Cell, Vol. 64, 961–969, March 8, 1991, Copyright © 1991 by Cell Press

DNA Binding and IκB Inhibition of the Cloned p65 Subunit of NF-κB, a *rel*-Related Polypeptide

Garry P. Nolan,* Sankar Ghosh,* Hsiou-Chi Liou,* Paul Tempst,† and David Baltimore*‡
*Whitehead Institute for Biomedical Research
Nine Cambridge Center
Cambridge, Massachusetts 02142
†Howard Hughes Medical Institute
and Department of Genetics
Harvard Medical School
Boston, Massachusetts 02215
‡Rockefeller University
New York, New York 10021

Summary

The sequence and biochemical properties of the product of the cloned cDNA for the p65 subunit of nuclear factor κB (NF-κB) have been determined. The cDNA has an open reading frame of 549 amino acids capable of encoding a 60 kd protein. NF-κB p65 contains an amino-terminal region of 320 amino acids with extensive similarity to the oncogene c-*rel* and lesser similarity to NF-κB p50. In vitro translated p65 forms a DNA-binding complex with NF-κB p50, and the binding of this complex can be specifically inhibited by purified IκB. Progressive carboxy-terminal deletions of p65 show that, contrary to previous assumptions, p65 does include a DNA-binding domain that in vivo might become activated only through hetero-oligomerization with p50. DNA binding by truncated p65 is inhibited by IκB, thus mapping the IκB interaction domain to the *rel*-homologous region and suggesting that IκB exerts its inhibitory effect upon NF-κB primarily through interaction with p65.

Introduction

NF-κB was originally discovered as a kappa immunoglobulin enhancer DNA-binding activity that correlated with κ gene transcription (Sen and Baltimore, 1986; Schlissel and Baltimore, 1989). Since then, NF-κB has been shown to act as a second messenger, activating transcription of a number of genes in multiple tissues (for review, see Lenardo and Baltimore, 1989), and has also been correlated to the activation of human immunodeficiency virus (Nabel and Baltimore, 1987). NF-κB preexists in the cytoplasm of most cells in an inactive form, complexed to an inhibitor, termed IκB (Baeuerle and Baltimore, 1988a, 1988b). Stimulation by a number of agents such as phorbol myristate acetate, LPS, or TNF-α results in the dissociation of the IκB–NF-κB complex and correlated transcription of target genes (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1988b; Libermann and Baltimore, 1990). This activation is thought to lead to release of IκB from the complex, with the subsequent translocation of NF-κB to the nucleus (Baeuerle and Baltimore, 1988b). In the nucleus, NF-κB binds to DNA motifs related to the sequence GGGGATTCC present in some enhancers or promoters and, in conjunction with other transcription factors, regulates the transcriptional activity of nearby genes.

NF-κB is a complex of two proteins of 50 and 65 kd that is thought to bind DNA either as a heterodimer of p50–p65 or as a heterotetramer of $(p50)_2(p65)_2$ (Baeuerle and Baltimore, 1989). $p50_2$ specifically bound to DNA in the absence of p65, whereas p65 alone did not specifically bind DNA (Baeuerle and Baltimore, 1989). Since p50 binds DNA with a DNA specificity similar to, but distinct from, the p50–p65 hetero-oligomer, it has also been suggested that p65 modifies the protein–DNA contacts of the NF-κB complex (Kieran et al., 1990). While containing no previously known DNA-binding capacity, p65 is necessary in vitro for IκB to inhibit the DNA-binding activity of NF-κB (Baeuerle and Baltimore, 1988b; Ghosh and Baltimore, 1990).

The gene encoding the p50 component of NF-κB was cloned by us and others (Ghosh et al., 1990, Kieran et al., 1990) and was found to be related to the proto-oncogene c-*rel* and the Drosophila pattern formation gene *dorsal*. In addition to the primary structure similarities, the products of these three genes all display cytoplasmic/nuclear partitioning that, in the case of p50 and *dorsal*, correlates with specific gene activation (for review, see Baueurle and Baltimore, 1990; Roth et al., 1989; Rushlow et al., 1989; Steward, 1989; Gilmore and Temin, 1986, 1988). NF-κB p50 was also identified putatively as KBF-1 (Kieran et al., 1990), a constitutively active nuclear-localized transcription factor that binds to NF-κB-like sites in the H-2 gene (Kimura et al., 1986; Israël et al., 1987; Baldwin and Sharp, 1987, 1988). The full-length cDNA-encoding p50 is 105 kd; it is possible that p50 is processed from its mature size of 105 kd by an undefined proteolytic mechanism (Ghosh et al., 1990; Kieran et al., 1990), although other mechanisms are possible. The 105 kd protein shows no protein-binding capacity, either alone or in the presence of purified p65. Removal of the carboxy-terminal half of the 105 kd protein in vitro to generate p50 activates DNA binding and complex formation with purified p65. Taken together, these findings suggest the simple hypothesis that p50, in association with p65, is the well-known NF-κB complex, and that p50 alone as KBF-1 (Kieran et al., 1990), or in concert with other as yet undefined components, constitutively resides in the nucleus to modulate the expression of a different subset of genes.

We have now cloned the gene that encodes p65 and have verified that the p65 molecule belongs to the *rel* family of genes, that it can form a DNA-binding complex with p50, and that the DNA-binding activity of this complex can be inhibited with IκB (Baeuerle and Baltimore, 1988b; Ghosh et al., 1990). In addition, we find that removal of the carboxyl terminus of p65 activates DNA binding as a presumptive homodimer, and that this DNA binding can also be inhibited by IκB. This suggests that hetero-oligomerization with p50 activates DNA binding in p65, that both p50 and p65 contribute to site specificity of DNA binding, that the inhibition by IκB of the NF-κB complex is mediated

primarily through the *rel* region of p65, and that the carboxyl terminus of p65 acts to suppress the DNA-binding capacity of the p65 *rel* region.

Results

Polymerase Chain Reaction (PCR) Cloning of Rabbit and Murine p65

The purification of the p65 component of NF-κB from rabbit lung was as previously described (Ghosh et al., 1990). Sequencing of several tryptic peptides gave unambiguous sequences that provided immediate identification of the *rel* homology in p65. Most peptides showed detectable homology to the N-terminal region of the *rel* gene family.

Using this information, primers were designed with known orientation for PCR-assisted cloning (Lee et al., 1988). Degenerate oligonucleotide primers corresponding to rabbit p65 peptides A and B were used in standard PCR on hexamer-primed reverse-transcribed poly(A)$^+$–containing mRNA from rabbit lung and the murine pre-B cell line 22 D6. These peptides were chosen from the several options available because of their limited homology to other *rel*-like sequences. Bands corresponding to a size predicted by *rel* homology, about 500 bp, were subcloned into pBluescript SK$^+$ and sequenced. The DNA sequence of several clones from both the rabbit and murine amplifications showed significant homology to murine *rel* (70%) (Grumont and Gerondakis, 1989). The murine and rabbit clones showed 92% DNA homology to each other over the region sequenced (data not shown) and greater than 97% amino acid homology. Therefore, using primers specific for rabbit p65, we were able to isolate virtually identical cDNAs from both rabbit lung and a murine hematopoietic cell line.

We used our murine PCR insert as a radiolabeled probe against a cDNA library prepared from the pre-B cell line 22 D6. Twenty five λ clones were isolated and of these, six were plaque-purified. Isolates λp65–5 and λp65–16, possessing inserts of 2.6 kb, were subcloned into pBSK$^+$ in both orientations.

The complete nucleotide sequence of λp65–5 is presented in Figure 1. An open reading frame of 549 codons is present, capable of encoding a protein of 60.2 kd. All of the other sequenced peptides from purified p65 that were not used in the design of PCR primers for amplification were also present in the translated sequence (Figure 1). This provided independent evidence that the cDNA isolate encodes the corresponding major protein isolated from the 65 kd region of the SDS gel and is therefore likely to be NF-κB p65.

The predicted p65 sequence shows strong similarity to oncogene *c-rel* and to other known *rel*-like molecules (Figure 2). Indeed, p65 is significantly more homologous to *c-rel* than it is to its partner p50 (73% versus 56% in the *rel*-homologous region, respectively). This is especially obvious in the region of amino acids 140 to 150 where *dorsal*, all *c-rel* homologs, and NF-κB p65 lack a region found in NF-κB p50. As the carboxyl terminus of *c-rel* does contain transcription-activating abilities (Hannink and Temin, 1989; Bull et al., 1990), it is possible that the carboxyl terminus of p65 contains such a domain, as previously proposed for p65 (Bauerle and Baltimore, 1989). A comparison of this region of p65 with the corresponding region in *c-rel* shows some charge distribution similarity (data not shown), perhaps indicating common roles for these regions.

Northern Analysis

The p50 and p65 subunits of the NF-κB complex are expected to be present in many tissues, either complexed to IκB as an inactive, although inducible, form or in a constitutively nuclear form, as in mature B cells. The murine PCR clone isolated above was radiolabeled and used as a probe for blot hybridization of electrophoretically separated RNA (Figure 3). A strong hybridization signal corresponding to a single 2.6 kb mRNA species was present in all tissues analyzed, with the exception of the mature T cell EL4. In EL4, weak hybridization was observed, indicating poor expression in this cell line for reasons that remain to be determined.

In Vitro Translated p65 Forms Biochemically Active Complexes with p50 and IκB

The characterized properties of p65 include its ability to hetero-oligomerize with p50 to form a DNA-binding p50–p65 complex and the ability of IκB to inhibit such binding when present. Therefore, to ascertain whether the cloned molecule has the properties of authentic p65, the clone was transcribed in vitro and translated alone or together with a p50 cDNA truncated at the HindIII site. This truncated p50 molecule is known to hetero-oligomerize with affinity-purified p65, and the complex formed binds to DNA and is inhibitable with purified IκB (Ghosh et al., 1990). SDS–PAGE analysis of the translated p65 protein (Figure 4A) showed a band migrating at approximately 65 kd and a series of faster migrating bands, which were probably premature translation termination products.

The translated proteins were analyzed for DNA binding and IκB inhibition by an electrophoretic mobility shift assay (EMSA; Figure 4B). Translated p50 formed a specific DNA-binding complex with radiolabeled cognate NF-κB sites (Figure 4B, lane 2). Translated p65 alone did not form a major DNA-binding complex, although there was weak formation of a complex that might represent p65 interaction with *rel*-like proteins present in the wheat germ lysate (Figure 4B, lane 3). Translated p65, however, hetero-oligomerized with cotranslated p50 to form a strong DNA-binding complex (Figure 4B, lane 6) with a mobility different from that of the p50 homodimer complex (lane 2). Importantly, the DNA binding of the cotranslated hetero-oligomeric p50–p65 complex, but not the p50 homodimer, could be specifically inhibited by the addition of IκB (Figure 4B, lanes 6, 7, and 8) as previously described for the affinity-purified components (Baeuerle and Baltimore, 1988b; Ghosh et al., 1990). A hetero-oligomeric complex formed readily when p50 and p65 were cotranslated but formed less efficiently when the components were translated separately and then mixed (Figure 4B, lanes 3–6). Competition experiments with unlabeled wild-type and mutant oligonucleotide binding sites showed that the binding of the cotranslated hetero-oligomeric complex was specific (not

```
   1  TTTACTTTAGCGCGCCGTGGGCTCAGCTGCGACCCCGGCCCCGCCCCCGGGACCCTGACCATGGACGATCTGTTTCCCCTCATCTTTCCCTCAGAGCCAGCC
                                                                  M  D  D  L  F  P  L  I  F  P  S  E  P  A     14
 103  CAGGCTTCTGGGCCTTATGTGGAGATCATCGAACAGCCGAAGCAACGGGGCATGCGATTCCGCTATAAATGCGAGGGGCGCTCAGCGGGCAGTATTCCTGGC
      Q  A  S  G  P  Y  V  E  I  I  E  Q  P  K  Q  R  G  M  R  F  R  Y  K  C  E  G  R  S  A  G  S  I  P  G     48
 205  GAGAGAAGCACAGATACCACCAAGACACACCCCACCATCAAGATCAATGGCTACACAGGACCAGGAACAGTTCGAATCTCCCTGGTCACCAAGGATCCACCT
      E  R  S  T  D  T  T  K  T  H  P  T  I  K  I  N  G  Y  T  G  P  G  T  V  R  I  S  L  V  T  K  D  P  P     82
 307  CACCGGCCTCATCCACATGAACTTGTGGGGAAGGACTGCCGGGATGGCTACTATGAGGCTGACCTCTGCCCAGACCGCAGTATCCATAGCTTCCAGAACCTG
      H  R  P  H  P  H  E  L  V  G  K  D  C  R  D  G  Y  Y  E  A  D  L  C  P  D  R  S  I  H  S  F  Q  N  L    116
 409  GGGATCCAGTGTGTGAAGAAGCGAGACCTGGAGCAAGCCATTAGCCAGCGAATCCAGACCAACAATAACCCCTTTCACGTTCCTATAGAGGAGCAGCGCGGG
      G  I  Q  C  V  K  K  R  D  L  E  Q  A  I  S  Q  R  I  Q  T  N  N  N  P  F  H  V  P  I  E  E  Q  R  G    150
 511  GACTATGACTTGAATGCAGTGCGCCTCTGCTTCCAGGTGACAGTGCGGGACCCAGCAGGCAGGCCCCTCCTCCTGACCCCTGTCCTCTCACATCCGATTTTT
      D  Y  D  L  N  A  V  R  L  C  F  Q  V  T  V  R  D  P  A  G  R  P  L  L  L  T  P  V  L  S  H  P  I  F    184
 613  GATAACCGGGCCCCCAACACTGCCGAGCTCAAGATCTGCCGAGTAAACCGGAACTCTGGGAGCTGCCTCGGTGGGGATGAGATCTTCTTGCTGTGCGACAAG
      D  N  R  A  P  N  T  A  E  L  K  I  C  R  V  N  R  N  S  G  S  C  L  G  G  D  E  I  F  L  L  C  D  K    218
 715  GTGCAGAAAGAAGACATTGAGGTGTATTTCACGGGACCAGGCTGGGAGGCACGAGGCTCCTTTTCTCAAGCTGATGTGCATCGGCAAGTGGCCATTGTGTTC
      V  Q  K  E  D  I  E  V  Y  F  T  G  P  G  W  E  A  R  G  S  F  S  Q  A  D  V  H  R  Q  V  A  I  V  F    252
 817  CGGACTCCTCCGTACGCCGACCCCAGCCTCCAGGCTCCTGTTCGAGTCTCCATGCAGCTACGGCGGCCTTCTGATCGCGAGCTCAGTGAGCCCATGGAGTTC
      R  T  P  P  Y  A  D  P  S  L  Q  A  P  V  R  V  S  M  Q  L [R  R  P  S] D  R  E  L  S  E  P  M  E  F    286
 919  CAGTACTTGCCAGACACAGATGATCGCCACCGGATTGAAGAGAAGCGCAAAAGGACCTATGAGACCTTCAAGAGTATCATGAAGAAGAGTCCTTTCAATGGA
      Q  Y  L  P  D  T  D  D  R  H  R  I  E  E [K  R  K  R] T  Y  E  T  F  K  S  I  M  K  K  S  P  F  N  G    320
1021  CCAACTGAACCCCGGCCTCCAACCCGGCGTATTGCTGTGCCTACCCGAAACTCAACTTCTGTCCCCAAGCCAGCCCCGCAGCCCTACACCTTCCCAGCATCC
      P  T  E  P  R  P  P  T  R  R  I  A  V  P  T  R  N  S  T  S  V  P  K  P  A  P  Q  P  Y  T  F  P  A  S    354
1123  CTCAGCACCATCAACTTTGATGAGTTTTCCCCCATGCTGTTACCATCAGGGCAGATCTCAAACCAGGCCCTGGCCTTAGCACCGTCCTCTGCCCCAGTCCTT
      L  S  T  I  N  F  D  E  F  S  P  M  L  L  P  S  G  Q  I  S  N  Q  A  L  A  L  A  P  S  S  A  P  V  L    388
1225  GCCCAGACCATGGTCCCTTCCTCAGCCATGGTACCTCTGGCTCAGCCCCCAGCTCCTGCCCCAGTTCTAACCCCGGGTCCTCCCCAGTCCCTGTCTGCACCT
      A  Q  T  M  V  P  S  S  A  M  V  P  L  A  Q  P  P  A  P  A  P  V  L  T  P  G  P  P  Q  S  L  S  A  P    422
1327  GTTCCAAAGAGCACCCAGGCTGGGGAAGGCACGCTGTCGGAAGCCCTGCTGCACCTGCAGTTTGATGCTGATGAAGACTTGGGGGCCTTGCTTGGCAACAGC
      V  P  K  S  T  Q  A  G  E  G  T  L  S  E  A  L  L  H  L  Q  F  D  A  D  E  D  L  G  A  L  L  G  N  S    456
1429  ACAGACCCAGGAGTGTTCACAGACCTGGCATCTGTGGACAACTCAGAGTTTCAGCAGCTCCTGAACCAGGGTGTGTCCATGTCTCACTCCACAGCTGAGCCC
      T  D  P  G  V  F  T  D  L  A  S  V  D  N  S  E  F  Q  Q  L  L  N  Q  G  V  S  M  S  H  S  T  A  E  P    490
1531  ATGCTGATGGAGTACCCTGAAGCTATAACTCGCCTGGTGACAGGGTCCCAGAGGCCCCCTGACCCAGCTCCCACACCCCTGGGGACCTCGGGGCTTCCCAAT
      M  L  M  E  Y  P  E  A  I  T  R  L  V  T  G  S  Q  R  P  P  D  P  A  P  T  P  L  G  T  S  G  L  P  N    524
1633  GGTCTCTCCGGAGATGAAGACTTCTCCTCCATTGCGGACATGGACTTCTCTGCTCTTTTGAGTCAGATCAGCTCCTAAGGTGCTGACAGCGACCCTGCTCAG
      G  L  S  G  D  E  D  F  S  S  I  A  D  M  D  F  S  A  L  L  S  Q  I  S  S  *                            549
1735  AGCACCAGGTTTCAGGGCACTGAAGCCTTCCCGAAGTGCGTACACATTCTGGGGAGTGTGCTCCAGCTGCCCCCGACTTGTTTGGGTGATCTCTCTGGGGCG
1837  GCACGTTTTACTCTTTATCTCGCTTTCGGAGGTGCTTTCGCAGGAGCATTAACCTCCTGGAGACGGAGCTGGGAGGACTCGGTGCATCCCTGTGTTGATAGC
1939  TCCTGCTTCGGTAGGGAACTCTGAGATCCTGCTTCCATCTCCAGCTTCTAGCACTCTCCTAGAGAGGGACAGACTGGAGCCATGCCTTAGGCCATATAGCCT
2041  TACTATCAAGTGTCTTCCTCCACGCGGATTCCTGTACACCTTGATCCAAAGCAGTGCTCCCAAGAGCAGCTCCTACGTGGTGCTGCCCGACACCAGCACATG
2143  AGGGGCCGCTCTTCTGTCCTGTGGAGCTCCTGCCCTGCCAGCTCTCCATGCTGAGCTGTGGCCAAGGGGAACAGGTGGGATGTTGCTGGCCGCCTTCAGAAT
2245  CAGGGGGAGTTTAGTCTGAGACATCCCTGCTCCCCCTTTTTTTCAAGTGCCTTAATAGCAGGGCAAACTGTAGAGTCAGGAGGGCAGGCTAGATGCTCAGCC
2347  ACAAGACAGCCTTTACTGAAAAAGCTATTGGACTCTTGCTCTTTCTAGCTCTGAACTAATAAATGTCTTATCACGCTGAAAAAAAAAAAAAAAAAAAAAAAA
2449  AAAAAAAA  2456
```

Figure 1. Nucleotide Sequence and Translation of p65 cDNA

The 2456 bp cDNA sequence of λp65–5 is presented along with the 549 amino acid–translated p65 sequence encoding a 60.2 kd protein. The methionine start codon conforms to the Kozak consensus (Kozak, 1989). The cDNA sequence of λp65–16 encodes an additional 200 bp of upstream 5′ untranslated sequence (not shown). Underlined are those regions corresponding to the peptides sequenced from p65. The heavy underlined region are those peptides used in the design of degenerate oligonucleotides used in the PCR cloning process. Boxed are the putative nuclear localization sequence (KKRK) and putative phosphorylation recognition sequence (RRPS). The nucleotide sequence is numbered on the left, and the amino acid sequence is numbered on the right.

shown). Taken together, these data confirm that the cDNA we have isolated manifests the biochemical criteria ascribed to NF-κB p65.

p65 Contains a DNA-Binding Domain That Can Be Inhibited by IκB

The weak DNA binding observed using p65 translated alone suggests that some p65 translation products (perhaps the prematurely terminated protein in Figure 4A, lane 1) are capable of binding specifically to cognate DNA. The idea that p65 might contain a DNA-binding domain is strengthened by the finding that truncations of v-*rel* can bind to KBF-1 sites (Kieren et al., 1990). To test whether truncated versions of p65 might bind to DNA, we prepared deletions of the p65 molecule starting at the carboxyl terminus of the molecule and ending at the BglII site in the *rel* homology region (Figure 5A). EMSA of these truncated proteins showed that the protein truncated at the BspH1 site, just after the putative nuclear localizing signal, was capable of binding the NF-κB DNA recognition sequence with high affinity (Figure 5B). The coincident migration of this band with the p50 homodimer band (Baeuerle and Baltimore, 1989; Ghosh et al., 1990; Kieran et al., 1990) suggests strongly that the deleted p65 binds as a dimer. Because p65 does contain a DNA-binding site, this suggests that p65, in oligomerization with p50, could contribute directly to DNA binding in the NF-κB complex.

Previous models held that p50 provided the major DNA-binding contacts made by the NF-κB complex, and that the role of p65 was to render the complex inhibitable by IκB (Baeuerle and Baltimore, 1989). However, it is unclear whether such inhibition is exerted directly through p65 or requires IκB binding to both p50 and p65. Therefore, we tested whether IκB could interact with and inhibit the DNA binding of truncated p65. Figure 6 clearly demonstrates that the activated DNA-binding property of the p65 truncation could be inhibited by the presence of IκB. The binding of p50, by contrast, is not significantly affected by IκB (Baeuerle and Baltimore, 1989; Ghosh et al., 1990; Figure 6). Therefore, the interaction of IκB with the NF-κB com-

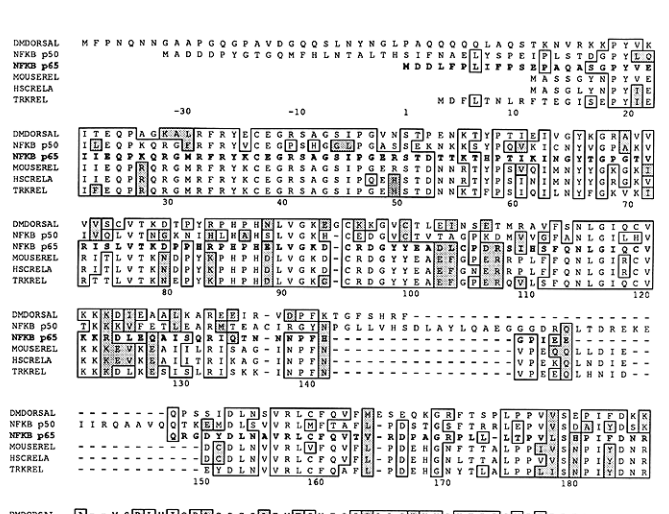

Figure 2. Homology of Amino Terminus of p65 to Other *rel*-Related Polypeptides

The NF-κB p65 amino terminal *rel* region is aligned to the other known members of the *rel* polypeptide family. The p65 sequence is in bold. The other *rel* members are divided into Drosophila *dorsal* (Steward, 1987) and NF-κB p50 (Ghosh et al 1990)—which are more related to each other than they are to *c-rel*—and the *rel* homologs from mouse (Grumont and Gerondakis, 1989), human (Brownell et al., 1989), and turkey (Wilhelmsen et al., 1984). p65 shares more identity with the *rel* homologs than it does with *dorsal* or p50. Numbering is with respect to the methionine start of p65. Open boxes represent identity to p65, and stippled boxes represent conservative amino acid replacement with respect to p65. Dashes indicate spacing introduced to maximize identity during alignment. Sequences are DMDORSAL: Drosophila *dorsal*; NF-κB p50: murine p50; NF-κB p65: murine p65; NF-κBrab: derived sequence from rabbit PCR-assisted p65 cloning; MOUSEREL: murine *c-rel*; HSCRELA: human *c-rel*; TRKREL: turkey *c-rel*.

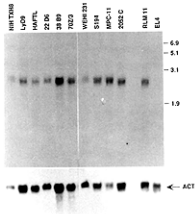

Figure 3. p65 Is a Widely-Expressed Message

Expression of p65 mRNA in cells representing different lineages: λp65–5 cDNA insert was labeled and used in a probe of a Northern blot (Oettinger et al., 1990) run with 16 μg of once-selected poly(A)-containing mRNA or 8 μg of twice-selected poly(A)$^+$ (22 D6) in the indicated lanes using standard techniques (Maniatis et al., 1982). NIH TXH8 is an NIH 3T3–derived cell line (Oettinger et al., 1990); Ly/D9 is a pre-B cell line; HAFTL, 22 D6, and 38 B9 are Abelson-transformed pre-B lines; WEHI 231 is a nonsecreting B cell lymphoma; S194 is a nonsecreting B-cell myeloma; MPC-11 is an imunoglobulin-secreting B cell myeloma; 2052 C is a pre-T thymoma; RLM11 is a terminal deoxynucleotide transferase-expressing immature T cell; EL4 is a mature T cell.

plex maps to the *rel* region of p65, and such a domain appears to be lacking in the *rel* region of p50.

Discussion

The cloned p65 subunit of the transcription factor NF-κB is shown in this article to encode a new *rel*-related polypeptide and to contain a DNA-binding domain that can be inhibited by IκB. By several biochemical criteria, the cloned cDNA corresponds to NF-κB p65. First, the cDNA was isolated using sequence information from peptides derived from affinity-purified, size-separated NF-κB p65. Second, peptides not used in the cloning procedure are encoded within the coding frame of the derived cDNA. Third, in vitro translated protein runs at approximately 65 kd and interacts to form a specific DNA-binding complex with NF-κB p50 (Baeuerle and Baltimore, 1989). Fourth, the binding of this complex to DNA is inhibited using purified IκB (Baeuerle and Baltimore, 1988b). Finally, Northern analysis shows the broad tissue distribution expected of this gene (Sen and Baltimore, 1986b; reviewed in Lenardo and Baltimore, 1989). p65 expression, however, is low, although not completely absent, from EL4, a CD4$^+$ murine lymphoma. As EL4 does have NF-κB activity as assayed by transient transfection of reporter constructs (Pierce et al., 1988) and expresses p50 (Ghosh et al., 1990), it is possible that another *rel*-like protein assumes some of the roles of p65 in EL4.

Deletion mapping of p65 identified a DNA-binding domain capable of recognizing the NF-κB recognition sequence with a specificity similar to $p50_2$ and p50–p65 oligomer. This suggests that p65 directly contributes to the DNA binding of the NF-κB complex. In support of this, the DNA contacts made by $p50_2$ were different from those made by p50–p65 (Kieran et al., 1990); we would interpret these differences as resulting from different polypeptide backbones contacting the DNA. Because p65 contains a DNA-binding domain, the DNA-binding form of NF-κB may be a heterodimer of p50 and p65. In this model, p50 and p65 would each bind a half-site of the NF-κB recognition sequence. A similar hypothesis has been proposed for $p50_2$ half-site binding to the KBF-1 site (Kieran et al., 1990). This is in conflict with a previous model that suggested, on the basis of gel filtration measurements, that p50 and p65 could associate in solution as a heterotetramer (Baeuerle and Baltimore, 1989). The heterotetramer model of NF-κB

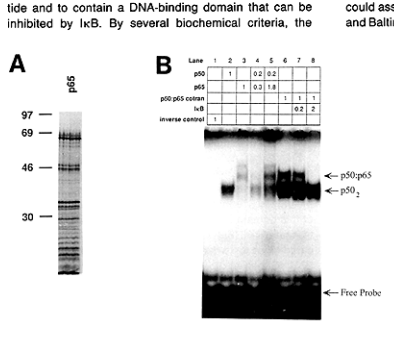

Figure 4. Heterodimeric Cloned p65–p50 Complex Specifically Binds DNA and Is IκB Inhibitable

(A) In vitro translation of cloned p65. Full-length p65 was translated and electrophoresed by 12.5% SDS–PAGE. Molecular weight size markers are as indicated.

(B) p65 forms specific p50–p65 heterodimers and is inhibited by IκB. Translated p50 (HindIII-truncated; Ghosh et al., 1990) and translated full-length p65 were run in EMSA in the combinations indicated using NF-κB site as specific probe (gift of Takeshi Fujita). Lane 1 is a control using translation mix with p65 RNA transcribed in the antisense orientation. Lanes 2 and 3 contain p50 and p65, respectively, translated alone. Lanes 4 and 5 are a titration of p65 using limiting amounts of p50. Lanes 6 through 8 are p50–p65 cotranslations inhibited by the indicated amounts of purified IκB (purified according to Ghosh and Baltimore, 1990). We interpret the weak binding of p65 translated alone to be interaction with *rel*-like proteins present in the wheat germ extract. Arrows show the positions of p50 homodimers and specifically inhibited p50–p65 heterodimer. Values given in the table are in microliters.

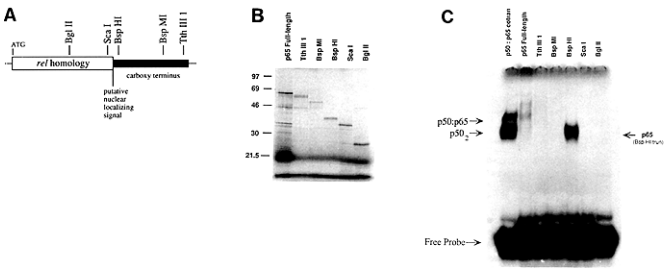

Figure 5. p65 Contains a DNA-Binding Domain That Is Suppressed by the Carboxyl Terminus

(A) Representation of p65 cDNA. Restriction endonucleases used in this deletion series and their relative positions in the p65 cDNA are as shown. The *rel* homologous region and putative nuclear localization are indicated.

(B) In vitro translation of carboxy-terminal p65 deletion series. pSK–p65* was digested with the restriction endonucleases indicated and then in vitro transcribed and in vitro translated as described in the Experimental Procedures. The in vitro translations were run on standard SDS–PAGE. Sizes of deletion products are as predicted from sequence. Molecular weight size markers are as indicated.

(C) EMSA of p65 deletion series. One microliter of each p65 deletion translation mix was used in standard EMSA using labeled NF-κB site. Restriction endonucleases used to make deletions are as indicated. The arrow points out the activation of a DNA-binding domain using the BspHI deletion. The faint bands seen in translations of full-length p65 may represent interaction of p65 with wheat germ extract *rel*-like proteins; these bands do not appear in cotranslations of p65 with p50.

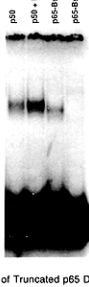

Figure 6. IκB Inhibition of Truncated p65 DNA Binding

BspHI-truncated p65 and HindIII-truncated p50 was run in EMSA with and without IκB as marked. The binding of truncated p65 (lane 3), but not of p50 (lane 1), is inhibited by IκB (lane 4), indicating that IκB specifically interacts with the rel region of p65.

DNA binding reconciled the DNA binding of $p50_2$ with the apparent lack of DNA-binding capacity for p65. In that model, $p50_2$ provided the known DNA-binding capacity, and each p65 bound one p50 without directly contacting DNA. It is possible that the gel filtration measurements, which are affected by molecular conformation, incorrectly determined the p50–p65 mass to be 250 kd. Glycerol gradient measurements conflicted with the possible existence of a heterotetramer, predicting a heterodimer (Baeuerle and Baltimore, 1989). Preformed p50 homodimers do not readily interact with p65 (Figure 4B), whereas cotranslation results in efficient p50–p65 complex formation. This strongly suggests that p65 requires free p50 to associate and form a p50–p65 DNA-binding complex. This also supports the possibility that p50 and p65 associate in a heterodimer via the same dimerization motifs present in their respective *rel* regions (Ghosh et al., 1990; Kieran et al., 1990) as do p50 homodimers. Because of this, and since it is now apparent that p65 contains a DNA-binding domain, we currently favor the simpler model that the DNA-binding form of NF-κB is a heterodimer. We cannot at this point, however, rule out the possibility that the DNA binding is accomplished as a heterotetramer.

Full-length, native p65 does not efficiently bind to DNA (Baeuerle and Baltimore, 1989), while truncated p65 does. A simple explananation for this would be that removal of the carboxyl terminus of p65 relieves an inhibitory function of this domain and allows for the DNA binding of dimeric truncated p65 (Figure 7). Full-length p50 must also be truncated close to its native size of 50 kd before it can bind to DNA (Ghosh et al., 1990; Kieran et al., 1990), and v-*rel* truncations bind DNA as homodimers (Kieran et al., 1990). However, full-length p65 interacts readily with p50 to form a hetero-oligomeric DNA-binding complex (Baeuerle and Baltimore, 1989) that we now interpret to be a heterodimer. The simplest model that accounts for this data posits that full-length p65 is prevented from forming a DNA-binding complex with itself but can form a complex with molecules

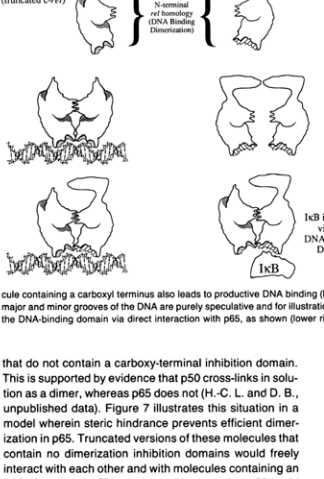

Figure 7. Steric-Constraint Model for *rel*-Related Polypeptide Interaction; How IκB Inhibits p50–p65 DNA Binding

The figure at the top left represents the in vivo processed form of p50 (or in vitro truncations). The figure at the top right represents full-length p65 (or *c-rel* or other similar molecules). Indicated are the *rel*-related regions, shared between p50 and p65, that contain a dimerization motif (fingers) and half-site DNA-binding domain (bottom of figurines). The relative positions of these domains to each other and their structural designation are speculation and for presentation purposes only. The carboxy-terminal region of p65 is shown as an inhibitory domain that acts by sterically hindering the interaction of p65 homodimers (or presumably p65–*c-rel* heterodimers) by preventing the contact of the dimerization motifs, thus preventing the joining of two half-site DNA-binding domains to form an active complex (right middle). Sterically unhindered dimerization occurs between two carboxy-terminal minus molecules (p50 homodimer, left middle), allowing for binding to DNA. Heterodimerization between a carboxyl terminus–deleted molecule and a molecule containing a carboxyl terminus also leads to productive DNA binding (lower left). The relative interaction of the DNA-binding domain with the major and minor grooves of the DNA are purely speculative and for illustration only. We propose that IκB inhibits NF-κB by binding to and masking the DNA-binding domain via direct interaction with p65, as shown (lower right).

that do not contain a carboxy-terminal inhibition domain. This is supported by evidence that p50 cross-links in solution as a dimer, whereas p65 does not (H.-C. L. and D. B., unpublished data). Figure 7 illustrates this situation in a model wherein steric hindrance prevents efficient dimerization in p65. Truncated versions of these molecules that contain no dimerization inhibition domains would freely interact with each other and with molecules containing an inhibition domain. Thus, one would expect that p50 could interact with *c-rel*, but that *c-rel* could not dimerize with itself or with molecules such as p65. Indeed, Ballard et al. have recently shown that HIVEN86A, a molecule that binds to the HIV enhancer, is in fact *c-rel*, and they suggest that when bound to the HIV enhancer it might complex with p50. The alternate hypothesis, that p65 homodimers do form but are incapable of binding to DNA (as found for E12 helix-loop-helix homodimers; Sun and Baltimore, 1991), seems unlikely, since we do not observe p65 homodimers in cross-linking experiments (H.-C. L. and D. B.).

We show in this article that IκB can specifically block the DNA binding of truncated p65 by interaction through the *rel* homology region (Figure 6B), whereas IκB has no effect upon the binding of p50 (Baeuerle and Baltimore, 1988b; Ghosh et al, 1990; Figure 6). Since IκB has no effect upon $p50_2$ binding, some subtle difference between the *rel* regions of p50 and p65 must provide the specificity of p65–IκB interaction. In the cell, IκB retains the NF-κB p50–p65 complex in the cytoplasm until IκB is inactivated, often apparently by cellular kinases (Ghosh and Baltimore, 1990), whereupon NF-κB is released to translocate to the nucleus. In vitro, however, IκB can inhibit the DNA-binding capacity of p50–p65 even after it has bound to DNA (Baeuerle and Baltimore, 1988b; Zabel and Baeuerle, 1990). Because IκB complexes to dimerized p50–p65 in the cytoplasm as a unit, and purified IκB–p50–p65 is incapable of binding DNA, it is reasonable to presume that IκB inhibits the DNA-binding activity of the complex by impairing the DNA-binding site of p65. Therefore, multiple protein–protein interaction domains exist in the *rel* region, besides the dimerization motif, that function to regulate factor activity. Since p65 shows a high degree of sequence similarity to *c-rel*, it is possible that IκB or similar molecules inhibit the activity of *c-rel* or other *rel*-related polypetides in an analogous manner. In support of this, *c-rel* and the oncogene *v-rel* are associated in the cytoplasm, but not in the nucleus, with a protein of 40 kd in avian cells (Simek and Rice, 1988; Morrison et al., 1989; Lim et al., 1990)—a size and distribution pattern reminiscent of IκB.

Several other polypeptide complexes that involve *rel*-related proteins have been postulated to have activities related to those of NF-κB. Recently, HIVEN86A, a DNA-binding activity that has been correlated to HIV regulation (Franza et al., 1997; Böhnlein et al., 1988), has been demonstrated to be the product of the *c-rel* oncogene (Ballard et al., 1990; Lee et al., submitted) and possibly acts by heterodimerization with p50. Two other proteins recognized by *v-rel* antisera, of molecular weights p55 and p75, also bound to NF-κB sites (Ballard et al., 1990). Thus, two previously recognized *rel* activities, NF-κB and a complex involving *c-rel*, might together regulate HIV transcription. If p50 acts as a common partner in complexing with either p65 and *c-rel*, it will be essential to determine the differential activities of these complexes. Kieran et al. (1990) have postulated that p50 homodimers constitute the KBF-1 transcription factor activity. If $p50_2$ is KBF-1, then p50 may also have a transcription-activating domain and might play a role in competing for sites recognized by NF-κB and the proposed complex of p50–*c-rel* (Ballard et al., 1990).

Besides the *rel* genes, several other gene families for transcription factors, such as the *jun/fos* family (for review, see Chiu et al., 1989 and Yang-Yen et al., 1990) or the basic helix-loop-helix family (Murre et al., 1989; Sun and Baltimore, 1991), also interact to form heterodimers. Interestingly, a significant outcome of such combinatorial association is that thresholds for gene activation can be built by the regulated titration of these factors in interaction with each other or with inhibitory binding proteins such as IκB or Id (Benezra et al., 1990; Fiering et al., 1990). Therefore, given the involvement of *rel*-related factors in several clinically and immunologically relevant processes, such as HIV activation and immunoglobulin transcription, it is crucial to determine the rules that govern how members of the *rel* protein family interact to target for activation specific genes and how these proteins fit into the signal-transduction schema of the cell.

Experimental Procedures

Design of Synthetic DNA Primers

Degenerate oligonucleotides corresponding to the underlined peptide sequence in peptides A and B were synthesized on an Applied Biosystems PCR-MATE DNA synthesizer. The sequences were:

A: GAPyGGNTTPyTAPyGAPuGCNGA (degeneracy 1/256)
B: GCPyTGNAPuNCCNGGPuTCNGCPuTA (degeneracy 1/4096).

PCR Isolation of p65

PCR of mRNA was performed according to the manufacturer's instructions with the following modifications. Poly(A) containing mRNA was purified from 0.5 grams of rabbit lung using Fast-Track (Invitrogen); poly(A)-containing mRNA from the murine pre-B cell 22 D6 was a gift of Marjorie Oettinger and was prepared as previously described (Oettinger et al., 1990). One microgram of each mRNA was reverse-transcribed with random hexamers (Pharmacia) under standard reaction conditions; after reverse-transcription the reaction was heat-inactivated, brought to 0.3 M sodium acetate, and precipitated with ethanol (to remove Mg^{2+} ions). Approximately 0.1 μg of each reverse-transcribed PCR reaction was run for 35 cycles in a total volume of 50 μl containing 5 U of Taq polymerase (Cetus Corporation), 10 mM Tris–HCl (pH 8.3), 0.5 mM $MgCl_2$, 100 μM dNTPs, 0.1 mg/ml BSA, and 100 pmol each of primers A and B. The melting temperature was 95°C, the annealing temperature was 60°C, and the extension temperature was 72°C. Reaction products were run on a 1.3% agarose gel; the expected 500 bp band was purified and cloned by standard techniques into pSK⁺ (Stratagene Cloning Systems).

Isolation of p65-Encoding cDNAs and Sequencing

Full-length cDNA clones corresponding to p65 were isolated from a 22 D6 λGT10 library (a gift of Carolyn Gorka, Marjorie Oettinger, and David Schatz) using cloned PCR-prepared p65 subclone as probe. Twenty-six plaques were isolated, and six were plaque-purified. Three clones contained an approximately 2.6 kb insert, which upon restriction analysis contained inserts that were clearly similar; all contained p65-specific inserts as determined by PCR (data not shown). Clones λp65–16 and λp65–5 were used for sequence analysis as presented in this article. The λp65–16 insert was subcloned into the NotI site of pSK⁺ in both orientations, and a deletion series of each orientation was prepared using the Exo/Mung kit as supplied by Stratagene Cloning Systems. Deletions were made in λp65–5 using restriction endonuclease digestion, determined by the sequence of λp65–16 and religation of the vector. Overlapping deletions were sequenced by the Sanger dideoxy method (Sanger et al., 1977), using the Sequenase kit as supplied by United States Biochemicals. Gaps in the sequence were filled using synthesized oligonucleotides determined from previously obtained sequences.

In Vitro Transcription and Translation of p65 and p50

p65 and p50 in pSK⁺ vector were in vitro transcribed using Stratagene Inc. kit #212301, using the T3 and T7 promoters and 7-methylguanosine capping structure according to the manufacturer's instructions. Vectors were linearized with XmnI for full-length p65 or with the enzyme indicated in Figure 5B for truncations. The RNA was purified and redissolved in 20 μl of H_2O, and 1 μl of this RNA was used in a wheat germ translation mix (Promega) using ^{35}S-methionine as label. Three microliters of reacted translation mix was prepared for standard SDS–PAGE and electrophoresed on a 12.5% gel.

Electrophoretic Mobility Shift Assay

Buffer conditions and oligonucleotide labeling were as per Baeuerle and Baltimore (1988a). One microliter of each in vitro translation was used per EMSA reaction. IκB inhibitions were run as per Baeuerle and Baltimore using 2 μl of purified IκB added to the mixture 10 min prior to addition of oligonucleotide label. Gels were run at constant voltage of 150 V for 2 hr.

Acknowledgments

The authors are indebted to Marjorie Oettinger, Daniel Silver, Drs. Mark Schlissel, Douglas Black, David Schatz, Rick Van Etten, and other members of the Baltimore laboratory for salient advice and gifts of reagents. We thank M. Oettinger for many helpful comments on the manuscript. We thank Ann Gifford and Lise Riviere for technical assistance. G. P. N. was supported by a fellowship from the National Institutes of Health; S. G. was supported by a fellowship from the Irvington Institute for Medical Research; H.-C. L. was supported by the Cancer Research Institute. This work was supported by NIH grant GM39458 to D. B.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received November 21, 1990; revised December 21, 1990.

References

Baldwin, A. S., and Sharp, P. A. (1987). Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse H-2K^b class I major histocompatibility gene. Mol. Cell. Biol. *7*, 305–313.

Baldwin, A. S., and Sharp, P. A. (1988). Two transcription factors, NF-κB and H2TF1, interact with a single regulatory sequence in the class I major histocompatibility complex promoter. Proc. Natl. Acad. Sci. USA *85*, 723–727.

Ballard, D. W., Walker, W. H., Doerre, S., Sista, P., Molitor, J. A., Dixon, E. P., Peffer, N. J., Hannink, M., and Greene, W. C. (1990). The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell *63*, 803–814.

Baeuerle, P. A., and Baltimore, D. (1988a). Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell *53*, 211–217.

Baeuerle, P. A., and Baltimore, D. (1988b). IκB: a specific inhibitor of the NF-κB transcription factor. Science *242*, 540–546.

Baeuerle, P. A., and Baltimore, D. (1989). A 65 kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. *3*, 1689–1698.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell *61*, 49–59.

Böhnlein, E., Lowenthal, J. W., Siekevitz, M., Ballard, D. W., Franza, B. R., and Greene, W. C. (1988). The same inducible nuclear protein(s) regulates mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. Cell *53*, 827–836.

Brownell, E., Mitterreder, N., and Rice, N. R. (1989). A human rel proto-oncogene cDNA containing an Alu fragment as a potential coding exon. Oncogene *4*, 935–942.

Bull, P., Morley, K. L., Hoekstra, M. F., Hunter, T., and Verma, I. M. (1990). The mouse c-rel protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. Mol. Cell. Biol. *10*, 5473–5485.

Chiu, R., Angel, P., and Karin, M. (1989). Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell *59*, 979–986.

Fiering, S., Northrop, J. P., Nolan, G., Magila, P. S., Crabtree, G. R., and Herzenberg, L. A. (1990). Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor. Genes Dev. *4*, 1823–1834.

Franza, B. R., Josephs, S. F., Gilman, M. Z., Ryan, W., and Clarkson, B. (1987). Characterization of cellular proteins recognizing the HIV enhancer using a microscale DNA-affinity precipitation assay. Nature *330*, 391–395.

Ghosh, S., and Baltimore, D. (1990). Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature *344*, 678–682.

Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P., and Baltimore, D. (1990). Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell *62*, 1019–1029.

Gilmore, T. D., and Temin, H. M. (1986). Different localization of the product of the v-*rel* oncogene in chicken fibroblasts and spleen cells correlates with transformation by REV-T. Cell *44*, 791–800.

Gilmore, T. D., and Temin, H. M. (1988). v-rel oncoproteins in the nucleus and in the cytoplasm transform chicken spleen cells. J. Virol. *62*, 703–714.

Grumont, R. J., and Gerondakis, S. (1989). Structure of a mammalian c-rel protein deduced from the nucleotide sequence of murine cDNA clones, Oncogene Res. *4*, 1–8.

Hannink, M., and Temin, H. M. (1989). Transactivation of gene expression by nuclear and cytoplasmic rel proteins. Mol. Cell. Biol. *9*, 4323–4336.

Israël, A., Kimura, A., Kieran, M., Yano, D., Kanellopoulos, J., Le Bail, O., and Kourilsky, P. (1987). A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and B_2 – microglobulin genes. Proc. Natl. Acad. Sci. USA *84*, 2653–2657.

Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A., and Israël, A. (1990). The DNA binding subunit of NF-κB is identical to factor KBF-1 and homologous to the *rel* oncogene product. Cell *62*, 1007–1018.

Kimura, A., Israël, A., Le Bail, O., and Kourilsky, P. (1986). Detailed analysis of the mouse H-2K^b promoter: enhancer-like sequences and their role in the regulation of class I gene expression. Cell *44*, 261–272.

Kozak, M. (1989). The scanning model for translation: an update. J. Cell Biol. *108*, 229–241.

Lee, C. C., Wu, X., Gibbs, R. A., Cook, R. G., Muzny, D. M., and Caskey, C. T. (1988). Generation of cDNA probes directed by amino acid sequence: cloning of urate oxidase. Science *239*, 1288–1291.

Lenardo, M. J., Kuang, A., Gifford, A., and Baltimore, D. (1988). NF-κB protein purification from bovine spleen: nucleotide stimulation and binding site specificity. Proc. Natl. Acad. Sci. USA *85*, 8825–8829.

Lenardo, M. J., and Baltimore, D. (1989). NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell *58*, 227–229.

Libermann, T. A., and Baltimore, D. (1990). Activation of interleukin-6 gene expression through the NF-κB transcription factor. Mol. Cell. Biol. *10*, 2327–2334.

Lim, M. Y., Davis, N., Zhang, J., and Bose, H. R. (1990). The v-rel oncogene product is complexed with cellular proteins including its proto-oncogene product and heat shock protein 70. Virology *175*, 149–160.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Morrison, L. E., Kabrun, N., Mudri, S., Hayman, M. J., and Enrietto, P. J. (1989). Viral rel and cellular rel associate with cellular proteins in transformed and normal cells. Oncogene *4*, 677–683.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., Weintraub, H., and Baltimore, D. (1989). Interactions between the heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537–544.

Nabel, G., and Baltimore, D. (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T-cells. Nature *326*, 711–713.

Oettinger, M. A., Schatz, D. G., Gorka, C., and Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science *248*, 1517–1523.

Pierce, J. W., Lenardo, M., and Baltimore, D. (1988). Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. USA *85*, 1482–1486.

Roth, S., Stein, D., and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the Drosophila embryo. Cell *59*, 1189–1202.

Rushlow, C. A., Han, K., Manley, J. L., and Levine, M. (1989). The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in Drosophila. Cell *59*, 1165–1177.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Schlissel, M. S., and Baltimore, D. (1989). Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. Cell *58*, 1001–1007.

Sen, R., and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell *46*, 705–716.

Simek, S., and Rice, N. R. (1988). p59 v-rel, the transforming protein of reticuloendotheliosis virus, is complexed with at least four other proteins in transformed chicken lymphoid cells. J. Virol. *62*, 4730–4736.

Steward, R. (1987). Dorsal, an embryonic polarity gene in Drosophila is homologous to the vertebral proto-oncogene, c-rel. Science *238*, 692–694.

Steward, R. (1989). Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. Cell *59*, 1179–1188.

Sun, X.-H., and Baltimore, D. (1991). An inhibitory domain of E12 transcription factors prevents DNA binding in E12 homodimers but not in E12 heterodimers. Cell *64*, 459–470.

Wilhelmsen, K. C., Eggleton, K., and Temin, H. M. (1984). Nucleic acid sequences of the oncogene v-rel in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-rel. J. Virol. *52*, 172–182.

Yang-Yen, H.-F., Chambard, J.-C., Sun, Y.-L., Smeal, T., Schmidt, T. J., Drouin, J., and Karin, M. (1990). Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein–protein interaction. Cell *62*, 1205–1215.

Zabel, U., and Baeuerle, P. A. (1990). Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor and its cognate DNA. Cell *61*, 255–265.

GenBank Accession Number

The GenBank accession number for the sequence reported in this article is M61909.

Note Added in Proof

Another EL4 subline does express p65 at levels comparable to other cell types. While this article was being prepared, M. B. Urban and P. A. Baeurle (1990) prepared a report also showing that p65 is a receptor for IκB. Genes Dev. *4*, 1975–1984. V. Bours et al. (1990) also recently published a paper describing the cloning of the gene encoding p50. Nature *348*, 76–80.