex of p50 and p65 and do not incorporate a significant amount of c-Rel proteins (1, 57). By contrast, Inoue et al. (28) showed that c-Rel is a part of NF-κB activities in pre-B cells. In addition, we show in this study that the lymphoid cell-specific nuclear factor IL-6κB BFII contains c-Rel but not p50 epitopes (Fig. 5C) (see below). Thus, there appears to be some preference in the interactions between p50, p65, and c-Rel in different types of cells.

As discussed above, the association of c-Rel with p50 is not readily detectable in T cells in vivo, through c-Rel proteins are generally more abundant in lymphoid cells (10, 12) and can readily associate with p50 in vitro (Fig. 1). This finding raises the possibility that c-Rel and p50 form discrete complexes in vivo. By using IL-6κB probes which have a high affinity for the c-Rel homodimer, we found the presence of a lymphoid cell-specific nuclear factor(s) (IL-6κB BFII) that specifically recognizes IL-6κB and some other related κB motifs (Fig. 3 and 4). IL-6κB BFII has not been detected previously, probably because of the lack of appropriate probes such as IL-6κB. The following observations indicate that IL-6κB BFII is a c-Rel-related factor. First, and most important, the DNA-binding activity of IL-6κB BFII was inhibited by anti-c-Rel antibodies (Fig. 5C). Incomplete inhibition by anti-c-Rel antibodies is presumably due to multiprotein complex formation as discussed below, but it is also possible that a fraction of IL-6κB BFII contains c-Rel-like factors, the antigenicity of which is distinct from that of c-Rel. In addition, lack of reactivity of IL-6κB BFII with anti-p50 antisera indicates that IL-6κB BFII does not contain significant amounts of p50 or its 110-kDa precursor. Second, IL-6κB BFII and c-Rel homodimers have similar DNA-binding specificities and bind preferentially to subsets of κB motifs such as IL-6 and IF κB motifs which have 5-bp AT-rich sequences in the middle of binding sites (Fig. 1, 3, and 4; Table 1). Third, the distributions of IL-6κB BFII and c-Rel in various types of cells are strikingly similar: their expression is primarily limited to lymphoid cells (Fig. 4) (10, 12). Interestingly, however, both IL-6κB BFII and c-Rel are present only at low levels in myeloma and phagocytic cell lines; MPC-11 cells, in particular, show only a trace of the IL-6κB BFII activity and c-*rel* mRNA (Fig. 4) (12, 24, 36). Taken together, these observations indicate that c-Rel homodimers are the integral part of IL-6κB BFII. However, it is unlikely that IL-6κB BFII is the c-Rel homodimer itself, since the mobility of the nucleoprotein complex mediated by IL-6κB BFII on EMSA is considerably slower than that of the c-Rel/DNA complex. In fact, the size of IL-6κB BFII estimated by gel filtration (>660 kDa) (unpublished data) is much larger than the estimated size of the c-Rel homodimer (<200 kDa). These results are also in agreement with the results of previous studies showing that the c-Rel protein associates with other cellular proteins in avian lymphoid cells (16, 36, 49). Interestingly, two different c-Rel complexes have been identified; the larger complex (400 to 800 kDa) lacked the p34-40 protein (16, 36), which is thought to be a chicken IκB homolog. IκB inhibits the DNA-binding activity of NF-κB and is thought to block its translocation to the nucleus, presumably through the p65 subunit (5). IκB likely interacts also with c-Rel and blocks its activity, since c-Rel is more homologous to p65 than to p50. Thus, we postulate that while a major part of c-Rel synthesized remains in the cytoplasm in a form complexed with IκB, a portion moves to the nucleus and associates with other cellular proteins to form IL-6κB BFII. The results that we could not detect IL-6κB BFII activity in the cytosol fraction and that IL-6κB BFII activity is sensitive to deoxycholate (unpublished data) further support this model. However, the precise subunit composition of IL-6κB BFII is not clear at present. Although it has been suggested that one of two large proteins (115 and 124 kDa) associated with c-Rel corresponds to the precursor of p50 (21), our results (Fig. 5C) do not support this possibility. In addition, Ballard et al. (8) have recently reported a novel Rel-related inducible nuclear factor. However, this factor appears to be distinct from

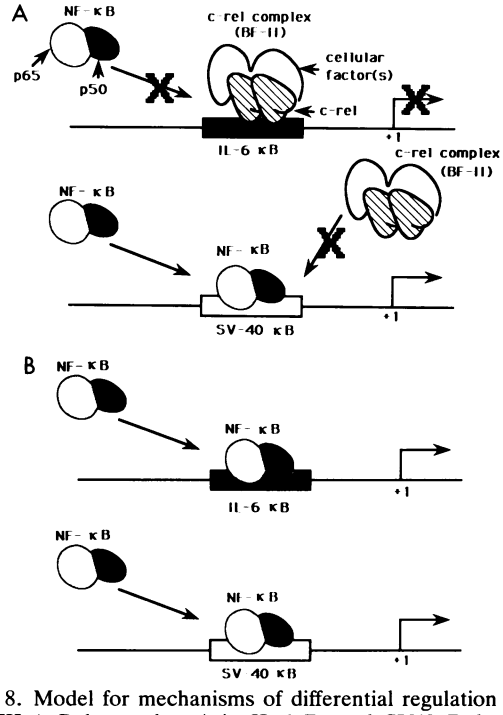

FIG. 8. Model for mechanisms of differential regulation by IL-6κB BFII (c-Rel complexes) in IL-6κB- and SV40κB-dependent transcriptional activation in lymphoid (A) and nonlymphoid (B) cells. It is postulated that while a p50/p65 heterodimer (NF-κB) is a major positive transcriptional factor, IL-6κB BFII is a constitutive IL-6κB specific repressor. IL-6κB BFII is depicted as a complex in which c-Rel homodimers function as a DNA-binding subunit of this complex. Because of the higher affinity of IL-6κB BFII for IL-6κB sites, NF-κB cannot displace IL-6κB BFII from IL-6κB sites and cannot activate transcription.

IL-6κB BFII, since IL-6κB BFII is a constitutive factor. Further work is required to establish the molecular structure of IL-6κB BFII.

We have previously shown that IL-6κB functions as a potent IL-1/TNF-responsive element in nonlymphoid cells (60). However, as shown in the present study, IL-6κB was virtually inactive in the TNF response in Jurkat T cells, though weak and significant transactivation of IL-6κB CAT reporter genes by HTLV-1 Tax was observed (Fig. 6). This unresponsiveness of IL-6κB in Jurkat cells cannot be ascribed to a lack of reactivity of IL-6κB with Jurkat NF-κB, since IL-6 and SV40 κB sites have comparable affinities for Jurkat NF-κB (Fig. 3A and unpublished data). Mutational analysis of the SV40κB motif revealed that the SV40κB mutants (mt2 and mt7) containing 5-bp AT-rich sequences bind IL-6κB BFII and behave like IL-6κB in TNF response in Jurkat cells (Fig. 7 and Table 1). Thus, the ability of various κB sequences to bind IL-6κB BFII appears to be correlated to their unresponsiveness to TNF in Jurkat cells, as summarized in Table 1. Although the MHCκB motif shows a weak affinity for IL-6κB BFII (Table 1) but functions as a TNF- and Tax-responsive element in Jurkat cells (Fig. 6), this result is explained by the fact that MHCκB sites have a much higher affinity for NF-κB. In IL-6κB-related sites such as IL-6κB, IFκB, SV40 mt2, and SV40 mt7, however, NF-κB could not displace IL-6κB BFII from these sites because of the higher affinity of IL-6κB BFII for these sites; IL-6κB BFII thereby could suppress the NF-κB-mediated transcription (Fig. 8). Although our model (Fig. 8)

postulates that the c-Rel-related IL-6κB BFII functions as a repressor for IL-6κB-related motifs, others have shown that turkey or mouse c-Rel has transcriptional activation domains (13, 30) and that cotransfection of mouse c-*rel* expression vectors with reporter genes containing six copies of MHCκB sites results in significant transactivation of reporter genes in nonlymphoid F9 or NIH 3T3 cells (28). Perhaps the transcriptional activity of c-Rel would be differentially regulated in different types of cells through interaction with other cellular factors as discussed above. The dorsal protein, a member of the NF-κB/Rel family (29, 62), and v-Rel, the viral counterpart of c-Rel (8, 18, 28), can indeed function both as positive and negative regulators. There are also other examples of transcription factors with dual activities (14, 22, 40). Thus, despite its transcriptional activating potentials, c-Rel may also function as a negative regulator.

Although IL-6 is produced by diverse types of cells (35), IL-6 production by normal T and B cells appears to be rare (31, 65). However, aberrant IL-6 production in several malignant cells of T- or B-cell lineages and its relationship to several diseases, including malignancy, have been reported (26, 33, 35, 65, 67). In particular, IL-6 has been purified, and its cDNA has been cloned from HTLV-1-infected T-cell lines (26). In the present study, we have shown that while the IL-6κB motif is virtually unresponsive to TNF in Jurkat T cells, the expression of HTLV-1 Tax results in a significant, albeit low, activation of IL-6κB reporter genes. In addition, we observed that an IL-6 promoter/CAT reporter gene having a defective IL-6κB site is unresponsive to Tax in Jurkat cells (unpublished data). These results suggest that aberrant IL-6 production by HTLV-1-infected T cells (26, 65) is mediated by Tax through IL-6κB as in the IL-2 receptor α gene activation by Tax, which is dependent on the IL-2 receptor κB motif (7, 41). However, it is not clear at present how Tax alleviates the repression of the IL-6κB activity in Jurkat cells, though TNF and Tax are apparently equally effective in the activation of NF-κB in Jurkat cells (7, 41, 45, 54). One provocative possibility is that Tax directly or indirectly modulates the formation or activity of IL-6κB BFII, which we postulate to be an IL-6κB-specific repressor. In this context, it is noteworthy that the IL-1β gene, which is not expressed normally in lymphoid cells but is aberrantly expressed in HTLV-1-infected T cells (66), has an AT-rich κB motif (15) (Table 1) similar to IL-6κB. In addition, several other cytokine genes (beta interferon, IP-10, and IL-8 genes; Table 1) also have IL-6κB-related κB motifs in their promoters (39, 46, 50). Thus, the expression of these cytokine genes, including the IL-6 gene, may be under similar negative control mechanisms through IL-6κB-related motifs in lymphoid cells.

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