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NF-κB p100 Is One of the High-Molecular-Weight Proteins Complexed with the v-Rel Oncoprotein in Transformed Chicken Spleen Cells

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The Rel/NF-κB family of proteins includes several interacting cellular transcription factors and the v-Rel oncoprotein of the avian Rev-T retrovirus. We report the isolation of a chicken cDNA for the NF-κB p52 precursor protein p100. Full-length p100 only weakly binds DNA in vitro; removal of the ankyrin-like repeats generates C-terminally truncated p100 proteins (like p52) that have an increased ability to bind an oligonucleotide containing a κB site. In addition, we show that chicken p100 is identical to a protein previously designated p115, which is found in a complex with v-Rel in transformed chicken spleen cells. Furthermore, p100 and v-Rel can form a complex when synthesized in vitro. Using cDNAs for chicken NF-κB p105, NF-κB p100, c-Rel, and v-Rel, we show that one of the complexes in v-Rel-transformed spleen cells can be reconstituted in vitro.

Transcriptional regulation of several cellular and viral genes has been shown to be affected by binding of nuclear factor κB (NF-κB) to a decameric *cis*-acting element, whose consensus sequence is GGGRNNYYCC (reviewed in reference 1). Active NF-κB is a heterodimer composed of 50-kDa (p50) and 65-kDa (RelA) subunits, both of which are members of the Rel/NF-κB family of transcription factors. Besides p50 and RelA, the Rel family includes the vertebrate proteins c-Rel, RelB, and NF-κB p100/p52, the retroviral oncoprotein v-Rel, and the *Drosophila melanogaster* ventral morphogen Dorsal (reviewed in references 1, 3, 14, and 16). Most Rel proteins can form homo- or heterodimers that have different intrinsic DNA-binding specificities and different abilities to affect transcription (20, 23, 24, 31).

Rel proteins are related through a common N-terminal domain called the Rel homology (RH) domain, which consists of approximately 300 amino acids (aa) and contains sequences important for dimerization, DNA binding, inhibitor binding, and nuclear localization. The C-terminal halves of Rel proteins generally have little sequence similarity but have been used to distinguish two classes of Rel proteins. One class includes NF-κB p100 and p105, which are precursor proteins to p52 and p50, respectively. It is thought that p100 and p105 require proteolytic processing to generate the mature p52 and p50 DNA-binding proteins in vivo (12, 22, 32); however, p52 may also arise by alternative splicing (35). p100 and p105 each have several ankyrin-like repeats in the C-terminal sequences that are removed by proteolysis, and full-length p105 and p100 molecules can retain Rel proteins such as c-Rel and RelA in the cytoplasm and can inhibit DNA binding by these proteins in vitro (23, 27, 28, 32). A second class of cellular Rel proteins includes c-Rel, RelA, RelB, and Dorsal. These proteins are not processed and bind DNA as full-length proteins. In addition, these Rel proteins all have transcriptional activation domains in their C-terminal halves (reviewed in references 16 and 30).

The v-*rel* oncogene of the highly oncogenic avian retrovirus Rev-T encodes a phosphoprotein of approximately 59 kDa (41, 44). v-Rel is a truncated and mutated form of avian c-Rel: compared with c-Rel, v-Rel is missing 2 aa at its N terminus and 118 aa at its C terminus and has several internal changes (reviewed in references 3 and 15). In transformed chicken spleen cells, v-Rel is located primarily in the cytoplasm, where it is complexed with at least five cellular proteins (p124, p115, p70, p68, and p40). Four of these proteins have been identified previously. p40 is a chicken IκB protein (IκBα) (10); p68 is c-Rel (21, 39); p70 is a constitutive form of the heat shock protein 70 (21); and recently we have shown that p124 is NF-κB p105, the precursor to NF-κB p50 (5). We have now isolated and characterized a full-length chicken NF-κB p100/p52 cDNA, and using this cDNA, we show that the p115 v-Rel-associated protein is NF-κB p100.

To isolate probes for screening of cDNA libraries for avian Rel/NF-κB cDNAs, we performed a reverse transcriptase-polymerase chain reaction (PCR) on RNA isolated from a v-Rel-transformed chicken spleen cell line as described by Xie and Rothblum (45). The primers used were RN2 [5′CG (A,T)TT(T,C)CG(G,A,T,C)TA(T,C)G(G,T)(G,A,C)TG(T,C) GA(G,A)GG] and KN1 [5′CGGAATTC(C,T)TT(C,T)TG(G, A,T,C)AC(C,T)TT(G,A)TC(G,A)CA)], which are degenerate oligonucleotides designed against the highly conserved amino acid sequences RFRY(VG)CEG and CDKVQKD, respectively, found in the RH domains of many Rel proteins.

Approximately 20 μg of total RNA was reverse transcribed by using avian myeloblastosis virus reverse transcriptase (Promega) with KN1 as the primer; PCR using KN1 and RN2 and *Taq* polymerase (Cetus) was then performed in an MJ Research thermocycler. Amplification was carried out for 5 cycles at 94°C (3 min), 50°C (1.5 min), and 75°C (1.5 min), followed by 30 cycles at 94°C (1.5 min), 50°C (1.5 min), and 75°C (1.5 min). Products of the PCR were then subcloned into pBluescript KS+ (Stratagene) and analyzed by double-stranded DNA sequencing by standard techniques (8, 34). DNA sequencing of 58 subclones revealed the presence of amplified cDNA sequences for many Rel proteins, including v-Rel, c-Rel, RelB, and NF-κB p105, and three identical probes for NF-κB p100.

A PCR-amplified NF-κB p100 cDNA probe was ^{32}P labeled by using random primers and was used to screen a chicken embryo fibroblast cDNA library constructed in λ Zap (Stratagene) as described previously (8) except that the final wash was in 0.1× SSPE–0.5% sodium dodecyl sulfate (SDS) con-

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taining 0.1 mg of sheared salmon sperm DNA per ml at 68°C for 30 min. Positive plaques were purified by three rounds of screening and were then excised from λ Zap as instructed by the manufacturer (Stratagene). The longest cDNA that showed sequence similarity to human NF-κB p100 was approximately 3.3 kbp, and a complete nucleotide sequence of this cDNA was determined by using subclones, partial cDNA clones, and designed primers.

The nucleotide sequence of the chicken NF-κB p100 cDNA and its predicted amino acid sequence are shown in Fig. 1 and can be obtained from GenBank (accession number U00111). This cDNA contains an open reading frame encoding a 906-aa protein, starting with an ATG at nucleotide position 53 and terminating with a TGA at position 2771 (Fig. 1). The predicted chicken p100 protein has a calculated molecular size of 99.7 kDa. The sequences flanking the first methionine codon conform to the consensus for initiator methionines (19), and this initiator methionine is in a position similar to that found in human p100 (4, 22, 29, 36). Other features of the chicken p100 cDNA include a short GC-rich 5′ nontranslated region and a 1.5-kb 3′ nontranslated region that harbors two polyadenylation signals (Fig. 1). All six NF-κB p100 cDNAs that we isolated were polyadenylated at the 3′-most site. Finally, there is an ATTTA sequence at position 2913; this motif has been shown to be involved in targeting mRNA for degradation (37).

The primary structure of the predicted chicken p100 protein is very similar to that of human p100 (4, 22, 29, 36). Each p100 protein has the following structure: there is a short N-terminal region (37 aa) preceding the RH domain; the RH domain extends from aa 38 to 340; this is followed by a glycine-rich region, which is thought to contain a site for proteolytic cleavage; there is a region from aa 466 to 752 that harbors six complete and two partial ankyrin-like repeats; and there is a C-terminal region of unknown function (Fig. 2). A conserved nuclear localization signal is found at the end of the RH domain (RNRKK, aa 336 to 340). However, a serine residue within a consensus protein kinase A recognition sequence (RRPS) found in the RH domains of most Rel proteins is not present in either human or chicken p100 (KRKR; aa 309 to 312).

Overall, the chicken p100 protein is approximately 65% identical to human p100 (Fig. 2), the only other species from which a p100 cDNA has been isolated. The highest degree of sequence similarity between these two proteins is in the RH domain, which has approximately 85% amino acid identity; the other p100 domains are all about 50 to 60% identical between the two species. The major differences between the human and chicken p100 proteins are that chicken p100 has a 22-aa insertion between the last two ankyrin-like repeats and human p100 has a 10-aa insertion in the glycine-rich region compared with chicken p100. It is not clear whether these represent species-specific differences or indicate that p100 can undergo alternative splicing in these regions.

Several groups have observed that DNA binding by in vitro-synthesized p100 and p105 is inhibited by the presence of the C-terminal half of the protein (4, 5, 13, 17, 22, 29). Therefore, to demonstrate that the chicken p100 cDNA encodes a functional DNA-binding protein, we generated plasmids for the in vitro expression of full-length p100 and of two truncated proteins (called p52K and p52P) that roughly contain the sequences that would be present in cellular p52 (Fig. 3). Plasmids for the in vitro expression of these p100 proteins were made by subcloning an *Eco*RI-to-*Eco*RI fragment for full-length p100 and an *Eco*RI-to-*Pst*I fragment or *Eco*RI-to-*Kpn*I fragment for the p52-like molecules, p52P or p52K, into the corresponding sites of pGEM4 (Promega) in the Sp6 orientation. These proteins were synthesized in vitro and were tested in an electrophoretic mobility shift assay for the ability to bind an oligonucleotide containing an NF-κB site from the chicken c-*rel* promoter as described previously (6, 38). As seen by others (4, 22, 29), the full-length in vitro-synthesized p100 protein could only weakly bind the κB site-containing probe. Both p52-like proteins bound more avidly to the κB site-containing probe; however, when equal amounts of in vitro-translated proteins were used, the ability of p52 to bind to this oligonucleotide was still less than that of v-Rel. The binding by the p52-like proteins to the κB site-containing probe could be specifically competed for by an unlabeled κB site-containing oligonucleotide included in the reaction but not by a mutant κB oligonucleotide or a heterologous (Sp1 site) oligonucleotide. These results demonstrate that the chicken NF-κB p100 cDNA encodes a protein with a functional DNA-binding domain.

Because two of the v-Rel-associated proteins in transformed chicken spleen cells are members of the Rel family (c-Rel and NF-κB p105) (5, 21, 39) and because mammalian c-Rel can complex with NF-κB p100 (23), we previously suggested that the unidentified p115 v-Rel-associated protein would likely be NF-κB p100 (5, 14, 15). To determine whether the v-Rel-associated protein p115 is NF-κB p100, we first compared the mobility on SDS-polyacrylamide gels of in vitro-synthesized chicken p100 with that of the p115 protein seen in v-Rel immunoprecipitates from transformed chicken spleen cells. As shown in Fig. 4A, in vitro-synthesized chicken NF-κB p100 and coprecipitated p115 comigrate on SDS-polyacrylamide gels. Furthermore, the partial peptide maps of in vitro-synthesized p100 and coimmunoprecipitated p115 were virtually identical (Fig. 4B). Therefore, p100 appears to be identical to the previously described p115 v-Rel-associated protein.

We have previously shown that chicken NF-κB p105 and v-Rel can form a complex in vitro (5). To determine whether p100 could also interact directly with v-Rel in vitro, p100 and v-Rel were cotranslated in vitro. When cotranslated p100 and v-Rel were incubated with anti-v-Rel antiserum, both v-Rel and p100 were immunoprecipitated (Fig. 5A). p100 by itself could not be immunoprecipitated with anti-v-Rel antiserum. Therefore, v-Rel and p100 can form a complex in vitro.

One of the v-Rel-containing complexes in transformed chicken spleen cells consists of v-Rel, c-Rel, NF-κB p105 (p124), and NF-κB p100 (p115) (9). Plasmids for the in vitro expression of v-Rel, chicken c-Rel, and chicken NF-κB p105 have been described previously (5, 25). To determine whether we could reconstitute this complex in vitro, we subjected approximately 0.2 to 1.0 μg of pGEM4 expression plasmids for all four proteins (v-Rel, c-Rel, p105, and p100, individually and together) to in vitro transcription-translation using Sp6 polymerase in the Promega TNT-coupled wheat germ system (Fig. 5B, lanes 1 to 5); this system has been shown to be useful for expressing high quantities of full-length Rel/NF-κB proteins in vitro (11). The cotranslation of all four proteins was then immunoprecipitated with preimmune or anti-v-Rel antiserum. All four proteins (v-Rel, c-Rel, p105, and p100) could be detected in immunoprecipitates using anti-v-Rel antiserum (Fig. 5B, lane 7). Since p105 and p100 cannot be recognized by the v-Rel antiserum used in these immunoprecipitations (Fig. 5A and reference 5), this result indicates that p100 and p105 are being immunoprecipitated because they are in a complex with v-Rel and/or c-Rel. Therefore, at least superficially, we can reconstitute the cellular complex containing v-Rel, c-Rel, p105, and p100 in vitro.

Our unambiguous identification of NF-κB p100 as the previously characterized v-Rel-associated p115 protein means

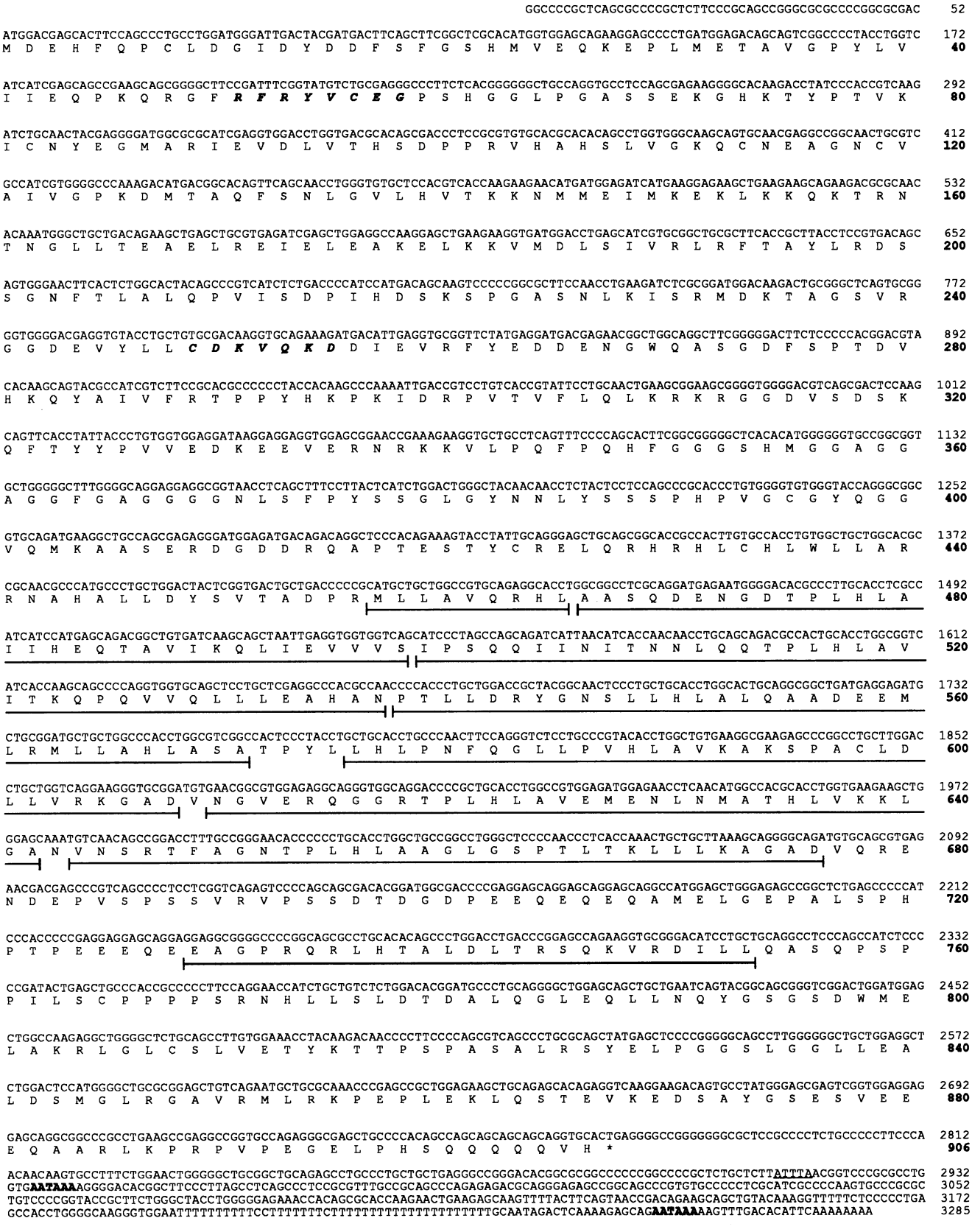

FIG. 1. Nucleotide and predicted amino acid sequence of a composite chicken NF-κB p100 cDNA. Numbers at the right indicate nucleotide and amino acid (in boldface) positions. The amino acid sequences against which degenerate PCR primers were made are indicated in boldface italics (aa 51 to 58 and 249 to 255). The ankyrin-like repeats are underlined with brackets and were aligned as phased by Bours et al. (4) and Neri et al. (29). Potential polyadenylation signals (AATAAA) are in boldface, and a possible destabilization signal (ATTTA) is underlined.

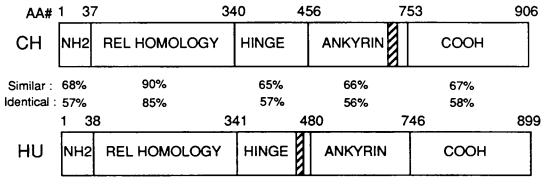

FIG. 2. Homology between human and chicken NF-κB p100 proteins. The human (HU) and chicken (CH) p100 proteins have been divided into five domains, and the amino acid identity and similarity (identical plus conserved amino acids) were determined for each domain. The domains for p100 are as follows: NH2, N-terminal region; Rel homology domain; HINGE, glycine-rich region; ANKYRIN, region containing ankyrin-like repeats; and COOH, C-terminal region. Numbers above the diagrams indicate the amino acid positions at the ends of the domains. Striped bars indicate the approximate positions of small insertions found in human and chicken p100 proteins.

that all of the major cellular proteins that associate with v-Rel in transformed chicken spleen cells have now been identified. Certain data suggest that v-Rel transforms cells by disturbing an equilibrium involving cellular Rel or IκB proteins: most

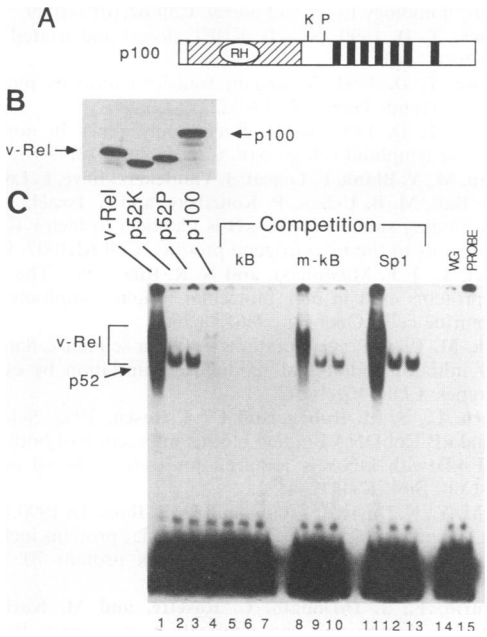

FIG. 3. DNA binding by chicken NF-κB p100. (A) Schematic of chicken p100. The approximate extent of the RH domain is shown by shading; the ankyrin-like repeats are indicated by black bars. The approximate positions of *Kpn*I (K) and *Pst*I (P) sites used to generate in vitro expression subclones for p100 proteins truncated at codons 397 and 427, respectively, are indicated. Plasmids for v-Rel, p100, and the two truncated versions of p100 (p52K and p52P) were subjected to coupled transcription-translation in vitro in the presence of [^{35}S]methionine and [^{35}S]cysteine. Approximately equal amounts of each radiolabeled protein (B) were then analyzed in an electrophoretic mobility shift assay (C), using a ^{32}P-labeled oligonucleotide containing a consensus NF-κB site from the chicken c-*rel* promoter as described previously (6, 38). The lane marked WG was performed by using a nonprogrammed wheat germ lysate, and the lane marked PROBE contains the radiolabeled probe alone. Competitions were performed with a 500-fold molar excess of the unlabeled κB site probe (kB), a mutant κB site probe (m-kB) (6), or an Sp1 site-containing probe (Sp1) (38). Positions of the relevant shifted complexes are indicated at the left.

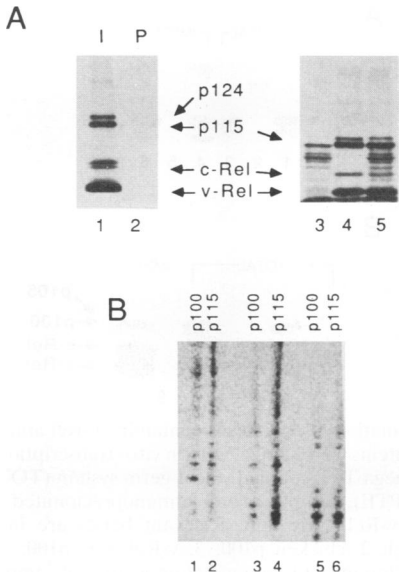

FIG. 4. Chicken NF-κB p100 is the v-Rel-associated protein p115. (A) Comigration of chicken p100 and p115 on SDS-polyacrylamide gels. Approximately 10^7 v-Rel-transformed spleen cells were labeled with Tran^{35}S-label (methionine plus cysteine; >1,000 Ci/mmol; ICN) for 6 h at 41.5°C, and detergent lysates containing SDS were immunoprecipitated with anti-v-Rel antiserum (I; lane 1) or preimmune serum (P; lane 2) as described previously (5). The chicken p100 cDNA was subjected to in vitro transcription followed by translation in a wheat germ lysate (lane 3) as described previously (5, 38) and immunoprecipitation with anti-v-Rel antiserum from v-Rel-transformed spleen cells (lane 4). Lane 5 contains a mixture of samples from lanes 3 and 4. All samples were analyzed on SDS–6% polyacrylamide gels that were treated with sodium salicylate after electrophoresis, and proteins were detected by fluorography. Positions of the relevant proteins are indicated by arrows. (B) Bands corresponding to p115 and to in vitro-translated p100 were excised from gels and subjected to partial proteolysis with 5 ng (lanes 1 and 2), 50 ng (lanes 3 and 4), or 500 ng (lanes 5 and 6) of V8 protease in situ in a SDS–12.5% polyacrylamide gel as described previously (5). An image was generated from the dried gel by using a Molecular Dynamics phosphorimager.

v-Rel is in the cytoplasm of v-Rel-transformed cells, mutations that abolish the nuclear localizing sequence of v-Rel do not affect its transforming function, and v-Rel complexed with p40, NF-κB p100, and p105 would not be expected to be able to bind DNA. In this model, the association of v-Rel with the NF-κB p100 and p105 precursor proteins and the IκB protein p40 could allow cellular Rel proteins to enter the nucleus and affect the transcription of genes involved in cellular growth control. In addition, v-Rel may associate with other cellular transcription factors, such as Sp1 and C/EBP, and affect their activity (38, 40). On the other hand, properties of v-Rel such as DNA binding and transcriptional activation may also be important for transformation (2, 7, 26, 35, 42, 43). Thus, the exact mechanism by which v-Rel causes transformation of avian spleen cells is currently unresolved, and v-Rel may very well transform cells by affecting multiple pathways, some that may involve an interaction with cellular proteins and some that may involve DNA binding. This, in turn, might result in an imbalance in the expression of genes that are crucial for the control of cell growth and proliferation.

A small percentage of human T-cell tumors have rearranged NF-κB p100 genes: in every case, the predicted p100 protein

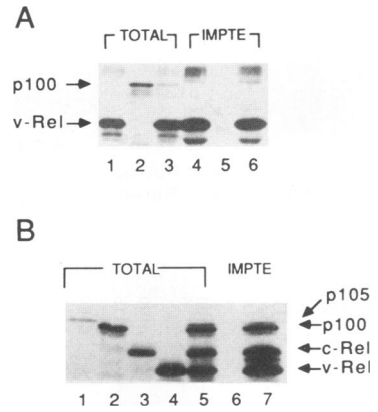

FIG. 5. Formation of complexes containing v-Rel and NF-κB p100 in vitro. All proteins were subjected to in vitro transcription-translation using the Promega TNT-coupled wheat germ system (TOTAL); where indicated (IMPTE), samples were immunoprecipitated with preimmune or anti-v-Rel antiserum. Relevant bands are indicated. (A) Lanes: 1, v-Rel; 2, chicken p100; 3, v-Rel plus p100; 4 through 6, immunoprecipitations with anti-v-Rel antiserum of samples shown in lanes 1 through 3, respectively. (B) Lanes: 1, chicken NF-κB p105; 2, chicken p100; 3, chicken c-Rel; 4, v-Rel; 5, cotranslation of p105, p100, c-Rel, and v-Rel; 6, immunoprecipitation with preimmune serum of cotranslation (i.e., as in lane 5); 7, immunoprecipitation with anti-v-Rel antiserum of cotranslation (as in lane 5).

would have a deletion of C-terminal ankyrin repeat sequences (8a, 29). However, it has not yet been possible to directly link these rearrangements in the human NF-κB p100 gene with the malignant state, and it is unclear at this point whether NF-κB p100 can function as a dominant-acting oncogene. We are currently attempting to determine whether overexpression of truncated forms of chicken p100 can transform avian lymphoid cells.

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