Interaction of the v-Rel Oncoprotein with NF- κ B and I κ B Proteins: Heterodimers of a Transformation-Defective v-Rel Mutant and NF- κ B p52 Are Functional In Vitro and In Vivo

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The v-Rel oncoprotein of the avian Rev-T retrovirus is a member of the Rel/NF- κ B family of transcription factors. The mechanism by which v-Rel malignantly transforms chicken spleen cells is not precisely known. To gain a better understanding of functions needed for transformation by v-Rel, we have now characterized the activities of mutant v-Rel proteins that are defective for specific protein-protein interactions. Mutant v- Δ NLS, which has a deletion of the primary v-Rel nuclear localizing sequence, does not interact efficiently with I κ B- α but still transforms chicken spleen cells approximately as well as wild-type v-Rel, indicating that interaction with I κ B- α is not essential for the v-Rel transforming function. A second v-Rel mutant, v-SPW, has been shown to be defective for the formation of homodimers, DNA binding, and transformation. However, we now find that v-SPW can form functional DNA-binding heterodimers in vitro and in vivo with the cellular protein NF- κ B p52. Most strikingly, coexpression of v-SPW and p52 from a retroviral vector can induce the malignant transformation of chicken spleen cells, whereas expression of either protein alone cannot. Our results are most consistent with a model wherein Rel homodimers or heterodimers must bind DNA and alter gene expression in order to transform lymphoid cells.

There is still surprisingly little known about the mechanism by which the various nuclear oncoproteins effect the malignant transformation of cells. The v-Rel nuclear oncoprotein (reviewed in references 7, 21, and 26) of the highly oncogenic avian Rev-T retrovirus malignantly transforms an avian cell that has the characteristics of an immature lymphoid cell but can differentiate into various lineages in vitro (4).

v-Rel is a member of the Rel/NF- κ B family of eukaryotic transcription factors, which also includes the proto-oncoprotein c-Rel, RelB, p52/p100, the NF- κ B subunits p50 and RelA (p65), and the *Drosophila melanogaster* Dorsal and Dif proteins (reviewed in reference 52). To date, v-Rel and derivatives of chicken c-Rel are the only members of this family that have been consistently shown to be transforming in vitro. However, rearranged c-rel and p52/p100 genes have been identified in certain human lymphoid cancer cells (17, 36, 45, 59, 67).

Rel proteins are related through an approximately 300-amino-acid (aa) N-terminal domain known as the Rel homology (RH) domain, which contains sequences important for dimerization, DNA binding, inhibitor binding, and nuclear localization (reviewed in reference 52). Almost all Rel transcription factors can form homodimers and heterodimers, and these various dimers show unique specificities for binding to target DNA. In addition, Rel complexes are regulated by interaction with a family of proteins known as the I κ B proteins (reviewed in reference 22). In general, the binding of an I κ B protein to a Rel complex inhibits both nuclear translocation and DNA binding by that complex.

v-Rel shows cell-type-specific subcellular localization. In nontransformed chicken embryo fibroblasts (CEF), v-Rel is located almost exclusively in the nucleus (23). In contrast, in transformed chicken spleen cells, v-Rel is primarily located in

the cytoplasm in high-molecular-weight complexes with several cellular proteins, including c-Rel, p52/p100, p50/p105, and p40 (chicken I κ B- α) (9, 12–14, 23, 54, 56, 65); however, a minority of v-Rel is also located in the nucleus of these cells (13).

Several studies have demonstrated that mutations in the RH domain that affect the ability of v-Rel to bind to DNA correspondingly affect its ability to transform cells (40, 41). Most strikingly, v-Rel mutants with a conditional ability to transform chicken spleen cells in vitro also show a conditional ability to bind to DNA (5, 11, 62). In addition, C-terminal sequences of v-Rel that are involved in transcriptional activation are also necessary for transformation (15, 50, 58). However, in many cases, mutations that affect the ability of v-Rel to bind DNA and transform cells also affect its ability to form complexes with cellular Rel proteins and/or I κ B- α (40–42, 64). Thus, several models have been proposed to explain how v-Rel transforms chicken lymphoid cells (7, 21, 26), including ones wherein v-Rel forms homodimers and/or heterodimers on DNA, sequesters cellular Rel transcription factors, or abrogates the inhibitory effect of I κ B- α .

In this report we have analyzed the activities of v-Rel mutants that are defective for the ability to form specific protein-protein interactions with Rel or I κ B- α proteins. Our results are most consistent with a model in which v-Rel must bind to DNA and alter the expression of cellular target genes in order to transform cells. In addition, we demonstrate for the first time that heterodimeric Rel complexes can also transform primary spleen cells.

MATERIALS AND METHODS

Plasmids. Plasmid GM282, a spleen necrosis virus plasmid vector for the expression of v-Rel, has been described previously (12). Mutant v- Δ NLS, containing a deletion of the v-Rel nuclear localization sequence (NLS), was created by site-directed mutagenesis as described previously (35, 62). Briefly, an oligonucleotide (5'-GATGAAGAGGACCCGCTCTGGCAACTCAACACTGGCTTG G3') that spans sequences encoding the v-Rel NLS was hybridized to single-stranded M13 DNA containing wild-type v-rel, second-strand DNA synthesis was performed, double-stranded DNA was transformed into *Escherichia coli* XL1-

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Blue cells (Stratagene, La Jolla, Calif.), and mutagenized clones were identified by restriction mapping. Screening for mutants was facilitated by the removal of a *Bam*HI restriction site located 5' to the deleted region. The relevant section of the NLS-deleted *v-rel* gene was then subcloned as a *Stu*I-to-*Bst*XI fragment replacing wild-type *v-rel* sequences in retroviral vector GM282BS+ (53).

Plasmid CG129 for the in vitro expression of wild-type v-Rel was a gift of C. Gélinas; plasmids Sv-SPW and Sc-Rel have been described previously (41). The plasmid for in vitro expression of full-length v- Δ NLS was made by subcloning an *Xba*I fragment containing the entire *v-rel* gene into pGEM4 (Promega Corporation, Madison, Wisc.) in the Sp6 orientation. This same *Xba*I fragment was also subcloned into retroviral vector JD214BS+ (53) such that it was in the same retroviral vector as wild-type *v-rel* (in GM282BS+ [53]). Plasmid SW253 contains a replication-competent proviral genome of helper virus Rev-A (61).

Plasmid MH105 (a kind gift of M. Hannink) is a spleen necrosis virus vector for the expression of wild-type *v-rel* from the 5' long terminal repeat and of *neo* from an internal ribosome entry site. To construct plasmid MHv-SPW, an *Xba*I fragment containing the mutant *v-rel* gene was inserted in place of wild-type *v-rel* in plasmid MH105. To construct plasmid MHv-SPW/p52, DNA sequences in p52K (54) were amplified by PCR using primers (5'-GCCCGCGCGCGCATGGACGAGCAC3') and 5'-GTAATACGACTCACTATAGGGC3') that span the p52 sequence. The amplified product was digested with *Nco*I and *Hinc*II to yield a fragment encoding aa 1 to 397 of chicken p52 that was subcloned downstream of the internal ribosome entry site in plasmid MHv-SPW that had been digested with *Rsr*II, treated with Klenow fragment, and then digested with *Nco*I. Plasmid MHp52 was constructed by deleting the *Xba*I fragment encoding v-SPW from plasmid MHv-SPW/p52.

κ B site-containing yeast reporter plasmid, Igk3, and plasmids for the expression of c-Rel and c-SPW in *Saccharomyces cerevisiae* have been described previously (33, 41). Plasmid Bp52 was made by subcloning a *Bam*HI fragment containing chicken p52 codons 1 to 397 into plasmid B2202, a high-copy-number plasmid containing the *LEU2* selectable marker and the *GAL1-10* inducible promoter (38).

Plasmid Sv-dH172, for the in vitro expression of aa 1 to 331 of wild-type v-Rel, has been described elsewhere (50). Plasmids for the in vitro expression of the Rel homology domain (aa ~1 to 331) of mutant v-Rel proteins (v-SPW and v- Δ NLS) were made by subcloning *Xba*I-to-*Hinc*II fragments from mutant genes into pGEM4 in the Sp6 orientation.

For amplification of proviral DNA, genomic DNA from transformed spleen cells was prepared according to standard procedures (1), and proviral DNA sequences were amplified by PCR using primers (5'-GGATCCTCTAGAAAGCTCCTGACAACCAAG 3' and 5'-CTTTCCTTCAGGGTTGACAGTGGGGA T3') flanking the RH domain of v-Rel (codons 1 to 331). Plasmids S5'v(PER) and S5'v-SPW(PER) for the in vitro expression of PCR-amplified *v-rel* and v-SPW proviral sequences were constructed by inserting *Xba*I-to-*Hinc*II fragments from the amplified DNA into pGEM4 in the Sp6 orientation.

Transformation of chicken spleen cells and growth of cells. Chicken spleen cell transformation assays were performed by electroporating *v-rel*-containing retroviral vectors and plasmid SW253 helper virus DNA directly into fresh chicken spleen cells as described previously (42). For colony assays, colonies were counted 10 to 14 days after seeding cells in soft agar at 41.5°C. For liquid transformation assays, electroporated cells were plated in 2.5 ml of Temin's modified Eagle's Medium (TMEM; Biologos Inc., Naperville, Ill.) supplemented with 10% fetal calf serum; cells were fluid changed every 3 days thereafter and monitored for the outgrowth of transformed cells (generally determined by acidification of the culture medium). To establish transformed cell lines, colonies were picked from soft agar and were propagated in liquid culture in TMEM supplemented with 10 to 20% fetal calf serum. CEF were purchased from Spafas, Inc. (Norwich, Conn.) and were cultured in TMEM containing 10% fetal calf serum.

In vitro transcription-translation and EMSAs. Coupled in vitro transcriptions and translations were carried out in the TNT wheat germ lysate system (Promega) in the presence of Tran³⁵S-Label (methionine plus cysteine; >1,000 Ci/mmol; ICN Biomedicals, Costa Mesa, Calif.) as described previously (10, 50, 54). Equalized amounts of v-Rel proteins were then used in electrophoretic mobility shift assays (EMSAs) performed with a radiolabeled probe containing a v-Rel binding site from the chicken *c-rel* promoter (10). EMSAs using proteins from transformed cells were performed with detergent lysates containing 40 μ g of total cellular protein as described previously (62). All samples were analyzed on 5% polyacrylamide gels, and complexes were detected by indirect autoradiography using intensifying screens, including one screen to block the ³⁵S emissions from radiolabeled proteins.

Protein analysis by immunofluorescence, in vitro protein association assays, immunoprecipitations, and immunoblotting. Indirect immunofluorescence was performed on transfected CEF or transformed chicken spleen cells, using an anti-v-Rel or anti-p52 (see below) primary antibody as described previously (12, 23, 62).

For metabolic labeling, ~10⁶ transformed chicken spleen cells were labeled with 750 μ Ci of Tran³⁵S-Label for 6 h at 41.5°C, and cleared lysates containing 0.3% sodium dodecyl sulfate (SDS) were immunoprecipitated with the anti-v-Rel antiserum as described previously (23). In vitro protein association assays between in vitro-translated v-Rel proteins and p40 were performed by mixing samples after translation as described previously (64). Associations between in

vitro-translated samples containing v-Rel and c-Rel or p52 were performed on proteins that had been cotranslated. All immunoprecipitations were performed with either a rabbit polyclonal anti-v-Rel antiserum (23) or an antiserum specific for the C terminus of v-Rel (a kind gift of R. Hrdličková) as described previously (23), and samples were analyzed on SDS-polyacrylamide gels containing 8 to 12% polyacrylamide.

Immunoblotting was performed on transformed spleen cell lysates with a monoclonal anti-p40 antiserum (HY95 [64]) at a dilution of 1:5,000, rabbit polyclonal anti-v-Rel antiserum (23) (1:500 dilution), or anti-p52 antiserum (1:500 dilution; antibody K27; Santa Cruz Biochemicals, Santa Cruz, Calif.). Complexes were then detected with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase, and immunoreactive proteins were visualized by using enhanced chemiluminescence detection as described by the manufacturer (Amersham, Arlington Heights, Ill.). Where indicated, membranes were stripped of bound antibodies with 100 mM 2-mercaptoethanol–2% SDS–62.5 mM Tris-HCl (pH 6.7) as described previously (64). Proteins were quantitated by scanning laser densitometry, and values were normalized to the amount of the given protein in wild-type v-Rel-transformed cell lysates.

Reporter gene assays in yeast cells. *S. cerevisiae* BJ2168 (a *leu2 trp1 ura3-52 pep4-3 prb1-122 prc1-407*) (33) was grown and transformed with plasmids by using standard techniques (1, 41). Cells were streaked on the appropriate selective media agar plates or grown in liquid culture containing glucose or galactose (as indicated) at 30°C for 24 to 72 h. Agar overlay assays for the detection of β -galactosidase activity and liquid β -galactosidase assays were performed as described previously (6, 39).

RESULTS

Interaction with p40 (I κ B- α) is not necessary for transformation by v-Rel. Most experiments to date have been unable to dissociate the abilities of v-Rel mutants to transform chicken spleen cells from their abilities to bind as homodimers to DNA or to interact with chicken I κ B- α (p40). That is, most non-transforming v-Rel mutants do not bind to DNA and do not associate with p40 (40, 41, 64).

v-Rel has two sequences that can direct it to the nucleus in CEF: a sequence of basic amino acids that is conserved among all Rel family proteins located at the C terminus of the RH domain and an undefined sequence that is located C terminal to the RH domain (24). The conserved NLS in the RH domain has been shown to be essential for interaction of several cellular Rel family proteins with I κ B- α (3, 18, 27). Therefore, to determine whether the interaction of v-Rel with I κ B- α is essential for its transforming function, we tested the ability of a v-Rel protein with deletion of the conserved NLS to transform chicken spleen cells. Mutant v- Δ NLS has a 6-aa deletion of the NLS that is located near the C terminus of the RH domain of v-Rel (Fig. 1A). v- Δ NLS had a reduced ability to localize to the nucleus in CEF compared with wild-type v-Rel when overexpressed from a retroviral vector (Fig. 1B). Nevertheless, v- Δ NLS transformed chicken spleen cells approximately as well as wild-type v-Rel did (Fig. 1A). v- Δ NLS was located primarily in the cytoplasm of transformed spleen cells, as is wild-type v-Rel (Fig. 1D; reference 23).

As a negative control, we also assayed the transforming ability of v-Rel mutant v-SPW, which has a Pro-Trp insertion in the middle of a conserved protein kinase A recognition sequence (Arg-Arg-Pro-Ser; v-Rel aa 272 to 275) in the RH domain (41, 42; see also Fig. 1A). Like wild-type v-Rel, v-SPW localizes to the nucleus of CEF (reference 42 and data not shown); however, v-SPW cannot form homodimers, bind to DNA, or transform chicken spleen cells (references 41 and 42; Fig. 1A and C). Therefore, consistent with previous results (24, 41, 42), a mutation in v-Rel that abolishes DNA binding also abolishes transformation, but mutations in v-Rel that disrupt the NLS do not affect transformation.

Because the deletion in v- Δ NLS is in the conserved RH DNA-binding/dimerization domain of v-Rel, we determined whether this mutation affected DNA binding. As shown in Fig. 1C, in vitro-synthesized v- Δ NLS binds to a κ B site-containing oligonucleotide as well as wild-type v-Rel does.

FIG. 1. Structures and activities of v-Rel mutants. (A) Structures of the v-Rel mutants. LTR indicates the 5' and 3' retroviral long terminal repeats. The amino acids above the structures designate the mutations in each protein: v-ΔNLS has a deletion of all 6 aa of the NLS (Lys-Ala-Lys-Arg-Gln-Arg); v-SPW has a 2-aa insertion (Pro-Trp) after aa 273 in the middle of a sequence (Arg-Arg-Pro-Ser) that is important for the formation of homodimers. S indicates a *Sst*I site in wild-type v-Rel; N indicates an *Nco*I site unique to mutant v-SPW. Transforming activity (TRANSF.) in chicken spleen cells was assayed by agar colony formation; it is relative to the value for wild-type v-Rel (set at 100) and is the average of four assays. (B) Subcellular localization of mutant v-Rel proteins in CEF. CEF were transfected with retroviral vectors expressing wild-type v-Rel or v-ΔNLS, and proteins were localized by indirect immunofluorescence using an anti-v-Rel primary antibody. (C) DNA binding by in vitro-translated mutant v-Rel proteins. The indicated v-Rel proteins (aa 1 to 331) were translated in vitro in the presence of [³⁵S]methionine and [³⁵S]cysteine (top). Equalized amounts of the in vitro-translated proteins were used in an EMSA using a ³²P-labeled κB site-containing probe. Samples containing free probe were analyzed in the first and last lanes of the EMSA gel. The position of the shifted complex is indicated. (D) Subcellular localization of v-ΔNLS in transformed spleen cells. As indicated, wild-type v-Rel- and v-ΔNLS-transformed spleen cells were analyzed by indirect immunofluorescence using an anti-v-Rel primary antibody. To visualize the nuclei, the same fields of cells were also stained with 4',6-diamidino-2-phenylindole (DAPI). The white arrows indicate reference cells for the corresponding fields.

To determine whether the NLS deletion affected the ability of v-ΔNLS to interact with chicken p40, we performed coimmunoprecipitation assays in vitro and in vivo. For the in vitro assays, v-Rel proteins and p40 were translated in vitro, mixed, and immunoprecipitated with an anti-v-Rel antiserum. In these experiments, wild-type v-Rel interacted with p40. In contrast, v-ΔNLS did not interact detectably with p40 under these conditions (Fig. 2A).

v-Rel has been shown to complex with and stabilize p40 in vivo in transformed chicken spleen cells (13, 30, 56, 64). To determine whether v-ΔNLS was impaired for interaction with p40 in vivo, lysates from cells transformed by v-Rel or v-ΔNLS were first immunoprecipitated with the v-Rel antiserum and

then immunoblotted with an anti-p40 antibody. As shown in Fig. 2B, immunoprecipitates of v-ΔNLS contained approximately 50 times less p40 than immunoprecipitates of wild-type v-Rel; moreover, the small amount of p40 seen in the v-ΔNLS immunoprecipitates is likely to be due to c-Rel complexes that are present in these immunoprecipitates because the antibody used in these experiments also recognizes chicken c-Rel (12). Therefore, the greatly reduced ability of v-ΔNLS to interact with p40 in vivo is consistent with its reduced ability to interact with p40 in vitro.

v-Rel is also thought to increase cellular levels of p40 by directly increasing transcription of the p40 gene (30). Therefore, we also quantitated the total levels of cellular p40 by

FIG. 2. Interaction of mutant v- Δ NLS with p40. (A) Mutant v- Δ NLS is disrupted for interaction with p40 in vitro. Equal amounts of in vitro-translated, radiolabeled wild-type v-Rel or v- Δ NLS were mixed with p40 (lanes 3 and 4, respectively), proteins were allowed to associate in vitro, and samples were immunoprecipitated with an anti-v-Rel antiserum. Lanes 1 and 2 contain in vitro-translated v-Rel and p40, respectively; lanes 5 and 6 are anti-v-Rel immunoprecipitations of v-Rel and of p40. The locations of v-Rel and p40 are indicated. (B) Mutant v- Δ NLS is disrupted for interaction with p40 in vivo. Cleared lysates prepared from spleen cell lines transformed by the indicated proteins were immunoprecipitated with the anti-v-Rel antiserum, and immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with an anti-p40 antiserum (panel 1). The nitrocellulose was stripped and reprobed with the anti-v-Rel antiserum (panel 2). (C) Reduced cellular levels of p40 in v- Δ NLS-transformed cells. Whole cell lysates prepared from the indicated transformed spleen cell lines were resolved by SDS-PAGE and immunoblotted with an anti-p40 antiserum (panel 1). The blot was stripped and reprobed with an anti-v-Rel antiserum (panel 2). In panels B and C, the positions of v-Rel and p40 are indicated and immunodetected proteins were quantitated by scanning laser densitometry. Relative Amt., relative amount of protein compared with the amount in the wild-type v-Rel lane.

immunoblotting of whole cell lysates from spleen cells transformed by wild-type v-Rel or v- Δ NLS. In these experiments, wild-type v-Rel-transformed cells were found to have only about four times more total p40 than cells transformed by v- Δ NLS (Fig. 2C). These results suggest that the increase in cellular p40 levels seen in cells transformed by v-Rel is likely to be due to both increased synthesis of p40 and increased stabilization of p40 by interaction with v-Rel.

Taken together, the results presented in this section indicate that the interaction of v-Rel with p40 does not correlate with its ability to transform chicken spleen cells. Therefore, we next sought to determine whether protein-protein interactions be-

tween v-Rel and cellular Rel proteins might be important for transformation.

v-Rel mutant v-SPW can form heterodimers with NF- κ B p52 in vitro. NF- κ B p100 and p105 undergo proteolytic processing in vivo to yield mature DNA-binding proteins p52 and p50, respectively (8, 16, 51). Although mutant v-SPW is unable to form homodimers and bind to DNA in vitro (41), we previously showed that v-SPW could interact with NF- κ B p105 in vitro almost as efficiently as wild-type v-Rel (9). Therefore, we wished to determine whether v-SPW could also interact with the processed chicken cellular Rel proteins p52 and p50. Because of the association of truncated forms of p100 (p52-like proteins) with human cancers (17, 45, 59, 67), we chose to focus initially on the association of v-SPW with p52.

To test for the formation of heterodimers between v-Rel proteins and p52, wild-type v-Rel and v-SPW were each co-translated in vitro with a truncated version of chicken NF- κ B p100, which roughly corresponds to the processed, mature DNA-binding protein p52. These samples were then immunoprecipitated with a v-Rel-specific antiserum. As shown in Fig. 3A, p52 could be coprecipitated with wild-type v-Rel and with v-SPW, indicating that wild-type v-Rel and v-SPW are both able to complex with p52.

To determine whether the complexes between v-SPW and p52 were able to bind to DNA, we used the in vitro-translated proteins in an EMSA using a κ B site from the chicken *c-rel* promoter (10). This site can be bound strongly by v-Rel homodimers but only weakly by homodimers of chicken p52 (9, 54). Consistent with previous results (42, 50), in vitro-translated v-SPW by itself did not bind to this site (Fig. 3B, lane 2). However, cotranslated samples containing v-SPW and p52 did generate a shifted complex in the EMSA (Fig. 3B, lane 9). These complexes contain v-SPW because the complexes could be supershifted with the v-Rel-specific antiserum (Fig. 3B, lane 10). As a control, the anti-v-Rel antiserum did not affect the binding of p52 homodimers, which only weakly bind to this site (Fig. 3B, lanes 5 and 6). Together, these results indicate that v-SPW and p52 can form heterodimers that can bind to DNA in vitro. (We have obtained similar results with v-SPW and p50 [46].)

Although mutant v-SPW cannot form homodimers in vitro, it was not clear whether v-SPW could form a dimer with a wild-type Rel molecule. For this reason and because DNA-binding complexes containing v-Rel and c-Rel are present in transformed cells (28), we determined whether v-SPW could form dimers with wild-type chicken c-Rel. v-SPW and chicken c-Rel were cotranslated in vitro and immunoprecipitated with an antibody against sequences unique to the C terminus of v-Rel; this antibody does not immunoprecipitate c-Rel (Fig. 3C). Wild-type v-Rel readily formed a complex with c-Rel in vitro. However, v-SPW did not form a heterodimer with wild-type c-Rel (Fig. 3C). These results indicate that v-SPW is unlikely to be able to form complexes with c-Rel in vivo.

c-SPW can form heterodimers with NF- κ B p52 and functionally bind to DNA in vivo. To determine whether an SPW/p52 heterodimer could bind to DNA in vivo, we tested the ability of chicken c-Rel containing the SPW mutation (c-SPW) to form a transcriptional activating complex with p52 in yeast cells, which are not known to contain endogenous Rel/NF- κ B proteins. Chicken c-Rel was used for this assay because the stronger C-terminal activation domain of c-Rel is needed to obtain efficient reporter gene activity in yeast cells (33), and chicken c-SPW is defective for homodimer formation and DNA binding in the same way as is v-SPW (42). As shown in Fig. 4, wild-type c-Rel efficiently induced transcription in yeast from a reporter plasmid (Igk3) containing c-Rel binding sites

FIG. 3. v-SPW and p52 form heterodimers that can bind to DNA in vitro. (A) Coimmunoprecipitation of in vitro-translated v-SPW and p52. The indicated proteins were translated in vitro (TOTAL), and samples were then immunoprecipitated with an anti-v-Rel antiserum (IMPTE.). The positions of v-Rel and p52 are indicated. (B) DNA binding by in vitro-translated v-SPW and p52. Equalized amounts of the indicated in vitro-translated proteins were analyzed in an EMSA using a radiolabeled κ B-binding site. Where indicated, supershift assays using an anti-v-Rel antiserum were performed. The first and last lanes contain free probe. The positions of the shifted and supershifted complexes are indicated. (C) v-SPW and chicken c-Rel do not form a complex in vitro. Equalized amounts of the indicated in vitro-translated proteins were either analyzed directly (TOTAL) or immunoprecipitated with an anti-v-Rel antiserum (specific for the C terminus of v-Rel) (IMPTE.). The positions of v-Rel and c-Rel are indicated.

FIG. 4. c-SPW/p52 heterodimers can activate transcription in yeast cells. Yeast strains containing plasmids for the expression of the indicated proteins were grown on the appropriate selective solid media containing either glucose or galactose for 24 to 72 h at 30°C, and β -galactosidase activity was detected by an agar overlay assay (6). (The expression of p52 is inducible by galactose.)

and the *lacZ* gene, as evidenced by the appearance of blue color in the presence of a 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal)-containing agar overlay. As expected, neither chicken p52, which lacks a C-terminal activation domain, nor c-SPW could by itself activate transcription from the reporter gene (Fig. 4). In contrast, cells expressing both p52 and c-SPW had increased levels of transcription from the reporter gene compared with control cells. However, liquid β -galactosidase assays of these cells indicate that the c-SPW/p52 heterodimers are approximately 12-fold less efficient at activating transcription from the reporter plasmid than wild-type c-Rel homodimers (Table 1). Nevertheless, these results indicate that c-SPW and p52 are able to form a functional activating complex on DNA in vivo.

TABLE 1. Activation of a κ B site reporter plasmid by v-SPW/p52 in *S. cerevisiae*

Plasmid ^a		Relative β -galactosidase activity ^b	
1	2	Glucose	Galactose
Vector	c-Rel	100	100
p52	Vector	ND	<1
Vector	c-SPW	ND	<1
p52	c-Rel	ND	93
p52	c-SPW	1	8

^a *S. cerevisiae* BJ2168 was transformed with both of the indicated expression plasmids, and all strains also contained the κ B site *lacZ* reporter plasmid Ig κ 3 (33). The expression of chicken p52 is inducible by galactose. Vector refers to the appropriate control vector lacking an inserted gene.

^b Yeast cells were grown in the appropriate selective liquid medium containing either glucose or galactose to an optical density at 600 nm of 0.5 to 1.0, and liquid β -galactosidase assays were performed as described in Materials and Methods. In each case, β -galactosidase activity is relative to that seen with wild-type c-Rel (assigned a value of 100). ND, not done.

FIG. 5. A v-SPW/p52 heterodimer can transform chicken lymphoid cells. (A) Schematic representation of avian retroviral vectors for the expression of v-Rel, v-SPW, p52, or v-SPW and p52. LTR, 5' or 3' viral long terminal repeat; IRES, the encephalomyocarditis virus internal ribosome entry site; *neo*, bacterial neomycin resistance gene. (B) Phenotypic appearance of v-SPW/p52-transformed chicken spleen cells. Wild-type v-Rel- or v-SPW/p52-transformed chicken spleen cells growing at 37°C in liquid culture were viewed under a phase-contrast microscope and were photographed at a magnification of $\times 400$.

Overexpression of NF- κ B p52 can rescue the transforming defect of v-SPW in vivo. Because Rel proteins that contain the SPW mutation can form functional heterodimers with chicken p52 in vitro and in vivo, we wished to determine whether coexpression of p52 could rescue the transforming function of v-SPW in chicken spleen cells. Therefore, we assayed the transforming activity of retroviral vectors expressing wild-type v-Rel, p52, v-SPW, or both v-SPW and p52 (Fig. 5A). Preliminary experiments indicated that the latter (v-SPW/p52) virus could induce the growth of transformed colonies in agar more slowly and at a greatly reduced level compared with wild-type v-Rel (data not shown). Consequently, as a more sensitive test for transformation, we used a semiquantitative liquid culture assay (see Materials and Methods) to measure the transforming potentials of these viruses. In this assay, virus encoding both v-SPW and p52 was consistently transforming (Table 2). Consistent with previous results (25, 47), neither p52 nor v-SPW by itself could transform chicken spleen cells in this liquid culture assay. These results indicate that v-SPW/p52 heterodimers can induce the transformation of chicken spleen cells.

Spleen cells transformed by the v-SPW/p52 virus grew in large clumps in liquid culture and were indistinguishable from cells transformed by wild-type v-Rel (Fig. 5B). However, we have not determined the cell-type-specific characteristics of cells transformed by v-SPW/p52.

To demonstrate that v-SPW and p52 were both expressed at elevated levels in v-SPW/p52-transformed cell lines, cell lysates

were subjected to immunoblot analysis with anti-v-Rel and anti-p52 antisera. As shown in Fig. 6A, v-SPW was expressed in these cells at approximately the same level as is v-Rel in cells transformed by wild-type v-Rel. Moreover, there were greatly elevated levels of p52 in v-SPW/p52-transformed cells compared with cells transformed by wild-type v-Rel (Fig. 6A). This is true both for independently derived v-SPW/p52-transformed cell clones and for pools of cells transformed by v-SPW/p52 virus. To avoid heterogeneity in transformed cells, we chose to characterize further a single clone of cells (clone A) transformed by v-SPW/p52.

To demonstrate directly that v-SPW/p52 complexes existed in vivo, lysates from radiolabeled transformed cell clone A were immunoprecipitated with an anti-v-Rel antiserum. The anti-v-Rel antiserum used in these experiments does not cross-react with chicken p52 (Fig. 6B, lane 4). As shown in Fig. 6B, a band that comigrates with in vitro-translated p52 was specifically present in v-Rel immunoprecipitates from cells transformed by the v-SPW/p52 virus. Therefore, a complex containing v-SPW and p52 is present in transformed cells.

To demonstrate that v-SPW/p52 DNA-binding complexes existed in transformed cells, lysates from transformed cells were used in EMSAs. As shown in Fig. 6C, lysates from wild-type v-Rel-transformed cells and from v-SPW/p52-transformed cells contain approximately equal amounts of DNA-binding activity for the κ B probe used in these experiments. The shifted complex from both lysates could be supershifted with the anti-v-Rel antiserum, indicating that v-Rel protein is present in these complexes. Because v-SPW and p52 can readily form heterodimers in vitro and in vivo, the results presented in Fig. 6C suggest that the majority of κ B site DNA-binding activity in v-SPW/p52-transformed cells is in the form of a v-SPW/p52 heterodimer.

To determine whether the subcellular locations of v-SPW and p52 proteins in transformed spleen cells were different from that of wild-type v-Rel in transformed spleen cells, v-SPW/p52-transformed cells were subjected to indirect immunofluorescence with an anti-v-Rel or anti-p52 antiserum. As shown in Fig. 7, v-SPW and p52 were both located primarily in the cytoplasm of transformed spleen cells, as is wild-type v-Rel.

We performed two experiments to demonstrate that the v-SPW gene in the v-SPW/p52-transformed cells was not a mutant form of v-SPW, for example, that it did not encode a revertant v-SPW protein that could now bind DNA. First, we

TABLE 2. Transformation of chicken spleen cells by v-SPW/p52

Plasmid	Virus structure ^a	Plates with outgrowth of cultures/total no. of plates examined			Days to transform ^b
		Expt 1	Expt 2	Expt 3	
MH105	v-Rel-I-Neo	2/2	5/5	3/5	11–19
MHp52	I-p52	0/2	0/5	0/5	— ^c
MHv-SPW	v-SPW-I-Neo	0/2	0/3	0/5	—
MHv-SPW/p52	v-SPW-I-p52	3/3	1/2	2/5	15–19

^a See Fig. 5A for virus structures. I, internal ribosome entry site.

^b Number of days after electroporation into culture was overgrown by transformed cells (see Materials and Methods).

^c —, no cells grew out in liquid culture even after 30 days in culture.

FIG. 6. Analysis of v-SPW/p52 heterodimers in transformed chicken spleen cells. (A) Expression of p52 and v-SPW proteins in v-SPW/p52-transformed cells. In panel 1, whole cell lysates prepared from wild-type v-Rel-transformed (lanes 3 and 5) or v-SPW/p52 clone A-transformed (lanes 4 and 6) cells were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with either an anti-v-Rel (lanes 3 and 4) or an anti-p52 (lanes 5 and 6) antiserum. As size standards, in vitro-translated v-Rel (lane 1) and p52 (lane 2) were analyzed on the same gel and detected by fluorography. The lower panels show the relevant sections of immunoblots of whole cell lysates prepared from cells transformed by wild-type v-Rel, v-SPW/p52 (clone A), or v-SPW/p52 (clone B) or a pooled sample of v-SPW/p52-transformed cells immunoblotted with an anti-v-Rel (panel 2) or anti-p52 (panel 3) antiserum. (B) Coprecipitation of v-SPW and p52 from transformed chicken spleen cells. In vitro-translated, ^{35}S -labeled v-Rel and p52 were analyzed directly (lanes 1 and 2) or were immunoprecipitated with an anti-v-Rel antiserum (lanes 3 and 4). Chicken spleen cells transformed by wild-type v-Rel (lane 5) or v-SPW/p52 (clone A; lane 6) were labeled with ^{35}S -Label for 6 h, and cleared lysates were immunoprecipitated (IMPTE.) with an anti-v-Rel antiserum. All samples were electrophoresed on the same SDS-polyacrylamide gel, and proteins were detected by fluorography. (C) κB site-binding activity in extracts from v-SPW/p52-transformed cells. Detergent lysates prepared from spleen cell lines transformed by the indicated proteins were analyzed in an EMSA using a radiolabeled κB site probe. Where indicated, supershift assays using an anti-v-Rel antiserum were performed. Probe alone was analyzed in the first lane. The positions of the shifted and supershifted complexes are indicated.

FIG. 7. Subcellular localization of v-SPW and p52 proteins in transformed spleen cells. v-SPW/p52-transformed spleen cells were analyzed by indirect immunofluorescence with an anti-v-Rel or anti-p52 antiserum as indicated. Panels marked DAPI show staining of nuclei in the same field of cells stained with the v-Rel or p52 antiserum. The white arrows indicate reference cells in the corresponding fields.

performed PCR using primers spanning codons 1 to 331 of *v-rel* to amplify proviral sequences from cells transformed by wild-type v-Rel or by v-SPW/p52. Wild-type *v-rel* has a unique *StuI* site in the RH domain; the insertion in v-SPW destroys the *StuI* site and inserts an *NcoI* site at the same position (Fig. 1A). As shown in Fig. 8A, amplified DNA from both cell types comigrated, and restriction enzyme digestion of the amplified DNA with *StuI* and with *NcoI* established that the v-SPW provirus still had the same mutation as the original v-SPW mutant plasmid DNA. Second, we subcloned the amplified proviral DNAs into in vitro expression vectors and assayed the ability of wild-type v-Rel and v-SPW to bind DNA in an EMSA. As shown in Fig. 8B, plasmid-encoded and provirus-encoded wild-type v-Rel readily bound the probe, whereas neither the original v-SPW protein nor the v-SPW protein encoded by the provirus in the v-SPW/p52-transformed cell line could bind to the κB site-containing probe. Third, virus harvested from v-SPW/p52-transformed cells transformed fresh spleen cells at a very low level, similar to results for the original virus (data not shown). These results indicate that the v-SPW gene has not undergone further mutations in the v-SPW/p52-transformed cell line.

DISCUSSION

Transformation of chicken spleen cells by v-Rel and v-SPW/p52. We have characterized the interaction of two v-Rel mutants with the cellular I κB - α protein p40 and with NF- κB p52. One v-Rel mutant, v- ΔNLS , transforms spleen cells and binds to DNA as well as wild-type v-Rel but is impaired for interaction with p40 (I κB - α), indicating that the interaction of v-Rel

FIG. 8. Structural and functional analysis of the v-SPW provirus. (A) Proviral sequences from wild-type v-Rel-transformed spleen cells or v-SPW/p52-transformed cells (clone A) were amplified by PCR using primers flanking the RH domain. Amplified DNA was directly examined (Uncut) or digested with *Nco*I or *Stu*I. Samples were electrophoresed on a 5% polyacrylamide gel, and DNA was visualized by staining with ethidium bromide. The large arrow indicates the undigested 1,018-bp product; the smaller arrows indicate the 844- and 174-bp fragments produced by restriction digest. DNA markers in lane 1 are listed in base pairs. (B) PCR-amplified v-SPW does not bind DNA in vitro. Equal amounts of in vitro-synthesized ³⁵S-labeled proteins (aa 1 to 331) of plasmid-encoded or PCR-amplified v-Rel or v-SPW were analyzed by SDS-polyacrylamide gel electrophoresis (top). These proteins were also used in an EMSA using a ³²P-radiolabeled κ B site probe (bottom). The position of the shifted complex is indicated. Probe alone was analyzed in the first and last lanes.

with p40 is not involved in transformation. A second v-Rel mutant, v-SPW, is defective for transformation, homodimer formation, and DNA binding, but v-SPW can form heterodimers with NF- κ B p52. The v-SPW/p52 heterodimers can functionally bind to DNA in vitro and in vivo and can transform chicken spleen cells.

Our demonstration that v-SPW/p52 heterodimers can transform primary chicken spleen cells shows for the first time that a heterodimeric Rel complex can induce transformation. This is especially relevant because rearranged *c-rel* and NF- κ B p100 genes have been found in a number of human lymphoid cancers (17, 36, 45, 59, 67). In each case, these rearrangements result in truncations of C-terminal sequences from the encoded Rel proteins. However, these truncated proteins do not by themselves contain transcription-activating sequences (17, 25), nor have they been reported to be transforming in vitro. Therefore, these abnormal human Rel proteins may contribute to oncogenesis by forming heterodimers with wild-type cellular Rel family proteins, such as c-Rel.

Overexpressed p52 and v-SPW proteins are not transforming by themselves. These results and those of others (37) suggest that there is a threshold of Rel dimer formation that is necessary for transformation. For example, it is likely that overexpressed v-SPW can form heterodimers with endogenous cellular Rel/NF- κ B proteins such as p50 and p52, but there may not be enough of these (processed) cellular proteins to form sufficient amounts of functional heterodimers with v-SPW to transform cells. Therefore, p52 must also be expressed at increased levels to rescue the transforming function of v-SPW.

The reduced transforming efficiency of a virus coexpressing v-SPW and p52 compared with that of a virus expressing wild-type v-Rel could be due to any of several factors. First, c-SPW/

p52 heterodimers are less efficient than c-Rel homodimers at inducing expression of reporter genes in yeast cells (Table 1), and the same is likely to be true of v-SPW/p52 heterodimers compared with v-Rel homodimers in chicken spleen cells. Because the ability of v-Rel to activate transcription can be correlated with its ability to transform (15, 34, 50), v-SPW/p52 heterodimers may be less transforming than v-Rel homodimers because v-SPW/p52 heterodimers are less potent transactivating complexes. Second, the optimal target sites bound by v-SPW/p52 are likely to differ slightly from those recognized by v-Rel homodimers. Because the abilities of v-Rel mutants to bind DNA can be correlated with their abilities to transform (2, 5, 11, 40, 42, 60), it may be that v-SPW/p52 heterodimers bind to relevant target genes less efficiently than v-Rel homodimers. Third, overexpressed v-SPW/p52 heterodimers may transform only a subset of the target cells transformed by wild-type v-Rel. For example, a v-Rel mutant with an alteration in its DNA-binding domain has been shown to have an altered target cell-transforming specificity (48). Lastly, we have used an artificial p52-like protein, created by recombinant DNA techniques, which may not have optimal heterodimer-forming abilities.

Our results and those of others indicate that the most important functions for transformation of chicken spleen cells by v-Rel are the abilities to form homodimers, bind to DNA, and activate transcription (2, 5, 11, 15, 40–42, 50, 58, 60, 62). These results are most consistent with a model in which transformation by Rel proteins is effected by the binding of Rel dimers to κ B sites, which affects the expression of relevant target genes. Although it is likely that v-Rel activates many of these target genes, there may also be some genes whose expression is decreased by the binding of v-Rel.

Several cellular genes whose expression appears to be regulated directly by v-Rel homodimers have been identified. These include the interleukin-2 gene, the interleukin-2 receptor α gene, major histocompatibility complex gene, HMG-14 gene, DM-GRASP, and several genes that have been identified by subtractive cDNA cloning (5, 15a, 29, 44, 66). It is not clear which, if any, of these genes are relevant to transformation by v-Rel. However, it will be interesting to determine which genes are also activated in cells transformed by v-SPW/p52, since such common genes may represent relevant target genes.

Although we favor a model wherein v-Rel must bind to DNA to effect transformation, certain observations are inconsistent with this model. For example, the majority of wild-type v-Rel is located in the cytoplasm of transformed chicken spleen cells (13, 23, 57). Thus, one must argue that a small portion of v-Rel is located in the nucleus of transformed spleen cells, either constitutively or during certain stages of the cell cycle when the nuclear envelope is not present.

In addition, our results do not exclude the possibility that activities of v-Rel besides direct regulation of transcription are involved in the transforming process. For example, v-Rel can interact with cellular transcription factor Sp1 and indirectly affect transcription (53, 55), and v-Rel can induce DNA synthesis (31).

Structural implications of v-SPW. Most Rel family proteins have a conserved consensus protein kinase A phosphorylation sequence located toward the C-terminal end of the RH domain. A number of studies have indicated the importance of this sequence (Arg-Arg-Pro-Ser) for the formation of homodimers in Rel and RelA. First, mutations that convert the serine residue to an acidic amino acid in v-Rel, c-Rel, and RelA greatly reduce the abilities of these proteins to form homodimers but not heterodimers (19, 41). Second, mutations in this sequence that convert the second arginine, which is

conserved in all Rel family proteins, to a histidine residue create temperature-sensitive (*ts*) mutants of Dorsal and v-Rel (32, 62); in v-Rel, these mutants are *ts* in vitro for transformation, DNA binding, and the formation of homodimers but are not *ts* for the formation of heterodimers with p50 (63). Third, the SPW mutation in Rel proteins, which inserts a proline and a tryptophan residue between the arginine and proline of this sequence, abolishes the abilities of v-Rel and c-Rel to bind to DNA and form homodimers (41) but does not affect their abilities to form functional heterodimers (reference 9 and Fig. 3). Similarly, mutations near this sequence in mouse p50 affect its ability to form homodimers but not heterodimers (49). Taken together, these results suggest that heterodimers between Rel family proteins are more stable (and therefore more resistant to mutations) than homodimers.

It should be noted, however, that although the amino acids of the consensus PKA recognition sequence are located near the predicted dimer interface of p50 homodimers bound to DNA, they are not obviously a direct part of the dimer interface (20, 43, 49). Therefore, alterations in this consensus phosphorylation sequence in v-Rel are likely to affect the dimer interface indirectly. Alternatively, but less likely, the dimer interface in v-Rel homodimers may differ from that in p50 homodimers.

In conclusion, current data suggest that most, if not all, retrovirally encoded nuclear oncoproteins must bind DNA and activate transcription to effect transformation of relevant target cells. For many of these nuclear transcription factors, including v-Rel, it appears essential that they form homodimers as part of this transforming process. More precisely, there is still no example of a v-Rel mutant that is, by itself, defective either for the formation of homodimers or for DNA binding that can transform chicken spleen cells. However, our present finding that overexpressed NF- κ B p52 can rescue a v-Rel mutant defective for the formation of homodimers indicates that Rel/NF- κ B heterodimers can also induce malignant transformation.

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