MOLECULAR AND CELLULAR BIOLOGY, Mar. 1993, p. 1769-1778 Vol. 13, No. 3
0270-7306/93/031769-10$02.00/0
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Differential pp40$^{I\kappa B\text{-}\beta}$ Inhibition of DNA Binding by *rel* Proteins

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Received 29 September 1992/Returned for modification 25 October 1992/Accepted 12 December 1992

Regulation of gene expression by members of the NF-κB/*rel* transcription factor family is a central component of signal transduction pathways utilized by many cellular processes, including lymphocyte activation, embryonic development, and oncogenesis. The members of the NF-κB/*rel* transcription factor family are regulated by association with a family of inhibitor (IκB) proteins. To address the importance of the association between *rel* and IκB proteins for oncogenesis by *rel* proteins, we characterized *rel*-IκB interactions in chicken embryo fibroblasts (CEF) infected with retroviral vectors encoding the avian c-*rel* (p68$^{c\text{-}rel}$), v-*rel* (p59$^{v\text{-}rel}$), and IκB-β (pp40$^{I\kappa B\text{-}\beta}$) proteins. In these experiments, the p59$^{v\text{-}rel}$:pp40$^{I\kappa B\text{-}\beta}$ ratio in coinfected CEF was nearly identical to the p59$^{v\text{-}rel}$:pp40$^{I\kappa B\text{-}\beta}$ ratio in v-*rel*-transformed cells. The avian IκB-β protein, pp40$^{I\kappa B\text{-}\beta}$, was able to associate with both the nononcogenic p68$^{c\text{-}rel}$ and the oncogenic p59$^{v\text{-}rel}$. Association of p68$^{c\text{-}rel}$ with pp40$^{I\kappa B\text{-}\beta}$ in coinfected CEF resulted in inhibition of the DNA-binding activity of p68$^{c\text{-}rel}$. Anti-pp40$^{I\kappa B\text{-}\beta}$ serum was able to restore DNA binding to p68$^{c\text{-}rel}$ in the presence of high levels of pp40$^{I\kappa B\text{-}\beta}$, indicating that pp40$^{I\kappa B\text{-}\beta}$ functions in a *trans*-acting manner to inhibit DNA binding by p68$^{c\text{-}rel}$. In contrast, sequence-specific DNA binding by the oncogenic v-*rel* protein, p59$^{v\text{-}rel}$, was not abolished by pp40$^{I\kappa B\text{-}\beta}$ in coinfected CEF. Anti-pp40$^{I\kappa B\text{-}\beta}$ serum did not immunoprecipitate the p59$^{v\text{-}rel}$-DNA adduct or alter the electrophoretic mobility of the p59$^{v\text{-}rel}$-DNA adduct, consistent with the idea that pp40$^{I\kappa B\text{-}\beta}$ and DNA are competitive inhibitors for the same or overlapping domains on *rel* proteins. Internal v-*rel*-derived sequences were identified that are responsible for loss of pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding by p59$^{v\text{-}rel}$. Loss of pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding by recombinant v/c-*rel* proteins was not sufficient for oncogenic activation of c-*rel*. Instead, removal of C-terminal c-*rel*-derived sequences in addition to loss of pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding was required for oncogenic activation of c-*rel*. These results demonstrate the presence of an interaction between internal and C-terminal regions of the c-*rel* protein that is important for the ability of c-*rel* to regulate the proliferation of lymphoid cells.

Posttranslational regulation of transcription factors provides for rapid alterations in gene expression in response to specific extracellular signals. A unique mechanism for posttranslational regulation of transcription factors has been convincingly demonstrated in the interaction between nuclear factor kappa B, NF-κB, and its inhibitor, IκB (3). NF-κB is one member of a ubiquitous transcription factor family that includes the c-*rel* proto-oncogene, the *dorsal* morphogen, and several structurally related proteins (1, 5, 8, 14, 17, 26, 28, 32–34, 37–40, 43). Several IκB proteins have been identified (10, 19, 20, 25, 35, 48), suggesting the existence of a family of inhibitor proteins in which each particular IκB protein may specifically regulate a particular member of the NF-κB/c-*rel* transcription factor family.

The central role of NF-κB in mediation of cellular responses to numerous external stimuli (27) suggests that disregulation of NF-κB/*rel* proteins would have profound consequences on cell growth and differentiation. Indeed, two members of the NF-κB/*rel* gene family, v-*rel* and *lyt-10*, were identified by virtue of their role in oncogenesis. The v-*rel* oncogene (42, 44, 47), isolated from the acutely oncogenic avian retrovirus reticuloendotheliosis virus strain T (Rev-T), is a structurally altered form of c-*rel*, while *lyt-10* is closely related to the gene encoding the p105 precursor of NF-κB (33). *bcl-3*, a member of the IκB gene family, has also been implicated as a potential oncogene (35).

The participation of IκB in regulation of NF-κB activity and the identification of the *rel*-associated protein pp40 as a member of the IκB-β family (9, 10, 25, 29, 41, 45) suggest an important role for pp40$^{I\kappa B\text{-}\beta}$ in gene regulation and oncogenesis by v-*rel*. One possible model for gene regulation and oncogenesis by v-*rel* is that association between the v-*rel* protein, p59$^{v\text{-}rel}$, and pp40$^{I\kappa B\text{-}\beta}$ results in sequestration of pp40$^{I\kappa B\text{-}\beta}$ by p59$^{v\text{-}rel}$ away from the endogenous NF-κB/*rel* proteins normally associated with pp40$^{I\kappa B\text{-}\beta}$ (7). This model, in which the sequestration of pp40$^{I\kappa B\text{-}\beta}$ by p59$^{v\text{-}rel}$ results in the release of the endogenous NF-κB/*rel* proteins normally associated with pp40$^{I\kappa B\text{-}\beta}$, is functionally analogous to the present model for activation of cellular NF-κB by phorbol esters in vivo, in which activation of NF-κB is thought to result from the phosphorylation-induced release of one or more IκB proteins (2, 3, 13).

There is substantial circumstantial evidence to support this type of indirect model for transformation by v-*rel*. First, p59$^{v\text{-}rel}$ is located predominantly in the cytoplasm of Rev-T-transformed cells (15), and neither the loss of its nuclear targeting sequence nor the addition of the nuclear targeting sequence from simian virus 40 T antigen affects the oncogenic properties of p59$^{v\text{-}rel}$ (16). Second, pp40$^{I\kappa B\text{-}\beta}$ was first identified by virtue of its association with p59$^{v\text{-}rel}$ (9, 29, 41, 45), and in vitro association of pp40$^{I\kappa B\text{-}\beta}$ with p59$^{v\text{-}rel}$ is able to inhibit DNA binding by p59$^{v\text{-}rel}$ (10, 25). Two other p59$^{v\text{-}rel}$-associated proteins have also been identified (7, 9, 29, 41), and one of these proteins is the avian homolog of the 105-kDa precursor of the 50-kDa subunit of mammalian NF-κB (7). Taken together, these lines of evidence support a model(s) in which the primary mode of action of p59$^{v\text{-}rel}$ in

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transformation is to perturb the normal regulation of endogenous NF-κB/*rel* proteins rather than acting as a DNA-bound transcription factor.

An alternative model for gene regulation and oncogenesis by v-*rel* is that $p59^{v\text{-}rel}$ is able to modulate gene expression by binding to cellular κB enhancer sequences. In this model, DNA binding by $p59^{v\text{-}rel}$ would be critically required for gene regulation and oncogenesis (46). There are two lines of evidence that support this type of direct model for transformation by v-*rel*. First, $p59^{v\text{-}rel}$ is the predominant κB-binding protein present in nuclear extracts from Rev-T-transformed cells (22; data not shown). Second, analysis of mutant v-*rel* proteins has provided a strong correlation between the ability of *rel* proteins to bind DNA and their oncogenic properties (1, 46). These two lines of evidence support a model in which transformation by $p59^{v\text{-}rel}$ results from its action as a DNA-bound modulator of gene transcription.

One important distinction between these two models is the nature and consequences of the association of $pp40^{I\kappa B\text{-}\beta}$ with both $p68^{c\text{-}rel}$ and $p59^{v\text{-}rel}$. Since overexpression of $p68^{c\text{-}rel}$ is not sufficient for oncogenic transformation, the direct model would explain the inability of $p68^{c\text{-}rel}$ to transform avian lymphoid cells as the result of inhibition of the DNA-binding activity of $p68^{c\text{-}rel}$ by $pp40^{I\kappa B\text{-}\beta}$ and predicts that mutant *rel* proteins that are not subject to inhibition of DNA binding by $pp40^{I\kappa B\text{-}\beta}$ would be activated for oncogenesis. In contrast, the indirect model would predict that oncogenic activation of $p68^{c\text{-}rel}$ would be the result of an increased affinity of mutant *rel* proteins for $pp40^{I\kappa B\text{-}\beta}$ with consequent perturbation of endogenous NF-κB or NF-κB-related proteins. We therefore undertook an analysis of *rel*-$pp40^{I\kappa B\text{-}\beta}$ interactions in order to understand the consequences of *rel*-$pp40^{I\kappa B\text{-}\beta}$ interactions for oncogenesis by *rel* proteins.

MATERIALS AND METHODS

Recombinant DNA methodology. All plasmids were constructed by standard recombinant DNA techniques. A spleen necrosis virus-derived retroviral expression vector essentially identical to pJD214 (11) was used to express the respective *rel* proteins and $pp40^{I\kappa B\text{-}\beta}$ in chicken embryo fibroblasts (CEF) and in primary avian spleen cells. The chicken c-*rel* gene was derived from a previously described cDNA clone containing the 3′ coding region of c-*rel* (18) and a polymerase chain reaction-generated cDNA containing the 5′ coding region of c-*rel*. The predicted amino acid sequence of the reconstructed c-*rel* cDNA is identical to that of the full-length chicken c-*rel* cDNA previously described (8). The c-rel^{581} and c-rel^{546} genes were constructed by unidirectional deletion from the 3′ end of the c-*rel* cDNA and then ligation of a termination linker containing TGA codons in all three reading frames. The superscript number indicates the last c-*rel*-derived amino acid. The proteins encoded by the c-rel^{581} and c-rel^{546} genes are predicted to contain two to four linker-encoded C-terminal amino acids. The c-rel^{543} and c-rel^{540} genes were constructed by introduction of a TGA codon following codon 543 or 540 of c-*rel*. The vvc-*rel*, vcc-*rel*, and vcv-*rel* genes are recombinants between c-*rel* and v-*rel* with conserved 5′ (*Cla*I) and 3′ (*Bst*XI) sites. The vvc-*rel* gene contains 428 codons from v-*rel* followed by 175 codons from c-*rel*; vcc-*rel* contains 147 codons of v-*rel* followed by 460 codons of c-*rel*; and vcv-*rel* contains 285 internal c-*rel*-derived codons in place of the corresponding 281 codons of v-*rel*. Certain of the biochemical and biological properties of the vvc-*rel*, vcc-*rel*, and vcv-*rel* proteins have been described previously (18). An additional recombinant v/c-*rel* gene, cvc-*rel*, that contains 281 internal v-*rel*-derived codons place of the corresponding 285 codons of c-*rel*, was constructed. Recombinant v/c-*rel* genes lacking C-terminal c-*rel*-derived sequences were also constructed. The proteins encoded by the vvc-rel^{542} and cvc-rel^{542} genes are predicted to contain four linker-derived amino acids at their C termini. To rule out the possibility that these linker-derived amino acids might influence the biochemical and biological properties of these proteins, we introduced a termination codon following amino acid 543 of the cvc-*rel* gene (all numbering is based on the wild-type c-*rel* coding sequence), forming cvc-rel^{543}. No differences in the biochemical or biological properties of cvc-rel^{542} (containing four linker-encoded amino acids following codon 542 of c-*rel*) or cvc-rel^{543} (containing a termination codon following codon 543 of c-*rel*) were detected (data not shown).

The v-*rel* gene used in these experiments is identical to that previously described (48). The $pp40^{I\kappa B\text{-}\beta}$ cDNA was obtained from H. R. Bose, Jr., University of Texas (10), and was inserted into a spleen necrosis virus-derived retroviral expression vector to provide for its expression in avian cells. The anti-*rel* antiserum and the anti-$pp40^{I\kappa B\text{-}\beta}$ have been previously described (15, 25).

Generation and use of virus stocks encoding *rel* proteins. CEF were cotransfected with plasmids containing retroviral expression vectors encoding the various *rel* and $pp40^{I\kappa B\text{-}\beta}$ proteins along with a plasmid containing a replication-competent DNA clone of Rev-A to provide helper functions. The plasmids were introduced into 2×10^5 CEF per 60-mm plate by the dimethyl sulfoxide-Polybrene protocol (24), and cell lysates and virus-containing media were collected 4 to 5 days after transfection. For most experiments, analysis of the DNA-binding properties of *rel* proteins was performed with cell lysates from the transfected cells. Since a replication-competent virus was included in these experiments, these cell lysates are considered to be infected. Several experiments were performed on cell lysates derived from CEF infected with virus obtained from the conditioned media of the transfected cells. There were no detectable differences in the biochemical properties of *rel* proteins in cell lysates from transfected or infected CEF. Typically, 70 to 90% of the cells were coinfected, as determined by indirect immunofluorescence (15, 18). Several experiments were also performed in the absence of helper virus DNA, and essentially identical results, compared to experiments performed in the presence of helper virus DNA, were obtained.

Virus stocks from the infected CEF were used to infect primary spleen cells. The infected spleen cells were incubated in RPMI 1640 medium containing 15% fetal calf serum for 2 days to maximize viral spread in the infected spleen cells prior to plating in RPMI 1640 containing 15% fetal calf serum, 1% chicken serum, 0.028% $NaHCO_3$, and 0.3% Bacto Agar. Infection of 3×10^7 primary spleen cells with v-*rel*-containing virus typically yielded 20 to 200 colonies of transformed cells. A v-*rel*-containing virus was prepared and assayed in parallel in all transformation assays. The transforming titers of the virus stocks were normalized to the level of *rel* proteins present in lysates of the transfected CEF as determined by immunoblot analysis.

Analysis of DNA binding by *rel* proteins. Cell lysates were typically collected 4 to 5 days posttransfection or postinfection. The cells were washed twice in phosphate-buffered saline and lysed in 400 μl of ELB buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8.0], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40)

containing 1.0 mM dithiothreitol and 5 μg of the following protease inhibitors per ml: antipain, aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor. Cell lysates were scraped into microcentrifuge tubes and centrifuged for 20 min to remove cell debris. The recovery of *rel* proteins from CEF lysed in this manner was essentially 100%, as determined by immunoprecipitation of soluble and pelleted fractions (data not shown). DNA binding was assayed by solution UV–cross-linking experiments. Typically, 2.0 μl of each lysate was incubated with 10^5 cpm of a ^{32}P-labeled oligonucleotide containing a palindromic κB site in a final reaction volumne of 25 μl of HDKE buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 1.0 mM EDTA, 5% glycerol) with 2 μg of poly(dI-dC) and 2 μg of pdN6 also present. The sense strand of the palindromic κB probe is 5′-CAACGGCAGGGGAAT TCCCCTCTCCTT-3′ and was radiolabeled as previously described with [^{32}P]dCTP and [^{32}P]dGTP and a 1:1 molar ratio of bromodeoxyuridine:thymidine (1). The reaction mix was irradiated with UV light for 5 min, and an equal volumne of 2× sodium dodecyl sulfate (SDS)-sample buffer was added. The samples were boiled for 3 min prior to electrophoresis on an SDS 7.5% polyacrylamide gel. Protein-DNA complexes were visualized by autoradiography. Sequence-specific binding of *rel* proteins to this palindromic κB oligonucleotide was confirmed by competition experiments with unlabeled palindromic κB oligonucleotide and with an oligonucleotide containing a mutant κB site. The DNA-binding reactions for electrophoretic mobility shift analysis were performed in an identical manner, except that the protein-DNA complexes were electrophoresed through a native 5% polyacrylamide gel.

Metabolic labeling, immunoprecipitations, and immunoblot analysis. CEF were metabolically labeled with 200 μCi of [^{35}S]methionine/cysteine (EXPRE^{35}S^{35}S; Dupont) per 60-mm dish for 4 h in RPMI 1640 medium lacking methionine and cysteine and containing 5% dialyzed fetal calf serum. The cells were washed twice with ice-cold PBS, and cell lysates were collected in 400 μl of RIPA buffer (10 mM sodium phosphate [pH 7.4], 150 mM NaCl, 1.0% Nonidet P-40, 1.0% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). Cellular debris was removed by centrifugation (20 min at 14,000 × *g*, 4°C), and 2.0 μl of v-*rel* antiserum was added to each supernatant. The immunoprecipitate was allowed to form on ice for 60 min and collected with fixed *Staphylococcus aureus* and then centrifuged (10 min, 3,000 × *g*, 4°C). The immunoprecipitated proteins were washed four times in RIPA buffer and resuspended in SDS-sample buffer after the final wash. The immunoprecipitated proteins were boiled for 2 min and electrophoresed through an SDS-polyacrylamide gel.

For immunoprecipitation analysis of protein-DNA adducts, the DNA-binding reaction mixture was diluted into 100 μl of ELB containing 2.0 μl of the appropriate antiserum. The extracts were incubated with the antiserum on ice for 1 h, collected with fixed *S. aureus*, and washed twice in ELB before SDS-polyacrylamide gel electrophoresis.

For immunoblot analysis of *rel* and pp40$^{I\kappa B-\beta}$ proteins, 20 μl of each extract was electrophoresed through an SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose. The nitrocellulose filters were incubated in PBS containing 1% nonfat dry milk to minimize nonspecific binding. The primary antisera were incubated with the nitrocellulose filter for 1 h at room temperature at a 1:1,000 dilution in PBS. The filters were washed three times with PBS and once with PBS containing 0.05% Tween 20. The filters were incubated with 1 μCi of ^{125}I-protein A (Dupont) for 1 h, washed, and exposed to film for 24 to 72 h.

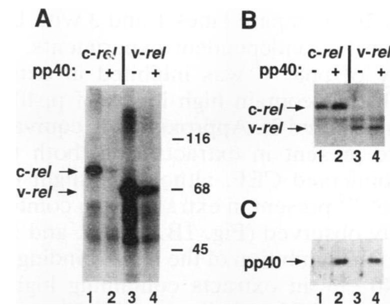

FIG. 1. pp40$^{I\kappa B-\beta}$ efficiently inhibits DNA binding by c-*rel* but not v-*rel*. (A) Solution UV–cross-linking of extracts from infected CEF. Whole-cell extracts were prepared from CEF infected with retroviral vectors encoding p68^{c-rel} (lane 1) or p59^{v-rel} (lane 3) or from CEF coinfected with retroviral vectors encoding pp40$^{I\kappa B-\beta}$ and p68^{c-rel} (lane 2) or pp40$^{I\kappa B-\beta}$ and p59^{v-rel} (lane 4). The presence of κB-binding proteins was determined by solution UV–cross-linking to an oligonucleotide containing a palindromic κB sequence. The protein-DNA adducts were electrophoresed through an SDS 7.5% polyacrylamide gel and visualized by autoradiography. Molecular size markers (in kilodaltons) are indicated on the right side of the figure. The arrows indicate the positions of the protein-DNA adducts containing p68^{c-rel} (lane 1) or p59^{v-rel} (lanes 3 and 4). In this experiment, p68^{c-rel} in lane 2 retained 12% of the DNA-binding activity of p68^{c-rel} in lane 1, while p59^{v-rel} in lane 4 retained 57% of the DNA-binding activity of p59^{v-rel} in lane 3. (B) Immunoblot analysis of *rel* proteins. Aliquots of the extracts used in the solution UV–cross-linking experiments were subjected to immunoblot analysis with anti-*rel* antiserum. The lanes are as indicated in panel A, and the arrows indicate the presence of p68^{c-rel} (lanes 1 and 2) or p59^{v-rel} (lanes 3 and 4). (C) Immunoblot analysis of pp40$^{I\kappa B-\beta}$. A portion of the extracts used in panel A were subjected to immunoblot analysis with anti-pp40$^{I\kappa B-\beta}$ serum. The lanes are as indicated in panels A and B, and the arrow indicates the presence of pp40$^{I\kappa B-\beta}$ encoded by the retroviral vector in lanes 2 and 4. The levels of endogenous pp40$^{I\kappa B-\beta}$ are below detectable levels in this experiment.

RESULTS

Differential DNA binding by p59^{v-rel} and p68^{c-rel} in the presence of pp40$^{I\kappa B-\beta}$. The ability of p59^{v-rel} and p68^{c-rel} to bind DNA in CEF either singly infected with retroviral vectors encoding p59^{v-rel} or p68^{c-rel} or coinfected with a retroviral vector encoding pp40$^{I\kappa B-\beta}$ was evaluated by solution UV–cross-linking experiments. Both p59^{v-rel} and p68^{c-rel} were able to bind DNA containing a palindromic κB sequence in extracts from CEF singly infected with retroviral vectors encoding either p59^{v-rel} or p68^{c-rel} (Fig. 1A, lanes 1 and 3). Equivalent levels of p59^{v-rel} and p68^{c-rel} were present in these extracts, as determined by immunoblot analysis with anti-*rel* serum (Fig. 1B, lanes 1 and 3). However, significantly greater levels of DNA binding by p59^{v-rel} relative to that of p68^{c-rel} were consistently observed, suggesting that the affinity of p59^{v-rel} for the palindromic κB sequence is greater than that of p68^{c-rel}.

A further difference in DNA binding between p59^{v-rel} and p68^{c-rel} was observed in extracts from CEF that were coinfected with a retroviral expression vector encoding pp40$^{I\kappa B-\beta}$. CEF that were coinfected with retroviral vectors encoding pp40$^{I\kappa B-\beta}$ and either p59^{v-rel} or p68^{c-rel} contained substantially increased levels of pp40$^{I\kappa B-\beta}$ relative to CEF singly infected with retroviral vectors encoding only p59^{v-rel}

or p68$^{c\text{-}rel}$ (Fig. 1C, compare lanes 1 and 3 with lanes 2 and 4). In more than five independent experiments, the DNA-binding activity of p68$^{c\text{-}rel}$ was inhibited in extracts from coinfected CEF that contain high levels of pp40$^{I\kappa B\text{-}\beta}$ (Fig. 1A, compare lanes 1 and 2). Approximately equivalent levels of p68$^{c\text{-}rel}$ were present in extracts from both the singly-infected and coinfected CEF, although a slight increase in the levels of p68$^{c\text{-}rel}$ present in extracts from coinfected CEF was consistently observed (Fig. 1B, lanes 1 and 2).

In contrast to the inhibition of the DNA-binding activity of p68$^{c\text{-}rel}$ by pp40$^{I\kappa B\text{-}\beta}$ in extracts containing high levels of pp40$^{I\kappa B\text{-}\beta}$, significant DNA binding by p59$^{v\text{-}rel}$ was present in extracts from CEF coinfected with vectors encoding p59$^{v\text{-}rel}$ and pp40$^{I\kappa B\text{-}\beta}$ (Fig. 1A, lane 4). In more than five separate experiments, p59$^{v\text{-}rel}$ present in CEF extracts that contained high levels of pp40$^{I\kappa B\text{-}\beta}$ retained 30 to 100% of the DNA-binding activity of p59$^{v\text{-}rel}$ present in CEF extracts that contained only low endogenous levels of pp40$^{I\kappa B\text{-}\beta}$. Equivalent levels of p59$^{v\text{-}rel}$ were present in both the singly-infected and coinfected extracts (Fig. 1B, lanes 3 and 4). The relative ratio of p59$^{v\text{-}rel}$ to pp40$^{I\kappa B\text{-}\beta}$ varied slightly from experiment to experiment (data not shown), and this variation is likely responsible for the variation in the level of DNA binding by p59$^{v\text{-}rel}$ that was retained in the presence of high levels of pp40$^{I\kappa B\text{-}\beta}$.

Equal levels of pp40$^{I\kappa B\text{-}\beta}$ were expressed in cells coinfected with retroviral vectors encoding pp40$^{I\kappa B\text{-}\beta}$ and p68$^{c\text{-}rel}$ or pp40$^{I\kappa B\text{-}\beta}$ and p59$^{v\text{-}rel}$ (Fig. 1C, lanes 2 and 4). Thus, the differential ability of p68$^{c\text{-}rel}$ versus p59$^{v\text{-}rel}$ to bind DNA in the presence of high levels of pp40$^{I\kappa B\text{-}\beta}$ is not due to differential expression of pp40$^{I\kappa B\text{-}\beta}$ in the respective coinfected cells.

The ability of p59$^{v\text{-}rel}$ to bind DNA in the presence of pp40$^{I\kappa B\text{-}\beta}$ in extracts from coinfected CEF contrasts with previous reports that purified pp40$^{I\kappa B\text{-}\beta}$ completely inhibits the DNA-binding activity of in vitro-synthesized p59$^{v\text{-}rel}$ (10, 25). One explanation for this discrepancy is that a posttranslational modification(s) of p59$^{v\text{-}rel}$ may contribute to reduced in vivo regulation of the DNA-binding activity of p59$^{v\text{-}rel}$ by pp40$^{I\kappa B\text{-}\beta}$. Alternatively, in experiments with nanogram amounts of purified pp40$^{I\kappa B\text{-}\beta}$ and in vitro-synthesized p59$^{v\text{-}rel}$, there will be a large excess of pp40$^{I\kappa B\text{-}\beta}$ relative to p59$^{v\text{-}rel}$, and the functional consequences of the pp40$^{I\kappa B\text{-}\beta}$-p59$^{v\text{-}rel}$ interaction are likely to be dependent on the stoichiometry of pp40$^{I\kappa B\text{-}\beta}$ and p59$^{v\text{-}rel}$. In our experiments, the ratio of pp40$^{I\kappa B\text{-}\beta}$ to *rel* proteins in coinfected CEF was equivalent to the ratio of pp40$^{I\kappa B\text{-}\beta}$ to p59$^{v\text{-}rel}$ in avian lymphoid cells transformed by p59$^{v\text{-}rel}$ (compare lanes 2 and 3 of Fig. 2A and B). DNA binding by p59$^{v\text{-}rel}$ is readily detectable in both nuclear and cytoplasmic extracts from transformed avian lymphoid cells (22; data not shown). Thus, expression of pp40$^{I\kappa B\text{-}\beta}$ at similar levels relative to p59$^{v\text{-}rel}$ in both coinfected CEF and in v-*rel*-transformed avian lymphoid cells does not eliminate the ability of p59$^{v\text{-}rel}$ to bind DNA. In contrast, the DNA-binding activity of p68$^{c\text{-}rel}$ in CEF is inhibited at a p68$^{c\text{-}rel}$:pp40$^{I\kappa B\text{-}\beta}$ ratio that is comparable to the p59$^{v\text{-}rel}$:pp40$^{I\kappa B\text{-}\beta}$ ratio present in v-*rel*-transformed lymphoid cells.

Importance of pp40$^{I\kappa B\text{-}\beta}$ association for inhibition of DNA binding by *rel* proteins. One explanation for the ability of p59$^{v\text{-}rel}$ to bind DNA in CEF extracts that contain high levels of pp40$^{I\kappa B\text{-}\beta}$ is a lack of association between p59$^{v\text{-}rel}$ and pp40$^{I\kappa B\text{-}\beta}$ in coinfected CEF. The ability of pp40$^{I\kappa B\text{-}\beta}$ to associate with p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ in coinfected CEF was directly determined. A protein of approximately 40 kDa was specifically coimmunoprecipitated with anti-*rel* antiserum from CEF coinfected with retroviral vectors encoding pp40$^{I\kappa B\text{-}\beta}$ and the respective *rel* proteins (Fig. 3, lanes 1, 3, and 5). These results demonstrate that pp40$^{I\kappa B\text{-}\beta}$ is capable of association with both wild-type and mutant c-*rel* proteins. Thus, the inability of pp40$^{I\kappa B\text{-}\beta}$ to inhibit efficiently the DNA-binding activity of p59$^{v\text{-}rel}$ in coinfected CEF is not simply due to an inability of pp40$^{I\kappa B\text{-}\beta}$ to associate with p59$^{v\text{-}rel}$ in CEF.

To determine whether pp40$^{I\kappa B\text{-}\beta}$ association is required for inhibition of DNA binding by p68$^{c\text{-}rel}$, we determined the effect of anti-pp40$^{I\kappa B\text{-}\beta}$ serum on the ability of p68$^{c\text{-}rel}$ to bind DNA in the absence or presence of high levels of pp40$^{I\kappa B\text{-}\beta}$. DNA binding by p68$^{c\text{-}rel}$ was readily detected in extracts from singly-infected CEF, and addition of anti-pp40$^{I\kappa B\text{-}\beta}$ serum to these extracts resulted in a slight increase in DNA

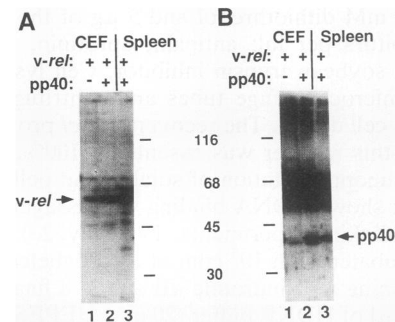

FIG. 2. Analysis of pp40$^{I\kappa B\text{-}\beta}$ and p59$^{v\text{-}rel}$ levels in coinfected CEF and v-*rel*-transformed spleen cells. Equivalent amounts of total cell protein from CEF (lanes 1 and 2) or v-*rel*-transformed spleen cells (lane 3) were subjected to immunoblot analysis with anti-*rel* antiserum (A) or with anti-pp40$^{I\kappa B\text{-}\beta}$ serum (B). The arrows indicate p59$^{v\text{-}rel}$ (A) and pp40$^{I\kappa B\text{-}\beta}$ (B), and the relative locations of molecular size markers (in kilodaltons) are indicated in the middle of the figure.

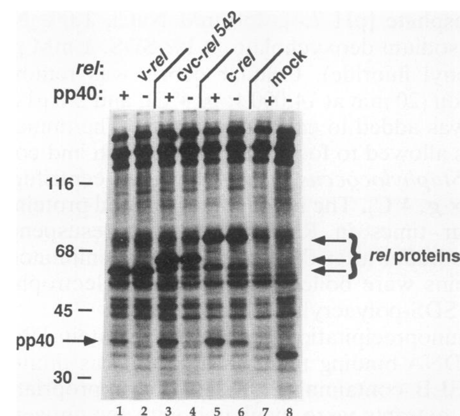

FIG. 3. Association of *rel* proteins with pp40$^{I\kappa B\text{-}\beta}$. CEF were infected with retroviral vectors encoding the indicated *rel* proteins (lanes 2, 4, and 6) or coinfected with retroviral vectors encoding the indicated *rel* proteins and pp40$^{I\kappa B\text{-}\beta}$ (lanes 1, 3, 5, and 7). The cells were labeled with [^{35}S]methionine/cysteine, and cell lysates were immunoprecipitated with anti-*rel* serum. The immunoprecipitates were electrophoresed through an SDS 10% polyacrylamide gel and visualized by fluorography. The arrows on the right side of the figure indicate the respective *rel* proteins, and the arrow on the left side of the figure indicates pp40$^{I\kappa B\text{-}\beta}$ that is coimmunoprecipitated with the respective *rel* proteins in lanes 1, 3, and 5. Numbers on left indicate relative molecular sizes (in kilodaltons).

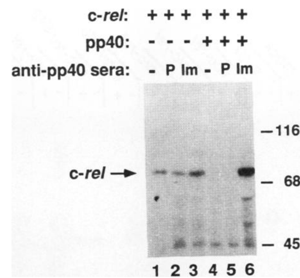

FIG. 4. Anti-pp40$^{I\kappa B\text{-}\beta}$ serum restores DNA binding to p68$^{c\text{-}rel}$ in the presence of pp40$^{I\kappa B\text{-}\beta}$. Extracts were prepared from CEF that were infected with a retroviral vector encoding p68$^{c\text{-}rel}$ (lanes 1 to 3) or from CEF coinfected with retroviral vectors encoding both p68$^{c\text{-}rel}$ and pp40$^{I\kappa B\text{-}\beta}$ (lanes 4 to 6). The presence of κB-binding proteins in these extracts was determined by solution UV–cross-linking and SDS-polyacrylamide gel electrophoresis and autoradiography. The ability of p68$^{c\text{-}rel}$ to bind DNA in the presence of no added serum (lanes 1 and 4), 1 μl of preimmune anti-pp40$^{I\kappa B\text{-}\beta}$ serum (lanes 2 and 5), or 1 μl of immune anti-pp40$^{I\kappa B\text{-}\beta}$ serum (lanes 3 and 6) was determined. The location of the p68$^{c\text{-}rel}$-DNA adduct is indicated by the arrow, and the locations of molecular size markers (in kilodaltons) are indicated on the right side of the figure. P indicates the presence of preimmune anti-pp40$^{I\kappa B\text{-}\beta}$ serum, and Im indicates the presence of immune anti-pp40$^{I\kappa B\text{-}\beta}$ serum.

binding by p68$^{c\text{-}rel}$ compared with the addition of preimmune serum or no antiserum (Fig. 4, lanes 1 to 3). The slight increase in DNA binding by p68$^{c\text{-}rel}$ in the presence of anti-pp40$^{I\kappa B\text{-}\beta}$ serum likely reflects a low level of endogenous pp40$^{I\kappa B\text{-}\beta}$ or another IκB-related protein in these extracts that has a slight inhibitory effect on DNA binding by p68$^{c\text{-}rel}$. Addition of anti-pp40$^{I\kappa B\text{-}\beta}$ serum to extracts from CEF coinfected with retroviral vectors encoding both pp40$^{I\kappa B\text{-}\beta}$ and p68$^{c\text{-}rel}$ resulted in restoration of a high level of DNA binding by p68$^{c\text{-}rel}$ (Fig. 4, lane 6). The ability of anti-pp40$^{I\kappa B\text{-}\beta}$ serum to allow DNA binding by p68$^{c\text{-}rel}$ in the presence of high levels of pp40$^{I\kappa B\text{-}\beta}$ indicates that pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding by p68$^{c\text{-}rel}$ in CEF extracts is the result of a direct interaction between pp40$^{I\kappa B\text{-}\beta}$ and p68$^{c\text{-}rel}$.

One possibility suggested by the ability of pp40$^{I\kappa B\text{-}\beta}$ to associate with but not inhibit efficiently DNA binding by p59$^{v\text{-}rel}$ is that pp40$^{I\kappa B\text{-}\beta}$ and DNA are competitive inhibitors for the same or overlapping domains on *rel* proteins. In this case, a single p59$^{v\text{-}rel}$ molecule would bind either pp40$^{I\kappa B\text{-}\beta}$ or DNA but not both at the same time. An alternative explanation is that the p59$^{v\text{-}rel}$-pp40$^{I\kappa B\text{-}\beta}$ complex is capable of binding DNA while the p68$^{c\text{-}rel}$-pp40$^{I\kappa B\text{-}\beta}$ complex is not able to bind DNA. To determine whether pp40$^{I\kappa B\text{-}\beta}$ remains associated with the p59$^{v\text{-}rel}$-DNA complex, we determined the ability of anti-*rel* serum or anti-pp40$^{I\kappa B\text{-}\beta}$ serum to immunoprecipitate the p59$^{v\text{-}rel}$-DNA complex from coinfected CEF. Only anti-*rel* serum was able to immunoprecipitate the p59$^{v\text{-}rel}$-DNA complex (Fig. 5, lane 5), while neither preimmune anti-*rel* serum nor immune pp40$^{I\kappa B\text{-}\beta}$ serum was able to immunoprecipitate the p59$^{v\text{-}rel}$-DNA complex (Fig. 5, lanes 4 and 6). In addition, immune anti-pp40$^{I\kappa B\text{-}\beta}$ serum had no effect on the electrophoretic mobility of the *rel*-DNA complex in electrophoretic mobility shift assays (Fig. 6). These results suggest that pp40$^{I\kappa B\text{-}\beta}$ and DNA are competitive inhibitors for the same or overlapping domains on *rel* proteins. The possibility that pp40$^{I\kappa B\text{-}\beta}$ and DNA are competitive inhibitors for binding to *rel* proteins is consistent with previous mapping experiments that have implicated the first 20 amino acids of p68$^{c\text{-}rel}$ in both pp40$^{I\kappa B\text{-}\beta}$ association and DNA binding (1, 25).

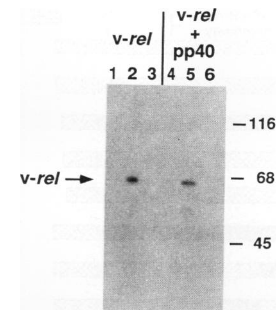

FIG. 5. Anti-pp40$^{I\kappa B\text{-}\beta}$ serum does not immunoprecipitate the p59$^{v\text{-}rel}$-DNA adduct. Extracts were prepared from CEF that were infected with a retroviral vector encoding p59$^{v\text{-}rel}$ (lanes 1 to 3) or from CEF coinfected with retroviral vectors encoding p59$^{v\text{-}rel}$ and pp40$^{I\kappa B\text{-}\beta}$ (lanes 4 to 6). The presence of κB-binding proteins in these extracts was determined by solution UV–cross-linking and immunoprecipitation with preimmune anti-*rel* serum (lanes 1 and 4), immune anti-*rel* serum (lanes 2 and 5), or immune anti-pp40$^{I\kappa B\text{-}\beta}$ serum (lanes 3 and 6). The presence of the p59$^{v\text{-}rel}$-DNA adduct is indicated by the arrow, and the locations of molecular size markers (in kilodaltons) are indicated on the right side of the figure.

Identification of v-*rel*-derived sequences that are sufficient for loss of pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding. There are multiple structural differences between p68$^{c\text{-}rel}$ and p59$^{v\text{-}rel}$ (8, 42, 47). These differences include substitution

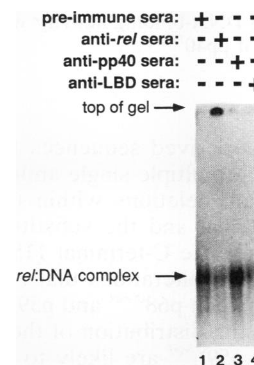

FIG. 6. Anti-pp40$^{I\kappa B\text{-}\beta}$ serum does not affect the electrophoretic mobility of the *rel*-DNA complex. Extracts were prepared from CEF coinfected with retroviral vectors encoding the cvc-*rel*^{542} protein and a recombinant pp40$^{I\kappa B\text{-}\beta}$ protein containing a 13-amino-acid epitope tag (LBD) at its C terminus. The extracts were incubated with 1 μl of the indicated antiserum before the addition of the ^{32}P-labeled κB oligonucleotide, and this was followed by electrophoresis through a native 4% polyacrylamide gel. The antisera used were preimmune serum (lane 1), anti-*rel* serum (lane 2), anti-pp40$^{I\kappa B\text{-}\beta}$ serum (lane 3), and anti-LBD serum (lane 4). The upper arrow indicates the top of the gel (and the location of a portion of the *rel*-DNA complex in the presence of anti-*rel* serum in lane 2), while the lower arrow indicates the location of the *rel*-DNA complex in the absence of antisera. The epitope tag (LBD) was derived from the ligand-binding domain of the platelet-derived growth factor receptor and consists of the sequence EVIVVPHSLPFML. A plasmid containing a segment of DNA encoding the epitope tag and antipeptide serum against the epitope tag were provided by Dan Donoghue (University of California at San Diego). The presence of the epitope tag does not significantly affect the ability of pp40$^{I\kappa B\text{-}\beta}$ to inhibit DNA-binding by p68$^{c\text{-}rel}$ (27a).

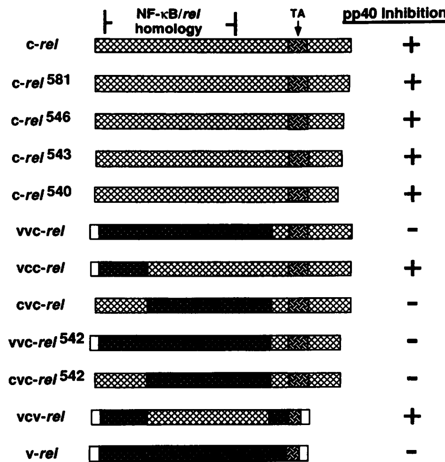

FIG. 7. Summary of $pp40^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding by *rel* proteins. The different *rel* proteins used in this study are represented diagrammatically. Sequences derived from the wild-type c-*rel* protein are represented by light cross-hatched rectangles, while sequences derived from the wild-type v-*rel* protein are represented by the dark cross-hatched rectangles. The open rectangles represent *env*-derived amino acids present at both termini of the v-*rel* protein. The amino-terminal region of homology with other NF-κB/*rel* proteins is indicated at the top of the figure. The brick-patterned portion ( ) of each rectangle indicates the transactivation domain (TA) that is present in the wild-type c-*rel* and mutant c-*rel* proteins used in these experiments. This domain is partially deleted (and is not functional) in the wild-type v-*rel* protein. The ability of $pp40^{I\kappa B\text{-}\beta}$ to inhibit DNA binding by each *rel* protein is indicated by the − or the +. A − indicates that little or no (10% or less) DNA binding by the indicated *rel* protein was retained in the presence of high levels of $pp40^{I\kappa B\text{-}\beta}$, while a + indicates that substantial (30 to 100%) DNA-binding activity was retained in the presence of high levels of $pp40^{I\kappa B\text{-}\beta}$.

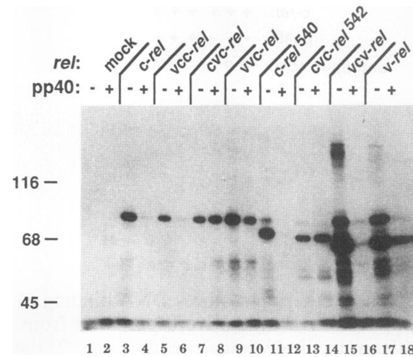

FIG. 8. Internal v-*rel*-derived sequences are sufficient for loss of $pp40^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding. Extracts from CEF infected with retroviral vectors encoding the indicated *rel* proteins (odd-numbered lanes) or extracts from CEF coinfected with retroviral vectors encoding the indicated *rel* proteins and $pp40^{I\kappa B\text{-}\beta}$ (even-numbered lanes) were assayed for κB-binding proteins by solution UV–cross-linking. The protein-DNA adducts were electrophoresed through an SDS 7.5% polyacrylamide gel and visualized by autoradiography. The locations of molecular size markers (in kilodaltons) are indicated on the left side of the figure.

of 11 N-terminal *env*-derived sequences for the first two amino acids of $p68^{c\text{-}rel}$, multiple single amino acid substitutions and several small deletions within the c-*rel*-derived regions of these proteins, and the substitution of 18 *env*-derived amino acids for the C-terminal 118 amino acids of $p68^{c\text{-}rel}$. The biochemical alterations that result from these structural changes between $p68^{c\text{-}rel}$ and $p59^{v\text{-}rel}$ are not fully understood, although the distribution of these changes suggest that $p68^{c\text{-}rel}$ and $p59^{v\text{-}rel}$ are likely to differ in several biochemical properties. In fact, several biochemical differences between nononcogenic and oncogenic *rel* proteins have been described, including differential DNA binding (30; this report) and transactivation properties (12, 18, 21, 23, 36). However, the importance of these biochemical differences for the oncogenic properties of $p59^{v\text{-}rel}$ is not clear. We therefore sought to identify those v-*rel*-derived sequences that allow DNA binding in the presence of high levels of $pp40^{I\kappa B\text{-}\beta}$ in order to correlate reduced $pp40^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding with oncogenic activation of c-*rel*.

The ability of a series of recombinant v/c-*rel* proteins to bind DNA in the presence of high levels of $pp40^{I\kappa B\text{-}\beta}$ was determined to define specific v-*rel*-derived sequences sufficient to confer loss of $pp40^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding (Fig. 7). Three recombinant v/c-*rel* genes were analyzed that contained portions of v-*rel* substituted for the corresponding regions in c-*rel*: vcc-*rel* contains 147 v-*rel*-derived codons in place of the first 138 codons of c-*rel*; cvc-*rel* contains 281 internal v-*rel*-derived codons in place of the corresponding 285 codons of c-*rel*; and vvc-*rel* contains 428 v-*rel*-derived codons in place of the first 423 codons of c-*rel*. The proteins encoded by each of these recombinant v/c-*rel* genes were able to bind DNA in extracts from singly-infected CEF at levels comparable to that of $p68^{c\text{-}rel}$ (Fig. 8, lanes 5, 7, and 9). However, only the cvc-*rel* and vvc-*rel* proteins were able to bind DNA in extracts from CEF that were coinfected with $pp40^{I\kappa B\text{-}\beta}$-encoding virus (Fig. 8, lanes 6, 8, and 10). These results demonstrate that internal v-*rel*-derived sequences are sufficient to confer loss of $pp40^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding to recombinant v/c-*rel* proteins.

To determine whether the corresponding c-*rel*-derived sequences were able to confer $pp40^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding, we determined the ability of the vcv-*rel* protein, containing the 285 internal c-*rel*-derived amino acids in place of the corresponding v-*rel*-derived amino acids, to bind DNA in the absence or presence of high levels of $pp40^{I\kappa B\text{-}\beta}$. The level of DNA binding by the vcv-*rel* protein in extracts from singly-infected CEF was comparable to that of $p59^{v\text{-}rel}$ and was markedly enhanced relative to that of $p68^{c\text{-}rel}$ (Fig. 8, lanes 3, 15, and 17). However, in contrast to $p59^{v\text{-}rel}$, DNA binding by the vcv-*rel* protein was completely inhibited in coinfected CEF extracts containing high levels of $pp40^{I\kappa B\text{-}\beta}$ (Fig. 8, lanes 16 and 18). Thus, 285 internal c-*rel*-derived amino acids are sufficient to confer $pp40^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding without significantly affecting the intrinsic DNA-binding activity of *rel* proteins.

Loss of $pp40^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding is not sufficient for oncogenic activation of $p68^{c\text{-}rel}$. The ability of several of these recombinant v/c-*rel* proteins (vvc-*rel*, vcc-*rel*, and vcv-*rel*) to transform avian spleen cells has been previously determined and is summarized in Table 1 (18). Only the vvc-*rel* protein was found to transform avian spleen cells with an efficiency comparable to that of v-*rel*. Substitution of internal c-*rel*-derived amino acids into either v-*rel* (to yield vcv-*rel*) or vvc-*rel* (to yield vcc-*rel*) reduced the oncogenic properties of the resultant proteins. To determine whether the presence of internal v-*rel*-derived sequences is sufficient for oncogenic activation of c-*rel*, we determined

TABLE 1. Summary of the biochemical and biological properties of *rel* proteins

rel protein[a]	pp40 Inhibition[b]	Localization[c]	Transformation[d]
c-*rel*	+	C	0.01[e-h]
c-*rel*^{581}	+	N/C	0.01[e]
c-*rel*^{546}	+	N/C	0.01[e]
c-*rel*^{543}	+	N/C	0.01[e]
c-*rel*^{540}	+	N/C	0.01[e]
vcc-*rel*	+	C	0.5[i]
cvc-*rel*	−	C	0.05[f]
vvc-*rel*	−	C	1.0[i]
vvc-*rel*^{542}	−	N/C	1.0[h]
cvc-*rel*^{542}	−	N/C	1.0[g]
vcv-*rel*	+	N	0.2[i]
v-*rel*	−	N	1.0[f-i]

[a] The *rel* proteins utilized in this study are listed. The superscript number represents the last c-*rel*-derived amino acid.

[b] The ability of pp40$^{I\kappa B\text{-}\beta}$ to inhibit DNA binding by the respective *rel* proteins is indicated by the − or +. A + indicates that little or no (10% or less) DNA binding by the respective *rel* protein was retained in CEF extracts containing high levels of pp40$^{I\kappa B\text{-}\beta}$, while a − indicates that substantial (30 to 100%) DNA-binding activity of the respective *rel* protein was retained in the presence of high levels of pp40$^{I\kappa B\text{-}\beta}$.

[c] The cellular localization of these *rel* proteins in CEF was determined by indirect immunofluorescence. These proteins were found to be exclusively cytoplasmic (C) or exclusively nuclear (N) or distributed between both the cytoplasm and the nucleus (N/C).

[d] The oncogenic properties of the respective *rel* proteins are given relative to that of v-*rel*. These results are the summary of more than 20 different experiments that always included c-*rel*- and v-*rel*-encoding viruses. However, not all the mutant proteins were characterized in each experiment. A further explanation of the transforming activities of the respective proteins, including actual colonies obtained in representative experiments, is given in footnotes *e* to *i*.

[e] Occasional colonies were observed with these viruses encoding wild-type and C-terminal-truncated c-*rel* proteins at a frequency of approximately 1%. This low frequency of colony formation by c-*rel*-encoding virus is consistent with a previous report describing the oncogenic properties of c-*rel* and certain mutant c-*rel* proteins (31).

[f] Two and 20 colonies were obtained with virus encoding the cvc-*rel* protein, while 90 and 200 colonies were obtained with v-*rel*-encoding virus, and no colonies were obtained with c-*rel*-encoding virus in two representative experiments.

[g] Thirty-three, 28, and 22 colonies were obtained with virus encoding the cvc-*rel*^{542} protein, while 13, 24, and 18 colonies were obtained with v-*rel*-encoding virus, and 2, 0, and 0 colonies were obtained with c-*rel*-encoding virus in three representative experiments. The two colonies obtained with the c-*rel*-encoding virus were small, and the cells from these colonies did not continue to proliferate upon transfer to liquid culture.

[h] Fifty-five colonies were obtained with virus encoding the vvc-*rel*^{542} protein, while 50 colonies were obtained with v-*rel*-encoding virus, and no colonies were obtained with c-*rel*-encoding virus in one representative experiment.

[i] The biological activities of the vvc-*rel*, vcc-*rel*, and vcv-*rel* proteins have been previously described (18). The biological activities of the vcc-*rel* and vcv-*rel* were reproduced in these experiments, with the minor difference that the vcv-*rel* protein was found to have a slightly higher transformation efficiency than previously reported (0.2 versus 0.1 as reported in reference 18).

the ability of the cvc-*rel* gene to transform avian spleen cells (Table 1). The oncogenic potential of cvc-*rel* was markedly reduced relative to that of v-*rel* and was only slightly greater than that of c-*rel*. These results indicate that, in the context of a full-length c-*rel* protein, loss of pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding is not sufficient for oncogenic activation.

Loss of pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding correlates with oncogenic activation of C-terminal-truncated c-*rel* proteins. In addition to the substitution of N-terminal v-*rel*-derived sequences for the corresponding c-*rel*-derived sequences, one other structural alteration that has been reported to be sufficient for oncogenic activation of c-*rel* is the substitution of 56 C-terminal c-*rel*-derived amino acids with vector-encoded sequences (23). To determine the importance of C-terminal c-*rel*-derived sequences for pp40-mediated inhibition of DNA binding, we constructed several mutant c-*rel* genes by the replacement of codons 581, 546, 542, and 540 with TGA termination codons. Several recombinant v/c-*rel* genes that lacked a portion of the C-terminal coding region of c-*rel* were also constructed (cvc-*rel*^{542} and vvc-*rel*^{542}). These genes were expressed in CEF with retroviral vectors, and their cellular localization was determined. In contrast to the exclusively cytoplasmic localization of p68$^{c\text{-}rel}$ in CEF, the truncated c-*rel* and v/c-*rel* proteins were distributed between the cytoplasm and the nucleus (data not shown). Thus, removal of as few as 18 C-terminal amino acids is sufficient for partial nuclear translocation of truncated c-*rel* proteins in CEF.

The ability of the encoded proteins to bind DNA in the absence and presence of high levels of pp40$^{I\kappa B\text{-}\beta}$ expression was determined. All these C-terminal-truncated *rel* proteins were able to bind DNA in singly-infected CEF extracts that contained only low levels of endogenous pp40$^{I\kappa B\text{-}\beta}$ (Fig. 8, lanes 11 and 13; and data not shown). DNA binding by the C-terminal-truncated c-*rel* proteins was completely inhibited in extracts from coinfected CEF that contained high levels of pp40$^{I\kappa B\text{-}\beta}$ (Fig. 8, lane 12; and Table 1). In contrast, the cvc-*rel*^{542} and vvc-*rel*^{542} proteins were able to bind DNA in the presence of high levels of pp40$^{I\kappa B\text{-}\beta}$ (Fig. 8, lane 14; and Table 1). The ability of pp40$^{I\kappa B\text{-}\beta}$ to differentially inhibit the DNA-binding activity of the C-terminal-truncated *rel* proteins was further investigated by using extracts from CEF transfected with constant amounts of the plasmid DNAs encoding the c-*rel*^{546} or c-*rel*^{542} protein and increasing amounts of plasmid DNA encoding pp40$^{I\kappa B\text{-}\beta}$. The DNA-binding activity of the c-*rel*^{546} gene product was inhibited by pp40$^{I\kappa B\text{-}\beta}$ in cotransfected CEF in a dose-dependent manner (Fig. 9, lanes 1 to 5). The DNA-binding activity of the endogenous p68$^{c\text{-}rel}$ (Fig. 9) was also inhibited in a dose-dependent manner. The residual DNA-binding activity by endogenous p68$^{c\text{-}rel}$ in lanes 5 and 10 is due to the fact that the transfection efficiency in these experiments is less than 100%. In contrast, the DNA-binding activity of the c-*rel*^{542} gene product was not affected by increasing levels of pp40$^{I\kappa B\text{-}\beta}$ expression (Fig. 9, lanes 6 to 10). These results are consistent with the notion that pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding is principally determined by internal c-*rel*-derived sequences.

The ability of the C-terminal-truncated c-*rel* genes to induce oncogenic transformation of avian lymphoid cells was determined. None of the C-terminal-truncated c-*rel* genes were able to induce oncogenic transformation of avian spleen cells. However, the cvc-*rel*^{542} gene was found to have a transforming efficiency equivalent to that of v-*rel* (Fig. 10 and Table 1). Therefore, in the context of a c-*rel*-derived protein that lacks C-terminal c-*rel* sequences, internal v-*rel*-derived sequences that are sufficient to confer loss of pp40-mediated inhibition of DNA binding are also sufficient for oncogenic activation.

DISCUSSION

pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding by *rel* proteins. Physical interactions between IκB and NF-κB/*rel* proteins are of fundamental importance for the ability of NF-κB/*rel* proteins to bind DNA and modulate gene expression. Our experiments have identified a region of p68$^{c\text{-}rel}$

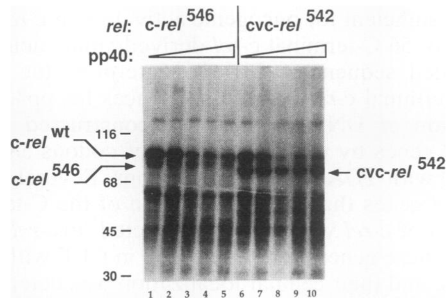

FIG. 9. Dose-dependent inhibition of DNA binding. Extracts from 2 × 10^6 CEF per 100-mm dish cotransfected with 20 μg of plasmid DNAs encoding either the c-rel^{546} (lanes 1 to 5) or cvc-rel^{542} (lanes 6 to 10) protein and with increasing amounts of a plasmid DNA encoding pp40$^{I\kappa B\text{-}\beta}$ were assayed for κB-binding proteins. The amount of plasmid DNA encoding pp40$^{I\kappa B\text{-}\beta}$ used in each sample was 0 μg (lanes 1 and 6), 5 μg (lanes 2 and 7), 10 μg (lanes 3 and 8), 20 μg (lanes 4 and 9), or 40 μg (lanes 5 and 10). The total amount of plasmid DNA was kept constant by the addition of the appropriate amount of a plasmid DNA that contained a retroviral vector with no coding sequence. A duplicate experiment in which 3 × 10^5 cells per 60-mm dish were used gave identical results. No virus replication occurred in this experiment, since a DNA clone encoding replication-competent Rev-A was not included in the transfection. The locations of molecular size markers (in kilodaltons) are indicated on the left side of the figure. The protein-DNA adducts containing the endogenous c-*rel* protein (c-rel^{wt}, lanes 1 to 10) and the c-rel^{546} protein (lanes 1 to 5) are indicated by the arrows on left side of the figure, while the protein-DNA adduct containing the cvc-rel^{542} protein (lanes 6 to 10) is indicated by the arrow on the right side of the figure.

(amino acids 138 to 423) that is critically important for pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding. The substitution of these c-*rel*-derived sequences for the corresponding sequences in p59$^{v\text{-}rel}$ does not affect the intrinsic DNA-binding activity of the recombinant vcv-*rel* protein but does provide for efficient pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding. Likewise, the presence of the corresponding v-*rel*-derived sequences in the recombinant vvc-*rel* and cvc-*rel* proteins results in loss of p40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding. There are multiple amino acid differences between p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ within the region that we have identified as important for pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding (8, 42, 47). It is possible that certain of these differences will affect the intrinsic affinity of *rel* proteins for DNA and that other differences will affect the affinity of *rel* proteins for pp40$^{I\kappa B\text{-}\beta}$. We are presently assessing the role of individual amino acid differences between p59$^{v\text{-}rel}$ and p68$^{c\text{-}rel}$ in this region for pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding.

pp40$^{I\kappa B\text{-}\beta}$ and DNA bind to overlapping domains on *rel* proteins. Our experiments are consistent with a model in which pp40$^{I\kappa B\text{-}\beta}$ and DNA are competitive inhibitors for the same or overlapping domains of *rel* proteins. The N-terminal limit of the DNA-binding domain of p59$^{v\text{-}rel}$ is within its N-terminal 29 amino acids (corresponding to the N-terminal 20 amino acids of p68$^{c\text{-}rel}$) (1). The N-terminal 51 amino acids of p68$^{c\text{-}rel}$ have previously been shown to be required for association with pp40$^{I\kappa B\text{-}\beta}$ (25). The C-terminal limit of the DNA-binding domain of p59$^{v\text{-}rel}$ is located between amino acids 272 and 293 (46), and our present results demonstrate that a region of p59$^{v\text{-}rel}$ extending from amino acid 147 to amino acid 428 (amino acids 138 to 423 of p68$^{c\text{-}rel}$) is important for the functional consequences of *rel*-pp40$^{I\kappa B\text{-}\beta}$ interactions. Thus, the region(s) of p68$^{c\text{-}rel}$ required for DNA binding overlap with those regions of p68$^{c\text{-}rel}$ important for pp40$^{I\kappa B\text{-}\beta}$ association and pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding. A model in which pp40$^{I\kappa B\text{-}\beta}$ and DNA are competitive inhibitors for the same or overlapping domains of *rel* proteins is further supported by the inability of anti-pp40$^{I\kappa B\text{-}\beta}$ serum to immunoprecipitate the p59$^{v\text{-}rel}$-DNA adduct or to perturb the mobility of the p59$^{v\text{-}rel}$-DNA complex in electrophoretic mobility shift analyses.

Regulatory interactions between internal and C-terminal regions of c-*rel*. Our experiments indicate that oncogenic activation of c-*rel* can be accomplished by coordinate alteration of two distinct regions of the protein. The presence of v-*rel*-derived sequences (amino acids 147 to 428) in combination with C-terminal truncation of c-*rel* protein results in oncogenic activation. However, in the context of a full-length c-*rel* protein, these v-*rel*-derived sequences are not sufficient for oncogenic activation of c-*rel*. These results indicate that the C terminus of p68$^{c\text{-}rel}$ contains a transformation-inhibitory activity that is dominant over the ability of the internal v-*rel*-derived region to confer loss of pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding.

Two distinct biochemical activities have been localized to the C terminus of p68$^{c\text{-}rel}$—a gene transactivation activity and a cytoplasmic retention activity (6, 8, 23, 36). The C-terminal gene transactivation domain of p68$^{c\text{-}rel}$ has been localized to amino acids 425 to 495 (36). Since the C-terminal gene transactivation domain is functional in both full-length and C-terminal-truncated recombinant v/c-*rel* proteins, and these recombinant v/c-*rel* proteins are able to transform avian lymphoid cells (18; this report), the C-terminal transactivation domain of p68$^{c\text{-}rel}$ is not likely to be responsible for the transformation-inhibitory activity that we identified. Instead, a more likely possibility is that the biochemical basis for the transformation-inhibitory activity of the C

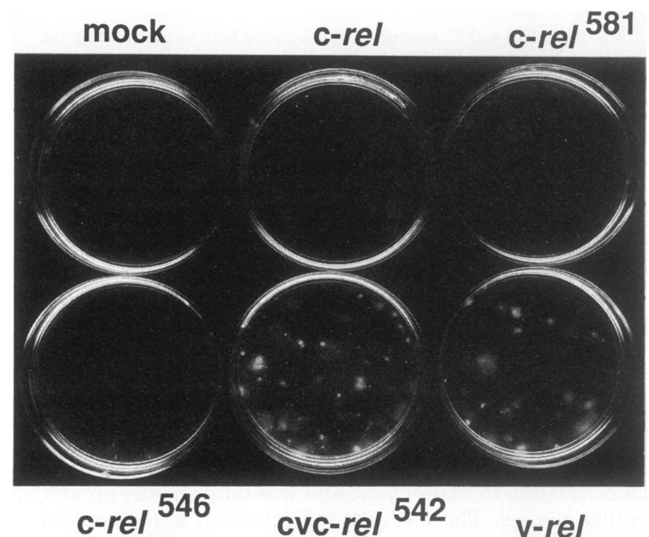

FIG. 10. Oncogenic transformation of avian spleen cells by *rel* proteins. Primary avian spleen cells (3 × 10^7) were infected with viruses encoding the indicated *rel* proteins and plated into soft agar. Colonies of transformed cells are clearly visible in plates containing cells that were infected by viruses encoding cvc-rel^{542} or v-*rel*, while no colonies of transformed cells are visible in plates containing cells that were infected by the other viruses. This photograph was taken 12 days after plating the infected cells in soft agar.

terminus of p68$^{c\text{-}rel}$ is the cytoplasmic anchoring property of the C terminus (8, 18). Cytoplasmic retention of *rel* proteins would necessarily prevent manifestation of those biological properties that are dependent on binding of *rel* proteins to cellular κB enhancer elements. Furthermore, this inhibitory effect of cytoplasmic retention would be dominant over the ability of those *rel* proteins, such as cvc-*rel*, that are able to bind DNA in the presence of high levels of pp40$^{I\kappa B\text{-}\beta}$. The C-terminal cytoplasmic retention domain of p68$^{c\text{-}rel}$ encompasses the C-terminal 103 amino acids (8), although removal of as few as 18 C-terminal amino acids is sufficient to allow partial nuclear translocation in CEF (this report). We suggest that a cytoplasmic retention activity (mediated by the C-terminal amino acids of p68$^{c\text{-}rel}$) and pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding (mediated by amino acids within the internal region of c-*rel*) cooperate to ensure that the growth-stimulatory property of c-*rel* is precisely regulated.

Oncogenic activation by N-terminal v-*rel*-derived sequences. The inability of the oncogenic vcc-*rel* protein to bind DNA in the presence of high levels of pp40$^{I\kappa B\text{-}\beta}$ would suggest that loss of pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding is not necessary for oncogenic transformation by *rel* proteins. Furthermore, the vcc-*rel* protein is located exclusively in the cytoplasm of CEF. These results present a quandary in understanding the mechanism of transformation by vcc-*rel*. One possibility is that vcc-*rel* does in fact utilize an indirect mechanism to induce oncogenic transformation of avian lymphoid cells. Although this possibility cannot be ruled out, previous studies have clearly established that DNA binding by v-*rel* is required for oncogenic transformation (46). Furthermore, DNA binding by the vcc-*rel* protein can readily be detected in nuclear extracts from vcc-*rel*-transformed cells (data not shown). A more attractive possibility is that, in vcc-*rel*-transformed cells, a sufficient level of nuclear vcc-*rel* protein that is capable of binding cellular κB enhancer elements is achieved. The importance of the N-terminal amino acids of *rel* proteins for pp40$^{I\kappa B\text{-}\beta}$ association, DNA binding, and nuclear translocation (1, 25; unpublished data) suggests that the N-terminal alterations present in vcc-*rel* might allow a level of nuclear vcc-*rel* protein in avian lymphoid cells that is sufficient for binding to cellular κB enhancer sequences and consequent cellular transformation. Cell-type-specific posttranslational modifications of either pp40$^{I\kappa B\text{-}\beta}$ or the vcc-*rel* protein might also contribute toward the ability of the vcc-*rel* protein to evade the actions of both the C-terminal cytoplasmic retention domain and pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding in vcc-*rel*-transformed avian lymphoid cells but not in vcc-*rel*-infected fibroblasts.

Multistep oncogenic activation of retrovirally transduced *rel* genes. Several different regions of the c-*rel* protein can participate in oncogenic activation of c-*rel*. We suggest that these apparently unrelated structural changes that can participate in oncogenic activation of c-*rel* have in common the attainment of a threshold level of nuclear *rel* protein sufficient to allow binding to specific κB enhancer elements in growth-regulatory genes. In this model, v-*rel* would, as the result of its multiple alterations with respect to c-*rel*, exceed the threshold level of DNA-binding nuclear *rel* protein required for transformation. This model is consistent with the results of the mutational analysis by Bhat and Temin (4), in which the substitution of single c-*rel*-derived amino acids into an otherwise wild-type v-*rel* protein had little effect on the oncogenic properties of v-*rel*. The minimal number of alterations sufficient for oncogenic activation of c-*rel* is not yet known. Our experiments suggest that any single alteration to c-*rel* will not be sufficient for oncogenic activation of c-*rel*, but rather that multiple alterations that affect cellular localization and pp40$^{I\kappa B\text{-}\beta}$-mediated inhibition of DNA binding (and perhaps other as-yet-unidentified biochemical properties) will be required.

ACKNOWLEDGMENTS

We thank Henry Bose, Jr., and Nathan Davis for their generous gift of the pp40$^{I\kappa B\text{-}\beta}$ cDNA and antisera and Deborah Vassar for laboratory assistance. We thank David Pintel, Bill Folk, and Mark Martin for critical and insightful discussions.

This work was supported by grant CA55027 from the National Cancer Institute, by grants from the University of Missouri Research Council and the Missouri Experimental Agriculture Station, and by the Molecular Biology Program at the University of Missouri. M.H. is grateful to the March of Dimes for a Basil O'Conner starter scholar award.

ADDENDUM IN PROOF

At a recent meeting, a new nomenclature for NF-κB/*rel*/IκB proteins was proposed; according to the new nomenclature, the avian pp40 protein should be considered a member of the IκB-α family.

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