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A Novel Mitogen-Inducible Gene Product Related to p50/p105-NF-κB Participates in Transactivation through a κB Site

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A Rel-related, mitogen-inducible, κB-binding protein has been cloned as an immediate-early activation gene of human peripheral blood T cells. The cDNA has an open reading frame of 900 amino acids capable of encoding a 97-kDa protein. This protein is most similar to the 105-kDa precursor polypeptide of p50-NF-κB. Like the 105-kDa precursor, it contains an amino-terminal Rel-related domain of about 300 amino acids and a carboxy-terminal domain containing six full cell cycle or ankyrin repeats. In vitro-translated proteins, truncated downstream of the Rel domain and excluding the repeats, bind κB sites. We refer to the κB-binding, truncated protein as p50B by analogy with p50-NF-κB and to the full-length protein as p97. p50B is able to form heteromeric κB-binding complexes with RelB, as well as with p65 and p50, the two subunits of NF-κB. Transient-transfection experiments in embryonal carcinoma cells demonstrate a functional cooperation between p50B and RelB or p65 in transactivation of a reporter plasmid dependent on a κB site. The data imply the existence of a complex family of NF-κB-like transcription factors.

NF-κB denotes a transcription factor which has been implicated in the induced expression of many genes and viruses (for reviews see references 2 and 27). NF-κB is presumed to exert its effect through binding to *cis*-acting, so-called κB elements present in the respective promoters-enhancers. The original κB site was defined as an essential functional component of the intronic immunoglobulin κ light-chain enhancer, and its decameric sequence is GGGAC TTTCC (the κB consensus sequence reads GGGRNNY YCC). Included among the NF-κB-responsive genes are many involved in immune responses and/or acute-phase reactions, such as the cytokines interleukin-2, interleukin-6, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor alpha, as well as the interleukin-2 receptor α, the immunoglobulin κ light chain, and angiotensinogen. In addition, certain viruses, including human immunodeficiency virus (HIV) and cytomegalovirus, are regulated in their expression by NF-κB. An array of different signals targets NF-κB to activate gene expression. Tumor necrosis factor alpha, phorbol esters, lectins, interleukin-1, and the human T-cell leukemia virus type I *tax* product, for example, have been reported to increase NF-κB DNA-binding activity in cells (for a review of NF-κB-regulated genes, binding sites, and stimulating agents of NF-κB, see reference 2). The activation of cytoplasmic NF-κB is thought to be mediated by the signal-dependent release from an inhibitory protein designated IκB (3). It has been reported that phosphorylation of IκB by protein kinase C releases NF-κB, thereby allowing the transcription factor to translocate to the nucleus and activate gene expression by binding to *cis*-acting regulatory DNA elements (12). Recently NF-κB has been recognized to exist as a heterodimer of a p50 and a p65 subunit (47). p50 can form homodimers, but it is the heterodimer that is thought to be the primary transactivating complex owing to potentially transactivating sequences present in p65 (34, 38).

The genes encoding NF-κB have recently been cloned. p50 is part of a larger precursor protein of 105 kDa. We have cloned this gene, originally designated 243, as a mitogen-induced cDNA selected from a collection of such clones which harbor immediate-early response genes (6), and others have cloned this gene by using nucleic acid probes based on partial peptide sequences of purified p50 protein (13, 24, 31). The 105-kDa precursor protein consists of two main domains, an amino-terminal region of about 300 amino acids related to the amino-terminal part of the c-Rel protein (7) and a carboxy-terminal portion containing several so-called cell cycle or ankyrin repeat structures. The carboxy-terminal domain inhibits binding of the precursor protein to DNA. Upon removal of the repeat domain by proteolytic cleavage, the amino-terminal region corresponding to the p50 protein and containing all sequences related to c-Rel specifically binds κB DNA elements (37). The p65 gene also encodes a Rel-related protein (34, 38). c-Rel, p50, and p65 share extensive homology in their amino-terminal regions, the so-called Rel homology domain, but differ elsewhere. c-Rel and p65 do not contain the cell cycle (ankyrin) repeats. The finding that p50 and p65 are related to c-Rel and bind κB sites has led to the recent discovery that the c-*rel* proto-oncogene and the v-*rel* oncogene products are also capable of binding κB DNA. However, only c-Rel transactivates, whereas v-Rel inhibits κB-dependent transcription (5, 19, 23, 36). In addition, a *rel*-related *drosophila* gene product, Dorsal, which is the essential morphogenic determinant for ventral-dorsal polarity, binds to sites closely resembling κB sites and activates gene expression (20, 45). These observations suggest a family of NF-κB-like complexes which together are responsible for the pleiotropic cellular effects originally ascribed to just one transcription factor complex.

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Recently the IκB-encoding gene and a related gene from chicken called pp40 have been cloned (10, 18). Both proteins contain cell cycle (ankyrin) repeats. The fact that these inhibitory proteins contain such repeats suggests that the repeats in the 105-kDa p50 precursor protein constitute a built-in inhibitor of this protein.

In the present report we describe the cloning and characterization of a novel gene encoding a κB DNA-binding activity. Like the p50 precursor gene (p105), this gene was discovered as a member of our collection of mitogen-inducible cDNA clones isolated from activated human T cells (the clone was originally designated 189). The encoded protein is most closely related to the p50 precursor (p105), harboring a Rel homology domain and a cell cycle (ankyrin) repeat domain. By analogy with the processed and full-length protein of p50/p105, we refer to the products of this novel gene as p50B/p97. p50B can form a previously unrecognized heteromeric κB-binding complex with the newly described mouse Rel homolog, RelB (39). This complex is distinct from any previously described NF-κB factor. Our results suggest that a complex family of NF-κB-like transcription factors exists in vivo.

MATERIALS AND METHODS

RNA analysis. Purification of peripheral blood T cells, extraction of RNA, fractionation on agarose-formaldehyde gels, and hybridization were performed as described previously (21, 50). The cells were stimulated with phytohemagglutinin (1 μg/ml) and phorbol-12-myristate-13-acetate (25 ng/ml) in the presence or absence of cycloheximide (10 μg/ml). The probe sequence lay between nucleotides 688 and 3113 (see Fig. 2A).

DNA sequencing. Nucleotide sequencing was performed by the dideoxy-chain termination method (Sequenase; U.S. Biochemical Corp.) as specified by the manufacturer. Both strands of several overlapping cDNA clones were sequenced.

Computer analysis. Computer searches were performed at the National Center for Biotechnology Information by using the Blast Network Service and the FASTA and FASTP programs. In addition, we used the PCGENE programs.

TAG constructs. The pMR-TAG plasmid (a generous gift from Mitchell Rosner) is a derivative of pSP64 into which a TAG epitope-encoding DNA segment was inserted downstream of the SP6 promoter. This DNA segment is identical to the one present in the vector pCGN (44): it contains a herpes simplex virus *tk* leader sequence/ATG followed by the sequence ASSYPYDVPDYASLGGPSR, encoding in part the TAG epitope derived from influenza virus hemagglutinin (11). The last two amino acids are encoded by an *Xba*I cloning site which is followed by a polylinker cloning region. By using polymerase chain reaction-generated clones of p50B/p97 into which convenient restriction enzyme sites were inserted as part of primers immediately upstream of the initiating methionine, p50B/p97 was cloned in frame downstream of the TAG epitope. Two constructs were generated: pMR-TAGp50B (truncated at the *Sac*I site [see Fig. 3A], used in the experiments in Fig. 3B, 4A, and 4B) and pMR-TAGp97 (used in the experiments in Fig. 3C, 3D, 4C, 5, and 7); these constructions added the codons for the amino acids QFTRVDPRSAAA (for the pMR-TAGp50B construct) or QF (for the pMR-TAGp97 construct) between the end of the epitope sequence and the original ATG initiator codon of p50B/p97, resulting in the total addition of 32 and 22 amino acids to the encoded proteins, respectively. The p50 gene (clone 243 truncated at the *Rsa*I site) was cloned into the same pMR-TAG vector by using the *Bam*HI site present at nucleotide 260 (6), inserting the amino acids QFTRV between the epitope and the p50 sequence (construct pMR-TAG p50). The sequences of the polymerase chain reaction-generated clones were confirmed by DNA sequencing, as were the cloning junctions of all constructs.

In vitro transcription and translation. An 8-μg portion of the pMR-TAG or Bluescript (Stratagene) vector bearing the gene of interest was linearized with the appropriate enzyme and used as a template for in vitro transcription with T3, T7 (Stratagene), or SP6 (Promega) polymerase as recommended by the manufacturer. After phenol-chloroform extraction and ethanol precipitation, the RNA was resuspended in 100 μl of diethyl pyrocarbonate-treated water. Then 0.5 to 2 μl of this solution was used to direct in vitro translation with wheat germ extract or rabbit reticulocyte lysate (Promega). To obtain heteromeric complexes between two different proteins, we routinely cotranslated their RNAs since mixing of separate translation products resulted in weaker associations.

The Bluescript plasmids used for the translations included those encoding human p65 (38), RelB (39), human p50 (the 243 gene encoding the precursor was truncated at the *Rsa*I site and cloned separately into the *Sma*I site of Bluescript [6]), and a p50B/p97 construct. The p50B/p97 construct contained a polymerase chain reaction-generated 9-nucleotide leader sequence (GCCGCCGCC) downstream of a *Bam*HI site and upstream of the ATG initiator to facilitate in vitro translations. In addition, we used the pMR-TAGp50B, pMR-TAGp97, and pMR-TAGp50 constructs described above.

EMSA. Double-stranded oligonucleotide probes were prepared for the electrophoretic mobility shift assay (EMSA) by annealing the appropriate single-stranded oligonucleotides at 65°C for 10 min in 10 mM Tris–1 mM EDTA–10 mM NaCl followed by slow cooling to room temperature. The probes were end labeled with ^{32}P-labeled ATP, GTP, CTP, and TTP by filling in 5′ overhangs with the Klenow fragment. The oligonucleotides used were as follows.

palindromic κB probe (PD-κB) (5)

wild type 5′-GATCCAACGGCAGGGGAATTCCCCTCTCCTTA
GTTGCCGTCCCCTTAAGGGGAGAGGAATCTAG-5′ and

mutant 5′-GATCCAACGGCAGATCTATTCCCCTCTCCTTA
GTTGCCGTCTAGATAAGGGGAGAGGAATCTAG-5′

HIV κB probe (32)

wild type 5′-GATCAGGGACTTTCCGCTGGGGACTTTCCAG
TCCCTGAAAGGCGACCCCTGAAAGGTCCTAG-5′ and

mutant 5′-GATCACTCACTTTCCGCTGCTCACTTTCCAG
TGAGTGAAAGGCGACGAGTGAAAGGTCCTAG-5′

One to three microliters of the in vitro-translated product (wheat germ extract) was incubated at room temperature for 30 min in a solution containing the incubation buffer, 0.5 to 2 μg of poly(dI-dC) (Pharmacia), and 0.2 ng of the labeled probe (100,000 cpm). The incubation buffer was either the buffer from the Stratagene Gelshift kit or buffer B (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 50 mM KCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, 0.1% Tween 20). Competition assays were performed with a 30-fold excess of unlabeled probe. For supershifts with antibodies, the in vitro-translated proteins and the antibody were incubated on ice for 30 min in incubation buffer plus poly(dI-dC) before the probe was added. Either an anti-TAG monoclonal antibody (Berkeley Antibody Co.) or an anti-RelB polyclonal antibody was used (39). The DNA-protein complexes were

separated on 4% polyacrylamide gels run at room temperature in 0.25× Tris-borate-EDTA (TBE) (41).

Stability assays. The in vitro-translated products were incubated with the probe with or without GTP as described. After 30 min of incubation, a sample was removed and loaded on a gel (time zero) and a 500- to 1,000-fold excess of the unlabeled probe was added. Subsequently aliquots were removed and loaded onto a continuously running gel (4°C) at the times indicated in Fig. 5. This experimental design ensured a minimum of experimental variation.

Immunoprecipitations. Immunoprecipitations were performed as described in the accompanying paper by Ryseck et al. (39). A 6-μl portion of each [^{35}S]methionine-labeled translation (Promega rabbit reticulocyte lysate system) was added to 0.6 ml of RIPA buffer without sodium dodecyl sulfate (SDS). Then 1.5 μl of RelB polyclonal antibody was added and mixed, and the mixture was allowed to stand on ice for 2 h. Ten microliters of a 1:1 slurry of protein A-Sepharose (Pharmacia) in RIPA buffer without SDS was added and mixed on a rotator at 4°C for 2 h. The protein A-Sepharose plus the antibody complex was then recovered by centrifugation; washed in succession with 0.6 ml of buffers A (twice), B, and C; resuspended in 10 mM Tris-HCl (pH 7.5); boiled in Laemmli sample buffer; and electrophoresed on an SDS-polyacrylamide gel electrophoresis (PAGE) gel (10% acrylamide). Buffer compositions are given in the accompanying paper (39).

Expression vectors and reporter plasmid. *relB*, p65, p50 (truncated at the *Xba*I site), and p50B (truncated at the *Xho*I site; see Fig. 3A for a map of the truncations) were cloned in the *Eco*RI site of the PMT2T expression vector (22). This plasmid contains an adenovirus major late promoter, a simian virus 40 enhancer, and a simian virus 40 polyadenylation site. The reporter plasmid, HIV-κB CAT, has a chloramphenicol acetyltransferase (CAT) gene driven by a minimal c-*fos* promoter which contains the two κB sites from the HIV enhancer inserted at the *Sal*I site at −56 (14, 26, 35). Another version of this construct containing two mutant κB sites was used also (J32).

Transfection of cell lines and CAT assay. Two million cells were transfected with 0.03 to 3 μg of each expression plasmid and 6 μg of the reporter plasmid. The DNA was diluted in 0.5 ml of 0.25 M $CaCl_2$, mixed with 0.5 ml of 2× BBS (BES-buffered saline), and then added to the medium [2× BBS buffer is 50 mM *N*,*N*-bis(2 hydroxyethyl)-2-aminoethanosulfonic acid (BES; pH 6.96) containing 280 mM NaCl and 1.5 mM Na_2HPO_4].

The cells were incubated for 14 to 16 h at 37°C, washed, and incubated further for 24 h before being harvested and lysed. Cell extracts were heated at 70°C for 10 min and incubated in a scintillation vial with a freshly prepared solution of chloramphenicol (3 mg/ml) and [^{3}H]acetyl coenzyme A (200,000 cpm) in 0.1 M Tris (pH 7.8). The reaction mixture was gently overlaid with 3 ml of Econofluor (Du Pont) (33). The scintillation vials were counted at selected time intervals.

RESULTS

Cloning and sequence of the cDNA encoding p50B/p97. We have previously constructed a subtractive cDNA library from peripheral blood T cells which was enriched for mitogen-inducible genes. Screening of this library with a subtracted probe, again enriched for genes activated early after mitogenic stimulation, yielded more than 60 distinct cDNAs (21, 50). Most of these clones were determined to be novel genes, and among them were a number of putative transcriptional regulatory proteins (6, 49; unpublished data). Recently we reported the characterization of one of these mitogen-induced genes, clone 243, encoding a c-Rel-related protein (6); 243 encodes the 105-kDa precursor of the p50 subunit of the NF-κB transcription factor complex (13, 24, 31). Analysis of an additional mitogen-induced clone designated 189 has now revealed a novel distinct *rel*-related gene. We will refer to the products of this gene as p50B/p97 by analogy with p50/p105-NF-κB (see below).

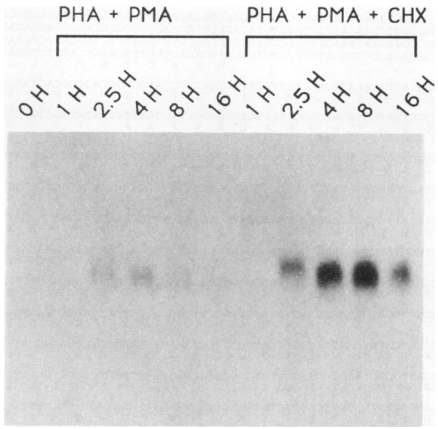
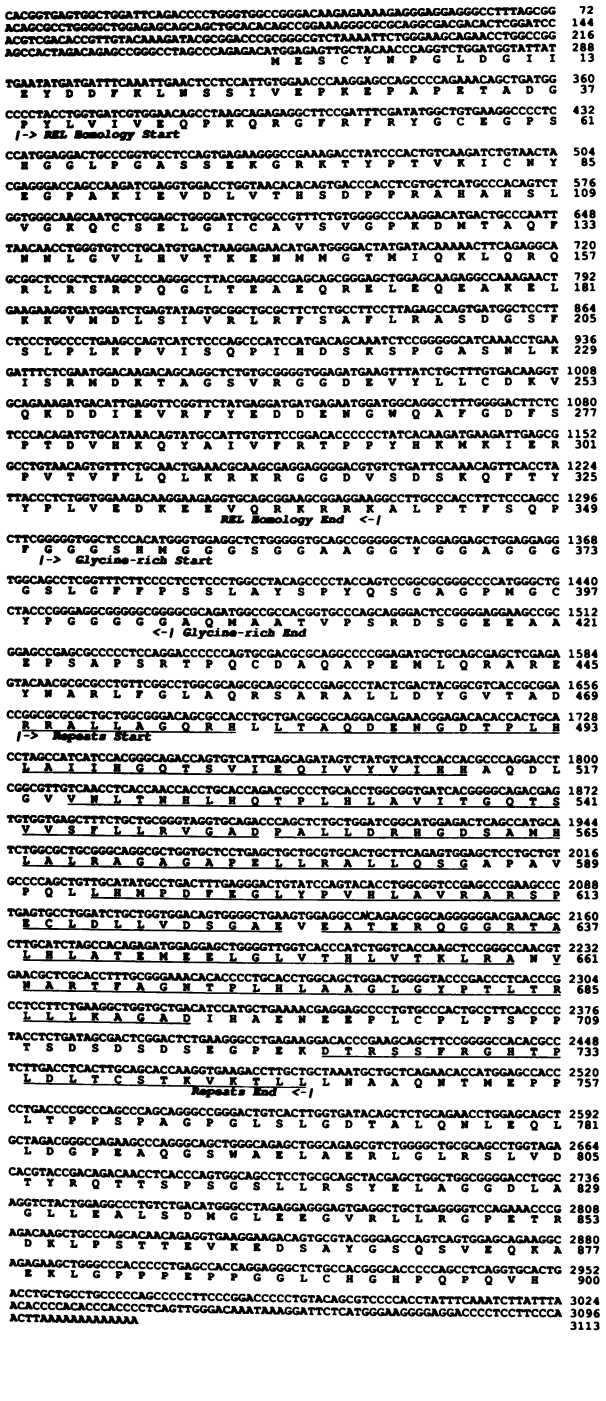


FIG. 1. Induction of p50/p97 mRNA. Peripheral blood T cells were stimulated with phorbol-12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA) in the presence or absence of cycloheximide (CHX) (see Materials and Methods). Total cellular RNA was extracted at the times indicated. Roughly equivalent amounts of RNA were loaded in each lane as confirmed by hybridization with a β_2-microglobulin probe (data not shown). We note that the size of the mRNA decreases slightly with time, possibly reflecting a progressive loss of the poly(A) tract.

Northern (RNA) blot analyses showed that the p50B/p97 mRNA induction in peripheral blood T cells closely mirrored that of p50/p105-NF-κB (clone 243) (6). The approximately 3,300-nucleotide mRNA appeared first between 1 and 2.5 h after stimulation of peripheral blood T cells with phytohemagglutinin and phorbol-12-myristate-13-acetate; it exhibited prolonged induction beyond 16 h, although the level began to decline after 4 h (Fig. 1). The protein synthesis inhibitor cycloheximide superinduced the mRNA as is the case for many genes activated early by mitogens (50). p50B/p97 mRNA was also inducible in other cells, including human peripheral blood B cells, the Jurkat and CEM T cell lines, mouse bone marrow-derived mast cells, and normal human lung fibroblasts stimulated with serum (data not shown).

The sequence of the p50B/p97 cDNA is shown in Fig. 2A. Its size is close to the estimated length of the mRNA. Several different cDNAs were isolated and analyzed to obtain the sequence. An ATG conforming to the Kozak consensus rules (25) is found at nucleotide 251, at the beginning of a large open reading frame encoding a 900-amino-acid polypeptide (molecular mass, 96.8 kDa). There are several in-frame and out-of-frame stop codons preceding the ATG at nucleotide 251. A polyadenylation signal is present at position 3055.

The predicted polypeptide encoded has extensive homology with other Rel-related proteins in its amino-terminal half (6, 7, 9, 13, 16, 24, 31, 34, 38, 42, 43, 48) and contains in its carboxy-terminal half six to seven cell cycle (ankyrin) re-



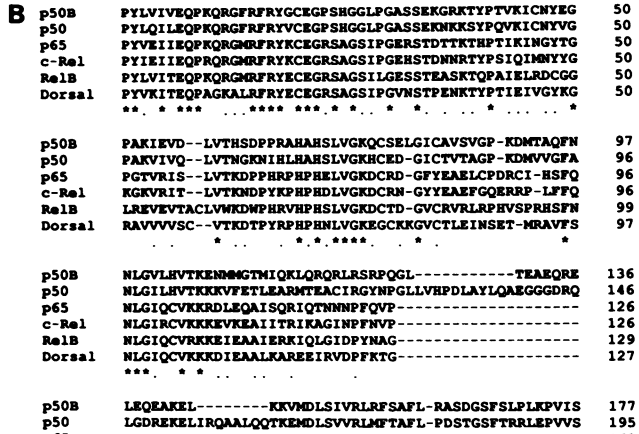


FIG. 2. (A) Nucleotide sequence and predicted amino acid sequence of p50B/p97 (clone 189). The Rel-related domain, the glycine-rich stretch, and the cell cycle (ankyrin) repeat motifs are indicated. The individual repeats are underlined. Numbering of nucleotides and amino acids is shown on the right. The predicted amino acid sequence differs from that in reference 40 at the following positions of our sequence: 144 (E, K), 213 (I, T), 396 (G, R), 433 and 434 (DA, EP), 455 to 470 (QRSARALLDYGVTAD, HAAPSPTRLLRHRG), 741 (K, L), 859 and 860 (TT, T), 891 (C, S), and 900 (H, T). In addition, our predicted sequence terminates after amino acid 900, whereas theirs continues for 35 more amino acids. A distinct cDNA clone of ours codes for a K instead of an E at position 144. (B) Amino acid comparison of the amino-terminal domains of p50B, p50/κBF-1/243 (6), human p65 (38), mouse RelB (39), human c-Rel (7), and Dorsal (43). Stars indicate amino acid identity among the six proteins, and dots indicate similar amino acids. Identity between the amino-terminal domain of p50B and these related proteins is 61.5% with p50, 44.1% with p65, 41.5% with RelB, 45.1% with c-Rel, 44.8% with v-Rel, and 40.8% with Dorsal.

peats (1, 6, 24, 30). These two domains are separated by a glycine-rich spacer region. Thus, the overall structure of p97 is very similar to that of the precursor of the p50 subunit (p105) (6, 13, 24, 31). p105 and p97 differ most significantly from the other Rel-related proteins (c-Rel, p65, RelB [39], and Dorsal) in that they contain cell cycle repeats. In vivo processing is thought to remove the repeat domain from p105, liberating a DNA-binding p50 polypeptide (37). The overall identity between p105 and p97 is 44%, and the identity in the amino-terminal Rel-related domain is 61.5%. The comparison of the amino-terminal domain of p97 (p50B) with the various Rel-related proteins is shown in Fig. 2B. Both p50 and p50B contain a short stretch of amino acids in the middle of the Rel homology region not found in c-Rel, p65, or Dorsal (Fig. 2B), but these additional amino acids are not well conserved between the two proteins. A further distinguishing feature of p50B is the absence of a putative phosphorylation site for protein kinase A (RRXS) found in all other Rel-related proteins; the equivalent amino acids in p50B are KRKR (starting with amino acid number 310). A nearby putative nuclear localization signal (15, 17) is present in all proteins of this family, including p50B (amino acids 337 to 341) (Fig. 2). This signal is located at the end of the Rel homology region. Apart from the Rel domain and the gly-

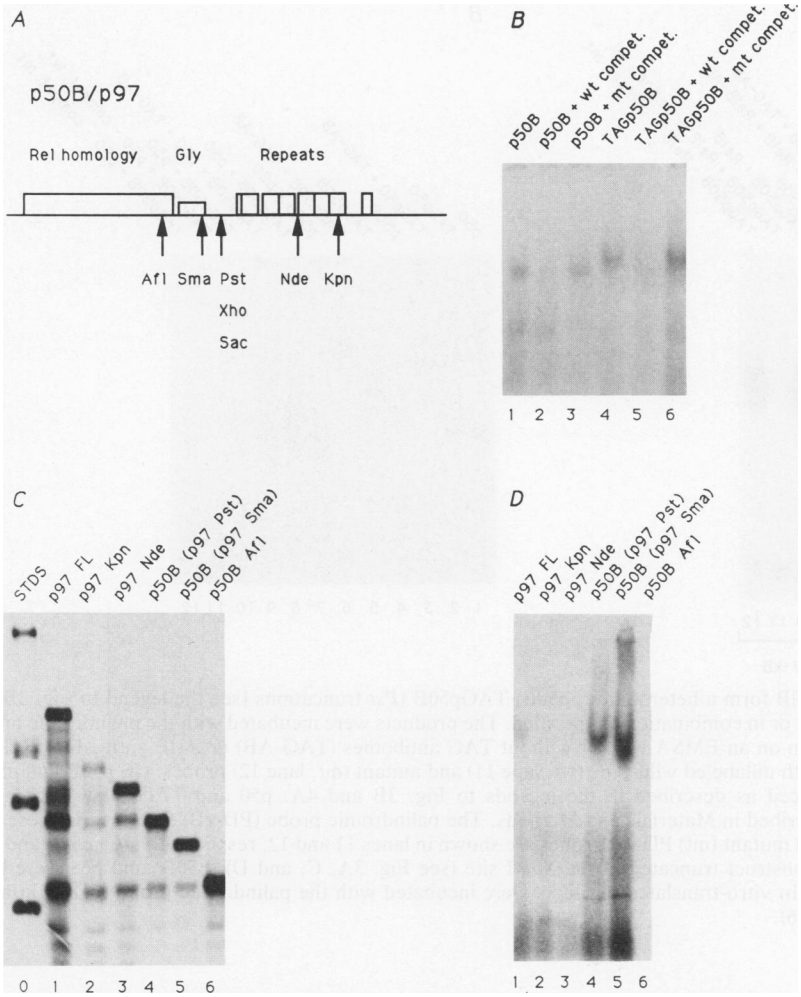


FIG. 3. (A) Restriction enzyme sites used in this paper to produce in vitro-truncated products and their relative positions with respect to the different domains of the p50B/p97 protein. These sites are located at the following nucleotide positions: *Afl*III, 1197; *Sma*I, 1444; *Pst*I, 1568; *Xho*, 1575; *Sac*I, 1578; *Nde*I, 2030; and *Kpn*1, 2286. (B) In vitro-translated p50B binds DNA. The Bluescript construct of p97 cut with *Pst*I (necleotide 1568) (p50B) and a *Pst*I-linearized version of the TAGp50B construct (pMR-TAGp50B) were used for in vitro transcription and translation (wheat germ extract). The translated product was incubated with a labeled PD-κB and run on an EMSA (see Materials and Methods). Lanes: 1, binding of p50B; 2, competition with an excess of the unlabeled probe; 3, competition with the mutant probe; 4, binding of the TAGp50B; 5 and 6, competition of the TAGp50B binding with wild-type (wt) and mutant (mt) unlabeled probes, respectively. (C) In vitro translations of truncated p50B/p97 proteins. The pMR-TAGp97 plasmid was linearized at different restriction sites and used for in vitro transcription and translation (wheat germ extract) to generate truncated proteins of progressively decreasing size. The restriction enzymes used were *Bam*HI, to generate a full-length TAGp97 protein (p97 FL, lane 1), *Kpn*I (p97 Kpn, lane 2) *Nde*I (p97 Nde, lane 3), *Pst*I (p50B [p97 Pst, lane 4]), *Sma*I (p50B [p97 Sma, lane 5]), and *Afl*III (p50B Afl, lane 6). Molecular mass markers (Rainbow markers; Amersham [200, 97.4, 69, and 46 kDa) are shown in lane 0. In addition to the major product, each lane shows several smaller species, which probably result from prematurely terminated translation products in the wheat germ system. (D) Binding of the variously truncated proteins to the PD-κB probe. The in vitro-translated products described in panel C were incubated with the PD-κB probe as described above. The resulting complexes were resolved on an EMSA.

cine-rich region, the homology between the p105 and p97 is confined almost entirely to the repeats (analysis not shown).

The amino-terminal half of p97 (p50B) binds κB DNA sites. Given the high degree of similarity to p50/p105-NF-κB, we investigated the binding to κB sites by a p97 truncated polypeptide analogous to p50 truncations (p50B). Wheat germ extracts programmed with in vitro-transcribed RNA of p97 truncated near the glycine-rich domain sequence (Fig. 3A shows a map of p50B/p97 and various truncations including the *Pst*I site used here) yielded a product (p50B) which could bind a palindromic κB oligonucleotide (PD-κB) in an EMSA (Fig. 3B, lane 1). The binding was specific, as shown by competition with an excess of wild-type (lane 2) or mutant (lane 3) oligonucleotide for the κB site; only the wild-type κB site competed. Since the specific band in this experiment migrated at the position of a weaker nonspecific band, competition appeared not to be complete. Complete competition was evident, however, when the same experiment was repeated with a slightly larger p50B protein, creating a band shift migrating more slowly than the nonspecific band (Fig. 3B, lanes 4 to 6). In this case a short TAG epitope-encoding sequence was fused in frame to the amino terminus of p50B, yielding a slightly bigger product (see Materials and Methods); this TAG construct was consistently more efficiently translated in vitro, probably owing to a different leader sequence, resulting in a stronger band shift (see also Fig. 4).

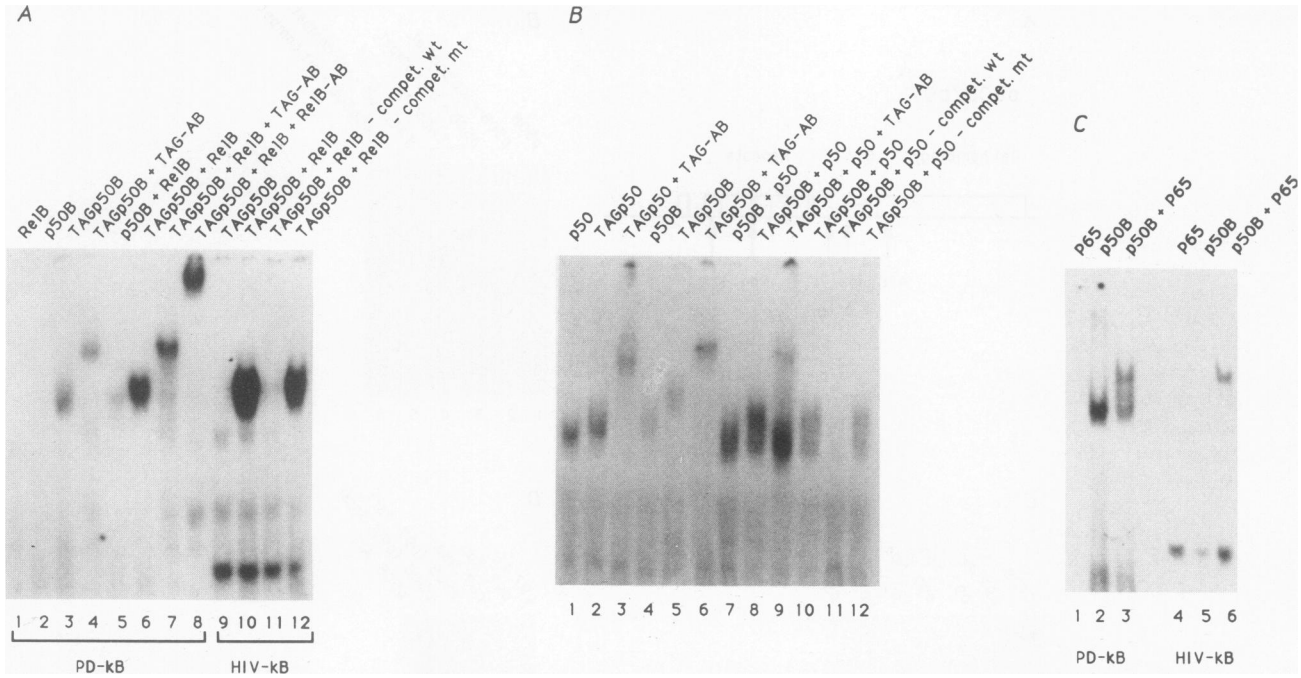


FIG. 4. (A) p50B and RelB form a heterodimer. p50B, TAGp50B (*Pst* truncations [see the legend to Fig. 3B]), and RelB were produced by in vitro translations alone or in combination as specified. The products were incubated with the palindromic probe (lanes 1 to 8) or the HIV probe (lanes 9 to 12) and run on an EMSA with or without TAG antibodies (TAG-AB) or RelB antibodies (Rel-AB) as indicated. Lanes 11 and 12 show competition with unlabeled wild-type (wt, lane 11) and mutant (mt, lane 12) probes. (B) p50B and p50 form a heterodimer. p50B and TAGp50B were produced as described in the legends to Fig. 3B and 4A. p50 and TAGp50 were generated from Bluescript and pMR-TAGp50 vectors, described in Materials and Methods. The palindromic probe (PD-κB) was used in these EMSAs. Competitions with unlabeled wild-type (wt) and mutant (mt) PD-κB probes are shown in lanes 11 and 12, respectively. (C) p50B and p65 form heterodimers. The full-length pMR-TAGp97 construct truncated at the *Sma*I site (see Fig. 3A, C, and D) (p50B) and p65 were translated either alone or in combination (wheat germ). In vitro-translated products were incubated with the palindromic probe (PD-κB) (lanes 1 to 3) or with the HIV probe (HIV-κB) (lanes 4 to 6).

The TAG construct was generated to permit unique identification of the encoded protein product by monoclonal antibodies directed at the TAG epitope (see below). We did not observe binding by p50B to the HIV-κB sites (see below), suggesting at least a much reduced affinity for this site.

We also tested the DNA-binding activity of in vitro-translated p97 truncated at a number of different sites to yield sequentially longer products. Previous work with the precursor p105 protein had shown that the carboxy-terminal domain inhibited DNA binding by the Rel homology domain (6, 13, 24, 31). The carboxy-terminal domain of p97 appears to possess a similar function. Protein translations of full-length p97 RNA or of truncations resulting in RNAs which preserve several of the cell cycle repeats were less able to bind the palindromic κB (PD-κB) probe (Fig. 3D, p97 FL, p97 Kpn, p97 Nde [lanes 1, 2, and 3, respectively]) than were the translations of truncations which removed these repeats (Fig. 3D, p50B [Pst truncation], p50B [Sma truncation] [lanes 4 and 5, respectively]) (Fig. 3C shows corresponding protein translations, and Fig. 3A gives the map). However, when the truncation removed part of the Rel homology domain, no binding was detected (Fig. 3D, p50B Afl [lane 6]). The small but detectable amount of binding observed for translations of the longer RNAs, and especially of the full-length RNA, was presumed to be the result of prematurely terminated protein made in the wheat germ system. Judging from its size, a prominent peptide species appeared to be terminated around the glycine-rich region. This species was seen in addition to the larger full-length proteins (Fig. 3C, p97 FL, p97 Kpn, and p97 Nde [lanes 1, 2, and 3, respectively]). Since the band shifts seen with the full-length protein translations migrated with a mobility approximately equal to that seen with the *Pst*I truncation (p50B), it is likely that the prematurely synthesized products were responsible; if the full-length protein had participated in the binding, a band shift of much slower mobility would be expected. In this regard it is relevant to note the difference in the mobility of the shifts generated by the closely spaced *Pst*I and *Sma*I truncations (Fig. 3D, p50B Pst and p50B Sma [lanes 4 and 5, respectively]).

p50B forms heteromeric complexes with other Rel-related proteins. In light of the binding of p50B to κB sites, we tested whether such a truncated protein could form κB-binding heteromeric complexes with RelB, p50, and p65. RelB is a novel Rel-related murine protein which is most homologous with c-Rel and p65 and has no cell cycle repeats (39). RelB did not generate a specific band shift with various κB oligonucleotides (Fig. 4A, lane 1) (39). However, when RelB was cotranslated with the TAG-bearing p50B (TAGp50B + RelB [lane 6]), a clear band shift appeared which migrated more slowly than the one created by TAGp50B alone (lane 3). This would be expected if TAGp50B (439 + 32 amino acids from the TAG) and the somewhat larger RelB protein (558 amino acids) formed a heterodimer, similar to the heterodimer formed between p50 and p65 (47). Heteromeric complexes between p50B/p97 and RelB could be shown independently by their coimmunoprecipitation (see below).

The p50B construct without the amino-terminal TAG sequence was translated more weakly here, and the slightly faster-migrating band shift created by its protein product was seen clearly only with longer exposures (Fig. 4A, lane 2; Fig. 4B). As expected, p50B without TAG also formed a complex with the RelB protein (lane 5), which migrated slightly faster than the corresponding one carrying the TAG leader (lane 6). Generally we noted a stronger band shift with the heteromeric complex relative to the homomeric p50B complex.

Additional experiments demonstrated directly that the distinct band shift seen with cotranslations of RelB and p50B did contain both proteins. Monoclonal antibodies to the TAG epitope present at the amino terminus of p50B supershifted the heteromeric complex (lane 7), as did polyclonal antibodies to the RelB protein (lane 8). In both cases the supershifting was nearly complete; little or no complex remained at the original position. The RelB antibodies did not cross-react with p50B (see below), and the monoclonal TAG antibodies did not react with RelB (data not shown).

Further evidence for a complex between p50B and RelB was revealed in experiments with the HIV-κB oligonucleotide probe: TAGp50B alone did not bind this probe (Fig. 4A, lane 9), nor did RelB alone (39; data not shown), but both together created a strong band shift (lane 10) which was specific for the κB site as shown by competition with an excess of unlabeled wild-type and mutant oligonucleotide (lanes 11 and 12). The better binding of the p50B homomeric complex to the PD-κB site than to the HIV-κB site mirrors what has been reported for the binding of the p50 homodimer (46). The p50 protein and the p65 protein apparently contact separate halves of the classical κB sites (47). Because the PD-κB (PD-κB) sequence is based on the half implicated in p50 binding, it explains the preferential binding of the p50 homodimer to the PD-κB site. RelB was able to form DNA-binding heteromeric complexes with p50 as well (39).

In experiments analogous to those above, we demonstrated that p50 and p50B could also associate. Cotranslation of p50B and p50 led to the formation of distinct heteromeric complexes (Fig. 4B, p50B + p50 and TAGp50B + p50 [lanes 7 and 8, respectively]) which migrated between the homomeric p50 complex (lane 1) and the corresponding homomeric p50B complex (Fig. 4B, p50B or TAGp50B [lane 4 or 5, respectively]). We could always discern the formation of some homomeric p50 complexes as well with these cotranslations, perhaps reflecting an excess of this protein and/or a less effective association with p50B. We noted the generally stronger binding observed with p50 homomeric complexes than with p50B homomeric complexes. When TAG monoclonal antibodies were added to the cotranslation of p50 and TAGp50B, the heteromeric complex was supershifted almost completely, while a considerable amount of p50 remained behind as a homomeric complex (Fig. 4B, lane 9). We also were able to supershift a heteromeric complex in which p50 rather than p50B carried the TAG epitope (data not shown). The heteromeric complex between p50B and p50 (lane 10) was sequence specific, as shown by competition with wild-type (lane 11) and mutant (lane 12) PD-κB oligonucleotides.

p50B also formed heteromeric complexes with p65 (Fig. 4C). Cotranslation of TAGp50B and p65 formed a new band shift not seen with either protein alone. The heteromeric complex could be supershifted with TAG antibodies (data not shown) and bound both the HIV-κB site and the PD-κB site. The relative mobilities of the homomeric and heteromeric complexes in all of our DNA-binding experiments are consistent with the conclusion that these complexes bind as dimers, although we have not directly demonstrated this. p50 and p65 are also thought to bind DNA as dimers (47). Taken together, the data suggest that many Rel-related proteins can associate with each other and bind κB sites, although they may do so differentially.

We investigated the stability on DNA of the p50B homomeric complex and the p50B/RelB heteromeric complex. As shown in Fig. 5A (lanes 1 to 6), the complex formed by p50B with the radioactive probe decreased with time during a challenge with a 500-fold excess of unlabeled probe, but was still detectable after 4 h. In the presence of 5 to 10 mM added GTP, stability was increased (Fig. 5A, lanes 7 to 12). GTP has been reported to increase the binding of complexes involving the p50 subunit of NF-κB (4, 28). Given our experimental design, the data suggest that, at least for p50B, it is the stability of the homomeric complex with κB sites which is enhanced. The apparent boost in the strength of the band shift with increasing time of competition in the presence of GTP probably occurs because the bands are less diffuse than those seen with the longer runs. It is also possible that the complexes are slightly unstable in gels during longer runs.

A test of the stability of the p50B/RelB heteromeric complex with the PD-κB probe in vitro revealed a less stable complex relative to the homomeric p50B complex (Fig. 5B, lanes 1 to 6). The presence of GTP significantly increased the amount of initial binding detected prior to competition (Fig. 5B, lane 7). The stability of the heteromeric complex increased as well (Fig. 5B, lanes 7 to 13), although it was less stable than the homomeric complex with GTP. A qualitatively similar result was obtained when the experiment was repeated with the HIV-κB probe (data not shown). The p50B/p65 heteromeric complex was also enhanced in its initial binding and in its stability by the addition of GTP (data not shown). Taken together, these experiments revealed that both homomeric and heteromeric p50B complexes are fairly stable, at least in the presence of GTP.

p50B/p97 and RelB form heteromeric complexes independent of DNA. In view of the potential for RelB and p50B to associate on DNA, we investigated whether various different truncated products of p50B/p97 could form heteromeric complexes with RelB in solution without DNA and, if so, whether the presence of the repeats prevented the formation of such complexes. Blocking a protein-protein interaction could be a means of inhibiting DNA binding by the repeats. Full-length and truncated RNAs of p50B/p97 were cotranslated with *relB* RNA in rabbit reticulocyte lysates, and then the products were immunoprecipitated with RelB antibodies. Figure 6 shows that full-length p97 protein and all truncations which occurred carboxy-terminal to the Rel domain could be coprecipitated with RelB antibodies (p97 FL, p97 Nde, and p50B [p97 Sma] [lanes 6, 10 and 14, respectively], see Fig. 3A for a map of the truncations), whereas a truncation ending within but near the end of the Rel-related domain did not complex with RelB (p50B Afl [lane 18]). Figure 6, lanes 5, 9, 13, and 17, shows the cotranslated products which were subjected to immunoprecipitation. RelB antibodies did not cross-react with the p50B/p97 products (lanes 4, 8, 12, and 16; lanes 3, 7, 11, and 15 show the translation products for p50B/p97). We conclude that p50B/p97 complex formation with RelB occurred independently of DNA, required a peptide sequence present in the carboxy-terminal end of the Rel homology domain, and was not interfered with by the cell cycle (ankyrin) repeats.

p50B cooperates with RelB and with p65 in stimulating transcription through the NF-κB site. RelB, p65, p50, and

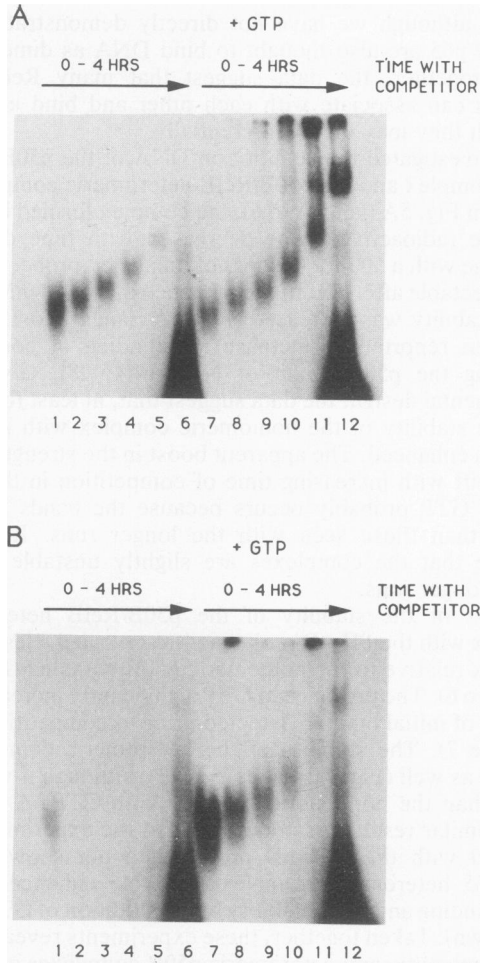


FIG. 5. Stability of the homomeric p50B (A) or the heteromeric p50B/RelB (B) complexes with DNA in the presence or absence of added GTP (5 mM) as indicated in the figure. p50B was generated by in vitro translation of a *SmaI* truncation of pMR-TAGp97 FL (Fig. 3A), and this product alone or cotranslated with RelB was incubated for 30 min with the palindromic probe (PD-κB). Subsequently a sample was removed and loaded on a gel (time 0) (lanes 1 and 7), and a 500-fold excess of unlabeled probe was added to the remaining solution. Thereafter, aliquots were removed and loaded on the gel after 15 min (lanes 2 and 8), 30 min (lanes 3 and 9), 1 h (lanes 4 and 10), 2 h (lanes 5 and 11), and 4 h (lanes 6 and 12).

p50B were each cloned into the expression vector PMT2T (22) to transiently transfect NTera-2 embryonal carcinoma cells. These cells have little or no endogenous NF-κB activity (see below). The expression plasmids were transfected alone or in combination into NTera-2 cells along with a reporter plasmid, HIV-κB CAT, in which the CAT gene is driven by a minimal c-*fos* promoter dependent on the HIV NF-κB sites (see Materials and Methods). Figure 7 shows the results recorded as the average of three to five independent transfection experiments. Although p50B or p50 caused little or no expression of the NF-κB site-dependent reporter plasmid, even at high concentrations, the combination of p50B with RelB or with p65 stimulated expression well beyond the weaker stimulation observed with RelB alone or with p65 alone. Interestingly, p50 and p50B were approximately equally effective in cooperating with p65 (Fig. 7) and with RelB (data not shown). The amounts of the transfected DNAs were chosen for optimal levels of cooperation. p65 alone at higher concentrations transactivated increasingly well by itself, whereas RelB alone transactivated only weakly even at high concentrations. The results may imply that p65 and RelB harbor a transactivation domain and that p50 and p50B provide high-affinity DNA binding (34, 38, 39). As a further control for these experiments, a reporter plasmid containing two mutant κB sites was not transactivated, supporting the conclusion that the observed positive transactivations were mediated through the κB site (data not shown). Transfection of the HIV-κB CAT reporter plasmid alone resulted in CAT values that were not significantly higher than background levels in untransfected cells, indicating that these cells had little or no intrinsic NF-κB activity (see legend to Fig. 7).

In additional experiments we observed cooperation in transactivation between p97 and RelB at higher concentrations of transfected DNAs (data not shown). It is possible that the full-length p97 protein was processed in vivo into an active form equivalent to p50B, which would account for this result.

DISCUSSION

This report describes the cloning and characterization of a novel mitogen-inducible gene product which is closely related to the p105 precursor protein for the p50 subunit of the classical NF-κB transcription factor complex. We have chosen the descriptive name p50B/p97 because of functional and structural similarity with p50/p105-NF-κB. By analogy with p50-NF-κB, p50B is the truncated form of the p97 protein, including the amino-terminal Rel-related domain but excluding the carboxy-terminal cell cycle (ankyrin) repeats. Like p50, p50B binds κB sites, forms heteromeric complexes with p65 and RelB, and synergizes with these proteins in transactivating through a κB site in vivo. The *rel* gene family can now be divided into two distinct groups, members of one encoding cell cycle (ankyrin) repeats (including p50/p105 and p50B/p97) and members of the other without such repeats (including c-*rel*, p65, *relB* [39], and dorsal).

We cloned both p50/p105 (clone 243) and p50B/p97 (clone 189) as cDNAs from a subtracted library enriched for genes which are mitogenically induced at an early time in human peripheral blood cells (6, 21, 50). The mRNAs of both genes have similar kinetics of induction in primary human T cells. In addition, *relB* and c-*rel*, as well as the inhibitor IκB-encoding gene, have been reported to be early response genes (8, 18, 39). NF-κB-like activity in cells is therefore likely to be controlled by transcriptional as well as posttranslational events. The transcriptional regulation of Rel-related and inhibitory genes may be coordinated during cellular activation. That all these genes are rapidly inducible in their expression demonstrates further that immediate-early activation genes include many important regulatory proteins which are central to the decision-making processes leading to proliferation and the expression of a differentiated phenotype.

The amino-terminal Rel-homologous domain and the carboxy-terminal cell cycle (ankyrin) repeat domain of p50/p97 are separated by a glycine-rich stretch. It has been suggested that an analogous glycine-rich region in the p50 precursor protein contains a proteolytic cleavage site for processing (37). It is therefore likely that p97 is processed into a protein of about 50 kDa as well (p50B), and preliminary findings in our laboratory support this (unpublished observation). A previous report has identified several Rel-related κB DNA-binding proteins detectable by UV cross-linking, proteins

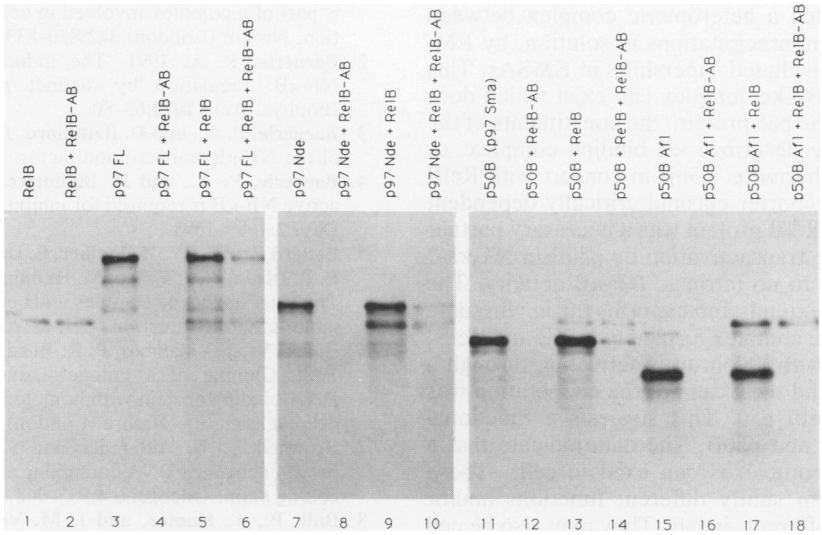


FIG. 6. Coimmunoprecipitation of p50B/p97 and RelB. Lanes on the SDS-PAGE gel contain [^{35}S]methionine-labeled in vitro translation products of the templates translated alone and in combination as indicated (RelB, full-length TAGp97, and various truncations thereof). See Fig. 3C and D for p50B/p97 constructs used and Fig. 3A for a map of the truncations. Odd-numbered lanes contain the translation products; even-numbered lanes contain the products recovered following immunoprecipitation with RelB antibody (AB).

with molecular masses of 50, 55, 75, and 85 kDa, the last of these being c-Rel (5). It is possible that an in vivo-processed form of p97 corresponds to the 50- or 55-kDa species, although the authors speculated that these two proteins may be related to each other and correspond to the p50/κBF-1 subunit of the classical NF-κB complex.

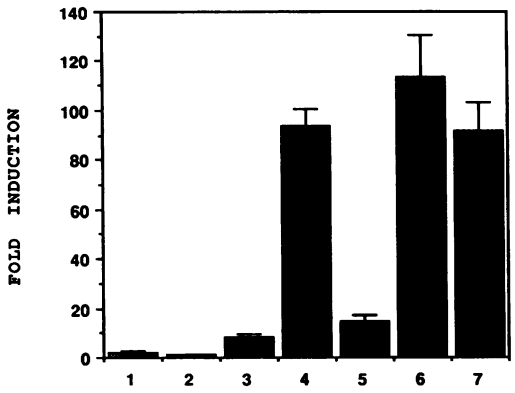


FIG. 7. p50B together with RelB or p65 stimulates transcription in vivo of a CAT reporter plasmid dependent on a κB site (HIV-κB CAT; see Materials and Methods). CAT assays were performed on cell extracts of NTera-2 cells after transfection with constructs expressing p50B, p50, RelB, or p65. These constructs were transfected separately or together as indicated. The figure shows the fold induction observed over the activity produced by a transfection with the HIV-κB CAT vector alone. The fold induction values represent the mean of three to five independent transfections after normalizing to the protein concentrations of the cellular extracts. Transfections involving RelB were done separately from those involving p65. The absolute counts obtained from the transfection of the CAT vector alone ranged from 1× to 1.9× above background levels in the absence of CAT vector. Amounts of DNAs used were as follows: 0.1 μg of RelB, 0.03 μg of p65, 0.1 μg of p50, and 0.1 μg of p50B. For p50 alone or p50B alone, 3 μg was transfected to show insignificant transactivation even at higher DNA concentrations. Tracks: 1, p50; 2, p50B; 3, RelB; 4, p50B-RelB; 5, p65; 6, p50-p65; 7, p50B-p65.

The Rel-related domain (p50B) is responsible for DNA binding and for protein-protein interaction to form homomeric or heteromeric complexes. The association between RelB and p50B depends on the carboxy-terminal portion of the Rel homology domain. Truncations removing this amino acid sequence abolish the interaction. These data are consistent with preliminary observations of site-directed mutations in the analogous region of the p50 protein which ablate dimerization (unpublished results). A recent report demonstrating that a deletion of the amino-terminal half of the Rel domain of p50 can still form dimers is in accord with our findings (29).

As observed for the p105 precursor protein of p50, the carboxy-terminal repeat domain of p97 apparently inhibits DNA binding. On the other hand, this domain does not interfere with dimerization, at least not with RelB. This suggests that the inhibitory activity inherent in the repeat domain may be directed at the DNA-binding domain of p50B/p97 rather than the dimerization domain; a similar scenario is likely for the p50 precursor. The very recent discovery that IκB proteins contain repeats confirms such a view (10, 18). The IκB protein is thought to inhibit the DNA binding of p65 but not its dimerization potential (34). The repeats may therefore be the common denominator for all proteins which have as a functional target the DNA-binding domains present in the Rel homology regions of the various proteins. This does not rule out other possible functions of the carboxy-terminal half of p97 and p105. For example, it has been suggested that the repeats may mediate an interaction of these proteins with cytoskeletal structures (6, 18, 30).

p50B does not possess a protein kinase A phosphorylation site near its nuclear translocation signal sequence. This distinguishes p50B from the other Rel-related proteins described to date, with the exception of RelB (39). It is not known whether the putative protein kinase A site present in p50, p65, c-Rel, and Dorsal is physiologically significant, but if it is, one could envision a role in nuclear translocation and/or function of these proteins.

We have demonstrated a heteromeric complex between p50 and RelB by immunoprecipitations in solution, by EMSAs, and by antibody-mediated supershifts in EMSAs. This indicates that an NF-κB-like complex can exist which does not involve the p50 or the p65 protein, the constituents of the classical, biochemically described κB-binding complex, or the c-Rel protein. Furthermore, p50B in concert with RelB transactivated a CAT reporter plasmid critically dependent on an NF-κB site. The RelB protein was a necessary partner to demonstrate a strong transactivation by p50B in NTera-2 cells, which have little to no intrinsic NF-κB activity. The functional cooperation extends and confirms the in vitro data on the novel NF-κB-like complex formed by p50B and RelB. p50B also cooperated with p65 in transactivating through a κB site in these cells, and the extent of the cooperation was similar to that seen with p50. This suggests a functional similarity between p50 and p50B. The data indicate that a family of NF-κB-like complexes can exist in cells. These complexes may perform subtly different functions and/or may be responsive to different signals. They may also be cell type specific, possibly depending on the amounts of the various constituents present. Further experiments are needed to determine in vivo which complexes exist in a given cellular milieu, what their specific functions are, and how they are regulated.

In vitro experiments showed that binding of heteromeric complexes between p50B and RelB or p65 was increased by the addition of GTP, as was the stability of these complexes when challenged with an excess of κB sites. On the other hand, the effect of GTP on the homomeric p50B complex was primarily one of stabilizing the complex in the presence of the competitive binding sites. GTP has been previously recognized to increase the binding of p50/p65 or of p50/p50 to DNA (4, 28). It is possible that the GTP effect seen in vitro reflects a physiologically relevant phenomenon; however, it is not known whether a ligand-induced change in binding occurs in vivo. Our data with p50B imply that responsiveness to GTP is not a unique property of the p50/κBF-1 subunit of NF-κB.

After the initial completion of the manuscript, the polymerase chain reaction-mediated cloning of a gene which appears to be identical to p50B/p97 was reported (40). We note several sequence differences which include changes of the protein structure in two extended regions (see the legend to Fig. 2A). Also, we did not detect the reported shorter alternatively spliced mRNA in our cells, which included primary lymphocytes. In accord with their data, we did observe cooperation between p50B (their p49 protein) and p65. However, we did not observe the reported dramatic difference in transactivation between p50B (their p49) and p50. In our assay system p50 cooperated at least as well as p50B did with either p65 or RelB.

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