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In Vivo Evolution of c-*rel* Oncogenic Potential

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The c-*rel* proto-oncogene belongs to the NF-κB/*rel* and IκB gene families, which regulate several inducible processes, including self-defense/repair and embryogenesis. Transduction of the c-*rel* transcription factor by the avian retrovirus resulted in the formation of a highly oncogenic virus, reticuloendotheliosis virus strain T (REV-T), that encodes the oncogene v-*rel*. To examine the oncogenic potential of c-*rel*, we inserted it into a REV-T-based retroviral vector, rescued virus [REV-C(CSV)], and infected 1-day-old chicks. All birds developed tumors, and all cell lines established from REV-C-induced tumors expressed c-*rel* proteins that lacked C-terminal sequences. These proteins, responsible for both in vivo and in vitro cell proliferation, were apparently selected for their oncogenic potential. In order to examine the cooperation of C-terminal deletions with other oncogenic alterations in vivo, point mutations present in the N-terminal and middle regions of v-*rel* were analyzed by a similar protocol. The data obtained support four conclusions. (i) c-*rel* proteins bearing any of three single-amino-acid mutations present in the N-terminal portion of v-*rel* were sufficiently oncogenic to induce tumor development in the absence of additional mutations. (ii) Combining a mutation from the N-terminal region of v-*rel* with a deletion of the C-terminal sequences of c-*rel* increases the oncogenicity of the protein in an additive manner. (iii) Mutations present in the middle of v-*rel* cooperated synergistically with C-terminal deletions to produce highly transforming viruses. (iv) Deletion of c-*rel* produced a variety of transforming *rel* proteins with sizes that extended from 42 to 65 kDa. The most frequently isolated *rel* deletion was 62 kDa in size. To examine the basis for the selection of different *rel* mutants, their ability to induce immunoregulatory surface receptors was analyzed. The data revealed a correlation between the induction capacity of these mutants and their corresponding contribution to in vivo tumorigenic potential. Moreover, an analysis of the subcellular localization of different *rel* proteins revealed an inverse correlation between the size of the protein and the proportion in the nucleus of lymphoid cells.

The proto-oncogene c-*rel* (13, 16, 34, 69) is a member of a system of vertebrate proteins that regulate transcription, encoded by the NF-κB/*rel* and IκB gene families, which includes RelA, RelB, NF-κB1, NF-κB2, IκBα, IκBβ, IκBγ, and Bcl-3 (33, 46a). An analogous system in *Drosophila melanogaster*, in which a *rel*-like transcription factor, *dorsal*, and its inhibitor, *cactus*, modulate the development of the dorsoventral axis, has been described (28, 41, 65). NF-κB/*rel* and IκB proteins function to regulate transcription during immune response reactions, healing/regeneration, and developmental processes (2, 9). These processes require a balance between proliferation and differentiation, and it is likely that disruption of this balance can lead to oncogenic transformation. Several members of the NF-κB/*rel* and IκB families, including NF-κB2, Bcl-3, and c-*rel*, have been implicated in human malignancies (48, 56, 57). Transduction of the turkey c-*rel* proto-oncogene by the avian retrovirus reticuloendotheliosis virus strain A (REV-A) produced the v-*rel* oncogene, a gene that produces hematopoietic disease with high efficiency, providing an animal model system for the analysis of oncogenic derivatives of these transcription factors (21, 61, 64).

c-*rel* contains an N-terminal *rel* homology (RH) domain that is conserved among all NF-κB/*rel* proteins (Fig. 1A). The RH domain is responsible for DNA binding, dimerization, and interaction with IκBα. The RxxRxRxxC amino acid motif within the N-terminal half of this domain has been shown to make direct contact with DNA (45). The carboxy half of the RH domain is required for dimerization between NF-κB/*rel* proteins (12, 47) so that the integrity of the entire RH domain is necessary for DNA binding (12, 30, 42, 47). The first 265 amino acids of c-*rel* are sufficient to establish binding with IκBα, but IκBα-mediated inhibition of c-*rel* DNA binding requires additional protein sequence, including a nuclear localization signal (8, 40). A conserved nuclear localization signal (32), as well as a consensus recognition sequence for cyclic AMP-dependent protein kinase, is located at the C-terminal end of the RH domain (53, 54). In addition to the RH domain, c-*rel* possesses an acidic region in the carboxy half of the protein that is involved in transcriptional activation (14, 39, 60, 68). Current data suggest the possibility of dividing this functional region into two distinct parts (39, 68), designated here domains I and II (Fig. 1A). The C-terminal sequences of c-*rel* contain a cytoplasmic anchor that overlaps the transactivation region (16, 35).

v-*rel* can be distinguished from c-*rel* by several structural changes (Fig. 1A). Transduction of c-*rel* resulted in the deletion of 2 N-terminal and 118 C-terminal amino acids from $p68^{c\text{-}rel}$ and their replacement by 11 and 18 *env*-derived amino acids, respectively. The C-terminal deletion removed a region responsible for cytoplasmic anchoring as well as sequences that encode transactivation domain II and part of domain I (16, 35, 39, 60). On the basis of a comparison of chicken and turkey c-*rel*, 13 point mutations and 3 small deletions are also found in v-*rel* (16, 35, 69). Despite these differences, v-*rel* shares two essential properties with c-*rel*: (i) binding to κB sites (5, 29, 38, 42, 44, 45, 51) and (ii) the formation of large molecular

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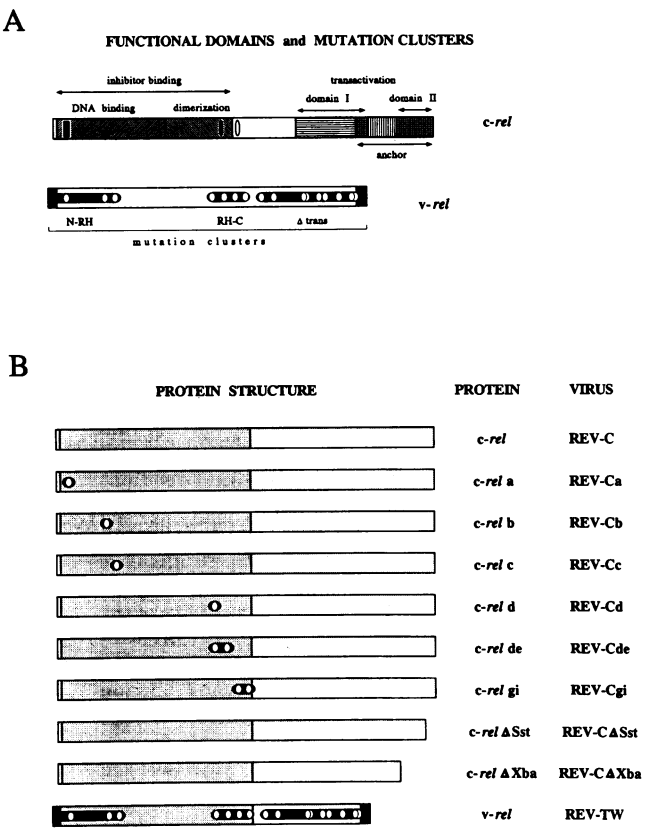

FIG. 1. Structures of c-*rel* and v-*rel* mutants. (A) Functional domains of c-*rel* and localization of mutation clusters present in the v-*rel* oncogene. Mutations by which v-*rel* differs from both turkey and chicken c-*rel* are indicated. The black box in the DNA-binding domain represents the RxxRxRxxC sequence. The white elipse indicates the position of the nuclear localization sequence, and the black ellipse indicates the position of the recognition site for cyclic AMP-dependent protein kinase. In the C-terminal part of c-*rel*, transactivation domains I and II overlap the domain responsible for cytoplasmic anchoring. The location of mutations in v-*rel* is divided into three regions: (i) the N-RH mutation cluster that contains point mutations *a*, *b*, and *c* as well as the N-terminal deletion of 2 amino acids of c-*rel* associated with the addition of 11 viral amino acids (black box); (ii) the RH-C cluster containing 4 amino acids substitutions (*d*, *e*, *g*, and *i*) in the carboxy region of the RH domain; and (iii) a mutation cluster designated here as Δ*trans*, representing a large C-terminal deletion of 118 amino acids and their replacement by 18 *env*-derived amino acids (black box) and several amino acids changes. (B) The protein structure of mutant *rel* proteins derived from parental c-*rel* by either site-directed mutagenesis or exchange of a homologous DNA fragment from v-*rel*. The darker amino half of the protein is the RH domain, while the lighter half represents the less conserved carboxy region. The protein nomenclature and corresponding viruses are shown.

complexes that contain several proteins, including NF-κB1 and IκBα (15, 24, 25, 43, 52).

The v-*rel* protein is highly oncogenic. REV-T infection of several distinct hematopoietic cells results in rapid tumor development in vivo and transformation in vitro (for a review, see reference 11). In contrast, similar retrovirus-mediated expression of c-*rel* possesses only marginal transformation potential (54). By comparing the properties of v-*rel* and c-*rel*, several attempts have been made to define functional alterations of v-*rel* that are responsible for its oncogenic potential. While differential subcellular localization of $p59^{v\text{-}rel}$, $p68^{c\text{-}rel}$, and several v-*rel* mutants has been noted, no correlation between localization and their ability to establish transformation has been described (31, 32). By using transient expression assays that measure transactivation, other analyses have established that c-*rel* possesses greater transactivation activity than v-*rel* (60). On the basis of apparent repression of transactivation mediated by v-*rel*, several studies have suggested that dominant inhibition of gene expression from κB sites was responsible for transformation (4, 5, 27, 38, 49, 60). It should be noted, however, that other experiments have described v-*rel* as possessing the ability to induce gene expression and/or transactivate (10, 29, 35, 63, 68).

Recent studies in this laboratory have demonstrated a correlation between the ability of v-*rel* or c-*rel* (and their hybrids) to induce endogenous gene expression and their transformation potential (36, 55). On the basis of these data, we proposed that gene expression that is both regulated by c-*rel* and essential for lymphocyte activation, lymphoblast formation, and cell proliferation is induced in a signal-independent manner through v-*rel* acting as a dominant positive mutant. This unregulated activity may be explained in part by a reduction in the ability of IκBα to inhibit $p59^{v\text{-}rel}$ binding to κB sites. The presence of mutations in the RH domain of v-*rel* that are responsible for this phenotype has been described (26).

Our analysis of the in vivo tumorigenic potential of c-*rel* generated a large number of C-terminal deletions of this gene. An examination of their properties has revealed a positive correlation between C-terminal deletion, tumorigenesis, and nuclear localization of the protein. We have examined the ability of several point mutations to promote tumor development through cooperation with c-*rel* proteins from which C-terminal sequences have been deleted. This experiment has defined two distinct mutation clusters in the RH region that can be distinguished genetically, suggesting that two different functions are altered in this domain.

MATERIALS AND METHODS

Plasmids. All plasmids were constructed by standard recombinant DNA techniques (62). c-*rel* mutants lacking 55 (ΔXba) or 16 (ΔSst) amino acids of C-terminal sequence were constructed as previously described (55). Point mutations *a* (Met-20→Thr), *b* (Asp-82→Gly), *c* (Arg-97→Glu), *d* (Arg-250→Gly), and *de* (Arg-250→Gly and Glu-269→Ala), mutations corresponding to alterations in v-*rel* (Fig. 1), were introduced into chicken c-*rel* (16) by site-directed mutagenesis with a Muta-gene Phagemid in vitro Mutagenesis Kit (Bio-Rad Laboratories, Inc., Richmond, Calif.). The mutated regions were sequenced to verify the amino acid alterations and cloned as *Xho*I-*Sca*I or *Hin*dIII-*Bam*HI fragments into a plasmid to replace the homologous region of c-*rel*. The double point mutation *gi* (Tyr-286→Ser and Leu-302→Pro) was constructed by cloning a *Bam*HI-*Hin*dII fragment from v-*rel* into the c-*rel* gene. All these c-*rel* mutants were constructed within the adaptor phagemid (pc-rel2) containing c-*rel*, and final constructs were assembled by transfer of *Xho*I-*Bss*HII fragments to the REV-T-based vector REV-0 (21, 55). REV-C and REV-TW express c-*rel* and v-*rel*, respectively (36). The virus REV-TCC expresses a hybrid *rel* protein that contains the amino-terminal one-third of v-*rel* and has been described previously (55). Construction of this chimeric gene was facilitated by exchanging homologous fragments of c-*rel* and v-*rel* generated by *Hin*dIII digestion. The nomenclature of other *rel* proteins and corresponding viruses is presented in Fig. 1. pCSV11S3 contains an infectious genome of chicken syncytial virus (36). pSW253 is an infectious genomic clone of REV-A in

pBR322 (20). pREVA6 is the REV-A genomic clone from pSW253 recloned as an *Eco*RI fragment into pUC19.

Tissue culture, cell lines, and viruses. Cells were cultured in vitro with Dulbecco's modified Eagle's medium supplemented with 5% bovine calf serum (HyClone Laboratories, Inc., Logan, Utah), 5% chicken serum (GIBCO Biologicals, Grand Island, N.Y.), and antibiotics unless otherwise stated. DT95 is an avian B-cell line derived from a chicken with avian leukosis virus-induced lymphoid leukosis (1). Chicken embryonic fibroblasts were prepared from 10-day-old embryos. Secondary cultures of chicken embryonic fibroblasts were used for transfection of plasmid DNAs by a modified calcium-phosphate method as described previously (18, 36). Viruses were harvested between 5 and 7 days after transfection, and infectious titers of both *rel*-expressing viruses and chicken syncytial virus (CSV) were determined by an immunochemical titration assay (36, 66).

Tumorigenesis assays and generation of cloned cell lines. The tumorigenesis assay was performed as described previously (55). One-day-old chicks were infected via intravenous injection of 0.2 ml containing 10^5 infectious units of *rel*-expressing retrovirus per ml. Birds infected with REV-TW(CSV) or CSV were sacrificed 7 days after infection. Birds infected with other viruses were killed 14 days postinfection. Body weight and spleen weight were determined for each bird. The spleen was passed through nylon mesh into Dulbecco's modified Eagle's medium supplemented with 5% bovine calf serum and 15% chicken serum. Cell suspensions, 20 mg of spleen per ml, were used to prepare fivefold dilutions [REV-TW(CSV)-infected spleens] or twofold dilutions (all other infected spleens). Two milliliters of suspension from each dilution was plated per 12 wells (96-well tissue culture plates). Cell growth was scored after 2 weeks. Wells were considered positive when at least one-third of the surface was covered by transformed cells. For each bird, clones from the last dilution positive for growth were examined by Western immunoblot analysis between 1 and 3 weeks after seeding to tissue culture.

Monoclonal antibodies, immunofluorescence, and flow cytometry. Cell surface protein expression was examined by indirect immunofluorescence and flow cytometry with specific monoclonal antibodies. The monoclonal antibody HY19 detects avian immunoglobulin M (IgM) protein (7). TCR-2 (19, 22) recognizes the avian 90-kDa α/β T-cell receptor (α/βTCR), and CT4 and CT8 (17) are specific for the avian homologs of CD4 and CD8, respectively. The antibodies F21-2 (23, 50), HY32 (6), and B337 (46) detect avian class I and class II proteins of the major histocompatibility complex (MHC) and the interleukin-2 receptor (IL-2R), respectively. HY87 is specific for avian c-*rel* and viral v-*rel* proteins (36). CSV expression was detected in titration assays with monoclonal antibody HY83 (36). Anti-mouse class-specific IgG antibodies were purchased from Southern Biotechnology Associates.

Indirect immunofluorescence was carried out by a standard protocol (36). Flow cytometric analysis of the live cells has been described previously (36). Briefly, cells were incubated with a specific monoclonal antibody and then fluorescein isothiocyanate-conjugated goat anti-mouse class-specific IgG antibody. Dead cells were eliminated by staining with propidium iodide (10 μg/ml). Analysis was provided by FACScan, using the LYSIS program (Becton-Dickinson, Fullerton, Calif.).

Western analysis. Western analysis was performed as described previously (36). Briefly, harvested cells were washed, resuspended in suspension buffer, boiled in sodium dodecyl sulfate (SDS) sample buffer, and separated on a SDS-polyacrylamide gel with a Mini-PROTEAN II or PROTEAN II xi apparatus (Bio-Rad). Proteins were transferred to nitrocellulose and sequentially reacted with monoclonal antibody HY87, goat anti-mouse IgG1 biotinylated antibody, and streptavidin-linked alkaline phosphatase (Boehringer Mannheim, Corp., Indianapolis, Ind.). Proteins were visualized by enzymatic reaction with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride as substrates (Boehringer Mannheim). Biotinylated molecular weight markers from Bio-Rad were utilized for construction of calibration curves.

Nuclear and cytoplasmic fractions of avian lymphoid cells were prepared with hypotonic buffer (50 mM Tris-HCl [pH 8.0], 1.1 mM magnesium chloride, 0.5% Triton X-100) as described by Morrison et al. (51).

Southern analysis. High-molecular-weight DNA (10 μg) was digested with 50 U of specific restriction enzyme. Digested DNA fragments were separated by agarose gel electrophoresis in 1× TBE (TBE is 0.09 M Tris-borate, 2 mM EDTA [pH 8.0]). The gels were treated in 0.25 N HCl for 15 min, in denaturing solution (0.4 N NaOH, 0.6 M NaCl) for 1 h, and then in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.5]) for 1 h. The DNA was transferred to an Immobilon N membrane (Millipore Corp., Bedford, Mass.) in 10× SSC (1× SSC is 15 mM sodium citrate plus 150 mM NaCl [pH 7.2]). DNA-membrane binding was fixed by air drying at room temperature. Specific fragments of plasmid DNA were labeled with [α-^{32}P]dCTP by a nick translation system (Bethesda Research Laboratories, Gaithersburg, Md.). *Xho*I-*Bss*HII (probe c-rel) and *Eco*RI-*Bss*HII (probe EcoCrel) fragments of pc-rel2 were used to detect *rel* sequences (see Fig. 3). Southern blots were prehybridized and hybridized in a solution of 0.5 M $Na_2HPO_4 \cdot NaH_2PO_4$ (pH 7.2), 5 mM EDTA, 0.1% bovine serum albumin, 0.1% Ficoll (molecular weight, 400,000), and tRNA (0.1 mg/ml) at 68°C. Filters were washed briefly in 2× SSC–0.5% SDS at room temperature and once for 30 min at 68°C and briefly in 0.1× SSC at 20°C.

RESULTS

Analysis of transformed cell lines obtained following in vivo infection with REV-C. In order to measure the tumorigenic potential of the c-*rel* gene, we infected 1-day-old chicks with REV-C, a recombinant retrovirus that expresses the avian c-*rel* gene and that replicates by using CSV as a helper. Within 2 to 3 weeks, all eight chickens developed splenic tumors from which 25 cell lines were established independently in vitro. Phenotypic analysis of these lines revealed that they all expressed T-cell markers (data not shown). Nine cell lines were analyzed in detail (Table 1). Eight of the nine lines expressed high levels of avian 90-kDa α/βTCR, in combination with either the CD4 or the CD8 protein. The prevalence of the $CD4^+$ $CD8^-$ phenotype suggested that the majority of cell lines immortalized by the weakly transforming retrovirus was derived from the population of helper T cells. Furthermore, all these cell lines expressed high levels of surface MHC class I and class II receptors as well as the IL-2R, indicating they have the phenotype of an activated T cell.

Western analysis revealed the presence of at least one species of deleted c-*rel* protein in each cell line (Fig. 2). Most of these deleted proteins were similar in size to c-*rel*ΔXba and c-*rel*ΔSst (Fig. 1B). However, one cell line (C1) possessed at least two c-*rel* mutants with deletions substantially larger than that present in v-*rel*. In several cell lines (C1, B1, 174/9, 174/4, 117/1, and 116/3), the intensity of a protein corresponding to the size of c-*rel* is significantly increased. Most likely this fact suggests the presence of an additional exogenous c-*rel* gene expressed from a proviral long terminal repeat. This explana-

TABLE 1. Phenotypic analysis of lymphoid cell lines established from splenic tumor of animals infected with REV-C(CSV) or REV-C(REV-A)

Cell line[a]	Staining intensity with monoclonal antibody against a designated antigen[b]						
	IgM	90-kDa α/βTCR	CD4	CD8	MHC class I	MHC class II	IL-2R
C1	−	++	+	++++	++++	++++	+++
D6	−	+++	+++	−	++++	++++	++++
B1	−	++++	++++	−	++++	+++	+++
200/4	−	++++	+++	−	++++	+++	+++
174/9	−	++	+++	−	++	++	++
174/4	−	++	+	++	++++	+++	+++
189/2	−	−	+++	−	++++	+++	+++
117/1	−	++++	++++	+	++++	++++	+++
116/3	−	+++	+	−	++++	++++	++

[a] The cell lines were derived from splenic tumors from chickens infected with REV-C(CSV) or REV-C(REV-A) (cell lines 174/9 and 174/4) and cultivated in vitro for at least 3 months before characterization. All cell lines were further analyzed by Western analysis (Fig. 2). Southern analysis of D6, 200/4, 174/1, 189/2, 117/1, and 116/3 cell lines is documented in Fig. 3.

[b] Cell lines were analyzed by indirect immunofluorescence staining with monoclonal antibodies specific for the antigens indicated. Staining intensity was classified by eye into five groups: ++++, very high; +++, high; ++, medium; +, low; −, negative. The cell lines were homogenous (90 to 100%) for the level of staining for any particular antigen. At least 10^3 cells were analyzed for each antigen.

tion was supported by Southern analysis (see Fig. 3, digest *Bam*HI-*Bst*UI, fragment 960 bp) that demonstrated the presence of unalterated REV-C provirus in these cell lines (data shown for cell lines 174/4, 117/1, and 116/3). A less likely alternative suggests that increased expression of c-*rel* protein was due to induction from an endogenous promoter.

Southern analysis designed to characterize the integration patterns of REV-C proviruses present in different cell lines established from one tumor revealed that many cell lines carried a large number of proviral structures, as many as 10 proviruses per clone integrated in different sites (Fig. 3, *Eco*RI digest). This finding revealed the polyclonal character of a tumor. Analysis of internal proviral structure with a *Bst*UI-*Bam*HI digest revealed that all lines possessed at least one REV-C provirus with a c-*rel* C-terminal deletion. In each line analyzed, an N-terminal *Bst*UI-*Bam*HI fragment (888 bp) containing exons 0 to 5 (69) was present, while a predicted *Bam*HI-*Bst*UI C-terminal c-*rel* fragment (960 bp) was missing or its intensity was significantly decreased. Each cell line contained a new fragment, usually with the same size for lines derived from the same animal. The majority of these aberrant fragments are larger than the expected REV-C *Bam*HI-*Bst*UI fragment (960 bp), indicating the loss of a *Bst*UI (*Bss*HII) site. In some cases, the intensity of these fragments was greatly reduced, suggesting a fragment with little *rel* homology. Cell lines 117/1 and 117/4 produced additional fragments smaller than 960 bp and further *Xho*I-*Bss*HII analysis (unique cloning sites for c-*rel* in the REV-0 vector) revealed an internal deletion of approximately 50 bp (data not shown). These data suggest that all cell lines derived from chicks infected with REV-C carried at least one REV-C provirus with a C-terminal deletion of c-*rel* and that the majority of cell lines established from a single bird contained a identical deletion. In addition, these findings suggested that the most likely explanation for the presence of deleted c-*rel* proteins in the analyzed lines (Fig. 2) is the large alteration of provirus involving coding sequence of c-*rel*.

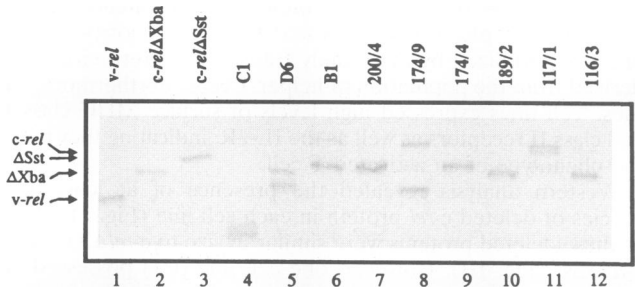

FIG. 2. Western analysis of cell lines derived from tumors obtained from REV-C(CSV)-infected birds. Lanes 1, 2, and 3, protein lysates from CEF infected with REV-TW(CSV), REV-CΔXba(CSV), and REV-CΔSst(CSV), respectively. The locations of these *rel* proteins, as well as the location of c-*rel*, are indicated on the left. Lanes 4 to 12, analysis of nine T-cell lines (Table 1).

Evolution of c-*rel* oncogenic potential on the background of single- or double-point mutations. v-*rel* appears to have acquired increased tumorigenic potential with the addition of several distinct amino acid alterations of c-*rel* (Fig. 1A). In order to evaluate the oncogenic potential of a particular mutation as well as its ability to cooperate with a C-terminal deletion, a set of point mutations was introduced into the backbone of c-*rel* (Fig. 1B). We focused on two clusters of mutations present in the RH domain of v-*rel*: (i) *a*, *b*, and *c* are located in the region of v-*rel* nearest the N terminus (N-RH), while (ii) *d*, *de*, and *gi* are present in the 3′ portion of this domain (RH-C). One-day-old chickens were infected with retroviruses expressing different *rel* protein and analyzed in order to (i) describe the pathologic effect, (ii) determine the ability of tumor cell growth in vitro, and (iii) examine the structural characteristics of the *rel* protein expressed by these clones.

(i) Pathologic effects. Chickens were sacrificed 2 weeks after infection, and autopsy data were collected (Fig. 4A and B). The influence of *rel*-expressing viruses on both body weight and the weight of the spleen, the site of extensive tumor development, was determined. A significant decrease in body weight was observed only in animals infected with REV-Cd, REV-Cde, REV-Cgi, REV-CΔXba, and REV-TW (Fig. 4A). Enlargement of the spleen from animals infected with c-*rel*-based constructs were 140 to 250% the size of spleens from CSV-infected birds, while those from REV-TW-infected birds were 460% of the size of the control organ. The most prominent effect among c-*rel*-based viruses was produced by REV-C itself or by REV-Cb and REV-Cd (Fig. 4B).

(ii) Tumor cell growth. Splenic tumors from the experiment described above were analyzed in the tumorigenic assay for the presence of transformed cells able to grow in vitro (Fig. 4C). Tumors from animals infected with virus expressing c-*rel*d, c-*rel*de, c-*rel*ΔXba and v-*rel* proteins produced more clones than c-*rel*-, c-*rel*a-, c-*rel*b-, c-*rel*c-, c-*rel*gi-, or c-*rel*ΔSst-derived tumors (Fig. 4C). Values, expressed with a logarithmic scale and compared with REV-TW-derived clones, revealed 100-fold differences in tumor development. We have shown previously that the difference between REV-TW(CSV)- and REV-C(CSV)-infected chicks is about 10^6-fold when the chicks are sacrificed at the same time (55). The comparison of these results with the autopsy data (Fig. 4A and B) demonstrated that the growth of in vitro-transformed cells correlates better with the decrease of body weight than with the increase of splenic tumor mass. The in vitro assay likely represents properties of tumor cells other than the local growth in spleen tumors.

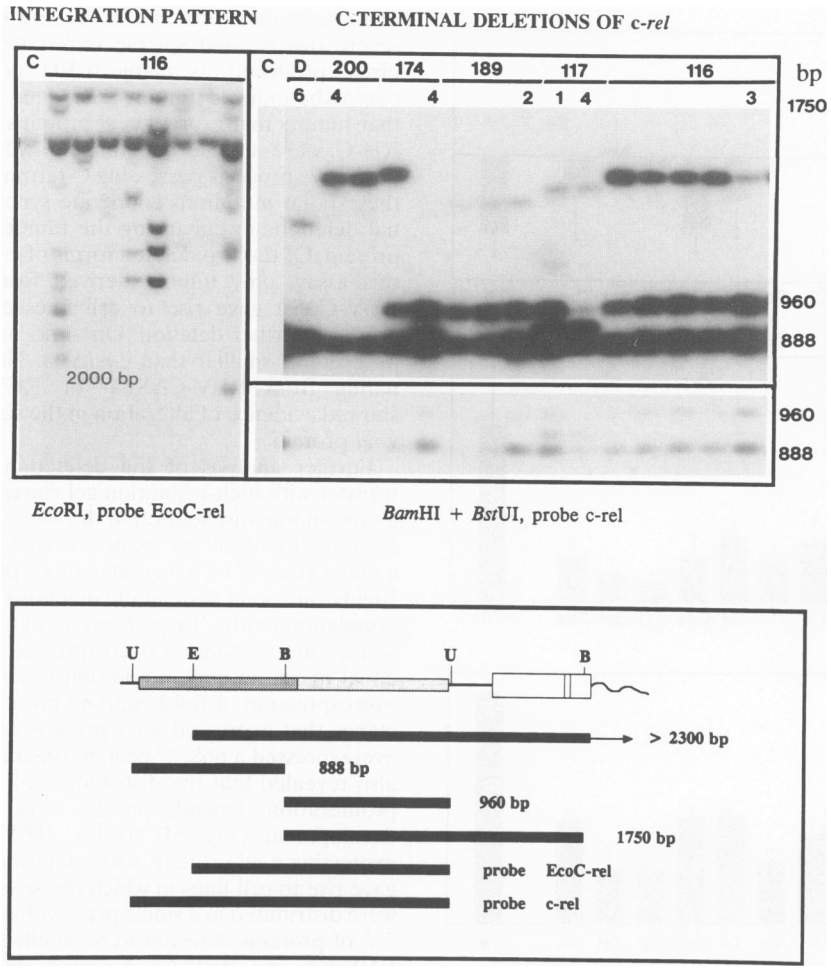

FIG. 3. Southern analysis of cell lines derived from c-*rel*-induced tumors. The left panel displays an *Eco*RI analysis of DNAs from different cell lines separated in a 0.7% agarose gel. The filter was hybridized with the EcoC-rel probe. The right panel shows an analysis by *Bst*UI-*Bam*HI separated in a 1.2% gel. The lower box displays part of the gel shown after a shorter exposure. Two parental c-*rel* *Bst*UI-*Bam*HI fragments (960 and 888 bp) are indicated on the right. The filter was hybridized with a c-rel probe. The lanes are designated by a number or letter (upper) that identifies the animal from which the clone was derived. In some cases, a number (lower) identifies a clone that is described in Table 1 and the legend to Fig. 2 (cell lines D6, 200/4, 174/4, 189/2, 117/1, and 116/3). The structure of c-*rel* inserted into the REV-0 vector and recognition sites for restriction enzymes used in the Southern analysis are shown. The filled two-tone box represents the c-*rel* gene inserted into the vector and the open tripartite box represents the viral long terminal repeat. Restriction enzyme sites: B, *Bam*HI; E, *Eco*RI; U, *Bst*UI. The c-rel and EcoC-rel probes are designated. Filled black boxes represent probes, and filled gray boxes illustrate DNA fragments detected by these probes. The 1,750-bp *Bam*HI fragment should not be detected in a *Bam*HI-*Bst*UI double digest, but deletion of the c-*rel* C terminus leading to loss of the internal *Bst*UI generates fragments between 960 and 1,750 bp in size. Fragments between 888 and 960 bp were generated by an internal deletion between the 5′ *Bam*HI and 3′ *Bst*UI sites (data not shown). Control DNAs from uninfected SC chicken (designated C) are shown in the first lane of each panel. *Bam*HI-*Bst*UI digestion produced fragments of endogenous c-*rel* larger than 2.8 kb which are not visible on the picture.

(iii) Structural characteristics of the *rel* protein. All cell lines developed from chicks infected by REV-C possessed deletions in the C terminus of c-*rel*, indicating that this type of alteration of c-*rel* is frequently selected for its ability to promote proliferation (Fig. 2 and 3). It was anticipated that other viruses expressing c-*rel* proteins into which mutations had been introduced by site-directed mutagenesis would also be subject to the acquisition of such deletions. The more oncogenic the original mutation, the less likely that C-terminal deletions would contribute to selection of a *rel* protein during one cycle of tumor development. However, the oncogenic potential of a virus expressing a *rel* protein with a point mutation that has also acquired a C-terminal deletion during retroviral replication will be a function of not only the original point mutation and the acquired deletion but also of any cooperative effects resulting from the coexpression of the two mutations in a single protein. In the following experiments, the frequencies with which different viruses expressing mutant c-*rel* proteins acquire C-terminal deletions were examined.

Western analysis was used to detect the presence of deleted *rel* proteins in cloned cell lines developed from chickens infected with the REV-based *rel* retroviruses examined in the tumorigenesis assay (Fig. 4C). Table 2 summarizes the data from 290 different clones. Consistent with earlier findings described in the legends to Fig. 2 and 3, 55 clones derived from chickens infected with REV-C expressed, without exception, deleted proteins derived from $p68^{c\text{-}rel}$. In contrast, a significant number of clones expressing c-*rel* proteins with point mutations *a*, *b*, or *c*, located in the distal N terminus of v-*rel*, in which the protein was apparently unaltered in size, were isolated

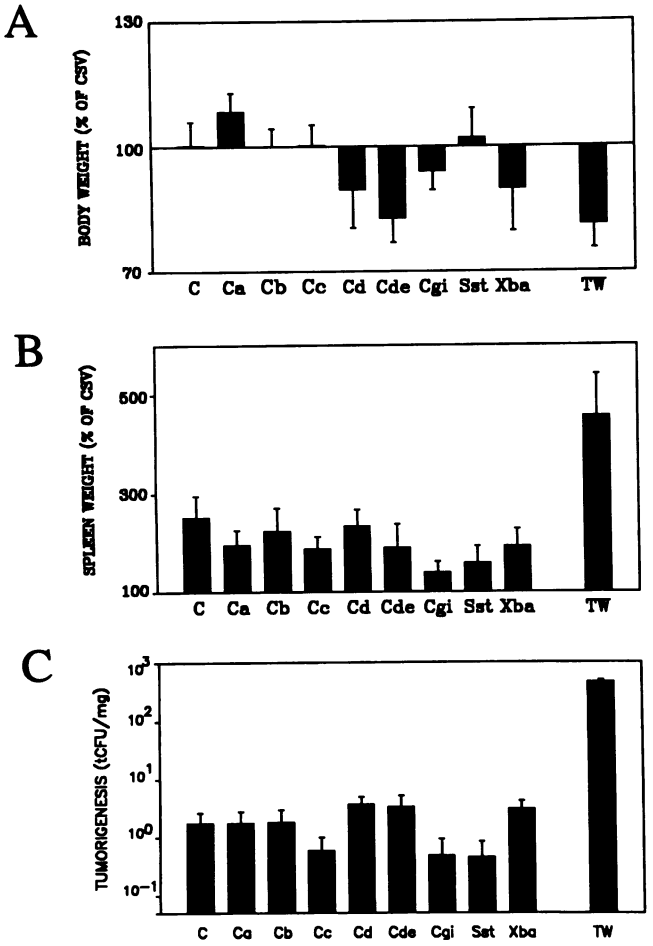

FIG. 4. Tumorigenic potential of viruses expressing different c-*rel* mutants. Birds were injected intravenously with 0.2 ml of the virus (10^5 infectious units/ml). To facilitate comparisons of these experiments with others previously reported, two chickens infected with REV-TW, a recombinant retrovirus that expresses v-*rel* and that replicates with CSV as a helper, were included as a positive control. REV-TW-infected birds were sacrificed 7 days after infection, just prior to death, and the autopsy data were compared with those of CSV-infected chickens of the same age. Other infected birds were sacrificed after 2 weeks. Each experimental group includes at least five animals. Viral abbreviations are presented in Fig. 1. Data are expressed as means and standard errors of the mean. (A) Whole-body weights were determined and expressed as percentages of average whole-body weight of CSV-infected birds. (B) Spleen weights were determined and expressed as percentages of average spleen weight of CSV-infected birds. The spleens from all birds were analyzed by tumorigenic assay. With the exception of spleens from CSV-infected birds, all spleens produced in vitro-growing clones. (C) Tumorigenic splenic assays were performed as described in Material and Methods. Assays were read after 2 weeks. The *y* scale is logarithmic. Results are expressed as tumor CFU (tCFU) per milligram of spleen. The tCFU is determined by end point dilution of a cell suspension from a tumorous spleen and is the quantity of splenic cells required to establish in vitro tumor cell growth.

(Table 2). Of these three proteins, c-*rel*b was found most frequently as a full-length protein (56% of the clones), indicating that among the changes examined, the Asp-82→Gly mutation had the greatest influence on oncogenicity. However, none of the proteins with mutations in the RH-C domain, including *d*, *de*, and *gi*, were isolated as 68-kDa molecules. Two of these mutant proteins, c-*rel*d and c-*rel*de, which produced severe disease and splenic tumor from both these infected birds, produced as many tCFU per milligram as did a c-*rel*ΔXba-induced tumor. The data support the conclusion that tumors that express c-*rel* proteins with point mutations in RH-C were unable to compete with tumors that express derivative proteins possessing C-terminal deletions. Therefore, these point mutations cooperate synergistically with C-terminal deletions in enhancing the tumorigenic potential of a *rel* protein. Of the two deleted forms of c-*rel* that were assessed in this assay, only tumors derived from chicks infected with REV-CΔSst gave rise to cell lines expressing proteins that arose by further deletion. One-fifth of these clones expressed *rel* proteins smaller than c-*rel*ΔSst. None of the clones originating from REV-CΔXba- or REV-TW-infected spleens showed evidence of alteration in the size of either c-*rel*ΔXba or v-*rel* protein.

Further analysis of the deleted *rel* proteins by Western transfer with high-resolution gel electrophoresis demonstrated a nonrandom distribution in the size of the acquired deletions. Examination of all the clones revealed a size distribution with a major peak of 62 kDa and a minor peak of 46 kDa (Fig. 5A). Eighty-one percent of all the deletions are present in the major population (with a range from 55 to 65 kDa). Seventy percent of the clones analyzed overexpressed a *rel* protein apparently equal in size to c-*rel* in addition to a deleted *rel* protein. This overexpression of full-length *rel* proteins was not random. All clones that expressed a *rel* protein smaller than 59 kDa also overexpressed a $p68^{c\text{-}rel}$ protein (data not shown). Our analysis also revealed that the distribution of *rel* deletions selected by proliferation depends on the virus used to induce tumor development (Fig. 5B to D). REV-C, as well as viruses expressing c-*rel* proteins with mutations in the RH-C domain, gave rise to cell lines in which the sizes of the deleted proteins were distributed in a single peak with a median of 61 kDa. Only 5% of proteins detected were smaller than 50 kDa. REV-Ca, REV-Cb, and REV-Cc, however, give rise to cell lines that express deleted *rel* proteins that are distributed into two classes: (i) 41 to 50 kDa (35%) and (ii) 55 to 63 kDa (65%).

Analysis of MHC class I, MHC class II, and IL-2R induction by c-*rel*, c-*rel* mutants, or v-*rel*. In previous studies from this laboratory, the analysis of c-*rel*, v-*rel*, and their hybrids has demonstrated that the transformation potential of a *rel* protein correlates with its ability to induce expression of c-*rel* target genes MHC class I, MHC class II, and IL-2R (36, 55). In this study, we have analyzed individual mutations in the context of c-*rel* for their contribution to tumor development. The same mutations were also examined for their ability to increase the induction capacity of the c-*rel* protein. DT95, an avian B-cell line, was infected with retroviruses that express different mutant *rel* proteins, and the expression of the same three surface immunoregulatory receptors was examined by flow cytometry 4 days after infection. The results demonstrate that all the mutations increased the ability of c-*rel* to induce the expression of MHC class I, MHC class II, and IL-2R (Fig. 6A). The most dramatic effect was produced by *b* and *d* point mutations and the C-terminal deletion of 55 amino acids (c-*rel*ΔXba). Data from flow cytometry used to construct the histogram in which the induction of all three receptors by REV-C and REV-Cb are compared are shown in Fig. 6B.

C-terminal deletions influence the subcellular localization of *rel* protein in DT95 and in *rel*-transformed cell lines. In the clones analyzed in this study, we identified the C-terminal deletion of a *rel* protein as providing a selective advantage for tumor growth. We examined these mutants further to determine whether this deletion influenced the subcellular localiza-

TABLE 2. Structural analysis of *rel* in clones proliferating in vitro[a]

Virus	No. of animals analyzed	No. of clones analyzed	% of clones with altered form of *rel*[b]	Avg no. of altered *rel* forms per animal	Size distribution of deletants (median [kDa])
REV-C	15	55	100	1.2	61
REV-Ca	3	26	58	4	56
REV-Cb	6	49	44	2.5	56
REV-Cc	3	14	78	2.5	57
REV-Cd	5	40	100	1	61
REV-Cde	3	25	100	3	62
REV-Cgi	3	30	100	2	61
REV-CΔSst	3	15	20	2	55
REV-CΔXba	4	20	0	NA[c]	NA
REV-TW	2	16	0	NA	NA

[a] Clones originated from the splenic tumors described in Fig. 4. Clones were derived from the last dilution positive for growth in a tumorigenesis assay. Clones were expanded to at least 10^6 cells and analyzed by Western analysis.
[b] Percentage of clones expressing alterated forms of the *rel* protein from all analyzed clones. The remaining clones expressed only full-length c-*rel* protein.
[c] NA, not applicable.

tion of *rel* proteins in target cells. Retroviruses expressing c-*rel*, c-*rel*ΔSst, c-*rel*ΔXba, or v-*rel* were used to infect DT95. Five days after infection, the cells were fractionated, and nuclear and cytoplasmic proteins were examined for the presence of *rel* proteins (Fig. 7A). The presence of *rel* protein in the nuclear fraction increased gradually with the size of the C-terminal deletion. For cells infected with REV-C, $p68^{c\text{-}rel}$ was predominantly cytoplasmic. In contrast, approximately 50% of $p59^{v\text{-}rel}$ was nuclear in cells infected with REV-TW. Results from an immunofluorescence analysis of the distribution of *rel* protein were in agreement with the findings described above (data not shown). The distribution of *rel* proteins in cells expressing c-*rel* altered by different point mutations was similar to that of $p68^{c\text{-}rel}$, that is, largely cytoplasmic (data not shown). This experiment made use of an avian lymphoid cell line so that localization of different *rel* proteins could be compared directly within the context of an identical cell background. The analysis was repeated, however, with different *rel*-transformed clones.

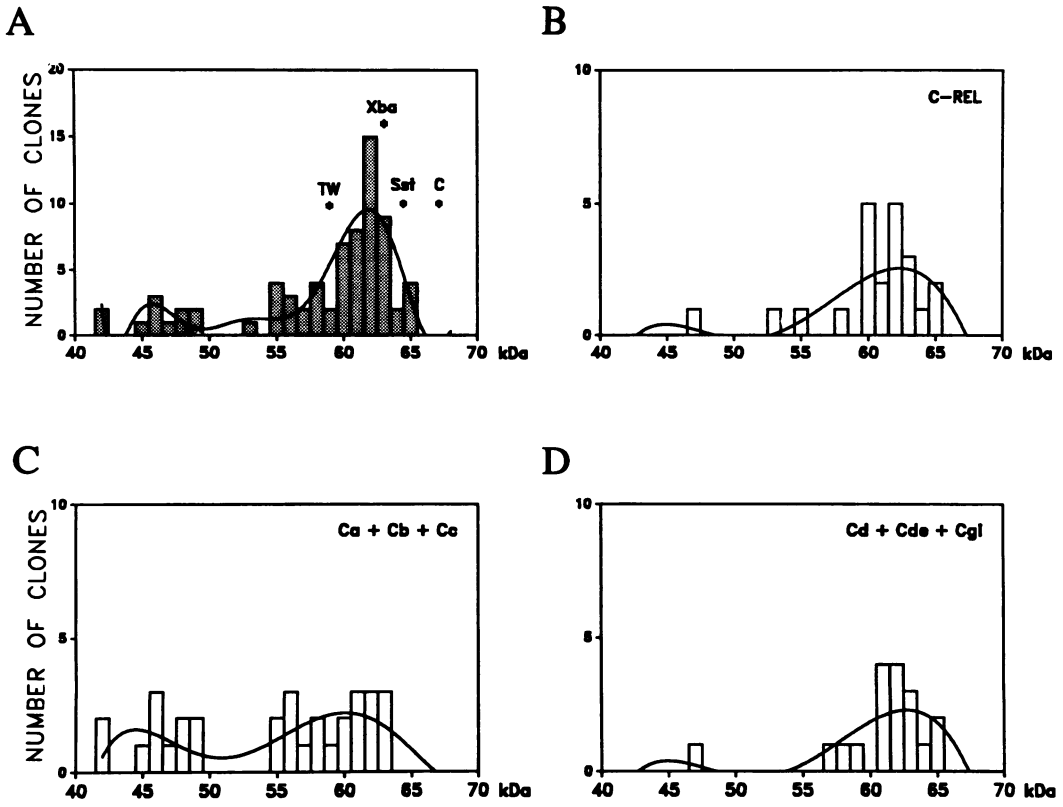

FIG. 5. Size distribution of c-*rel* deletion mutants. All clones containing *rel* deletions were analyzed by Western transfer with a high-resolution 8% polyacrylamide gel. Calibration curves were constructed with biotinylated protein markers. (A) Size distribution in kilodaltons of all deletion mutants characterized in this study. The size distribution of *rel* deletion mutants produced by different viruses, REV-C (B); REV-Ca, REV-Cb, and REV-Cc (C); and REV-Cd, REV-Cde, and REV-Cgi (D), is plotted separately. The regression curves (a ninth-order curve [A] and a third-order curve [B to D]) were generated from the data by computer analysis with Sigma-Plot.

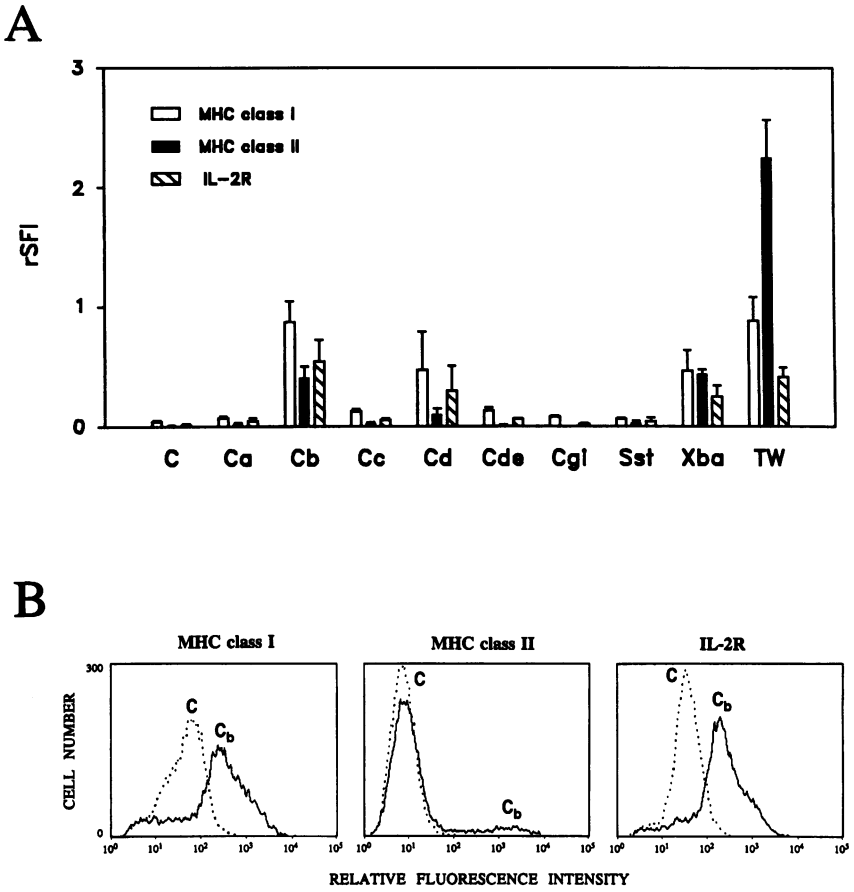

FIG. 6. Induction of MHC class I, MHC class II, and IL-2R on DT-95 by viruses expressing c-*rel* or different c-*rel* mutants. (A) The DT95 cell line was infected with the virus indicated (Fig. 1) at a multiplicity of infection of 2. Four days after infection, expression of surface proteins was analyzed by flow cytometry, and summary immunofluorescence intensity (SFI) was measured. The SFI of cells infected with CSV alone has been subtracted, and results are designated as relative SFI (rSFI). The data represent a mean SFI from three independent experiments. Western analysis of DT95 infected in this experiment failed to detect any alterated form of the *rel* protein (data not shown). (B) Induction of MHC class I, MHC class II, and IL-2R proteins by REV-Cb (Cb) is compared with induction by REV-C (C) and shown as an example of the data used in generating the histogram. The *x* axis represents the relative fluorescence intensity (log scale); the *y* axis represents cell number.

The experiment yielded the same conclusions (Fig. 7B). Moreover, the analysis revealed a substantial increase in the amount of $p68^{c\text{-}rel}$ in the nucleus of a cell line coexpressing a deleted form of *rel* smaller than 50 kDa (Fig. 7B, ΔTCC). This observation suggests that deleted forms of c-*rel* possess a transporter-like function.

DISCUSSION

In vivo evolution of highly oncogenic retroviruses encoding *rel* proteins. Retroviruses are characterized by a high mutation rate. Two processes contribute the majority of these mutations: (i) the low fidelity of reverse transcriptase and (ii) recombination during the process of reverse transcription (37, 58, 59, 70). The high mutation rate of retroviruses creates a large population of viruses from which oncogenic variants able to promote in vivo and subsequent in vitro proliferation of permissive target cells can be selected. Twenty-five cell lines were established from birds infected with REV-C, a retrovirus expressing wild-type $p68^{c\text{-}rel}$. Southern and Western analysis of the cell lines revealed that all cell lines contained altered proviral structures that expressed *rel* proteins with C-terminal deletions. These observations, therefore, indicate that tumor development associated with the expression of $p68^{c\text{-}rel}$, a weakly transforming protein, requires significant alteration of its structure and the selection of a protein variant with increased oncogenic potential. This is consistent with the reported observation that a retrovirus expressing a c-*rel* deletion mutant lacking 55 carboxy-terminal amino acids possessed transforming activity (39). On the other hand, analysis of the in vitro transforming activity of a set of c-*rel* C-terminal deletion mutants revealed no difference when compared with c-*rel*, perhaps because of a lack of sensitivity in the in vitro assay (26).

Southern analysis revealed a second observation. The overwhelming majority of c-*rel* deletion mutants were the result of the loss of large segments of coding sequence frequently associated with the acquisition of helper virus restriction enzyme sites by the long terminal repeat of the defective virus (data not shown). These data suggest that the principal mechanism by which C-terminal deletions were acquired was nonhomologous recombination and not the introduction of translation termination codons resulting from single-base alterations.

Oncogenic potential of c-*rel* and its evolution in vivo are influenced by single-amino-acid alterations. It has been shown

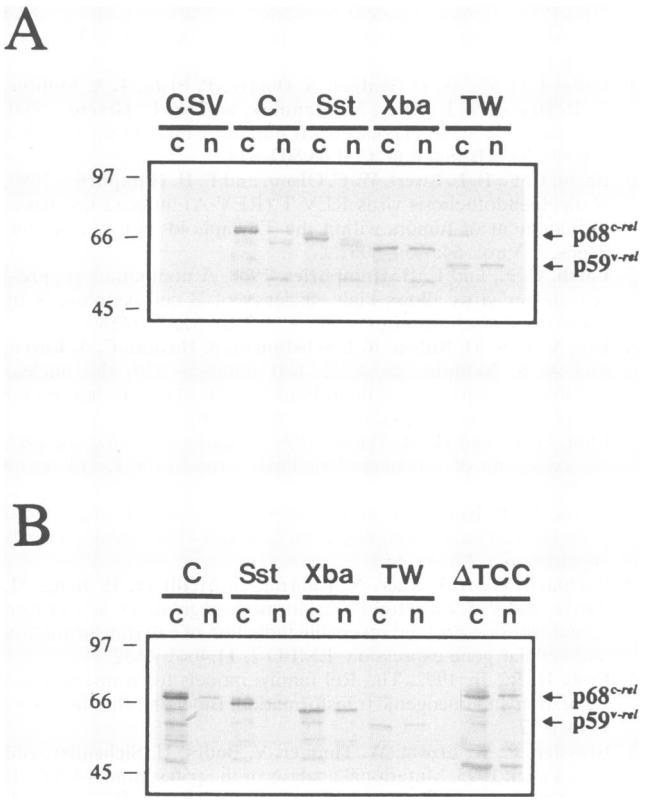

FIG. 7. Influence of C-terminal deletions of *rel* on subcellular localization in the lymphoid cell line DT95 and in *rel*-transformed cell lines. (A) The DT95 cell line was infected with CSV or with viruses expressing c-*rel*, c-*rel*ΔSst, c-*rel*ΔXba, or v-*rel*. Cells were harvested after 5 days, and cytoplasmic (c) and nuclear (n) proteins were prepared for Western analysis. One lane contains protein from subcellular fractionation of 10^5 cells. (B) Cytoplasmic and nuclear fractions were prepared from the cell clones derived from chickens infected with retroviruses expressing c-*rel*ΔSst, c-*rel*ΔXba, or v-*rel*. c-*rel*-transformed cell lines were derived in vitro by infection of splenic cells. The analysis also includes cell clones derived from birds infected with REV-TCC(CSV) and expressing the protein designated tcc-*rel* or derived deletions. Abbreviations: C, c-*rel*; Sst, c-*rel*ΔSst; Xba, c-*rel*ΔXba; TW, v-*rel*; ΔTCC, tcc-*rel*. The locations of $p59^{v\text{-}rel}$ and $p68^{c\text{-}rel}$ are shown on the right. The migration of molecular size markers (in kilodaltons) is shown on the left. The experiments were repeated at least three times, and representative results are shown.

previously that all three point mutations in the N terminus of v-*rel* (designated *a*, *b*, and *c*) as well as several other mutations in the middle of the protein contribute to the transformation potential of $p59^{v\text{-}rel}$ (8a). The analysis presented here distinguishes the oncogenic potential of point mutations in the distal N-RH (*abc*) from those in RH-C (*degi*). First, the autopsy data demonstrate that mutations in the RH-C domain produce significant body weight loss during *rel*-mediated disease. On the other hand, viruses encoding c-*rel* mutants with amino acid changes in the N-RH domain, for example, REV-Ca, produced less-severe disease. Furthermore, birds infected with REV-Cd or REV-Cde yielded the greatest frequency of splenic cells capable of in vitro proliferation. Second, Western analysis demonstrated that all clones expressing a *rel* RH-C mutant also expressed a *rel* protein with a C-terminal deletion. In contrast, chickens infected with retroviruses expressing *rel* with mutations from the N-RH cluster produced a substantial number of clones expressing only the original mutant *rel* protein. Lastly, it is noteworthy that the deletions derived from c-*rel* N-RH mutants displayed a greater degree of variation than those derived from c-*rel* RH-C mutants (Fig. 5C and D). These data are consistent with the conclusion that mutations in the N-RH domain are highly oncogenic and that they do not necessarily require cooperation with C-terminal deletions of c-*rel* to establish tumor development. Furthermore, our observations support the conclusion reported previously that a mutation in the RH-C domain cooperated synergistically with a C-terminal deletion, producing a highly oncogenic protein (26). Taken together, our data support a more general conclusion that mutations in the RH domain of c-*rel* alter two different functions.

Characterization of the C-terminal deletions. The evolutionary origin of a few C-terminal deletions of the c-*rel* gene has been described previously. A transformed splenic cell line expressing a 65-kDa *rel* protein as well as cell lines expressing full-length c-*rel* proteins were derived from an in vitro splenic infection with a retrovirus expressing c-*rel* (54). The detection of a fusion protein with a substantial deletion of c-*rel* C-terminal sequences in a human lymphoma suggests a possible contribution of this alteration to the oncogenic process (48).

In the study reported here, we undertook a systematic analysis of C-terminal deletions of c-*rel* in order to characterize the permissible range for such deletions as well as to identify a deletion of c-*rel* optimal for expression of its tumorigenic potential. The results indicate that transforming mutants of c-*rel* can be as small as 42 kDa and as large as 65 kDa. The optimal size for a transforming derivative of c-*rel* was between 60 and 63 kDa. This size is most likely a compromise between increasing nuclear localization and reducing transactivation, both of which result from increasing the size of the deletion. A second group of deletion mutants were smaller than 50 kDa (Fig. 5A), a size that is large enough to contain an intact RH domain that includes (i) the nuclear localization signal and domains required for (ii) DNA binding and (iii) dimerization and as well as (iv) complex formation with 124-, 115-, and 36- to 40-kDa proteins (16, 51, 63). While *rel* proteins of this size probably lack transactivation activity (14, 60, 63, 68), our analysis revealed an increase in the translocation of full-size c-*rel* into the nuclei of cells that express these smaller *rel* proteins. The observation that all clones with a deleted form of c-*rel* shorter than 59 kDa also express elevated levels of a 68-kDa *rel* protein (apparently from another provirus) suggests a functional cooperation between a full-size *rel* protein and a deleted form of the same gene. A requirement for such cooperation to effect transformation of avian hematopoietic target cells would be similar to that reported earlier for the human c-*rel* protein and its deleted form (67). Therefore, despite the identification of an optimal size of 62 kDa for a single *rel* protein with sufficient transactivation capacity to establish transformation, proteins with substantially smaller size, retaining only the RH domain, may possess a transporter-like function with which they are able to cooperate with other nondeleted NF-κB/*rel* proteins to establish tumor development.

Basis for the evolution of c-*rel* variants. Our analysis distinguishes at least three types of individual mutations in c-*rel* that contribute to the oncogenic potential of this protein. These studies indicate that *b* and *d* are the dominant mutations in the N-RH and RH-C domains, respectively. A strong selection for a C-terminal deletion of c-*rel* to yield a 62-kDa protein nearly corresponding to the c-*rel*ΔXba mutant was observed. Our previous studies have demonstrated that tumorigenesis and transformation potential of *rel* proteins correspond with their ability to induce expression of three c-*rel* target genes: MHC

class I, MHC class II, and IL-2R (36, 55). The tumorigenic potential of the mutants examined in this study, as well as of the additional mutants selected in vivo during tumor development, also correlates with their ability to induce expression of these genes and is consistent with our earlier findings.

The ability to induce c-*rel* target genes may be the most essential property for *rel*-mediated tumor induction. The ability to induce expression may be affected by alterations in several distinct functional domains of c-*rel*. Differential inhibition of *rel* protein-DNA binding by IκBα has been proposed as one possible mechanism (26). Mutations within the RH-C region have been shown to be responsible for this effect. At the same time, mutations within the N-RH domain do not appear to influence this interaction. It is likely that mutations in the N-RH domain will alter general DNA-binding affinity and/or specificity.

A third possibility relates to the consequence of deleting C-terminal sequences of c-*rel*. This deletion has a major effect on the location of this protein. Anchoring of *rel* in the cytoplasm of nontransformed cells (chicken embryonic fibroblasts) has been reported to be a function of the distal C terminus of c-*rel* (16, 31, 35). However, since a majority of v-*rel* was found in the cytoplasm of the transformed cell lines, the significance of v-*rel* in the nucleus in these cells may have been underestimated (25, 31, 32, 35, 51). Data presented here suggest a relationship among tumorigenesis, C-terminal deletions, and subcellular localization of *rel* proteins in lymphoid cells. We have demonstrated (i) that a wide range of C-terminal deletions contribute to tumorigenesis of *rel* proteins; (ii) that an inverse correlation exists between the size of *rel* protein and the proportion of the protein that is in the nucleus of transformed cells; (iii) that for c-*rel* and the c-*rel*ΔXba and c-*rel*ΔSst mutants, the relative amount of *rel* protein in the nucleus correlates with the ability of the mutant to induce expression of target genes; and (iv) that an increased amount of $p68^{c\text{-}rel}$ is nuclear in cell lines that expressed mutants of c-*rel* that are smaller than 50 kDa. Finally, it should be emphasized that our analysis indicates that there is a selective advantage for C-terminal deletion mutants of c-*rel*. This advantage increases as the amount of *rel* protein in the nucleus increases, thereby strongly supporting the hypothesis that v-*rel* directly modulates gene expression. Since translocation of NF-κB/*rel* proteins from the cytoplasm to the nucleus is an essential feature regulating their activity (3), the disruption of this regulation can activate the tumorigenic potential of these genes. It is likely that this type of alteration contributes to human tumorigenesis by the same mechanism as avian c-*rel* deletion mutants (48, 56).

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