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p105, the NF-κB p50 Precursor Protein, Is One of the Cellular Proteins Complexed with the v-Rel Oncoprotein in Transformed Chicken Spleen Cells

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Active NF-κB-like transcription complexes are multimers consisting of one or two members of a family of proteins related to the c-Rel proto-oncoprotein. We have isolated a chicken cDNA encoding p105, the precursor protein for the p50 subunit of NF-κB. Sequence analysis shows that chicken p105 is approximately 70% identical to the mouse and human p105 proteins, containing the Rel homology domain in its N-terminal 370 amino acids and several ankyrinlike repeats in the C-terminal portion of the protein. The Rel homology domain is particularly highly conserved between chicken and mammalian p50, and an in vitro-synthesized, truncated chicken p105 protein, containing sequences that correspond to the predicted p50 protein, bound to a consensus κB site in an electrophoretic mobility shift assay. In v-Rel-transformed chicken spleen cells, v-Rel is found in high-molecular-weight complexes which include cellular proteins of approximately 124 kDa (p124) and 115 kDa (p115). Here we report that in vitro-produced p105 comigrates with p124 from v-Rel-transformed spleen cells and that p105 and p124 appear to be identical by partial proteolytic mapping with V8 protease. Furthermore, both p105 and p50 can complex directly with v-Rel and chicken c-Rel in vitro. However, in vitro association with p105 by v-Rel does not necessarily correlate with transformation, since one nontransforming v-Rel mutant can associate with p105 in vitro.

NF-κB was first identified as a transcription factor which bound to the decameric DNA sequence GGGACTTTCC, found in the enhancer of the mouse immunoglobulin κ light-chain gene (58). Although originally associated with lymphoid-specific genes, NF-κB-binding sites have now been identified in genes expressed in cells of lymphoid and nonlymphoid origin as well as many viral enhancers (reviewed in reference 1). The NF-κB complex that binds to the κ light-chain enhancer is a heterodimer consisting of subunits of 50 kDa (p50) and 65 kDa (p65) (62).

It is now apparent that NF-κB-like complexes can consist of any two of a number of proteins related to the c-Rel proto-oncoprotein. The Rel family includes p50/p105, p49/p100, p65, c-Rel, RelB, the viral oncoprotein v-Rel, and the *Drosophila melanogaster* dorsal gene product (reviewed in references 18 and 19). It is likely that all mammalian Rel proteins can form homo- and heterodimers that can bind sites that fall within the consensus sequence for a κB site (GGGRNNYYCC, where R is a purine, Y is a pyrimidine, and N is any nucleotide). However, there are also non-Rel proteins that can bind to this sequence (4, 15, 49).

Rel proteins are related through a domain of approximately 300 to 350 amino acids (aa), called the Rel homology (RH) domain. The RH domain contains sequences responsible for DNA binding, dimerization, inhibitor binding, and nuclear localization. However, Rel proteins are generally unrelated in sequences C-terminal to the RH domain.

cDNAs for mouse and human NF-κB p50 have recently been characterized (7, 17, 32, 40). The sequences of these cDNAs and biochemical evidence (16) reveal that the p50 subunit of NF-κB is a processed form of a 105-kDa precursor protein (p105) and that p50 is derived from the N-terminal portion of p105, which contains the RH domain. The C-terminal portion of p105 contains several ankyrinlike repeats, which have been found in proteins including erythrocyte ankyrin, cell cycle control proteins in *Saccharomyces cerevisiae*, the transcription factor GABPβ, and the IκBβ inhibitor proteins MAD-3 and pp40 (14, 24). Neither the function nor the fate of the C-terminal portion of p105 is completely understood, but it may be involved in cytoplasmic retention (6), inhibition of DNA binding by p50 homodimers (25, 31), and/or transcription activation (40a). In addition, the C-terminal p105 sequences can be expressed from an alternatively spliced mRNA and can function as an IκB protein (IκBγ) in vitro (25). Although the p49/p100 protein is also likely to be processed, other cellular Rel proteins (p65, c-Rel, RelB, and Dorsal) do not appear to be processed and contain strong transcription activation domains in their unique C-terminal sequences (8, 29, 43, 47, 50, 51, 56).

NF-κB-like complexes are regulated, in part, by subcellular localization. For example, the p50-p65 NF-κB complex is sequestered in the cytoplasm in an inactive form through the interaction of IκB with p65 (2, 3). NF-κB can be induced to translocate to the nucleus by a wide variety of mitogens, cytokines, and other proliferation signals, which cause the dissociation of IκB from the p50-p65 complex (reviewed in reference 57).

v-Rel, the oncoprotein of the avian retrovirus Rev-T, transforms avian lymphoid cells in vitro and in vivo (reviewed in references 19 and 46). v-Rel shows cell type-specific subcellular localization in that it is a nuclear protein in chicken embryo fibroblasts (CEF) and is found predominantly in the cytoplasm of v-Rel-transformed spleen cells (20, 45, 61). In contrast, c-Rel is an exclusively cytoplasmic protein when overexpressed in CEF (10). v-Rel is a trun-

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cated and mutated form of the proto-oncoprotein c-Rel; the most obvious alteration in v-Rel is the loss of 118 C-terminal aa that are involved in transcription activation by and cytoplasmic retention of c-Rel (10, 22, 29, 63).

Both v-Rel and c-Rel exist in multiple complexes involving one or more cellular proteins in v-Rel-transformed spleen cells. v-Rel is found exclusively in high-molecular-weight complexes in both the nucleus and the cytoplasm of these cells (13, 33, 41, 60). The cellular proteins complexed with v-Rel have apparent molecular masses (M_r) on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAG) of approximately 124, 115, 70, 68, and 40 kDa. The 70-, 68-, and 40-kDa proteins have been identified as an avian heat shock protein (p70), c-Rel (p68), and an avian IκB (pp40), respectively (14, 37, 60). The identities of p124 and p115 have not been determined previously.

Kieran et al. (32) have shown that human p50 can dimerize with a truncated v-Rel protein in vitro. On the basis of this result and the size of the p50 precursor protein, we have previously suggested that one of the higher-M_r v-Rel-associated proteins could be the NF-κB p50 precursor protein p105 (18, 19). Here we report the sequence and a functional characterization of a cDNA encoding the avian NF-κB p50 precursor p105. In addition, we show that p105 and p50 can complex with v-Rel in vitro and that the p124 protein associated with v-Rel in vivo is p105.

MATERIALS AND METHODS

Cloning and sequence analysis of a cDNA encoding chicken p105. An eightfold-degenerate oligonucleotide (RHP8X:TG TGA[C/T]AA[A/G]GGTCA[G/A]AAA) was used to screen a CEF cDNA library constructed in Lambda Zap (10). This sequence corresponds to the aa sequence CDKVQK (aa 277 to 282 of chicken p105; Fig. 1) found in the RH domain of all known Rel family proteins. Approximately 150,000 plaques were screened under conditions of low stringency (final wash: 2× SSPE [53], 0.3% SDS, 5× Denhardt's solution, 0.1 mg of salmon testis DNA per ml at 25°C). Eleven positive clones were plaque-purified, excised from Lambda Zap according to the manufacturer's (Stratagene) protocol, and sequenced by using both the RHP8X and universal (reverse and forward) oligonucleotides as primers. The largest clone that showed similarity to the human and mouse p105 cDNAs was characterized further. The entire sequence of the chicken p105 cDNA was determined by creating a series of nested deletions with the Erase-a-Base system (Promega) and also by using internal, designed primers. Sequencing of the double-stranded templates was performed by the method of Sanger et al. (54) with Sequenase (United States Biochemical).

Plasmid constructions and in vitro transcription-translation. All recombinant DNA techniques were done by standard protocols (53). Enzymes were purchased from New England Biolabs, unless otherwise indicated, and used according to the specifications of the manufacturer.

Plasmid Sp6Chp105 was constructed by first subcloning a *Bst*UI fragment into the *Eco*RV site of pBS (Stratagene), to remove much of the 5′ GC-rich nontranslated sequences. The p105 coding region was then subcloned into the *Eco*RI and *Kpn*I sites of pGEM4 (Promega), in the SP6 orientation for transcription.

Plasmid pSG3′Chp105 was created by first subcloning an *Nsi*I fragment containing the 3′ two-thirds of the p105 cDNA into the *Pst*I site of pBS; a *Sma*I-*Kpn*I fragment encoding aa 415 to 984 was then subcloned into *Bam*HI- and Klenow fragment-treated and *Kpn*I-digested pSG424 (52). pSG3′M p105 (mouse p105 aa 437 to 971) and pSG3′Mc (mouse c-Rel aa 265 to 588) are described elsewhere (40a). pSG424 and the GAL4 reporter plasmid pG5BCAT, which contains five GAL4 binding sites upstream of the E1b minimal promoter, were kindly provided by I. Verma (8, 36, 52).

Plasmid Sp6Chp105 was used to produce in vitro-transcribed RNAs encoding p105 and p50 by using SP6 RNA polymerase (Promega). RNA encoding full-length p105 was produced after linearizing pSp6Chp105 with *Xho*I; to express p50 (truncated form of p105), pSp6Chp105 was linearized with *Nsi*I and then treated with T4 DNA polymerase to remove the 3′ overhang. RNA encoding v-Rel was produced in vitro from plasmid CG129 (a kind gift of C. Gélinas) as described elsewhere (9). Plasmids for the in vitro expression of v-Rel mutants v-SPW (42) and dStu/Hinc (21) were created by substituting mutant sequences for wild-type v-*rel* sequences in CG129. RNA for chicken c-Rel was produced from Sp6Chc-Rel (kindly provided by S. Sarkar). In vitro-transcribed RNAs were translated in either wheat germ or rabbit reticulocyte lysates according to the manufacturer's (Promega) protocols, including 30 to 60 μCi of [^{35}S]methionine (>800 Ci/mmol; Amersham) or Tran^{35}S-label (methionine plus cysteine; ICN; >1,000 Ci/mmol). Proteins were separated on a 9% SDS–PAG, soaked in 1 M sodium salicylate, and visualized by fluorography at −70°C.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (9). Briefly, 4 μl of in vitro-translated protein was incubated with either ^{32}P-end-labeled oligonucleotide containing the c-*rel* promoter κB site (GGGAAATTCC [9]) or an *Eco*RI-*Hin*dIII restriction fragment from plasmid p7/25 containing the H2-κB site (GGGGATTCCCC [39]). p50 and v-Rel proteins were quantified by excising the relevant bands from a PAG and counting the samples by liquid scintillation counting; protein samples were equalized after taking into account the predicted number of methionines and cysteines in each protein. DNA-binding reactions with in vitro-translated proteins and target DNA were performed in 20 μl of reaction mix (10 mM Tris [pH 7.4], 50 mM NaCl, 1 mM dithiothreitol, 5% glycerol, 0.5% Nonidet P-40, 2 μg of [dI-dC]:[dI-dC], 1 μg of salmon sperm DNA) at room temperature for 20 min, and DNA-protein complexes were resolved by electrophoresis on a 5% PAG. Gels were dried, and autoradiography was performed at −70°C with an intensifying screen.

CAT assays. CEF were prepared and cultured as described previously (10). Approximately 2 × 10^6 cells were transfected with both reporter and producer plasmids (indicated in the figure legends) by the dimethyl sulfoxide-Polybrene method (30). Approximately 48 h after transfection, cells were harvested and lysed in 25 mM Tris (pH 7.4) by several cycles of freeze-thawing. Chloramphenicol acetyltransferase (CAT) activity in the lysates was determined as described previously (9, 47) and corresponds to the amount of radioactivity in the acetylated form of chloramphenicol compared with the total amount of radioactivity in the acetylated and nonacetylated forms of chloramphenicol. All CAT assay results were normalized to β-galactosidase activity by including plasmid MSV-βgal in the transfection mixes (9, 47).

Cell labeling and V8 peptide mapping. v-Rel-transformed spleen cells were maintained in culture as described by Mosialos et al. (42). Cells were labeled with [^{35}S]methionine or Tran^{35}S-label essentially as described by Gilmore and Temin (20). Briefly, 10^7 v-Rel-transformed spleen cells were incubated for 6 h at 40.5°C with 300 to 750 μCi of [^{35}S]methionine or Tran^{35}S-label in 1 ml of Dulbecco's modified Eagle's medium minus methionine (or minus methionine and

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CGCGGCGGCCGTGCGGGGCGGTGCCGCTCGCGTGCCTCGCCAGCGGGCCGGCGGCGGGGCCGAGGAGGGAAAAACGGGGGAAAGTCCGCGAGAGACGGGGCGCGGCGGGTCACCGCGTGA  120
CATGGGCGGCGCTGAGGGGTGCGCGGCCGGGACCTGGAGGCGACGACGGCGACGACGGCGCGGCTCAACCAGCCCTGCGCTGATCCAGGACTTAAAATGGCAGGAGAGGATCCGTATATT  240
                                                                                                    MetAlaGlyGluAspProTyrIle    8

ATGGGAGTGTCAGATCCCCAAATGTTTGCAATGGACCAGCTCATGGGAATGAGTACAATATTTAATAATACAGGTTACATCACTTCAGATTTACCACTACGAACAGCAGATGGACCGTAT  360
MetGlyValSerAspProGlnMetPheAlaMetAspGlnLeuMetGlyMetSerThrIlePheAsnAsnThrGlyTyrIleThrSerAspLeuProLeuArgThrAlaAspGlyProTyr   48

CTTCAAATCATTGAACAGCCAAAACAGAGGGGTTTTCGATTCCGATACGTATGCGAAGGGCCTTCACATGGTGGACTTCCTGGTGCTTCTAGTGAAAAGAACAAAAAGTCATACCCCCAA  480
LeuGlnIleIleGluGlnProLysGlnArgGlyPheArgPheArgTyrValCysGluGlyProSerHisGlyGlyLeuProGlyAlaSerSerGluLysAsnLysLysSerTyrProGln   88

GTCAAAATTTGCAACTATGTTGGACCTGCAAAAGTAATCGTCCAGTTAGTTACTAATGGGAAATACCGTCACTTACATGCTCACAGCTTGGTGGGTAAATTTTGTGAAGACGGAGTATGC  600
ValLysIleCysAsnTyrValGlyProAlaLysValIleValGlnLeuValThrAsnGlyLysTyrArgHisLeuHisAlaHisSerLeuValGlyLysPheCysGluAspGlyValCys  128

ACAGTCAACGCAGGACCTAAAGACATGGTTGTTGGGTTTGCAAACCTGGGAATACTTCACGTAACAAAGAAGAAGGTGTTTGAAACTCTGGAAACACGCATGATCGATGCTTGCAAAAAA  720
ThrValAsnAlaGlyProLysAspMetValValGlyPheAlaAsnLeuGlyIleLeuHisValThrLysLysLysValPheGluThrLeuGluThrArgMetIleAspAlaCysLysLys  168

GGATACAACCCAGGACTCTTGGTGCATCCTGAACTTGGCTATCTGCAGGCTGAAGGATGTGGAGACAGACAGCTAACTGAACGAGAAAGAGAAATTATTCGCCAGGCAGCAGTACAGCAG  840
GlyTyrAsnProGlyLeuLeuValHisProGluLeuGlyTyrLeuGlnAlaGluGlyCysGlyAspArgGlnLeuThrGluArgGluArgGluIleIleArgGlnAlaAlaValGlnGln  208

ACAAAAGAAATGGATCTCAGTGTGGTGCGTCTTATGTTTACTGCCTTTCTTCCTGACAGTAACGGTGGTTTCACACGGAGGCTTGATCCTGTTATATCGGATGCAATATATGACAGCAAA  960
ThrLysGluMetAspLeuSerValValArgLeuMetPheThrAlaPheLeuProAspSerAsnGlyGlyPheThrArgArgLeuAspProValIleSerAspAlaIleTyrAspSerLys  248

GCCCCCAATGCCTCCAACCTTAAAATTGTAAGAATGGACAGGACAGCGGGGTGCGTAACAGGAGGGGAAGAGATTTACCTTCTCTGTGACAAGGTTCAGAAAGATGATATTCAAATACGT 1080
 AlaProAsnAlaSerAsnLeuLysIleValArgMetAspArgThrAlaGlyCysValThrGlyGlyGluGluIleTyrLeuLeu***CysAspLysValGlnLys***AspAspIleGlnIleArg  288

TTCTATGAAGAGGATGAGAATGGTGGTATGTGGGAAGGCTTTGGAGACTTTTCTCCTACAGATGTACATAGACAATTTGCTATTGTGTTCAAAACGCCCAAGTATCGAGATGTCAACATC 1200
PheTyrGluGluAspGluAsnGlyGlyMetTrpGluGlyPheGlyAspPheSerProThrAspValHisArgGlnPheAlaIleValPheLysThrProLysTyrArgAspValAsnIle  328

ACAAAGCCAGCATCTGTATTTGTACAGCTACGTCGGAAATCTGATCTAGAAACGAGTGAACCAAAGCCTTTTCTCTACTATCCAGAAATCAAAGATAAAGAAGAAGTGCAGAGGAAGAGG 1320
ThrLysProAlaSerValPheValGlnLeuArgArgLysSerAspLeuGluThrSerGluProLysProPheLeuTyrTyrProGluIleLysAspLysGluGluValGlnArgLysArg  368

CAGAAGCTCATGCCAAACTTCTCCGATGGCTACGGTGGAGGCAGCGGCGCAGGCGGCGGTGGCATGTTTGGAGGAGGAGGCGGCGGTGCTGGCTCTGGGTTCAGTTATCCTTCCTATGGC 1440
GlnLysLeuMetProAsnPheSerAspGlyTyrGlyGlyGlySerGlyAlaGlyGlyGlyGlyMetPheGlyGlyGlyGlyGlyGlyAlaGlySerGlyPheSerTyrProSerTyrGly  408

TACTCTGCTTTTGGAGGAATGCATTTCCACCCTGGCACAACGAAGTCCAATGCTGGAATGAAACACGAACTGTCAAATAGCACAGTCAAAAAAGATGAAGAGAGCTCTGATAAACAAAGT 1560
TyrSerAlaPheGlyGlyMetHisPheHisProGlyThrThrLysSerAsnAlaGlyMetLysHisGluLeuSerAsnSerThrValLysLysAspGluGluSerSerAspLysGlnSer  448

GACAAATGGGACACAAAACATGACGTGAAAGTGGAAACTGTGGAGAAGAATGAGTGCAGAACCTCTGGTCATAATGAGGAGAAAGAGGATGCTTCGTTGTGCTGTAAAGATGAAGGAAAC 1680
AspLysTrpAspThrLysHisAspValLysValGluThrValGluLysAsnGluCysArgThrSerGlyHisAsnGluGluLysGluAspAlaSerLeuCysCysLysAspGluGlyAsn  488

AAACCAAAGTGTGGTTGTCAGGATGGTCTGTTCCTGGAGAAGGCTATGCAGCTTGCCAAGCGTCACTGCAACCGTCTCTTTGATTATGCTGTAACTGGAGATGTGAGGATGCTGCTAGCT 1800
LysProLysCysGlyCysGlnAspGlyLeuPheLeuGluLysAlaMetGlnLeuAlaLysArgHisCysAsnArgLeuPheAspTyrAlaValThrGlyAspValArgMetLeuLeuAla  528

GTTCAGCGCCATCTCACTGCTGTCCAGGATGACAATGGAGACAATGTCCTTCATTTGTCAATTATCCACCTTCATAGAGAACTGGTTAAAAACCTCTTGGAAGTGATGCCAGATATGAAT 1920
ValGlnArgHisLeuThrAlaValGlnAspAspAsnGlyAspAsnValLeuHisLeuSerIleIleHisLeuHisArgGluLeuValLysAsnLeuLeuGluValMetProAspMetAsn  568

TATAACAACATTATCAACATGAGAAATGACCTTTACCAGACCCCGCTGCACCTGGCAGTAATCACAAAGCAGGCAGAGGTGGTAGAAGACCTGCTGAAGGCTGGAGCAAATGTCAACCTT 2040
TyrAsnAsnIleIleAsnMetArgAsnAspLeuTyrGlnThrProLeuHisLeuAlaValIleThrLysGlnAlaGluValValGluAspLeuLeuLysAlaGlyAlaAsnValAsnLeu  608

TTGGATCGCCATGGTAATTCTGTCTTGCACTTAGCTGCTGCTGAAGGAGATGACAAGATTCTGAGTCTGCTCCTCAAGCATCAGAAGGCATCTTCAATGATCGACCTTTCCAATGGTGAA 2160
LeuAspArgHisGlyAsnSerValLeuHisLeuAlaAlaAlaGluGlyAspAspLysIleLeuSerLeuLeuLeuLysHisGlnLysAlaSerSerMetIleAspLeuSerAsnGlyGlu  648

GGTCTCAGTGCAATTCACATGGTGGTGACCGCCAATAGCTTGTCCTGCCTGAAACTACTGATTGCTGCTGGAGTTGATGTCAATGCTCAGGAACAAAAATCTGGACGAACGGCATTGCAT 2280
GlyLeuSerAlaIleHisMetValValThrAlaAsnSerLeuSerCysLeuLysLeuLeuIleAlaAlaGlyValAspValAsnAlaGlnGluGlnLysSerGlyArgThrAlaLeuHis  688

TTAGCTGTTGAACAAGAGAACGTCCCCTTGGCAGGCTGCCTTTTGCTTGAGGGTGATGCAGATGTGGACAGCACTACATACGATGGCACAACGCCTCTTCACATAGCAGCTGGAAGGGGC 2400
LeuAlaValGluGlnGluAsnValProLeuAlaGlyCysLeuLeuLeuGluGlyAspAlaAspValAspSerThrThrTyrAspGlyThrThrProLeuHisIleAlaAlaGlyArgGly  728

TTCACAAAACTGGCAGCAGTTCTCAAAGCAGCAGGCGCAGATCCTCACGTTGAGAACTTTGAGCCGCTCTTTGATGTAGAAGAAGATGTGAAAGATGATGATGATGATGAAGGAATCGTA 2520
PheThrLysLeuAlaAlaValLeuLysAlaAlaGlyAlaAspProHisValGluAsnPheGluProLeuPheAspValGluGluAspValLysAspAspAspAspAspGluGlyIleVal  768

CCGGGAACAACACCACTGGATATGGCAGCCAACTGGGAGGTGTATGACATATTAAATGGCAAACCCTACATAGCAGCAGCGGCAGTTTCAGAGGATTTACTGTCACAAGGCCCTCTGAGA 2640
ProGlyThrThrProLeuAspMetAlaAlaAsnTrpGluValTyrAspIleLeuAsnGlyLysProTyrIleAlaAlaAlaAlaValSerGluAspLeuLeuSerGlnGlyProLeuArg  808

GAGCTGAATGAAAGCTCCAAGCAGCAGCTTTACAAGCTATTGGAAACGCCTGATCCAAGCAAGAACTGGTCTACTCTAGCAGAAAAGCTGGGTCTTGGCATTCTTAATAATGCCTTCCAG 2760
GluLeuAsnGluSerSerLysGlnGlnLeuTyrLysLeuLeuGluThrProAspProSerLysAsnTrpSerThrLeuAlaGluLysLeuGlyLeuGlyIleLeuAsnAsnAlaPheGln  848

TTGAGCCCTTCACCCTCAAAAACTCTGCTGGATAACTATAAGATTTCTGGAGGTACAGGTCAAGAGCTGATCGCTGCTTTCACACAGATGGATCACACAGAAGCTATTGAAGTAATTCAG 2880
LeuSerProSerProSerLysThrLeuLeuAspAsnTyrLysIleSerGlyGlyThrGlyGlnGluLeuIleAlaAlaPheThrGlnMetAspHisThrGluAlaIleGluValIleGln  888

AAAGCACTATCTAGCTCACAAAGGCAGTCTCACCAGGAGGACAAGACAATCGAAGCTTTTCCTTCATTATCACCAACCAGTTTTGCAAAGGAAGAGACAGGTGAACTTTACAATCACAAA 3000
LysAlaLeuSerSerSerGlnArgGlnSerHisGlnGluAspLysThrIleGluAlaPheProSerLeuSerProThrSerPheAlaLysGluGluThrGlyGluLeuTyrAsnHisLys  928

TTTCAAGACCCTGAAAGCACGTGTGACAGCGGCGTAGAGACCTCCTTCCGCAAACTGAGCTTTACTTATTCAGACTCTCTGAACAGCAAGTCATCCATAACGCTTAGCAAGATGACCCTG 3120
PheGlnAspProGluSerThrCysAspSerGlyValGluThrSerPheArgLysLeuSerPheThrTyrSerAspSerLeuAsnSerLysSerSerIleThrLeuSerLysMetThrLeu  968

GGCTACGGCAGGAAAGCTCAGTGCAAAGCAGTTATATACCTAACTAGGTAACTTCAATTAGCAATATATGCAGTTACAGGAACAACCCTGACATTTAGAATTACTTTGGTATTGTCACAG 3240
GlyTyrGlyArgLysAlaGlnCysLysAlaValIleTyrLeuThrArgEnd                                                                        984

CTGGAGGGAAGTAATTCACAACCAGAAATGCATTGGAGGACCACACACAACTGAACCAGTGCGACTCCTCAACCTCACTTCCTTACTCAAGTACTGTTCATTTGTAAGCCTCGACTGAGA 3360
ACCAGGCTCCACATGAATACAAAGGACTGGACACTGAAGGACTGACGGAGCTCACCTGGTTTGTGAGGATTTTCTTTTATAGATGAAAGTCACTCCTTGTTAGTGTGTAATATCAGCTCA 3480
CTTAATTCAAAGAGGTAAAGCACACAGCAATCAAAGGATGGTTTCTGGCTGTTCCCTTCAGAAGTTTTGCAGTACGGTTGTCTGGAAAAAGCTCCTAAAATCGCTGGCAAAGAAAAACAG 3600
TGACTTTAACTGCAACAGTGACAACTCTGGAAGACAAAAGAATCCTAAAAAAAA                                                                     3654
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FIG. 1. Sequence of a cDNA encoding the avian NF-κB p50 precursor p105. Nucleotide sequence was determined as described in Materials and Methods. The predicted aa sequence is shown below the nucleotide sequence. The ankryinlike repeats are underlined with solid lines. The conserved aa sequence (aa 277 to 282) used to make probe RHP8X is indicated in boldface italic letters.

cysteine) and supplemented with 6% dialyzed fetal bovine serum. Cells were lysed in lysis buffer (20 mM Tris [pH 7.4], 5 mM $MgCl_2$, 0.1 M NaCl, 1% Nonidet P-40, 0.5% SDS, 1% aprotinin [Sigma]) and immunoprecipitated with anti-v-Rel antiserum as described previously (20).

Partial V8 peptide maps were generated by the method of Cleveland et al. (11) and others (23, 44). Briefly, immunoprecipitated proteins were electrophoresed on a preparative gel, and bands were excised from the gel. Gel slices were soaked in equilibration buffer (1.25 M Tris [pH 6.8], 0.1%

SDS, 1 mM EDTA) containing 20% glycerol and stuffed into the wells of a 12.5% SDS–PAG. The gel slices were overlaid with a solution of equilibration buffer containing 10% glycerol and 5 ng of V8 protease (Sigma). Samples were allowed to digest for 30 min with the power off after they had been electrophoresed into the stacking gel. The samples were then electrophoresed until the tracking dye reached the bottom of the gel. The gel was then dried, and an image was generated on a phosphorimager (Molecular Dynamics), or the gel was soaked in a solution of 1 M sodium salicylate, dried, and subjected to fluorography.

Immunoprecipitation of in vitro-prepared proteins. In vitro-translated proteins were diluted to 400 μl in lysis buffer and incubated with 2 to 5 μl of anti-v-Rel antiserum for 1 h to overnight on ice. Protein A-Sepharose was then added, and samples were incubated on ice for 1 h. The Sepharose beads were then washed five times with lysis buffer, and the immune complexes were removed from the beads by boiling in 2× SDS-sample buffer (0.125 M Tris [pH 6.8], 20% glycerol, 10% 2-mercaptoethanol, 4.6% SDS, 0.1% bromphenol blue). The proteins were then resolved by PAG electrophoresis (PAGE) on a 9% SDS–PAG. The gels were soaked in 1 M sodium salicylate and dried, and fluorography was done at −70°C.

Nucleotide sequence accession number. The sequence of the chicken p105 cDNA can be obtained from GenBank (accession number M86930).

RESULTS

Cloning and sequence analysis of a cDNA encoding the chicken NF-κB p50 precursor, p105. A CEF cDNA library was screened with a degenerate oligonucleotide corresponding to a highly conserved aa sequence found in the RH domain of all Rel proteins identified to date, as described in Materials and Methods. We isolated six clones that showed significant similarity to cDNAs for the human and mouse NF-κB p50 precursor proteins (7, 17, 32, 40), and the largest of these cDNAs was characterized further.

Figure 1 shows the complete nucleotide and aa sequences of a 3,654-bp cDNA for the chicken NF-κB p50 precursor protein, p105. The cDNA contains a 216-bp 5′ GC-rich nontranslated region, followed by an open reading frame of 2,952 bp, and a 478-bp 3′ nontranslated region [excluding the poly(A) sequences]. The open reading frame begins with an ATG at position 217, which fits well with the consensus sequence for initiation of translation in eukaryotes (34). In addition, there is an upstream in-frame termination codon at position 133, making the ATG at position 217 the most likely ATG for initiation. The predicted 984-aa protein has a calculated M_r of approximately 108 kDa, which is similar to the M_r of both human and mouse p105 proteins. To be consistent with the nomenclature of mammalian NF-κB proteins, we will designate the chicken NF-κB p50 subunit precursor p105.

Figure 2 shows a diagram of the structural regions of chicken p105. At the extreme N terminus, there is a sequence of 45 aa that is not conserved among Rel proteins. The RH domain is contained between aa 46 and 370. Like mammalian p105 proteins, chicken p105 contains ankyrinlike repeats in the C-terminal aa; there are six full ankyrin repeats followed by a half repeat, and the sequence between the sixth and seventh ankyrin repeats is highly acidic.

Between the RH and ankyrin domains, there is a glycine-rich hinge region. The proteolytic cleavage site for processing of p105 to p50 is thought to lie within this region (i.e., aa 371 to 427 of chicken p105). There are several sequences within the glycine-rich region of chicken p105 that resemble

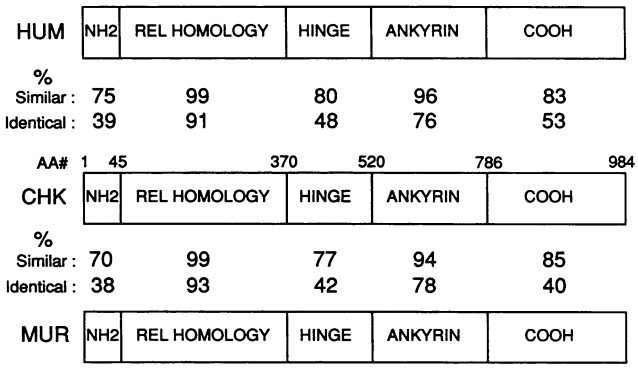

FIG. 2. Homology between chicken (CHK) and mammalian (human [HUM] and mouse [MUR]) NF-κB p105 proteins. The p105 proteins have been divided into five domains: N terminus (NH_2; aa 1 to 45 in chicken p105), RH (aa 46 to 370), hinge (aa 371 to 520), ankyrin repeat (aa 521 to 786), and C terminus (COOH; aa 587 to 984). The N-terminal extent of the hinge region is based on the definition of this region in mammalian p105 proteins, but aa 520 to 540 of chicken p105 are not included as part of the ankyrin repeats in Fig. 1 because of the limited homology to the consensus ankyrin repeat in these 20 aa. The aa numbers which define the various regions are indicated for chicken p105 above the figure corresponding to chicken p105. The aa sequences between chicken p105 and human or mouse p105 were compared by using the GenBank FASTA homology search program (kindly provided to GenBank by Pearson and Lipman); the values correspond to the aa sequence similarity (including conservative changes) or aa sequence identity for any two homologous regions between chicken p105 and the mammalian p105 proteins.

FPXYG, a proteolytic processing signal found in many viral polyproteins (1, 48); the most similar sequence in chicken p105 is YPXYG (aa 404 to 408), which would yield a protein of approximately 45 kDa following processing.

The overall sequence identity at the aa level between chicken p105 and either human or mouse p105 proteins is approximately 70% (Fig. 2). To look more closely at the extent of homology between these proteins, we have divided the proteins into five regions: N terminus (NH_2), RH, hinge, ankyrin repeat, and C terminus (COOH). As described above, the regions of highest sequence identity between chicken and mammalian p105 proteins are the RH domain and the ankyrin repeat domain, which are 93 and 78% identical, respectively, between chicken and mouse p105. The percentages of identity of the N and C termini as well as the hinge region are much lower.

Characterization of the chicken p105 protein. To show that our cDNA clone encoded a functional κB site-binding protein, we performed EMSAs with in vitro-produced protein. Full-length p105, a truncated version of p105, and full-length v-Rel were translated in wheat germ extracts, as shown in Fig. 3A. Truncated p105 corresponds to a protein of approximately 50 kDa (aa 1 to 414) and contains the entire RH domain. We then performed an EMSA with the in vitro-translated proteins and a consensus κB site from the chicken c-*rel* promoter (9). As shown in Fig. 3B, v-Rel and chicken p50 bound to the κB site. v-Rel produced a large smear, as has been seen previously by us and others (9, 26, 31), whereas p50 produced a tightly shifted complex. The wild-type κB site-containing oligonucleotide competed for both v-Rel and p50 DNA binding; however, an unrelated oligonucleotide did not compete (Fig. 3B). p50 and v-Rel also bound to the κB site from the mouse major histocompatibil-

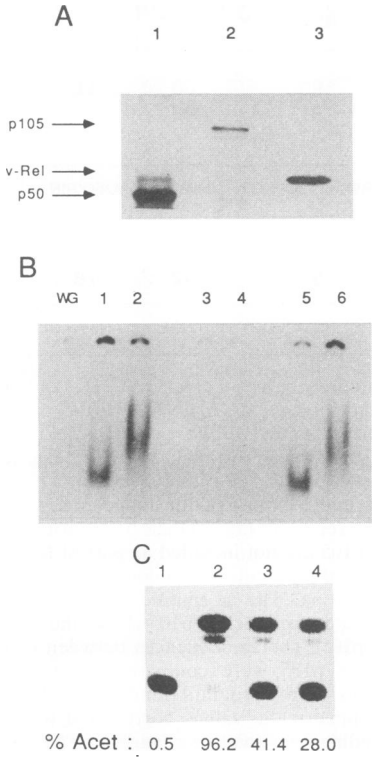

FIG. 3. Characterization of the protein encoded by the p105 cDNA. (A) In vitro-synthesized mRNAs encoding chicken p50 (truncated p105) (lane 1), full-length p105 (lane 2), and full-length v-Rel (lane 3) were translated in a wheat germ lysate as described in Materials and Methods. The specific polypeptides are indicated by arrows to the left of the figure. (B) Approximately equal amounts of in vitro-synthesized p50 and v-Rel (see panel A) were analyzed in an EMSA with an oligonucleotide corresponding to a κB site from the chicken c-*rel* promoter as described in Materials and Methods. WG, EMSA with an unprogrammed wheat germ lysate; lane 1, EMSA with p50; lane 2, EMSA with v-Rel; lanes 3 and 4, p50 and v-Rel, respectively, which were first incubated with a 100-fold molar excess of cold wild-type κB oligonucleotide prior to addition of the radiolabeled oligonucleotide; lanes 5 and 6, p50 and v-Rel, respectively, which were preincubated with a 100-fold molar excess of a cold oligonucleotide containing GC boxes III and IV from the simian virus 40 early promoter, which contain four binding sites for the Sp1 transcription factor (kindly provided by Said Sif). Only the parts of the figures containing the specific shifted complexes are shown. (C) Transcription activation by chicken p105 C-terminal sequences. CEF were transfected with GAL4 site-containing reporter plasmid G5BCAT and producer plasmids, and CAT assays were performed as described in Materials and Methods. Lanes: 1, SG424 (GAL4 aa 1 to 147 alone); 2, SG3'Mc (GAL4 aa 1 to 147; mouse c-Rel aa 265 to 588); 3, SG3'Mp105 (GAL4 aa 1 to 147; mouse p105 aa 437 to 971); 4, SG3'Chp105 (GAL4 aa 1 to 147; chicken p105 aa 415 to 984). CAT activity is presented as percent acetylation (% Acet), determined as described previously (47).

ity complex (data not shown). Consistent with the results of others (17, 32), full-length p105 did not bind to either κB site (data not shown); however, the amount of in vitro-produced p105 was consistently low in wheat germ extracts (see Fig. 3A).

Like other Rel family proteins, both mouse and human p105 precursor proteins contain sequences in the C-terminal halves of the proteins that can function as transcription activation domains when fused to heterologous DNA-binding proteins (40a). To determine whether the chicken p105 protein also contained a C-terminal activation domain, we constructed a GAL4-p105 fusion gene encoding aa 1 to 147 of GAL4 and aa 415 to 984 of chicken p105. Figure 3C shows the results of a CAT assay with extracts of CEF cotransfected with a CAT reporter plasmid containing GAL4-binding sites upstream of a minimal promoter and producer plasmids expressing GAL4 fusions to C-terminal mouse c-Rel aa (pSG3'Mc), C-terminal mouse p105 aa (pSG3'Mp105), C-terminal chicken p105 aa (pSG3'Chp105), or GAL4 aa 1 to 147 by themselves (pSG424). Both mouse and chicken p105 C-terminal aa sequences (Fig. 3C, lanes 3 and 4) have transcription-activating domains, which in this assay are weaker than that of the mouse c-Rel (lane 2). GAL4 aa 1 to 147 alone showed no appreciable transcription activation (lane 1). Thus, C-terminal chicken p105 sequences can activate transcription when fused to GAL4 DNA-binding sequences.

Association of p105 with v-Rel in vivo and in vitro. In immunoprecipitates from avian lymphoid cells, v-Rel and c-Rel are stably associated with several cellular proteins, including proteins of about 124 and 115 kDa (12, 13, 33, 41, 60). Since human NF-κB p50 has been shown to associate with a truncated v-Rel protein in vitro (32), we thought that one of these higher-M_r proteins could be chicken p105. The results in Fig. 4A show that in vitro-translated p105 comigrates with the larger (p124) protein present in an anti-v-Rel immunoprecipitate from v-Rel-transformed spleen cells.

To determine whether the p105 protein and the v-Rel-associated protein (p124) were related, gel slices were subjected to partial proteolysis with V8 protease. The partial peptide maps of in vitro-prepared p105 were virtually indistinguishable from those of the v-Rel-associated protein (p124) from the immunoprecipitate, whereas the lower band (p115) did not show any obvious similarity to p105 (Fig. 4B). Therefore, p105 appears to be identical to the previously described p124 v-Rel-associated protein.

To demonstrate that p105 interacts directly with v-Rel, we performed immunoprecipitation experiments with in vitro-translated proteins (Fig. 5A and B). When v-Rel was cotranslated with p105 or p50 and immunoprecipitated with anti-Rel antiserum, bands corresponding to p105 (Fig. 5B, lane 5) or p50 (Fig. 5B, lane 8) were observed in addition to v-Rel. Neither preimmune serum nor anti-v-Rel antiserum recognized chicken p105 or p50 in these in vitro immunoprecipitation experiments. These results indicate that v-Rel complexed with p105 and p50 and that this complex was precipitated by the anti-v-Rel antiserum. Additionally, chicken c-Rel also formed complexes with p105 in vitro (Fig. 5C).

To determine whether mutations that abolish spleen cell transformation by v-Rel also affected association between v-Rel and p105, we assayed two nontransforming v-Rel proteins for their ability to associate with p105 in vitro. As shown in Fig. 6, a nontransforming v-Rel protein with a 58-aa deletion near the end of the RH domain (dStu/Hinc [21]) did not complex with p105 in vitro, but a nontransforming mutant with a 2-aa insertion in the RH domain (v-SPW [42]) still associated with p105 in vitro.

The results presented in this section identify chicken p105 as one of the in vivo v-Rel-associated proteins. Furthermore, they demonstrate that v-Rel can associate with both the precursor and processed forms of chicken NF-κB p50 in vitro. However, the ability of v-Rel to associate with p105 is not precisely correlated with its transforming ability, since there is one nontransforming v-Rel mutant that can still associate with p105 in vitro.

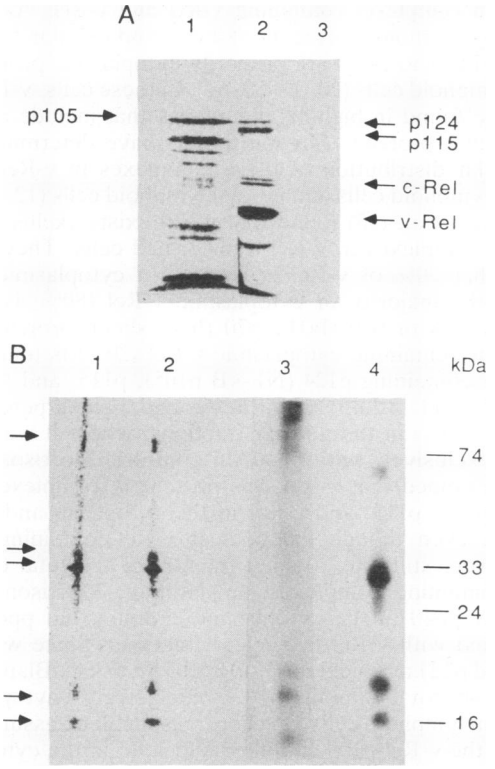

FIG. 4. Chicken p105 is the v-Rel-associated protein p124. (A) Lane 1 is an in vitro translation of chicken p105 in a rabbit reticulocyte lysate. Lanes 2 and 3 are immunoprecipitates from v-Rel-transformed spleen cells with anti-v-Rel antiserum and preimmune serum, respectively. These three samples were run on adjacent lanes of the same SDS-PAG, but the exposure times are different for lane 1 versus lanes 2 and 3 to compensate for the greater number of counts in lane 1. The comigrating in vitro-synthesized p105 band and the previously designated p124 band in the immunoprecipitate are indicated by arrows. v-Rel and the cellular proteins complexing with v-Rel are also designated. (B) Partial proteolysis with V8 protease. Bands corresponding to in vitro-synthesized chicken p105 (lanes 2 and 4), p124 from a v-Rel immunoprecipitate (lane 1), and p115 from a v-Rel immunoprecipitate (lane 3) were excised from preparative SDS-PAGs and digested with 5 ng of V8 protease in a 12.5% SDS–PAG as described in Materials and Methods. The proteolytic fragments shared between p105 and p124 are indicated by the arrows to the left side of the picture. The image in lanes 1 and 2 was generated by phosphorimaging of the dried gel. The image in lanes 3 and 4 was generated by fluorography, and these samples were run on a different SDS-PAG than lanes 1 and 2, which is why the fragments in lanes 2 and 4 (in vitro-synthesized p105) do not match precisely. Sizes of molecular mass markers are also indicated to the right of the figure for lanes 3 and 4.

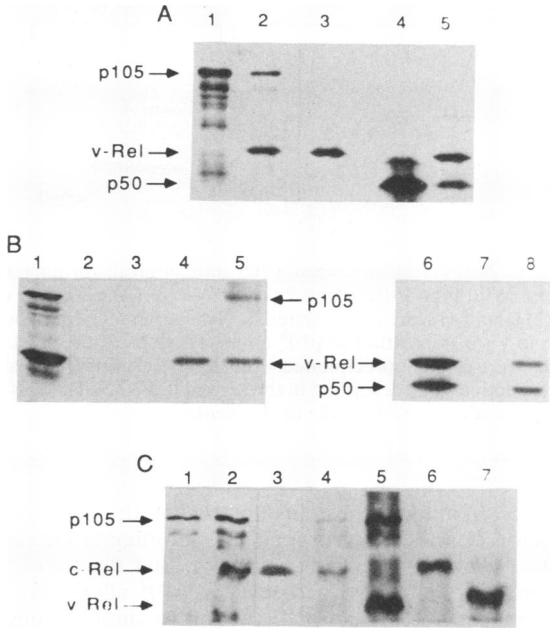

FIG. 5. v-Rel and c-Rel complex with p105 in vitro. Proteins were synthesized in vitro in rabbit reticulocyte lysates and, where indicated, immunoprecipitated with either preimmune or anti-v-Rel antiserum. (A) In vitro synthesis of p105, p50, and v-Rel. Lane 1, translation of p105; lane 2, cotranslation of p105 and v-Rel; lane 3, translation of v-Rel; lane 4, translation of p50; lane 5, cotranslation of p50 and v-Rel. (B) Complexing of v-Rel and p105 or p50. v-Rel was cotranslated with p105 (lane 1) or p50 (lane 6). Lanes 2 to 5, 7, and 8 are immunoprecipitations of in vitro translations: lane 2, cotranslation of p105 and v-Rel (preimmune serum); lane 3, p105 translation (anti-v-Rel antiserum); lane 4, v-Rel translation (anti-v-Rel); lane 5, cotranslation of p105 and v-Rel (anti-v-Rel); lane 7, cotranslation of v-Rel and p50 (preimmune serum); lane 8, cotranslation of p50 and v-Rel (anti-v-Rel). (C) c-Rel complexes with p105. Lanes 1 to 3 are total in vitro translation reactions: lane 1, p105; lane 2, cotranslation of p105 and c-Rel; lane 3, c-Rel. Lanes 4 to 7 are immunoprecipitations with anti-v-Rel antiserum of in vitro translations of the following: lane 4, cotranslation of p105 and c-Rel; lane 5, cotranslation of p105 and v-Rel; lane 6, c-Rel translation; lane 7, v-Rel translation. Pertinent proteins are indicated with arrows to the sides of the figures.

DISCUSSION

Sequence analysis of the chicken NF-κB p50 precursor p105. Sequence analysis of a cDNA encoding the chicken NF-κB p50 precursor protein p105 reveals that chicken p105 is approximately 70% identical to mouse and human p105 proteins. Because of the high degree of sequence identity in the RH domain of p50 (93% identical between chicken and mouse), it is almost certain that the proteins from chickens and mammals would show virtually identical biochemical and cellular properties in vitro and in vivo. On the other hand, the RH domain of chicken p50 is only approximately 50% identical to chicken c-Rel and v-Rel. These differences may account for differences that are seen in the ability of p50 and c-Rel to recognize different κB half sites.

Chicken p50, like mammalian p50, has additional sequences in the RH domain compared with other Rel proteins: there are three small insertions of 16 aa (aa 172 to 187), 13 aa (aa 198 to 210), and 5 aa (aa 292 to 296). The functional significance of these small insertions is not known. One possibility is that these insertions interfere with the ability of IκB to inhibit DNA binding by p50; even though IκBβ (pp40) can interact with p50, it does not prevent DNA binding by p50 homodimers as IκBβ does with complexes containing other Rel family proteins, such as p65 and c-Rel (26a, 31). Another possibility is that these p50-specific sequences confer a transcription activation function on the RH domain of p50, since it has been shown that p50 homodimers can activate transcription in vivo as the factor known as KBF1 (27).

Within the C-terminal portion of chicken p105 (aa 541 to 789), as in mammalian p105 proteins, there are six full ankyrinlike repeats followed by a seventh half repeat. In the

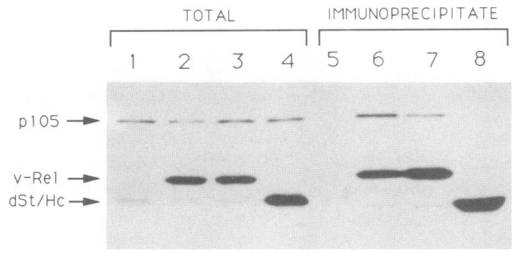

FIG. 6. Association between p105 and nontransforming v-Rel mutants. Wild-type v-Rel (lane 2), v-SPW (42) (lane 3), and dStu/Hinc (21) (lane 4) were cotranslated in vitro with chicken p105; lane 1 is an in vitro translation of p105 alone. Portions of the same four samples were immunoprecipitated with anti-v-Rel antiserum (lanes 5 to 8, respectively) as described in the legend to Fig. 5. The pertinent bands are indicated to the side of the figure.

erythrocyte, ankyrin proteins interact with both the cytoskeleton (via the ankyrin repeats) and integral membrane proteins. Blank et al. (6) have shown that deletion of the sixth and seventh ankyrin repeats in p105 allows the normally cytoplasmic human p105 protein to enter the nucleus in Cos cells. Therefore, the p50/p105 and p49/p100 precursor proteins could be sequestered in the cytoplasm by virtue of an interaction between their ankyrin repeats and the cytoskeleton or, more likely, an intramolecular interaction between the ankyrin repeats and the RH domain. The c-Rel-like proteins (p65, c-Rel, and RelB), which do not contain ankyrin repeats, could be retained in the cytoplasm through interaction with IκB, which contains its own set of ankyrin repeats.

In addition, there may be other mechanisms for retention of the c-Rel-like proteins in the cytoplasm. For example, deletion of C-terminal aa from chicken c-Rel allows it to enter the nucleus of CEF (10), even though this deleted c-Rel protein could still likely bind to IκB proteins via its intact RH domain. Furthermore, when overexpressed in CEF from a retroviral vector, pp40 is primarily a nuclear protein, as judged by immunofluorescence (40a). These types of results suggest that the C terminus of c-Rel is directly masking the nuclear localization signal and that cytoplasmic retention of c-Rel in CEF occurs through a mechanism that is independent of IκB association.

Cytoplasmic retention is probably not the only function for the C terminus of p105. That is, the C-terminal sequences of mouse p105 can specifically inhibit DNA binding of p50 homodimers (25, 31). In addition, we have shown that chicken, mouse, and human p105 C-terminal aa have sequences that can function as a transcription activator when fused to the DNA-binding domain of GAL4 (40a) (Fig. 3C). The transcription-activating function maps to the acidic aa located between the sixth and seventh ankyrin repeats in mouse p105 (40a). Since these acidic aa are conserved in chicken p105, it is likely that these sequences are responsible for transcription activation by chicken C-terminal p105 sequences in SGCh3′p105. Along these lines, it is interesting that ankyrinlike repeats have been found in a protein, GABPβ, known to be involved in transcription activation (35). In GABPβ, the ankyrin repeats are involved in heterodimerization with GABPα. Thus, it is possible that the C-terminal aa of p105 are, in certain circumstances, involved in transcription activation, as are the C-terminal aa of other Rel proteins, such as c-Rel, RelB, p65, and Dorsal (8, 29, 47, 51, 56).

Protein complexes containing v-Rel and c-Rel. As determined by immunofluorescent staining and cellular fractionation, v-Rel and c-Rel are primarily cytoplasmic proteins in avian lymphoid cells (20, 45, 59, 61). In these cells, v-Rel and c-Rel are found in high-M_r complexes that include at least five cellular proteins. Several groups have determined the subcellular distribution of these complexes in v-Rel-transformed lymphoid cells and normal lymphoid cells (12, 13, 33, 41). Davis et al. (13) found that v-Rel exists exclusively in high-M_r complexes in v-Rel-transformed cells. They determined that 90% of v-Rel is present in cytoplasmic complexes: the majority of cytoplasmic v-Rel (80%) is found complexed with p40 (IκB), p70 (heat shock protein), and c-Rel; the remaining cytoplasmic v-Rel (20%) is found in a complex containing p124 (NF-κB p105), p115, and the majority of c-Rel. Additionally, they found a small percentage of v-Rel (10%) in the nuclear fractions, where it was complexed exclusively with pp40. In contrast, Morrison et al. (41) determined that v-Rel was present in complexes containing p124, p115, and c-Rel in the cytoplasm and in the nucleus, even though the nuclear v-Rel-containing complexes were still only a small fraction of the total cellular v-Rel-containing complexes. In addition, Morrison et al. only saw pp40 in the cytoplasmic complex; no pp40 was complexed with v-Rel in nuclear fractions. Since we have identified p124 as chicken NF-κB p105 and since Blank et al. (6) have shown that mouse p105 is exclusively a cytoplasmic protein in lymphoid cells, it seems reasonable to assume that most of the v-Rel-p105 complex is located in the cytoplasm of transformed avian lymphoid cells.

In contrast to v-Rel, chicken c-Rel in normal lymphoid cells is primarily complexed with p124 and p115, and only a small percentage is complexed with pp40 (12). Thus, the ability of v-Rel to form complexes with these cellular proteins may be different from that of c-Rel in vivo. This is supported by data which show that c-Rel does not appear to be complexed with any proteins in MSB-1 cells (an avian T-cell line transformed by Marek's disease virus) (59). However, when v-Rel is expressed in MSB-1 cells, cellular proteins, including p124 (p105), can be found complexed to c-Rel and v-Rel (33).

We have also shown that NF-κB p50 can complex with v-Rel in vitro. However, p50 has never been detected in immunoprecipitates from v-Rel-transformed cells with anti-v-Rel antibody. This is probably because the majority of p50 in unstimulated cells is likely to be in the precursor form, and therefore the amount of p50 complexed with v-Rel may be quite low. On occasion, we have detected faint bands of approximately 50 kDa in v-Rel immunoprecipitates, and we believe that it is likely that v-Rel also complexes with p50 in vivo.

p115 is the only member of the v-Rel complex which has not yet been identified. Since we detected no obvious similarity between p115 and p105 by partial V8 proteolysis mapping, it is unlikely that p115 is an alternate form of p124 (p105). It seems more reasonable to suspect that the p115 v-Rel-associated protein is the nonprocessed form of the p50/p105-related protein p49/p100 (55).

Transformation by Rel proteins. v-Rel primarily transforms cells of lymphoid origin. However, c-Rel is only approximately 2% as transforming as v-Rel for avian lymphoid cells (42). It is probable that v-Rel transforms lymphoid cells by disrupting the normal role of c-Rel, and possibly other proteins in NF-κB-like complexes, in the control of lymphoid cell growth. The exact role of c-Rel in lymphoid cells is not known. However, it seems likely that

c-Rel regulates lymphoid cell proliferation and/or differentiation by activation or repression of transcription of specific genes.

v-Rel is an inactive version of the c-Rel protein in terms of transcription activation, and v-Rel can interfere with transcription activation by c-Rel and related proteins in a dominant-negative fashion (5, 26, 29, 47). Although v-Rel can bind to DNA in vitro, the bulk of the data indicate that it is unlikely that v-Rel effects transformation by binding directly to DNA. First, the majority of v-Rel is located in the cytoplasm of v-Rel-transformed cells, and a v-Rel protein with a mutated nuclear localizing sequence is fully transforming and appears to be located exclusively in the cytoplasm of transformed spleen cells (21). Second, since the overwhelming majority of v-Rel in transformed spleen cells is complexed with p105 (p124) or pp40, it is unlikely that either of these forms is competent for DNA binding. That is, pp40 (IκB) inhibits κB site binding by complexes containing c-Rel (14), and p105 does not bind to DNA (17, 32). Still, there is some v-Rel in cells which can bind to DNA, since extracts from v-Rel-transformed spleen cells do contain κB site-binding activity that includes v-Rel (28).

We favor a model for transformation in which v-Rel is disrupting the regulation of NF-κB complexes, through either the formation of altered Rel family protein heterodimers or the titration of an inhibitor such as pp40 (IκB). Our identification of avian NF-κB p105 as one of the v-Rel-associated proteins, our demonstration that v-Rel can complex with p50 in vitro, the characterization of dominant-negative mutants of p50 and c-Rel (26, 38, 47), and our suggestion that the remaining unidentified v-Rel-associated protein p115 is likely to be the NF-κB p49/p100 precursor protein all support this type of model. This model would predict that multiple types of mutations could activate the transforming potential of c-Rel, and there is some evidence for this (22, 29). Similarly, it would suggest that overexpression of mutant versions of other Rel family proteins would be transforming, provided that they could form the same types of cellular complexes as mutant c-Rel proteins. However, it is clear that association with p105 or pp40 is not sufficient for transformation, since v-SPW, a nontransforming v-Rel mutant, can still associate with p105 (Fig. 6), and both v-SPW and dStu/Hinc can associate with pp40 in vitro (8a). Furthermore, c-Rel associates with p105 and p40, yet c-Rel is not highly transforming when overexpressed in avian spleen cells (42).

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REFERENCES

1. **Baeuerle, P. A.** 1991. The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim. Biophys. Acta **1072**:63–80.
2. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242**:540–546.
3. **Baeuerle, P. A., and D. Baltimore.** 1989. A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3**:1689–1698.
4. **Baldwin, A. S., Jr., K. P. LeClair, H. Singh, and P. A. Sharp.** 1990. A large protein containing zinc finger domains binds to related sequence elements in the enhancers of the class I major histocompatibility complex and kappa immunoglobulin genes. Mol. Cell. Biol. **10**:1406–1414.
5. **Ballard, D. W., W. H. Walker, S. Doerre, P. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hannink, and W. C. Greene.** 1990. The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63**:803–814.
6. **Blank, V., P. Kourilsky, and A. Israël.** 1991. Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. EMBO J. **10**:4159–4167.
7. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature (London) **348**:76–80.
8. **Bull, P., K. L. Morley, M. F. Hoekstra, T. Hunter, and I. M. Verma.** 1990. The mouse c-*rel* protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. Mol. Cell. Biol. **10**:5473–5485.

8a. **Capobianco, A. J., and T. D. Gilmore.** Unpublished results.

9. **Capobianco, A. J., and T. D. Gilmore.** 1991. Repression of the chicken c-*rel* promoter by vRel in chicken embryo fibroblasts is not mediated through a consensus NF-κB binding site. Oncogene **6**:2203–2210.
10. **Capobianco, A. J., D. L. Simmons, and T. D. Gilmore.** 1990. Cloning and expression of a chicken c-rel cDNA: unlike $p59^{v\text{-}rel}$, $p68^{c\text{-}rel}$ is a cytoplasmic protein in chicken embryo fibroblasts. Oncogene **5**:257–265.
11. **Cleveland, D. W., S. G. Fischer, M. W. Krischner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
12. **Davis, J. N., W. Bargmann, and H. R. Bose, Jr.** 1990. Identification of protein complexes containing the c-*rel* proto-oncogene product in avian hematopoietic cells. Oncogene **5**:1109–1115.
13. **Davis, N., W. Bargmann, M.-Y. Lim, and H. R. Bose, Jr.** 1990. Avian reticuloendotheliosis virus-transformed lymphoid cells contain multiple $pp59^{v\text{-}rel}$ complexes. J. Virol. **64**:584–591.
14. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H. R. Bose, Jr.** 1991. Rel-associated pp40: an inhibitor of the Rel family of transcription factors. Science **253**:1268–1271.
15. **Fan, C.-M., and T. Maniatis.** 1990. A DNA-binding protein containing two widely separated zinc finger motifs that recognize the same DNA sequence. Genes Dev. **4**:29–42.
16. **Fan, C.-M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature (London) **354**:395–398.
17. **Ghosh, S., A. M. Gifford, L. R. Rivière, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell **62**:1019–1029.
18. **Gilmore, T. D.** 1990. NF-κB, KBF1, *dorsal*, and *rel*ated matters. Cell **62**:841–843.
19. **Gilmore, T. D.** 1991. Malignant transformation by mutant Rel proteins. Trends Genet. **7**:318–322.
20. **Gilmore, T. D., and H. M. Temin.** 1986. Different localization of the product of the v-*rel* oncogene in chicken fibroblasts and spleen cells correlates with transformation by REV-T. Cell **44**:791–800.
21. **Gilmore, T. D., and H. M. Temin.** 1988. v-*rel* oncoproteins in the nucleus and in the cytoplasm transform chicken spleen cells. J. Virol. **62**:703–714.

22. **Hannink, M., and H. M. Temin.** 1989. Transactivation of gene expression by nuclear and cytoplasmic *rel* proteins. Mol. Cell. Biol. **9**:4323–4336.
23. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Haskill, S., A. A. Beg, S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. S. Baldwin, Jr.** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65**:1281–1289.
25. **Inoue, J.-I., L. D. Kerr, A. Kakizuka, and I. M. Verma.** 1992. IκBγ, a 70 kD protein, identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell **68**:1109–1120.
26. **Inoue, J.-I., L. D. Kerr, L. J. Ransone, E. Bengal, T. Hunter, and I. M. Verma.** 1991. c-rel activates but v-rel suppresses transcription from κB sites. Proc. Natl. Acad. Sci. USA **88**: 3715–3719.
26a. **Inoue, J.-I., L. D. Kerr, D. Rasheed, N. Davis, H. R. Bose, Jr., and I. M. Verma.** Direct association of pp40/IκBβ with rel/NF-κB proteins: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA, in press.
27. **Israël, A., A. Kimura, M. Kieran, S. Yano, J. Kanellopoulos, O. Le Bail, and P. Kourilsky.** 1987. A common positive transacting factor binds to enhancer sequences in the promoter of mouse H-2 and β2-microglobulin genes. Proc. Natl. Acad. Sci. USA **84**:2653–2657.
28. **Kabrun, N., J. W. Hodgson, M. Doemer, G. Mak, B. R. Franza, and P. J. Enrietto.** 1991. Interaction of the v-rel protein with an NF-κB DNA binding site. Proc. Natl. Acad. Sci. USA **88**:1783–1787.
29. **Kamens, J., P. Richardson, G. Mosialos, R. Brent, and T. Gilmore.** 1990. Oncogenic transformation by vRel requires an amino-terminal activation domain. Mol. Cell. Biol. **10**:2840–2847.
30. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4**:1172–1174.
31. **Kerr, L. D., J.-I. Inoue, N. Davis, E. Link, P. A. Baeuerle, H. R. Bose, Jr., and I. M. Verma.** 1991. The Rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. Genes Dev. **5**:1464–1476.
32. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62**:1007–1018.
33. **Kochel, T., J. F. Mushinski, and N. R. Rice.** 1991. The v-*rel* and c-*rel* proteins exist in high molecular weight complexes in avian and murine cells. Oncogene **6**:615–626.
34. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
35. **LaMarco, K., C. C. Thompson, B. P. Byers, E. M. Walton, and S. L. McKnight.** 1991. Identification of Ets- and Notch-related subunits in GA binding protein. Science **253**:789–792.
36. **Lillie, J. W., and M. R. Green.** 1989. Transcriptional activation by the adenovirus E1a protein. Nature (London) **338**:39–44.
37. **Lim, M.-Y., N. Davis, J. Zhang, and H. R. Bose, Jr.** 1990. The v-*rel* oncogene product is complexed with cellular proteins including its proto-oncogene product and heat shock protein 70. Virology **175**:149–160.
38. **Logeat, F., N. Israël, R. Ten, V. Blank, O. Le Bail, P. Kourilsky, and A. Israël.** 1991. Inhibition of transcription factors belonging to the rel/NF-κB family by a transdominant negative mutant. EMBO J. **10**:1827–1832.
39. **Mauxion, F., and R. Sen.** 1989. Alteration of a single nucleotide allows efficient binding of H2 TFI/KBFI to the immunoglobulin κ enhancer B motif. Mol. Cell. Biol. **9**:3548–3552.
40. **Meyer, R., E. N. Hatada, H.-P. Hohmann, M. Haiker, C. Bartsch, U. Röthlisberger, H.-W. Lahm, E. J. Schlaeger, A. P. G. M. van Loon, and C. Scheiderleit.** 1991. Cloning of the DNA-binding subunit of human nuclear factor κB: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor alpha. Proc. Natl. Acad. Sci. USA **88**:966–970.
40a. **Morin, P. J., and T. D. Gilmore.** The C terminus of the NF-κB p50 precursor and an IκB isoform contain transcription activation domains. Nucleic Acids Res., in press.
41. **Morrison, L. E., N. Kabrun, S. Mudri, M. J. Hayman, and P. J. Enrietto.** 1989. Viral *rel* and cellular *rel* associate with cellular proteins in transformed and normal cells. Oncogene **4**:677–683.
42. **Mosialos, G., P. Hamer, A. J. Capobianco, R. A. Laursen, and T. D. Gilmore.** 1991. A protein kinase A recognition sequence is structurally linked to transformation by $p59^{v\text{-}rel}$ and cytoplasmic retention of $p68^{c\text{-}rel}$. Mol. Cell. Biol. **11**:5867–5877.
43. **Nolan, G. P., S. Ghosh, H.-C. Liou, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a rel-related polypeptide. Cell **64**:961–969.
44. **Radke, K., T. Gilmore, and G. S. Martin.** 1980. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell **21**:821–828.
45. **Rice, N. R., T. D. Copeland, S. Simek, S. Oroszlan, and R. V. Gilden.** 1986. Detection and characterization of the protein encoded by the v-*rel* oncogene. Virology **149**:217–229.
46. **Rice, N. R., and R. V. Gilden.** 1988. The rel oncogene, p. 495–512. *In* E. P. Reddy, A. M. Skalka, and T. Curran (ed.), The oncogene handbook. Elsevier Science Publishing, Inc., New York.
47. **Richardson, P. M., and T. D. Gilmore.** 1991. v-Rel is an inactive member of the Rel family of transcriptional activating proteins. J. Virol. **65**:3122–3130.
48. **Rivière, Y., V. Blank, P. Kourilsky, and A. Israël.** 1991. Processing of the precursor of NF-κB by the HIV-1 protease during acute infection. Nature (London) **350**:625.
49. **Ron, D., A. R. Braser, and J. F. Habener.** 1991. Angiotensin gene-inducible enhancer-binding protein 1, a member of a new family of large nuclear proteins that recognize nuclear factor κB-binding sites through a leucine zipper. Mol. Cell. Biol. **11**:2887–2895.
50. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C.-H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a *rel*-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. Science **251**:1490–1493.
51. **Ryseck, R.-P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobranski, and R. Bravo.** 1992. RelB, a new Rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. **12**:674–684.
52. **Sadowski, I., and M. Ptashne.** 1989. Vector for expressing GAL4 (1–147) fusions in mammalian cells. Nucleic Acids Res. **18**:7539.
53. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
54. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
55. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352**:733–736.
56. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10**:3805–3817.
57. **Schmitz, M. L., T. Henkel, and P. A. Baeuerle.** 1991. Proteins controlling the nuclear uptake of NF-κB, Rel and dorsal. Trends Cell Biol. **1**:130–137.
58. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a post-translational mechanism. Cell **47**:921–928.
59. **Simek, S., and N. R. Rice.** 1988. Detection and characterization of the protein encoded by the chicken c-*rel* protooncogene. Oncogene Res. **2**:103–119.
60. **Simek, S., and N. R. Rice.** 1989. $p59^{v\text{-}rel}$, the transforming protein reticuloendotheliosis virus, is complexed with at least

four other proteins in transformed chicken lymphoid cells. J. Virol. **62**:4730–4736.
61. **Simek, S. L., R. M. Stephens, and N. R. Rice.** 1986. Localization of the v-*rel* protein in reticuloendotheliosis virus strain T-transformed lymphoid cells. J. Virol. **59**:120–126.
62. **Urban, M. B., R. Schreck, and P. A. Baeuerle.** 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. EMBO J. **10**:1817–1825.
63. **Wilhelmsen, K., K. Eggleton, and H. M. Temin.** 1984. Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. J. Virol. **52**:172–182.