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Both N- and C-Terminal Domains of RelB Are Required for Full Transactivation: Role of the N-Terminal Leucine Zipper-Like Motif

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RelB, a member of the Rel family of transcription factors, can stimulate promoter activity in the presence of p50-NF-κB or p50B/p49-NF-κB in mammalian cells. Transcriptional activation analysis reveals that the N and C termini of RelB are required for full transactivation in the presence of p50-NF-κB. RelB/p50-NF-κB hybrid molecules containing the Rel homology domain of p50-NF-κB and the N and C termini of RelB have high transcriptional activity compared with wild-type p50-NF-κB. The N and C termini of RelB cooperate in transactivation in *cis* or *trans* configuration. Alterations in the structure of the leucine zipper-like motif present in the N terminus of RelB significantly decrease the transcriptional capacity of RelB and of different RelB/p50-NF-κB hybrid molecules.

Serum stimulation of quiescent fibroblasts induces the expression of more than 100 genes, including several proto-oncogenes encoding for transcriptional factors belonging to the Jun, Fos, and Rel families (2, 11, 13, 24, 34–36, 38).

The Rel family of transcription factors is defined by the Rel homology domain (RHD), a highly conserved region of approximately 300 amino acids, which is considered to function as a DNA binding and protein dimerization domain. The first identified members of this gene family were the v-*rel* oncogene (55, 58) and its cellular homolog c-*rel* (61). Another member of this family is the maternal gene *dorsal* of *Drosophila melanogaster*, which is involved in establishing the dorsal ventral axis (56). Recently, a number of genes encoding for the transcriptional complex NF-κB were identified as members of this family. These include p50-NF-κB (also known as KBF1) with its precursor p105-NF-κB (10, 17, 32, 40), p50B-NF-κB (also known as p49 and *lyt*-10) with its precursor p97-NF-κB (9, 41, 52), p65-NF-κB (42, 48), and *rel*B (51), also named I-*rel* (49). All of the protein products of this gene family have the capacity to bind to κB sites found in the regulatory regions of many genes involved in the immunological response and/or acute-phase reactions such as in the immunoglobulin kappa (Igκ), interleukin-2, interleukin-6, beta interferon, and other genes (3, 8, 18, 19, 20, 22, 37, 42, 54).

Another characteristic shared by members of the Rel family is their ability to interact with members of the IκB family. The inactive NF-κB/IκB complexes are localized in the cytoplasm (4, 5). After stimulation of cells with serum factors, cytokines, antigens for lymphocytes, or many other stimuli, NF-κB dissociates from IκB and translocates to the nucleus, where it functions as a transcriptional activator (43). In vitro IκB inhibits the binding of the NF-κB complexes to DNA. This IκB effect originally demonstrated for p50-/p65-NF-κB heterodimers (16, 62) and c-Rel (14, 31) has been recently extended to p50-NF-κB/RelB heterodimers (57) and p50-NF-κB homodimers (27). Interestingly, the activity of the *D. melanogaster* protein dorsal is similarly regulated by the inhibitory factor cactus (28). The different homodimers and heterodimers formed between the members of the Rel family regulate gene expression in vivo (20, 22, 42) and in vitro (15, 33). The activating domains of p65-NF-κB (50, 53) and c-Rel (12) have been mapped to their C-terminal regions.

Recently we reported the characterization of *rel*B, an immediate-early gene isolated from our collection of growth factor-induced genes (2). Serum stimulation in NIH 3T3 cells leads to the induction of RelB protein to an extent comparable to the induction of p105-NF-κB. We further demonstrated that RelB interacts with p50- and p50B-NF-κB, forming heterodimers able to bind to κB sites. These complexes, in contrast to p50- and p50B-NF-κB homodimers, activate the transcription of a promoter containing κB sites in vivo (9, 51). These data indicate that RelB, like p65-NF-κB, provides the heterodimers with an activating domain.

In this report, we analyze by transfection into mammalian cells the RelB domains which contribute to its transactivating potential. Surprisingly, the acidic C-terminal region alone does not efficiently transactivate in mammalian cells, nor does its removal completely abolish the activity of RelB. Using a number of RelB mutants and hybrid molecules between p50-NF-κB and RelB, we demonstrate that the N-terminal region of RelB is involved in transcriptional activation. We show further that the structural integrity of a leucine zipper-like motif located in the N terminus is essential for the transactivating potential of this region.

MATERIALS AND METHODS

Cell culture and transfections. F9 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics (100 U of penicillin per ml and 50 μg of streptomycin per ml). Transfections were performed by using the standard calcium phosphate coprecipitation method (21) with minor modifications. Typically, 2 μg of reporter vector and 4 μg of each of the expression vectors were used. The reporter vector 2 × κB-tk-CAT (51), containing two κB binding sites from the mouse immunoglobulin κB enhancer, was used in all experiments. To rule out any

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variations due to possible titration of transcriptional factors, the total amount of DNA was always adjusted to 15 μg with pMexneo. In some experiments, pBAG (45), containing the β-galactosidase gene under the control of the Moloney murine leukemia virus long terminal repeat, was cotransfected as an internal control.

Jurkat cells were grown in RPMI medium supplemented with 10% fetal calf serum and antibiotics (100 U of penicillin per ml and 50 μg of streptomycin per ml). Transfections were performed by electroporation at 220 V and 960 μF, using a Bio-Rad electroporator. Typically, 10^7 cells were electroporated with 2 μg of reporter plasmid and 4 μg of each of the expression vectors. Cells were harvested 46 h after transfection and processed as described previously (21).

Chloramphenicol acetyltransferase (CAT) assays were performed as described previously (21). Reactions were normalized for β-galactosidase levels and/or protein concentrations. All assays were quantitated by using a PhosphorImager (Molecular Dynamics).

Constructs for in vitro transcription. The constructs used for the in vitro transcription of RelB and p50-NF-κB have been described elsewhere (51). RelB ΔC2 (amino acids 1 to 392) was constructed by digesting *rel*B with *Ava*I (position 1171) and *Xba*I (in Bluescript polylinker) and inserting the oligonucleotide

TCGGGATCATGACAGCTACGGTGTGGACAAGAAGCGAAAGAAGCTTTAGT
CTAGTACTGTCGATGCCACACCTGTTCTTCGCTTTCTTCGAAATCAGATC

as a linker, introducing at the end of the nuclear localization signal of the RHD a *Hin*dIII restriction site followed by a stop codon.

RelB ΔN1 (amino acids 103 to 558) was constructed by digesting *rel*B with *Hin*dIII (in polylinker) and *Sph*I (position 385) and inserting two oligonucleotides in the following order:

AGCTTCTGTGTTCACTAGCAACCTCAAACAGACACCATGCGCATG
AGACACAAGTGATCGTTGGAGTTTGTCTGTGGTACGC

CCGTACCTGGTCATCACAGAGCAGCCAAAGCAGCGTGGCATG
GTACGGCATGGACCAGTAGTGTCTCGTCGGTTTCGTCGCACC

The first oligonucleotide, derived from the β-globin gene, provides the initiation codon for efficient translation plus two amino acids; the second restores the N terminus of the RelB RHD.

The clone expressing RelB RHD (amino acids 103 to 392) was generated by a combination of RelB ΔC2 and RelB ΔN1, using a unique *Bgl*II site in the RHD.

The hybrid molecule p50/RelB 5 was constructed by digesting p50-NF-κB with *Hin*dIII (position 1380) and *Xba*I (polylinker) and recombining it with a polymerase chain reaction (PCR) product that was generated by using the oligonucleotide GAAGCGAAGCTTGGACTGCCTGATG, starting at position 1215 and finishing at the T7 primer corresponding to the C terminus of RelB. Thus, in the hybrid the first 364 amino acids derived from p50-NF-κB, and the last 167 amino acids from RelB.

RelB/p50 6 is a combination of RelB ΔC2 (partial digestion with *Hin*dIII and complete with *Sac*I) and p50-NF-κB (*Hin*dIII and *Sac*I), which corresponds to the first 392 amino acids from RelB and the last 92 from p50-NF-κB (amino acids 363 to 455).

RelB/p50 1 was constructed by recombining two PCR products. The first fragment was generated by using the T3 primer and the oligonucleotide CCAGGTAAGGCCTTGGG CACGGGCCG, corresponding to positions 331 to 358 of *rel*B, introducing a *Stu*I restriction site. The second fragment was generated by using the T7 primer and the oligonucleotide GTCAACAGATTGGCCATACCTTCAAATATTAG, corresponding to positions 409 to 427 of the murine p50-NF-κB, introducing an *Msc*I restriction site. After digestion and recombination, the resulting clone encodes the first 102 amino acids of RelB followed by p50-NF-κB amino acids 40 to 455.

To generate RelB/p50 2, a PCR product of p50-NF-κB generated by using the T7 primer and the oligonucleotide GAGGGGCATGCGATTCCGCTATGTG (positions 451 to 465) containing a *Sph*I restriction site was recombined with the corresponding *Sph*I site (position 385) of *rel*B. The resulting construct contains amino acids 1 to 116 of RelB and 54 to 455 of p50-NF-κB.

To generate the N-terminal region of I-Rel (49), poly(A)$^+$ RNA was isolated from Daudi cells, cDNA was synthesized by using oligo(dT) as the initial primer, and PCR was performed with the oligonucleotide pair CTCCCGGGAAT TCGGGCCCCGCGTGC**ATG**CTTCGG and TGCTGCGG ATCCAGCGGCCCTCGCACTCGTAGCGG, the first of which delivers the ATG as described by Ruben et al. (49). The resulting product was digested with *Eco*RI and *Sph*I and used to replace the corresponding region of RelB, creating an I-Rel/RelB hybrid which contains the first 148 amino acids of I-Rel and the last 442 amino acids of RelB.

To confirm the reading frames, all constructs were checked by sequencing.

Site-directed mutagenesis. Mutagenesis was performed by using a kit from Promega Biotec according to the manufacturer's protocol. The leucine-to-proline change (amino acid 36) and the ΔELE deletion (amino acids 35, 36, and 37) in RelB were introduced with oligonucleotides CCAGGACCA CAGATGAACCAGAAATCATCGACGAATAC and GGT CTCCAGGACCACAGATATCATCGACGAATACATTA AG, respectively. Successful in vitro mutagenesis was confirmed by sequencing.

Expression vectors. Expression vectors were constructed by recloning the corresponding coding regions from the Bluescript vector into the mammalian expression vector pMexneo (39). The identity of the constructs was confirmed by sequencing.

In vitro synthesis of proteins. RNA for in vitro translation was generated by using the T7 and T3 polymerase kit from Ambion according to the manufacturer's protocol. For efficient capping, the original 20-μl reaction was diluted to 100 μl and adjusted to 6 mM KCl, 50 mM Tris-HCl (pH 7.9), and 50 mM *S*-adenosylmethionine. After incubation with 1.5 U of guanylyltransferase (GIBCO-BRL) at 37°C for 1 h followed by DNase I digestion for 10 min, the RNA was phenol-chloroform extracted, precipitated in the presence of 1 M ammonium acetate with ethanol, and resuspended in 100 μl of water.

Two microliters of RNA was in vitro translated in a wheat germ extract as directed by the manufacturer (Promega Biotec) in the presence of [^{35}S]methionine (Amersham).

Electrophoretic mobility shift assay (EMSA). Oligonucleotides corresponding to the two strands of the double-stranded κB sequence of the mouse Igκ enhancer were annealed as described previously (51). The sequences of the oligonucleotides used were the following:

TCGACAGAGGGGACTTTCCCAGAGGC
GTCTCCCCTGAAAGGGTCTCCGTCGA

For EMSA, 1 pmol of the double-stranded oligonucleotide was end labeled with [α-^{32}P]dCTP by filling in the overlap-

ping ends with the Klenow fragment of DNA polymerase I. The oligonucleotide was separated from the unincorporated nucleotides by using a Sephadex G-25 column. One hundred picograms of the oligonucleotide probe was incubated with 1 μl of in vitro-translated protein in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 8% glycerol, 0.5 mM dithiothreitol, 0.5% Nonidet P-40, and 0.1 μg poly(dI-dC) as a nonspecific DNA competitor in a final volume of 40 μl. Reaction mixtures were incubated for 30 min at room temperature, loaded onto a 6% acrylamide-bisacrylamide (40:1) gel, and run in 0.25× TBE buffer (22 mM Tris base, 22 mM boric acid, 0.5 mM EDTA).

Protein gels. Gel separation of in vitro-translated proteins was performed in a 10% acrylamide-bisacrylamide (100:1) gel as described previously (51).

RESULTS

RelB requires both the N and C termini for its transcriptional activity. Previously, we demonstrated that RelB is able to transactivate transcription via a κB binding site in complexes with p50- or p50B-NF-κB (9, 51). In addition to the RHD and a nonconserved C-terminal region present in the Rel proteins, RelB contains a unique N terminus of 102 amino acids. This domain is significantly longer than the N-terminal region present in other members of this family.

To determine which parts of RelB are necessary for its activating potential, N- and C-terminal deletion mutants were constructed. The RHD was kept intact, as it is essential for dimerization and DNA binding. The capacity of the different RelB mutants to enhance the transcription of a κB-dependent promoter was analyzed in cotransfection assays in combination with p50-NF-κB and a CAT reporter construct containing two copies of a κB site upstream of the thymidine kinase promoter. As previously shown, when the CAT reporter plasmid is cotransfected with a RelB expression vector into F9 cells, a weak but consistent transcriptional activation is observed (Fig. 1a and c, lanes B). In contrast, the reporter plasmid cotransfected with a p50-NF-κB expression vector results in no significant increase in CAT activity (Fig. 1a and c, lanes A). The low transactivation by RelB alone can be explained by its inability to form homodimers and therefore the lack of DNA binding activity in the absence of a partner (51). Thus, the observed increase in transcriptional activity is most likely due to the association of RelB with endogenous p50-NF-κB. EMSAs using nuclear extracts from F9 cells have demonstrated that these cells contain p50-NF-κB homodimers (not shown). Cotransfection of both RelB and p50-NF-κB expression vectors significantly increases CAT activity over that observed with p50-NF-κB alone, confirming that these proteins cooperate in the transactivation of a promoter dependent on a κB site (Fig. 1a and c, lanes C).

To examine the contribution of the C terminus of RelB in transactivation, vectors expressing a C-terminally truncated version of RelB (RelB ΔC2) and p50-NF-κB were cotransfected with the CAT reporter construct (Fig. 1a and c, lanes D). The results show that the C-terminal deletion mutant in combination with p50-NF-κB activates transcription to a lesser extent than does wild-type RelB. This was expected from our previous results showing that the acidic C-terminal domain of RelB confers transcriptional activity to a GAL4 fusion protein in yeast cells (51). However, the CAT activity in F9 cells following cotransfection of RelB ΔC2 together with p50-NF-κB is clearly higher than that observed after

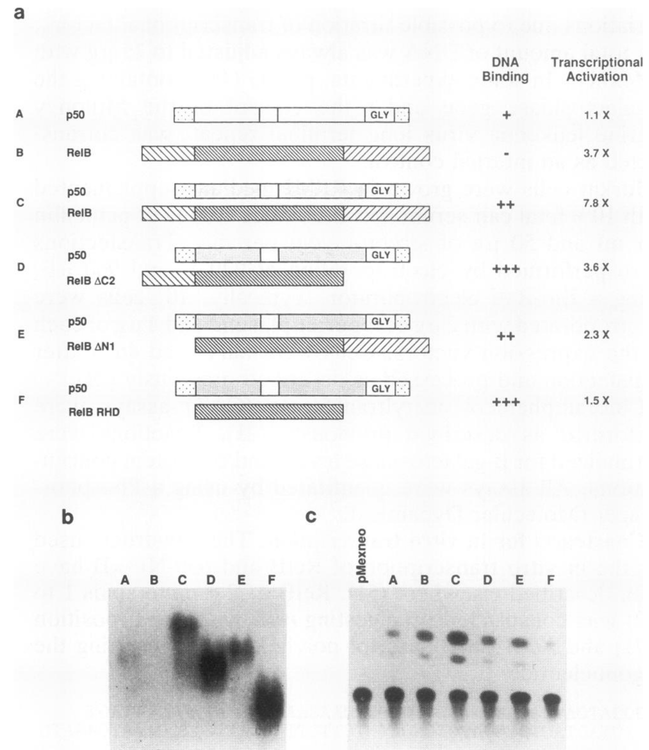


FIG. 1. Evidence that RelB requires the N and C termini for its transcriptional activity. (a) Schematic representation of the different RelB mutants cotransfected with the p50 expression vector and the 2 × κB-tk-CAT reporter plasmid. The relative DNA binding and transcriptional activation of the resulting dimers are based on EMSA of in vitro-translated proteins (b) and CAT assays (c), respectively. The transcriptional activity was calculated as an average of four independent experiments quantitated in a PhosphorImager. Transfection of pMexneo alone serves as a control and represents onefold activation. The thin hatched boxes represent the RHD of RelB; the broad hatched boxes indicate the N- and C-terminal domains of RelB. The shaded boxes represent the RHD of p50; the dotted boxes are the N and C termini of p50. An empty box within p50 RHD represents the hinge not present in RelB. The GLY box indicates the glycine-rich region of p50. (b) EMSA analysis of the DNA binding activity of the different dimers represented in panel a. Proteins were cotranslated in the presence of [^{35}S]methionine in the wheat germ system, and comparable amounts, as determined by gel electrophoresis, were tested in DNA binding assays using the murine Igκ gene κB binding site as described in Materials and Methods. Lanes A to F correspond to the combinations depicted in panel a. (c) Representative CAT assays of transfection experiments summarized in panel a. F9 cells were cotransfected with 4 μg of each of the expression vectors (A to F) and 2 μg of reporter plasmid. The total amount of DNA transfected was adjusted to 15 μg with pMexneo. Cells were processed as described in Materials and Methods.

transfection of p50-NF-κB alone, suggesting that RelB ΔC2 can still cooperate with p50-NF-κB to enhance transcription. This finding prompted us to investigate the role of the N terminus of RelB in transcriptional activation. Although cotransfection of vectors expressing the N-terminally deleted RelB (RelB ΔN1) and p50-NF-κB still increases CAT activity, the levels are consistently lower than that observed with RelB ΔC2 (Fig. 1, lanes E). Therefore, truncation of the N terminus of RelB has a more significant effect than does deletion of the C-terminal region. This finding is in contrast

to our results obtained in yeast cells, in which case the N terminus of RelB confers no detectable transcriptional activity to a GAL4 fusion protein (51). To determine whether the RHD of RelB also contains a transactivating domain, we cotransfected vectors expressing only this region, RelB RHD, and p50-NF-κB (Fig. 1a and c, lanes F). The results show that such heterodimers do not activate transcription of the CAT reporter construct, suggesting that the N- and C-terminal regions, but not the RHD of RelB, contain transcriptional activation domains.

The observed differences in the transcriptional activities of the various heterodimers could be due to variations in their ability to dimerize or to bind to a κB site. Therefore, all proteins were synthesized by in vitro translation in the presence of [^{35}S]methionine by using a wheat germ extract, and their dimerization as well as DNA binding activities were determined by immunoprecipitation (not shown) and EMSA, respectively, using equivalent amounts of proteins as confirmed by gel electrophoresis. In agreement with previously reported results (33), p50-NF-κB homodimers have a weak DNA binding activity, and RelB alone is not able to bind to the asymmetric κB site present in the mouse Igκ enhancer (Fig. 1b, lanes A and B, respectively). However, when RelB and p50-NF-κB are cotranslated, heterodimers that strongly bind DNA are formed. This can be clearly seen in the EMSA by the decreased mobility of the RelB/p50-NF-κB heterodimer DNA complex compared with that of the complex containing p50-NF-κB homodimers (Fig. 1b, lane A versus lane C). All RelB mutants, including RelB RHD, which has no transcriptional activity in the presence of p50-NF-κB, dimerize with p50-NF-κB (not shown). These heterodimers show binding affinities for the κB site similar to that of the heterodimer containing the complete RelB protein (Fig. 1b, lanes D to F). This finding indicates that the observed decrease in transcriptional activation of the various RelB deletion mutants is not due to a reduction in heterodimer formation or to differential binding of these heterodimers to a κB site.

These results demonstrate that both the N and C termini are necessary for the full transcriptional activity of RelB.

Roles of the N- and C-terminal domains of RelB in transcriptional activation. To further characterize the transactivating potential of the N- and C-terminal domains of RelB, hybrids between RelB and p50-NF-κB were constructed by replacing the N or C terminus of p50-NF-κB with the corresponding region of RelB but leaving the RHD of p50-NF-κB intact. We have chosen this approach over the GAL4 fusion system because it also enables us to replace the N and C termini simultaneously and to determine their transactivating properties in a more natural context. These molecules were then tested for their potential to transactivate a κB site-dependent reporter construct by transfecting the hybrid construct alone or in combination with p50-NF-κB, RelB, or its derivatives into F9 cells.

As shown in Fig. 2a and c (lanes A), a p50-NF-κB construct containing the C-terminal region of RelB (p50/RelB 5) slightly increases CAT activity. This finding indicates that the C terminus of RelB itself is only a weak transcriptional activator in mammalian cells, despite being present twice in the resulting homodimer. Alternatively, the lower activation might be due to the weak binding of p50/RelB 5 to the κB binding site (Fig. 2b, lane A). To investigate this possibility, we cotransfected p50/RelB 5 with the RelB deletion mutant lacking the N terminus, RelB ΔN1. The transcriptional activation by the resulting RelB ΔN1/p50-NF-κB heterodimer is weak, despite the fact that it binds very efficiently to the κB binding site (Fig. 2, lane E). This result further demonstrates that the C terminus alone functions only as a weak transactivation domain either in RelB or when fused to a heterologous molecule such as p50-NF-κB.

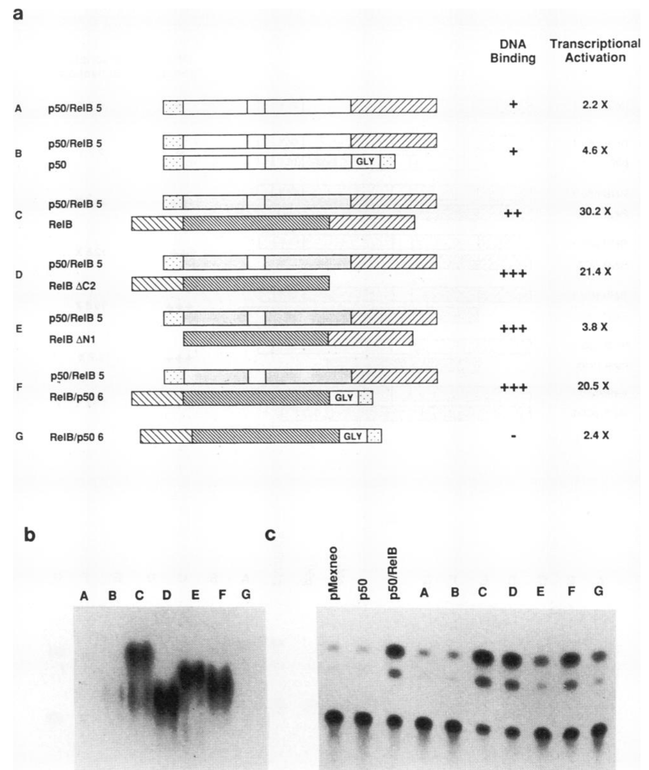


FIG. 2. Functional characterization of the C terminus of RelB. (a) Expression vectors encoding RelB, p50, and their derivatives were cotransfected in combinations as schematically indicated together with the 2 × κB-tk-CAT reporter plasmid. The relative DNA binding and transcriptional activation of the resulting dimers are based on the EMSA analysis of in vitro-translated proteins and CAT assays as shown in panels b and c, respectively. All other details are as for Fig. 1. (b) EMSA analysis of the DNA binding activity of the different molecules depicted in panel a. Proteins were cotranslated in the wheat germ system, and comparable amounts were tested in binding assays using the κB binding site present in the murine Igκ gene. A to G correspond to the dimers depicted in panel a. (c) Representative CAT assays from transfection experiments summarized in panel a. F9 cells were cotransfected with the different constructs shown in panel c as described for Fig. 1.

Cotransfection of p50/RelB 5 together with wild-type RelB leads to a strong transactivation that is 10-fold higher than that observed with p50/RelB 5 and RelB ΔN1 (Fig. 2a and c, lanes C). These data further demonstrate that the N-terminal region of RelB is necessary for efficient transactivation. It is important to note that p50/RelB 5 together with RelB presents a much higher transcriptional activation than does wild-type p50-NF-κB together with RelB, probably because of the additional contribution of the C terminus present in the p50/RelB 5 hybrid molecule. To investigate whether this strong activation was due to the presence of two RelB C termini in the heterodimers or mainly to a unique contribution by the C terminus in the p50/RelB 5 molecule, the latter was cotransfected with RelB ΔC2, which lacks the C terminus (Fig. 2a and b, lanes D). There is a decrease in the transactivating capacity of this heterodimer in comparison

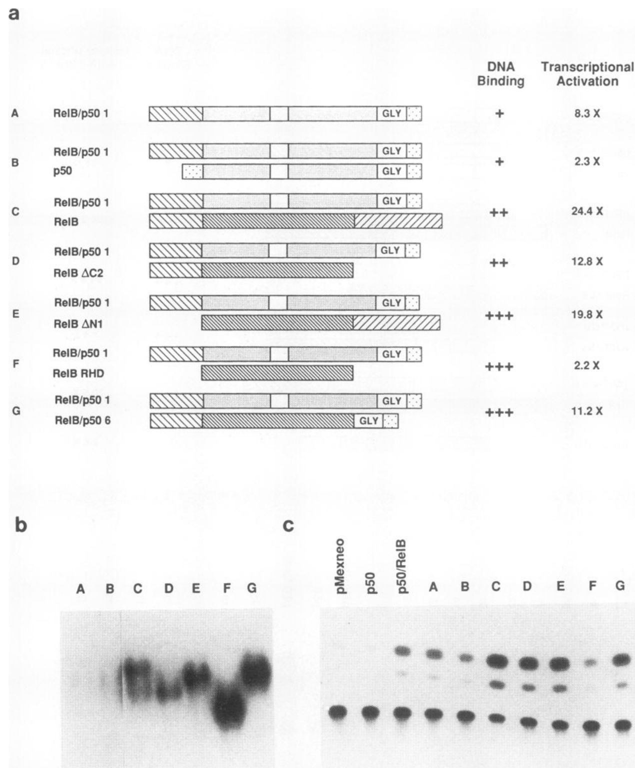


FIG. 3. Evidence that the N terminus of RelB contains a transactivating domain. (a) Schematic representation of p50, RelB, and derivatives cotransfected in combinations together with the 2 × κB-tk-CAT reporter plasmid. The relative DNA binding and transcriptional activation of the resulting dimers are based on the EMSA analysis of in vitro-translated proteins and CAT assays as shown in panels b and c, respectively. All other details are as for Fig. 1. (b) EMSA analysis of the DNA binding activity of the different dimers depicted in panel a. Proteins were cotranslated in the wheat germ system, and comparable amounts were tested in binding assays as described for Fig. 1. A to G represent the combinations depicted in panel a. (c) Representative CAT assays of transfections summarized in panel a. Cotransfections were performed as described for Fig. 1.

with p50/RelB 5 and RelB; however, it is still significantly more active than the combination of both wild-type molecules.

To test whether the glycine-rich region of p50-NF-κB has any significant effect on transactivation, a construct of RelB with its C terminus replaced by that of p50-NF-κB, named RelB/p50 6, was cotransfected with p50/RelB 5 (Fig. 2a and c, lanes F). The increase in CAT activity is comparable to that observed for p50/RelB 5 and RelB ΔC2, indicating that the C terminus of p50-NF-κB has little or no influence on the transactivation activity already provided by the N and C termini of RelB in these heterodimers. Since RelB/p50 6 has the RelB dimerization domain, it does not form homodimers and consequently cannot bind to DNA (Fig. 2b, lane G). As expected, this construct transfected alone stimulates transcription weakly (Fig. 2c, lane G).

The role of the N-terminal region of RelB was investigated in another series of experiments by using a p50-NF-κB hybrid, RelB/p50 1, in which the N terminus of p50-NF-κB was replaced with that of RelB (Fig. 3a, lane A). This hybrid molecule homodimerizes (not shown) and binds with low affinity to the κB site, comparable to the wild-type p50-NF-κB molecule (Fig. 3b, lane A). Interestingly, the RelB/p50 1 homodimer has a transactivating potential that is significantly higher than that observed with p50/RelB 5, which contains the C-terminal part of RelB (Fig. 2a and c, lanes A), and is comparable to that of the heterodimer p50-NF-κB/RelB (Fig. 1c). This finding suggests that the N terminus of RelB is a positive modulator of transcription which is functional on a heterologous molecule. However, in this configuration, efficient transactivation occurs only when each molecule of the dimer contains the N-terminal domain, since the activity of RelB/p50 1 is consistently lower when the construct is cotransfected with p50-NF-κB (Fig. 3a and c, lanes B). This conclusion is supported by the data obtained when RelB/p50 1 was cotransfected with RelB ΔC2 or RelB RHD. In the first case, the N-terminal region is present in both molecules of the dimer, which confers a high transcriptional activation (Fig. 3a and c, lanes D). This can be explained by the observation that this heterodimer binds better to a κB binding site than does the RelB/p50 1 homodimer (Fig. 3b; compare lanes A and D). If only one of the molecules contains the N terminus, as in the combination RelB/p50 1 with RelB RHD, there is a strong decrease in the activity compared with RelB/p50 1 alone, although the resulting heterodimers efficiently bind to DNA (Fig. 3, lanes F). These results indicate that the N terminus when present on both molecules of the dimer is sufficient to transactivate, whereas a single copy of this domain cannot efficiently activate transcription. It is important to note that this conclusion is valid only for molecules lacking the C-terminal domain. For example, cotransfection of RelB/p50 1 with RelB ΔN1, which generates a heterodimer with only one N and one C terminus (in *trans*), produces a strong increase in CAT activity (Fig. 3a and c, lanes E). The high activity conferred in *trans* by these domains functions similarly when the domains are present on either the p50-NF-κB or RelB molecule. As expected, RelB/p50 1 with wild-type RelB leads to an activation that is even higher than that with RelB ΔN1 (Fig. 3a and c, lanes C).

These observations confirm that the N terminus of RelB is a stronger transcriptional activator than is the C terminus and demonstrate that these two regions of RelB can cooperate in transactivation in *cis* and *trans* configurations.

The N-terminal leucine zipper-like motif is involved in transcriptional activation. To determine whether the leucine zipper-like motif present in RelB (51) is involved in transcriptional activation, we introduced two independent mutations in this region. In the first case, the third leucine was changed to proline, RelB(L-P); in the second case, a small deletion was introduced by eliminating the sequence glutamic acid-leucine-glutamic acid, RelBΔELE (Fig. 4a), resembling a possible alternatively spliced product as suggested by the analysis on the genomic structure (unpublished data). These two mutations would significantly alter the structure of the motif.

When tested in the CAT expression system, the two mutants have a small but consistently lower transactivating potential than does the wild-type RelB cotransfected with p50-NF-κB (Fig. 4). It is important to note that the effect of these mutations on the transcriptional activity of RelB is comparable to the effect of deletion of the entire N terminus, as shown in Fig. 1. These data suggest that the structural integrity of the leucine zipper-like motif is important for the transactivating potential of the RelB N terminus. The remaining transactivating activity is due to the presence of the C-terminal region.

To further investigate whether the leucine zipper-like

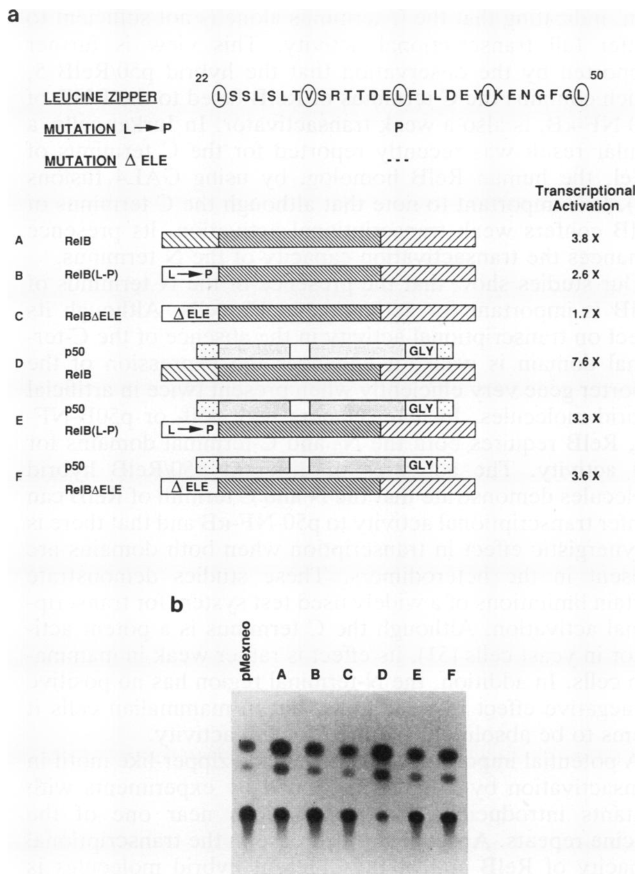


FIG. 4. Involvement of the N-terminal leucine zipper-like motif in transactivation. (a) Schematic representation of two independent mutants of the N-terminal leucine zipper-like motif used in the transfection experiments. The first mutant represents a leucine-to-proline change (L-P); the other mutant contains a three-amino-acid deletion (ΔELE) which includes the third leucine residue of the leucine zipper-like motif. Transcriptional activities of the mutants were compared with that of the wild-type RelB by transfecting the corresponding expression vectors either alone or with a p50-expressing plasmid together with the 2 × κB-tk-CAT reporter plasmid. All other details are as for Fig. 1. (b) Representative CAT assays of the transfection experiments summarized in panel a. The DNA transfections were performed as described for Fig. 1.

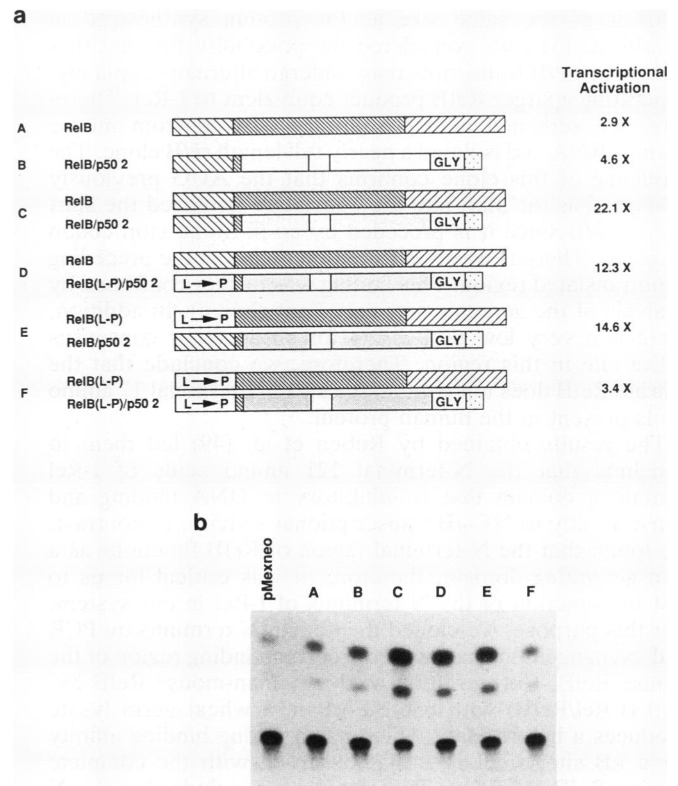


FIG. 5. Evidence that the leucine zipper-like motif is essential for the transcriptional activity of the N-terminal domain of RelB. (a) Expression vectors encoding RelB, RelB/p50 2, and their counterparts carrying the leucine-to-proline mutation (L-P) were cotransfected in different combinations as schematically indicated together with the 2 × κB-tk-CAT reporter plasmid. All other details are as for Fig. 1. (b) Representative CAT assays of F9 transfection experiments summarized in panel a. The DNA transfections were performed as described for Fig. 1.

motif is responsible for the transactivating potential of the N terminus, two p50-NF-κB/RelB hybrids were constructed, RelB/p50 2 and RelB(L-P)/p50 2, in which the first 116 amino acids of RelB with and without the change of leucine to proline, respectively, were joined to amino acids 54 to 455 of p50-NF-κB (Fig. 5a). Cotransfection of RelB together with RelB/p50 2 leads to a strong transactivation, as expected from the presence of two RelB N termini and a C terminus in the heterodimer (Fig. 5, lane C). In contrast, cotransfection of RelB with the hybrid RelB(L-P)/p50 2, which contains the mutation leucine to proline, leads to a weaker transactivation compared with RelB/p50 2 (Fig. 5, lane D). These data further support the notion that the region comprising the leucine zipper-like motif of the N terminus is critical for transcriptional activation. A similar decrease in transcriptional activity was observed when RelB/p50 2 was cotransfected with RelB(L-P), indicating that the structural alteration of the leucine zipper-like motif in either of the two molecules in the dimer has the same effect (Fig. 5, lane E). The remaining activation is due to the contribution of the C and the wild-type N termini, which are present either in *cis* (Fig. 5, lane D) or in *trans* (Fig. 5, lane E). The most dramatic decrease in transcriptional activity is observed when RelB(L-P)/p50 2 and RelB(L-P) are cotransfected. These heterodimers present a nearly eightfold reduction in activity in comparison with RelB/p50 2 and RelB (Fig. 5, lane F). The remaining transcriptional activity is similar to that of heterodimers lacking the N terminus. Taken together, these data indicate that the structural integrity of the region comprising the leucine zipper-like motif present in the N-terminal region is essential for full transcriptional activity of RelB.

Comparison of RelB and its human homolog I-Rel. While this work was in progress, Ruben et al. (49) reported the cloning of I-*rel*, the human homolog gene of the murine *rel*B gene (51). In contrast to our results, it was found that the complete I-Rel is an inhibitor of DNA binding when complexed with p50-NF-κB, whereas an N-terminally truncated I-Rel is able to bind to DNA in the presence of p50-NF-κB. The human gene product contains an additional 17 amino acids in the N terminus that could be responsible for the inhibitory function proposed by these authors. Although our previous data have demonstrated that in vitro-translated

RelB is of the same size as the protein synthesized in fibroblasts (51), we considered the possibility that in other cell types, *rel*B transcripts may undergo alternative splicing, generating a larger RelB product equivalent to I-Rel. Therefore, we screened a cDNA library derived from mouse thymus RNA and isolated a nearly full length *rel*B clone. The sequence of this clone confirms that the AUG previously described as the start site for translation is indeed the start site of *rel*B since it is preceded by an in-frame stop codon (Fig. 6a). There is no other initiation codon in the preceding 5′ untranslated region. This finding was further confirmed by analysis of the genomic sequence (not shown). In addition, there is a very low probability for an acceptor consensus splice site in this region. Therefore, we conclude that the murine RelB does not contain the extra N-terminal 17 amino acids present in the human protein.

The results obtained by Ruben et al. (49) led them to conclude that the N-terminal 121 amino acids of I-Rel contain a domain that is inhibitory to DNA binding and consequently to NF-κB transcriptional activity. In contrast, we found that the N-terminal region of RelB functions as a transactivating domain; therefore, it was critical for us to test the function of the N terminus of I-Rel in our system. For this purpose, we cloned the human N terminus by PCR and exchanged this part with the corresponding region of the mouse RelB. Cotranslation of this human-mouse RelB hybrid (I-Rel/RelB) with p50-NF-κB in a wheat germ lysate produces a heterodimer which has a strong binding affinity for a κB site, similar to that observed with the complete murine RelB (Fig. 6b). Therefore, we conclude that the N terminus of I-Rel is not inhibitory for DNA binding activity. This conclusion is further supported by comparing the transactivating potential of RelB with that of the hybrid I-Rel/RelB upon cotransfection with p50-NF-κB into Jurkat cells. As shown in Fig. 6c, both RelB and I-Rel/RelB, when transfected alone, activate the transcription of the 2 × κB-tk-CAT reporter construct in Jurkat cells two- to threefold. Upon cotransfection with p50-NF-κB, the expression of the reporter construct increases approximately 20- to 22-fold, confirming that the N terminus of I-Rel is not inhibitory to NF-κB activity. Similar results were obtained for F9 cells (not shown). The activities of RelB and I-Rel/RelB were also compared in combination with the hybrid molecule p50/RelB 5 or RelB/p50 1 (not shown). In all cases examined, RelB and I-Rel/RelB activated transcription to very similar levels, further proving that the two molecules are functionally indistinguishable. These results strongly suggest that human RelB, similarly to mouse RelB, is indeed a transcriptional activator.

DISCUSSION

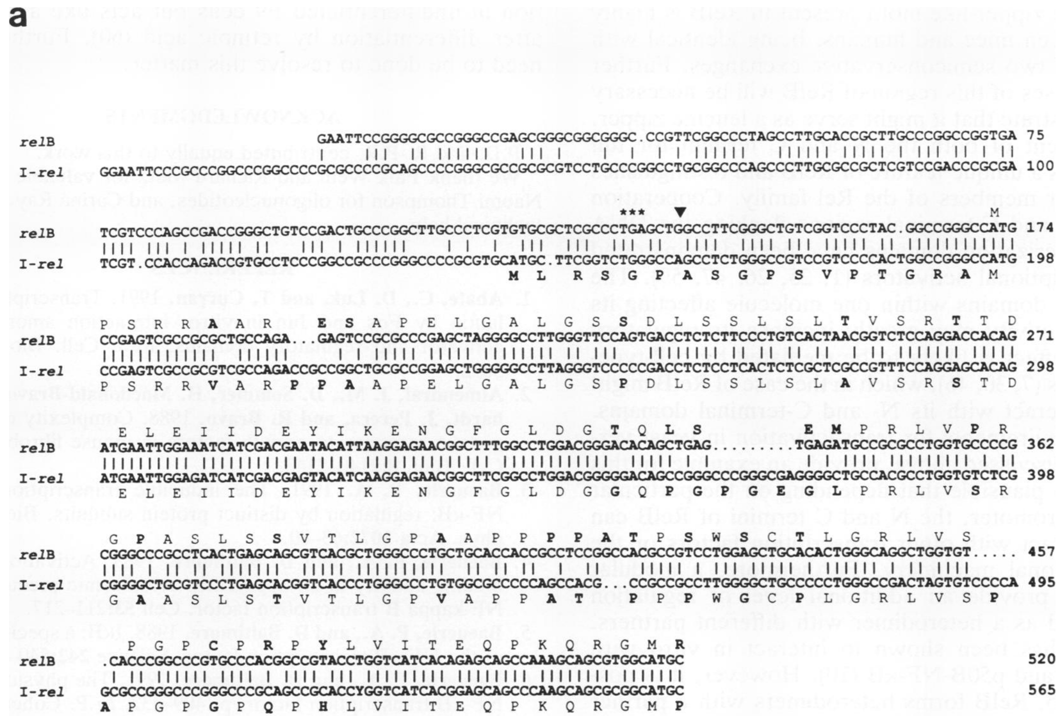
We have determined the capacity of RelB, a protein belonging to the Rel family of transcription factors, to transactivate a promoter containing a κB site in mammalian cells. To identify the domains of RelB involved in transcriptional activation, we used a series of deletion mutants and RelB/p50-NF-κB hybrid molecules. Interestingly, the results indicate that the N and C termini of RelB independently confer transcriptional activity. However, both termini are required for full transactivation.

The highly negatively charged C terminus of RelB has been previously shown to function as a potent transcriptional activation domain when fused to a GAL4 expression vector in yeast cells (51). However, in F9 cells, RelB ΔN1 together with p50-NF-κB only weakly activates CAT expression, indicating that the C terminus alone is not sufficient to confer full transcriptional activity. This view is further supported by the observation that the hybrid p50/RelB 5, which contains the C terminus of RelB fused to the RHD of p50-NF-κB, is also a weak transactivator. In Jurkat cells, a similar result was recently reported for the C terminus of I-Rel, the human RelB homolog, by using GAL4 fusions (49). It is important to note that although the C terminus of RelB confers weak transcriptional activation, its presence enhances the transactivation capacity of the N terminus.

Our studies show that the presence of the N terminus of RelB is important for activation in F9 cells. Although its effect on transcriptional activity in the absence of the C-terminal domain is weak, it enhances the expression of the reporter gene very efficiently when present twice in artificial hybrid molecules. In heterodimers with p50- or p50B-NF-κB, RelB requires both the N- and C-terminal domains for full activity. The activities of different p50/RelB hybrid molecules demonstrate that the N and C termini of RelB can confer transcriptional activity to p50-NF-κB and that there is a synergistic effect in transcription when both domains are present in the heterodimers. These studies demonstrate certain limitations of a widely used test system for transcriptional activation. Although the C terminus is a potent activator in yeast cells (51), its effect is rather weak in mammalian cells. In addition, the N-terminal region has no positive or negative effect in yeast cells, but in mammalian cells it seems to be absolutely required for full activity.

A potential importance for the leucine zipper-like motif in transactivation by RelB is suggested by experiments with mutants introducing drastic alterations near one of the leucine repeats. A significant decrease in the transcriptional capacity of RelB and of the different hybrid molecules is observed when the structure of the leucine zipper-like motif is altered either by changing a leucine to a proline or by deleting three amino acids, suggesting that this domain may interact with specific cofactors involved in transcriptional activation. An alternative explanation for these results is that the region comprising the leucine zipper-like motif functions as an acidic activating domain since its overall charge is negative. It is important to note that our experiments do not distinguish between these two interpretations. However, the fact that in a fusion protein with GAL4, the N-terminal region of RelB is not able to activate transcription in yeast cells (51) suggests that this region does not behave as a general acidic activation domain. The identification of the putative factors interacting with the N-terminal region of RelB will be essential to understand its function. Characterization of these factors will show whether they contain leucine zipper-like motifs capable of interacting with RelB.

A comparison of the transcriptional activation domains of RelB, c-Rel, and p65-NF-κB reveals that they all contain a highly acidic C-terminal domain initially identified as a transactivating domain in c-Rel (12, 29). The transactivation domain of human p65-NF-κB can be subdivided in two transactivating areas, TA_1, which contains a predicted α-helical area, and TA_2, composed of a leucine zipper-like motif of three heptad-arranged leucines (53). Interestingly, the destruction of this motif by in vitro mutagenesis eliminates the transcriptional activity of p65-NF-κB (50). However, in contrast to what is expected for an essential motif, it is not highly conserved in the murine molecule, in which the second heptad contains an additional residue that alters the repeat (42). This finding has been confirmed by the genomic sequence (50a). Therefore, it seems unlikely that this region,



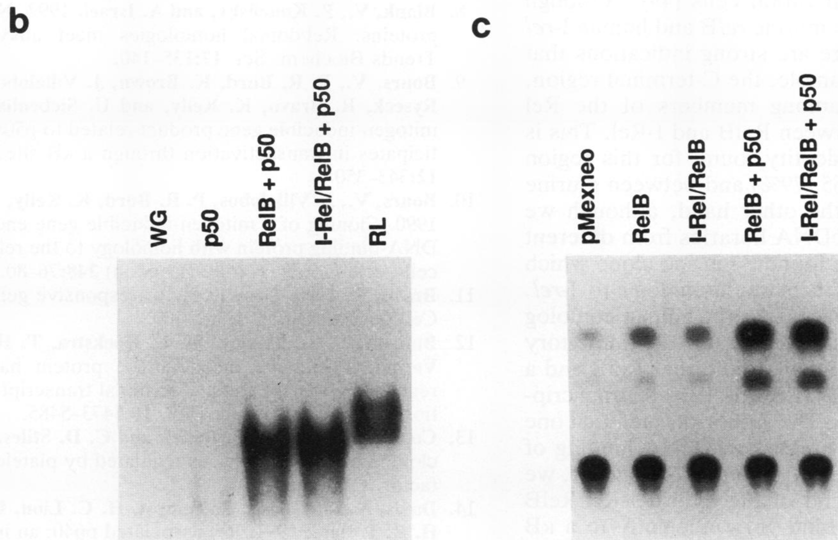


FIG. 6. Comparison of murine *rel*B and its human homolog I-*rel*. (a) Homology of the 5′ end of a *rel*B cDNA isolated from mouse thymus and the corresponding part of I-*rel* (49). The part encoding the I-Rel region used for the construction of the I-Rel/RelB hybrid is shown. Amino acids in bold are different between the two genes. The three asterisks represent an in-frame stop codon upstream of the initiation codon for RelB. The closed triangle indicates the beginning of the previously published cDNA *rel*B sequence (51). (b) EMSA analysis of the DNA binding activity of an I-Rel/RelB hybrid compared with that of murine RelB in the presence of p50-NF-κB, using the murine Igκ gene κB binding site. WG, 4 μl of wheat germ extract as a negative control; RL, 1 μl of reticulocyte lysate (Promega) containing as a positive control the endogenous p50/p65-NF-κB activity. (c) Representative CAT assays of transfection experiments performed in the Jurkat T-cell line. The expression vector for RelB and the hybrid I-Rel/RelB were transfected either alone or in combination with p50-NF-κB as described in Materials and Methods.

although important for transcriptional activation, interacts with other proteins via a leucine zipper-like motif. In contrast, the leucine zipper-like motif present in RelB is highly conserved between mice and humans, being identical with the exception of two semiconservative exchanges. Further mutational analyses of this region of RelB will be necessary to finally demonstrate that it might serve as a leucine zipper.

The requirement of both the N and C termini for full transactivation is a unique feature of RelB and distinguishes it from the other members of the Rel family. Cooperation between the N- and C-terminal regions flanking the DNA binding and dimerization domains have been demonstrated in other transcriptional activators (1, 25, 26, 47, 59). The presence of two domains within one molecule affecting its transcriptional activity may greatly increase its regulatory potential. This effect is likely to be mediated by cell-type-specific cofactors (7, 30, 46), which in the case of RelB might differentially interact with its N- and C-terminal domains. The different requirements for transactivation in yeast versus F9 cells, as discussed above, provide an example for this model. It is also plausible that depending on the particular context of the promoter, the N and C termini of RelB can specifically interact with other transcription factors or the basic transcriptional machinery. Furthermore, a modular structure might provide an additional level of regulation when complexed as a heterodimer with different partners. To date, RelB has been shown to interact in vitro with p50-NF-κB (51) and p50B-NF-κB (10). However, it is possible that in vivo, RelB forms heterodimers with a partner that also contains an activating domain. An example of such a type of Rel heterodimer is the recently identified in vivo c-Rel/p65-NF-κB complex (23).

In a recent report, the putative human homolog of RelB, I-Rel, was described to act as a suppressor of transcriptional activity via κB binding sites in Jurkat cells (49). Although there is no direct proof that the murine *rel*B and human I-*rel* genes are truly homologs, there are strong indications that this is indeed the case. For example, the C-terminal region, usually the least conserved among members of the Rel family, shows 85% identity between RelB and I-Rel. This is significantly higher than the identity found for this region between murine and human p65 (79%) and between murine and human c-Rel (61%). On the other hand, although we have screened several mouse cDNA libraries from different cellular origins, we have never found a murine clone which being related to *rel*B presents a better homology to I-*rel*. Therefore, we are convinced that I-*rel* is the human homolog of the murine *rel*B gene. In contrast to the inhibitory function described for I-Rel (49), we found that RelB and a hybrid between I-Rel and RelB (I-Rel/RelB) are transcriptionally active in this T-cell line. The authors argued that one of the functions of I-Rel is to prevent the DNA binding of p50-NF-κB by forming inactive heterodimers. However, we find that complexes of RelB and of the hybrid I-Rel/RelB together with p50-NF-κB can bind very efficiently to a κB site. Therefore, we conclude that the mouse and human RelB proteins activate transcription in the presence of p50-NF-κB. This view is further supported by the recent observations that I-Rel does not inhibit the transcriptional activity via κB sites in NTera-2 cells (54a). Presently, we have no clear explanation for the discrepancy between our results and those of Ruben et al. (49); however, one possibility is the different contexts of the promoter and of the κB binding sites in the reporter genes used. For example, it has been shown that dorsal activates *twist* but represses *zerknüllt* gene expression (44). Another possibility is that the differentiation state of cells influences the activity of RelB. This effect has been recently described for v-Rel, which activates transcription in undifferentiated F9 cells but acts like a suppressor after differentiation by retinoic acid (60). Further studies need to be done to resolve this matter.

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P.D. and R.-P.R. contributed equally to this work.

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