JOURNAL OF VIROLOGY, Jan. 1995, p. 403–413
0022-538X/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 69, No. 1

The Relocalization of v-Rel from the Nucleus to the Cytoplasm Coincides with Induction of Expression of *Ikba* and *nfkb1* and Stabilization of IκB-α

RADMILA HRDLIČKOVÁ,[1] JIŘÍ NEHYBA,[1] AVIJIT ROY,[2] ERIC H. HUMPHRIES,[2,3]† AND HENRY R. BOSE, JR.[1]*

Department of Microbiology and the Cell Research Institute, The University of Texas at Austin, Austin, Texas 78712-1095,[1] and Mary Babb Randolph Cancer Center[3] and Department of Microbiology and Immunology,[2] West Virginia University, Morgantown, West Virginia 26506-9300

Received 29 July 1994/Accepted 5 October 1994

The v-Rel oncogene induces the expression of major histocompatibility complex class I and II proteins and the interleukin-2 receptor more efficiently than does c-Rel (R. Hrdličková, J. Nehyba, and E. H. Humphries, J. Virol. 68:308–319, 1994). The kinetics with which these immunoregulatory receptors are induced in B- and T-lymphoid cell lines and chicken embryo fibroblast cultures expressing c-Rel or v-Rel have been examined. v-Rel induced the expression of major histocompatibility complex classes I and II and interleukin-2 receptor more efficiently than did c-Rel at later times after infection. In all three cell types, this increased efficiency was accompanied by a shift in the majority of v-Rel from the nucleus to the cytoplasm. The concomitant relocalization of v-Rel was also demonstrated during the in vitro transformation of spleen cells. The translocation coincided with increased steady-state levels of IκB-α. Coinfection by retroviral vectors expressing v-Rel, IκB-α, or NF-κB1 demonstrated that either IκB-α or NF-κB1 can contribute to the shift of v-Rel to the cytoplasmic compartment. The induction of *nfkb1* and *Ikba* mRNA and the stabilization of IκB-α by v-Rel were shown to be responsible for these effects. In comparison with c-Rel, the expression of v-Rel was associated with lower levels of transcription of these genes. However, the ability of v-Rel to stabilize IκB-α remained unchanged. The ability of v-Rel to stabilize IκB-α but poorly induce *Ikba* mRNA expression relative to c-Rel may play a role in regulating gene expression, thereby leading to transformation.

c-Rel belongs to a group of transcription factors designated the Rel/NF-κB family. These transcripion factors regulate genes which function during defense responses, in healing and regeneration processes, and during development (1, 34). The activity of Rel/NF-κB is regulated by a small family of inhibitors termed IκB. These proteins are present in inactive cytoplasmic complexes in a large number of different cell types. Following extracellular stimulation and intracellular signal transduction, a modified protein complex enters the nucleus, where modulation of gene expression from promoters containing a κB-binding site(s) is effected (34, 58). It is likely that functional interactions between Rel/NF-κB and IκB proteins have coevolved to regulate specific gene expression.

Two major structural features define the members of this family: the highly conserved RH (Rel homology) domain in the transcription factors and the presence of ankyrin repeats in the inhibitory molecules. Rel/NF-κB proteins, including c-Rel, RelA (p65), RelB, NF-κB1 (p105/p50), NF-κB2 (p100/p52), and the *Drosophila melanogaster* morphogen dorsal share homology in the N-terminal RH domain (34, 58). This domain is responsible for DNA-binding and protein-protein interactions that result in dimerization and inhibitor binding (5, 8, 31, 32, 46, 51, 53, 60). At the 3′ end of the RH domain, a nuclear localization sequence has also been identified (33). The carboxy region has been shown to possess transactivating activity in c-Rel, RelA, RelB, and dorsal (10, 25, 48, 49, 75, 79, 89). In addition, the distal portion of the C terminus of c-Rel and dorsal contains a domain that functions in cytoplasmic anchoring (13, 48). In contrast, NF-κB1 and NF-κB2 contain C termini with several ankyrin repeats that are removed by proteolytic cleavage and/or alternative splicing, thereby producing p50 (from NF-κB1) or p52 (from NF-κB2) (28). The ankyrin repeats are also a major structural feature of IκB-α (22, 38, 88), Bcl-3 (29, 50), and the *D. melanogaster* IκB analog cactus (30, 52). Two functional activities have been associated with the IκB ankyrin repeats: (i) inhibition of DNA binding of Rel/NF-κB proteins in vitro (30, 38, 39, 41, 45, 51, 52, 59, 94) and (ii) regulation of nuclear localization in vivo (5, 30, 74, 95).

Rel/NF-κB proteins function to regulate the specific expression of a large number of target genes. Several mechanisms with the potential to modulate Rel/NF-κB and IκB protein activity have been described. These include phosphorylation (31, 52, 57, 71, 85), proteolytic degradation (28, 40, 62, 63), and alternative splicing (45, 68). Furthermore, it is likely that reactive oxygen intermediates are involved directly or indirectly in the signal transduction pathway leading to NF-κB activation (80). Following signal transduction, the pool of Rel/NF-κB and IκB proteins in the cytoplasm is depleted. The induction of transcription of *rel/NF-κB* as well as *Ikba* RNA after signal transduction is likely to play an important function in reestablishing cell responsiveness to additional exogenous signals (88). The presence of functional κB sites in the promoters of *nfkb1*, c-*rel*, and *Ikba* indicates that these genes operate in an autoregulatory pathway (9, 12, 16, 18, 23, 35, 55, 81, 86, 87).

Disruption of the regulation of the Rel/NF-κB network may play a role in the development of some human malignancies (61, 70, 72, 90). An extensively altered form of avian c-*rel*, the oncogene v-*rel*, efficiently induces a variety of neoplastic dis-

* Corresponding author. Mailing address: Department of Microbiology, The University of Texas at Austin, Austin, TX 78712-1095. Phone: (512) 471-5525. Fax: (512) 471-2130.

† Deceased 11 December 1993.

eases in the chicken (76). The major alteration in c-Rel to form v-Rel involves a truncation of the C terminus that eliminated both a large part of the transactivation domain and a cytoplasmic anchoring sequence (13, 36, 49, 75). The deletion of the C terminus results in an increase in the amount of Rel in the nucleus of transformed cells and substantially contributes to the tumorigenesis of v-Rel (43). Further, v-Rel differs from turkey c-Rel by an additional 14 single-amino-acid changes and three small in-frame deletions (92). The majority of these small mutations contribute to the transformation potential of v-Rel (6). Mutations in the C-distal end of the RH domain appear to be responsible for decreased inhibition of v-Rel DNA binding by IκB-α (24). The contributions of other mutations in v-Rel to the disruption of Rel/NF-κB and IκB autoregulation and subsequent transformation are not known.

We have demonstrated previously that v-Rel induces the surface expression of major histocompatibility complex (MHC) classes I and II as well as the interleukin-2 receptor (IL-2R) on the avian B-cell line DT95 and the T-cell line MSB-1 more efficiently than c-Rel does (44). In this study we demonstrate that the increased induction of these immunoregulatory proteins by v-Rel was accompanied by a partial relocalization of v-Rel protein from the nucleus to the cytoplasm. This shift coincided with the appearance of increased steady-state levels of IκB-α. Two processes responsible for these effects were analyzed: the induction of *Ikba* and *nfkb1* and the stabilization of IκB-α by Rel proteins. While the ability of v-Rel to stabilize IκB-α was not changed, the ability of v-Rel to induce *Ikba* and *nfkb1* mRNA was decreased relative to that of c-Rel. Finally, coinfection experiments with retroviral vectors expressing v-Rel, IκB-α, or NF-κB1 demonstrated that either IκB-α or NF-κB1 was capable of retaining v-Rel in the cytoplasm.

MATERIALS AND METHODS

Plasmids. All recombinant DNA techniques were performed by conventional methods (77). Construction of pREV-0, pREV-TW, pREV-C, and pCSV11S3 has been described previously (44, 69). pREV-TW plasmid contains v-*rel* (15) cloned into an REV-T-based vector, REV-0. pREV-C is the same vector expressing chicken c-*rel* (13). Similarly, pREV-I is REV-0 into whose *Xho*I-*Bss*HII sites the coding sequence of chicken *Ikba* (22) has been cloned as a 1,060-bp *Eco*RI-*Nde*I fragment by using an adaptor. The retroviral vector 214p105 (a kind gift from A. Capobianco and T. D. Gilmore) was derived from spleen necrosis virus (26) and carries the coding region of chicken *nfkb1* (11). pCSV11S3 contains an infectious genomic clone of chicken syncytial virus (CSV).

Cell lines, transfection, and virus titer determinations. Cells were grown in 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 (100 U of penicillin per ml and 50 μg of streptomycin per ml). The DT95 cell line was derived from an avian leukosis virus-induced bursal lymphoma and expresses surface immunoglobulin M receptor (3). MSB-1 is a chicken T-cell line established from a Marek's disease virus-induced lymphoma (2). Chicken embryo fibroblasts (CEF) were prepared from 10-day-old SC embryos (Hyline International Hatcheries, Dallas Center, Iowa) by standard methods.

Transfection of CEF with plasmids containing different retroviral genomes was performed by a modified calcium phosphate method as described previously (14). Infectious titers of helper CSV and replication-defective viruses were determined by a modified immunocytochemical assay with monoclonal antibodies HY83, HY87, and HY95 (44, 84).

Antibodies, indirect immunofluorescence, and flow-cytometric analysis. Monoclonal antibodies HY87 (44) and 3C1 (42) specifically recognize both avian c-Rel and v-Rel. Monoclonal antibody HY83 recognizes CSV antigen (data not shown). Monoclonal antibody HY95 was prepared against bacterially expressed protein (amino acids 49 to 273) of avian IκB-α (22) by a standard method (37, 91). Monoclonal antibodies F21-2 (19, 64), HY32 (4), and B337 (56) specifically bind avian class I and class II proteins of MHC and IL-2R, respectively. The rabbit anti-chicken Rel antiserum IV raised against a 262-amino-acid segment of $p59^{v\text{-}Rel}$ (83) and anti-chicken IκB-α antisera 1275 and 1276 developed against the N and C termini were used for immunoprecipitation. All three antisera were the kind gift of N. R. Rice.

Indirect-immunofluorescence staining of cell cytospots was performed as follows. Cells were fixed and permeabilized for 5 min with methanol-acetone (1:1), washed twice with phosphate-buffered saline (PBS), and incubated for 30 min with PBS–0.1% bovine serum albumin. The cells were then incubated for 1 h with one or two monoclonal antibodies as indicated, washed with PBS, and incubated for 1 h with fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse antibodies directed against specific subclasses (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Cells were washed thrice with PBS, given a final overnight wash, and mounted with Fluoromount-C (Southern Biotechnology Associates).

Induction of cell surface receptor expression on cell lines was analyzed by staining live cells and quantified by flow cytometry analysis as described previously (44).

Western analysis. Western immunoblot analysis was performed as described previously (44). Briefly, cells were washed, resuspended in suspension buffer, and immediately boiled in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and sequentially probed with the specific mouse monoclonal antibody, goat anti-mouse immunoglobulin G biotinylated antibody, and streptavidin-linked alkaline phosphatase. Staining was developed with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride as substrates.

Nuclear and cytoplasmic fractions of avian lymphoid cells were prepared in hypotonic buffer (50 mM Tris-HCl [pH 8.0], 1.1 mM $MgCl_2$, 0.5% Triton X-100) as described by Morrison et al. (66).

Immunoprecipitation. Pulse-chase experiments were performed as described by Scott et al. (81). DT95 cells (8×10^6/ml) were incubated for 10 min in methionine-free medium supplemented with 10% dialyzed chicken serum and then labeled for 30 min with [^{35}S]methionine (200 μCi/ml; NEN, Boston, Mass.). At the end of the labeling period, the cells were washed and transferred to complete Dulbecco's modified Eagle's medium supplemented with 5% chicken serum and 5% calf serum. Cells (4×10^6) were harvested at each time point and lysed in 1 ml of RIPA buffer (25 mM Tris-HCl [pH 7.4], 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.5 mM EDTA, 150 mM NaCl, 1 μg of aprotinin per ml, 100 μg of phenylmethylsulfonyl fluoride per ml). Each lysate was precleared by centrifugation 10 min at $12,000 \times g$. Lysates were frozen at −70°C. Before immunoprecipitation, each lysate was precleared by incubation with 5 μl of rabbit preimmune serum for 30 min at 4°C. Then 40 μl of protein G-Sepharose 4 Fast Flow (Pharmacia LKB) precoated with cold lysate from DT95 cells was added; after an additional 30 min it was collected by centrifugation. The supernatant fluid was divided between two Eppendorf tubes; one was incubated with 5 μl of anti-Rel rabbit antiserum IV for 3 h, and the other was incubated with anti-IκB-α rabbit antisera (2.5 μl of 1275; 5 μl of 1276) for 6 h. The immune complexes were collected by a 30-min incubation with 30 μl of protein G-Sepharose beads precoated with cold DT95 lysate. The beads were washed twice with RIPA buffer, twice with high-salt buffer (1% Triton X-100, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.6]), and twice with low-salt buffer (1% Triton X-100, 10 mM NaCl, 20 mM Tris-HCl [pH 7.6]). The pellets were resuspended in 50 μl of protein sample buffer (100 mM Tris-HCl [pH 6.8], 2% SDS, 0.05% bromophenol blue, 10% glycerol, 5% β-mercaptoethanol) and boiled for 10 min, and a 10-μl sample was analyzed in an SDS-polyacrylamide gel and visualized by autoradiography.

Northern (RNA) analysis and probes. Total RNA was isolated by acidic guanidinium thiocyanate-phenol-chloroform extraction (17). Total RNA was separated by electrophoresis in a 1% agarose gel in 20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer and transferred to an Immobilon N membrane (Millipore Corp., Bedford, Mass.). Parallel samples with ethidium bromide were analyzed under identical conditions, and the gel was photographed. Filters were hybridized with DNA fragments labeled with [α-^{32}P]dCTP by nick translation (Bethesda Research Laboratories, Gaithersburg, Md.).

The *Hpa*II 1,848-bp fragment derived from the chicken cDNA encoding c-*rel* (13) was used to detect endogenous and exogenous *rel* mRNA. A probe consisting of a mixture of the *Bgl*I 278-bp fragment from the N-terminal portion of the coding region and the *Nde*I-*Eco*RI 658-bp fragment from the nontranslated region (22) was used to detect *Ikba* mRNA. *nfkb1* mRNA was detected by hybridization with a *Bam*HI-*Sst*I 1,314 bp fragment containing the coding sequence of the *rel*-related domain (11). A chicken MHC class II β chain gene was detected by a 260-bp *Pst*I-*Hae*III fragment containing almost the whole exon β2 of the pCCII-4 gene (93).

RESULTS

Analysis of Rel-induced expression of immunoregulatory receptors and subcellular localization of Rel in cells infected with REV-C(CSV) or REV-TW(CSV). In avian lymphoid cells in which endogenous c-Rel is activated and in cells overexpressing c-Rel, the number of MHC class I and II molecules and the IL-2R are upregulated (44). The surface expression of these immunoregulatory molecules is also induced in cells expressing v-Rel but at levels significantly higher than in cells

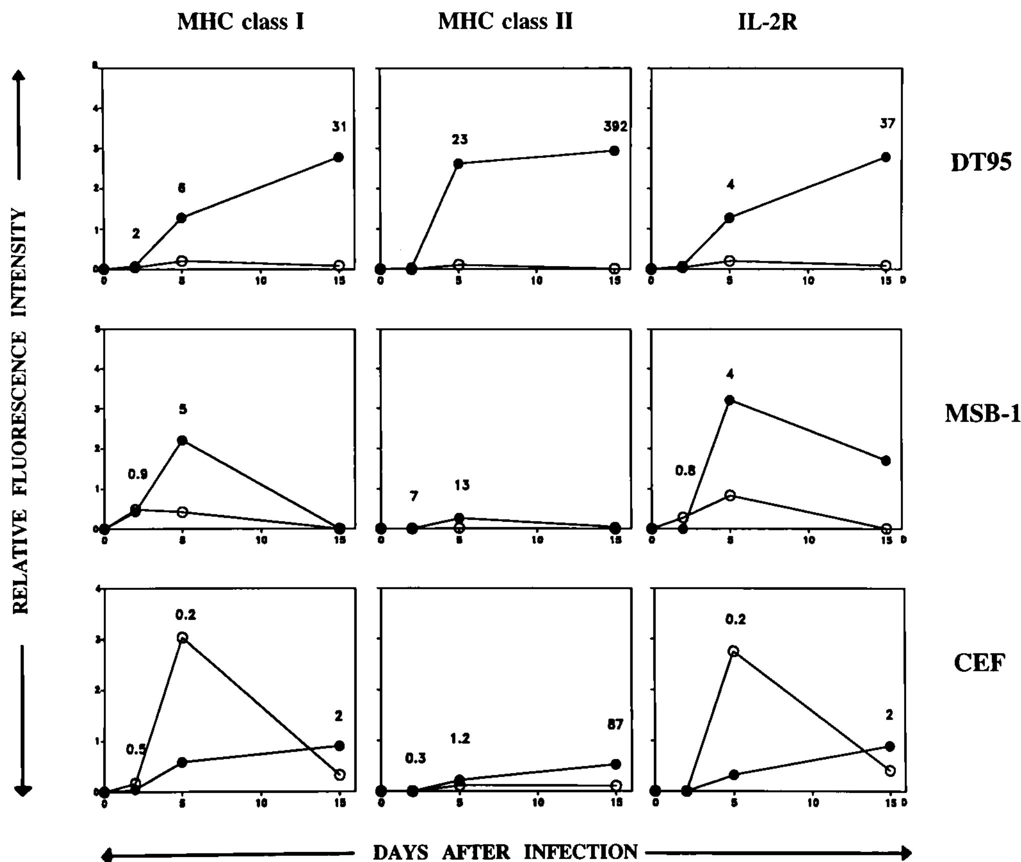

FIG. 1. Kinetic analysis of the expression of MHC class I and II and IL-2R on the surface of the avian B-cell line DT95, T-cell line MSB-1, and CEF following infection with REV-C(CSV) or REV-TW(CSV). DT95, MSB-1, and CEF were infected by Rel-expressing viruses at a multiplicity of infection of 2. At 2, 5, and 15 days after infection, live cells were stained and analyzed by flow cytometry. The x axis represents the time (days) after infection; the y axis represents a summary of relative fluorescence intensity (SFI) of 10^4 cells infected with REV-C(CSV) or REV-TW(CSV) after subtraction of SFI of cells infected with CSV helper virus alone. The y axis scale is the same for the DT95 and MSB-1 cell lines but reduced 10-fold for CEF. The solid circles represent SFI values of cells infected with REV-TW(CSV), and the open circles represent SFI values of cells infected with REV-C(CSV). The numbers inside the graph express the v-Rel/c-Rel ratios of the induced SFI for the protein indicated. The high c-Rel/v-Rel ratios, especially in MHC class II induction in DT95 cells and CEF, reflected the extremely poor induction of this receptor by c-Rel. This experiment was repeated three times with similar results. The data presented in Fig. 2 to 5 were obtained as a result of analyses of this kinetic experiment.

expressing c-Rel. The expression of MHC class I and II proteins and that of IL-2R in DT95, MSB-1, and CEF cultures were examined following infection with REV-TW(CSV) (v-Rel) or REV-C(CSV) (c-Rel), two recombinant retroviruses that replicate with CSV as a helper virus. At the same time, the kinetics of subcellular localization of v-Rel and c-Rel was examined and compared with the kinetics of induction of these immunoregulatory receptors. v-Rel-dependent induction of MHC proteins and IL-2R in DT95 and MSB-1 cultures increased with time, while c-Rel-dependent induction decreased or remained constant (Fig. 1). This difference was most pronounced in the comparative analysis of the induction of MHC proteins and IL-2R in DT95 cells. Western analysis at 2, 5, and 15 days after infection demonstrated that the expression of v-Rel was comparable to or somewhat lower than that of c-Rel in all three cell types (Fig. 2). This finding confirms previous results indicating that, directly or indirectly, v-Rel induces the expression of the MHC proteins and IL-2R more efficiently than c-Rel does (44). The expression of Rel proteins in CEF cultures resulted in 10-fold-less induction of the three immunoregulatory receptors than in the avian lymphoid cell lines. This analysis also demonstrated that 5 days after infection of CEF cultures, c-Rel was a more efficient inducer of MHC class I and IL-2R than was v-Rel. This relative efficiency was only transitory, however, so that 10 days later, v-Rel induced twofold-higher levels of these two receptors than did c-Rel. Rel/NF-κB family members are expressed at significantly lower levels in avian fibroblasts than in hematopoietic cells (27). The C-terminal truncation of v-Rel removed a large part of its transactivation domain (75). Therefore, v-Rel most probably requires the cooperation of other Rel/NF-κB factors to activate gene transcription. This may explain why v-Rel, when expressed in fibroblasts, is initially less efficient than c-Rel in induction of the immunoregulatory receptors. Finally, the levels of both v-Rel and c-Rel proteins decreased in MSB-1 cells between days 5 and 15 after infection, and the expression of all three receptors also decreased or was beneath the limits of detection (Fig. 1, second row). This loss in receptor expression appeared to result from the selection of a population of cells that did not express Rel and for which there was a significant proliferative advantage.

Indirect-immunofluoresence analysis conducted on the kinetic study described in Fig. 1 was used to examine the subcellular localization of c-Rel and v-Rel following infection. The analysis provided several observations that were common to all three types of cells examined (Fig. 3). At the earliest time that c-Rel was detected (12 h after infection), it was observed only in the nucleus (data not shown). This compartmentaliza-

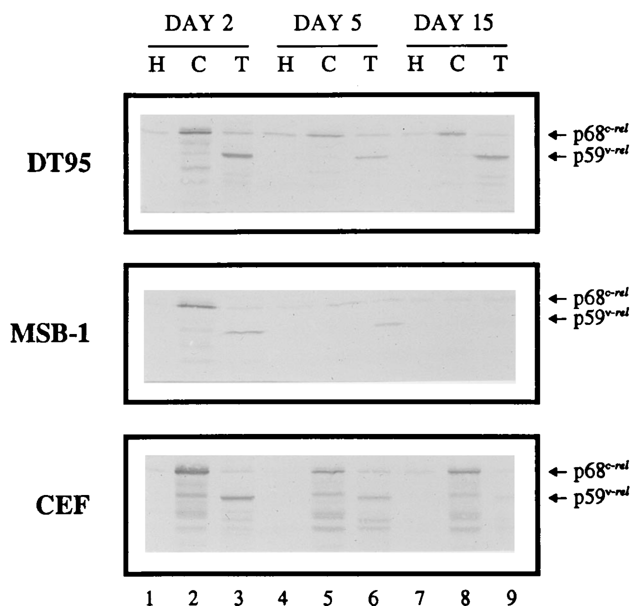

FIG. 2. Western analysis of Rel protein expression following infection of the DT95, MSB-1, and CEF cells with REV-C(CSV) or REV-TW(CSV). The cells (5×10^4) from the experiment described in the legend to Fig. 1 were analyzed by Western transfer. Rel proteins were detected with the monoclonal antibody HY87. The locations of v-Rel and c-Rel are indicated on the right-hand side of the panel. T, cells infected by REV-TW(CSV); C, cells infected by REV-C(CSV); H, cells infected by CSV helper virus. The time (days) after infection is indicated above the panels.

tion was very brief, and a significant accumulation of nuclear protein was not detected. At 24 h after infection, the vast majority of c-Rel was located in the cytoplasm. Its localization did not change at later times after infection (2, 5, and 15 days). In contrast, 1 and 2 days after infection, v-Rel was nuclear in all cell types examined. Five days after infection, a substantial amount of v-Rel was detected in the cytoplasm. The rate at which v-Rel appeared in the cytoplasm and the distribution between the two compartments differed among the cell types. Specifically, v-Rel accumulated most rapidly in the cytoplasm of MSB-1 cells, with a final distribution that was almost entirely cytoplasmic. It accumulated more slowly in the cytoplasm of DT95 cells, with a significant amount remaining in the nucleus. The nuclear/cytoplasmic transition occurred even more slowly in CEF cultures, in which a large fraction of v-Rel remained nuclear 15 days after infection (data not shown).

Kinetic analysis of *Ikba*, *nfkb1*, and MHC class II expression in chicken cell lines following infection with REV-C(CSV) or REV-TW(CSV). The transcriptional activity of the Rel/NF-κB family is regulated, in part, as a result of their association with inhibitory molecules, which sequester them as inactive complexes in the cytosol. The movement of both c-Rel and v-Rel from the nucleus to the cytoplasm as described here (Fig. 3) is consistent with their inactivation through such a regulatory mechanism. The observation that v-Rel accumulates more slowly in the cytoplasm and that a fraction remains nuclear suggests that v-Rel is inactivated less effectively than c-Rel. The presence of NF-κB1 and IκB-α in cytoplasmic complexes that contain c-Rel as well as v-Rel (11, 22, 67), together with their known inhibitory functions, suggested that IκB-α and

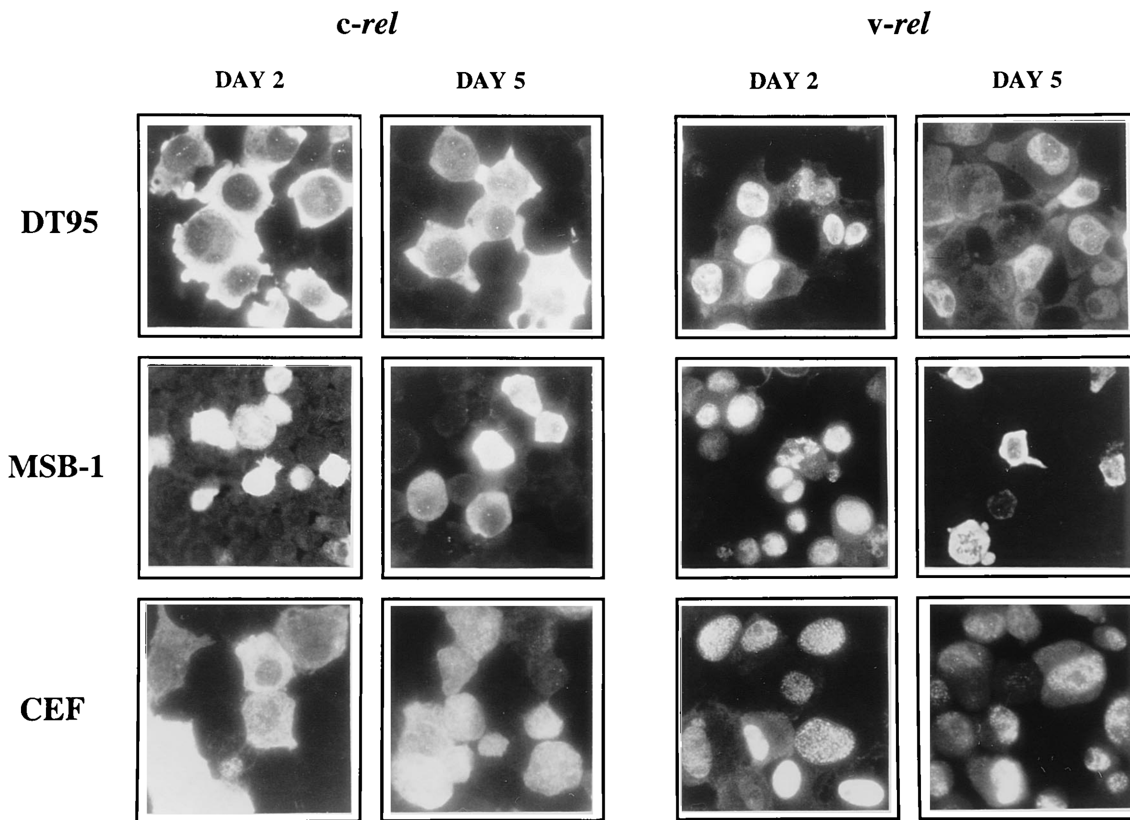

FIG. 3. Kinetic analysis of the subcellular localization of Rel protein expression following infection of DT95 and MSB-1 cells and CEF with REV-C(CSV) (c-*rel*) or REV-TW(CSV) (v-*rel*). Cells from the experiment described in the legend to Fig. 1 were analyzed by indirect immunofluorescence with the Rel-specific monoclonal antibody 3C1. The staining from days 2 and 5 after infection is shown.

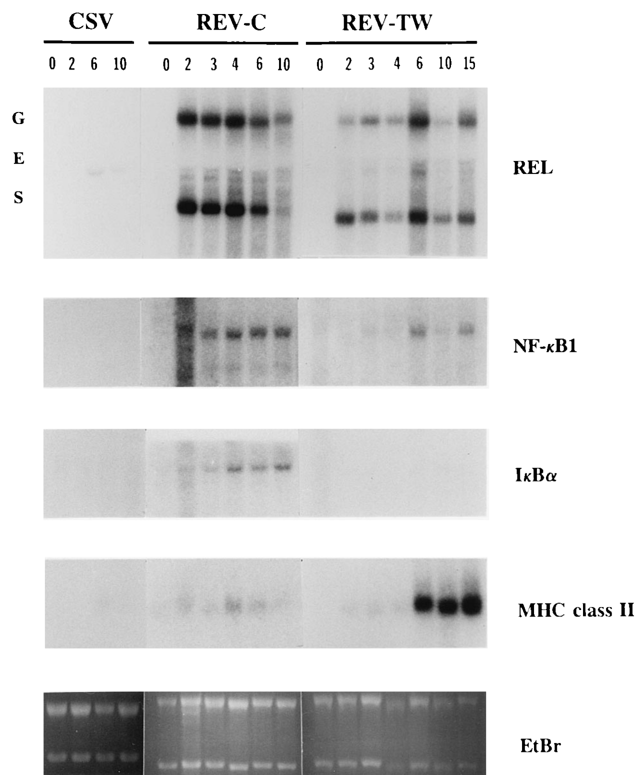

FIG. 4. Northern analysis of the expression of *Ikba*, *nfkb1*, and *rel* mRNA in DT95 cells following infection with REV-C(CSV) or REV-TW(CSV). DT95 cells (30 × 10^6) were infected with REV-C(CSV), REV-TW(CSV), or helper CSV alone at a multiplicity of infection of 2. Cellular RNA was prepared on the days indicated. Each line contains 10 μg of total RNA. The probes used to detect different mRNAs (*rel*, *nfkb1*, *Ikba*, and MHC class II) are described in Materials and Methods. The intensity of rRNA stained with ethidium bromide is shown in the bottom panel (EtBr). (The viral genomic [G] and spliced [S] RNAs and the endogenous c-*rel* [E] mRNA are indicated.)

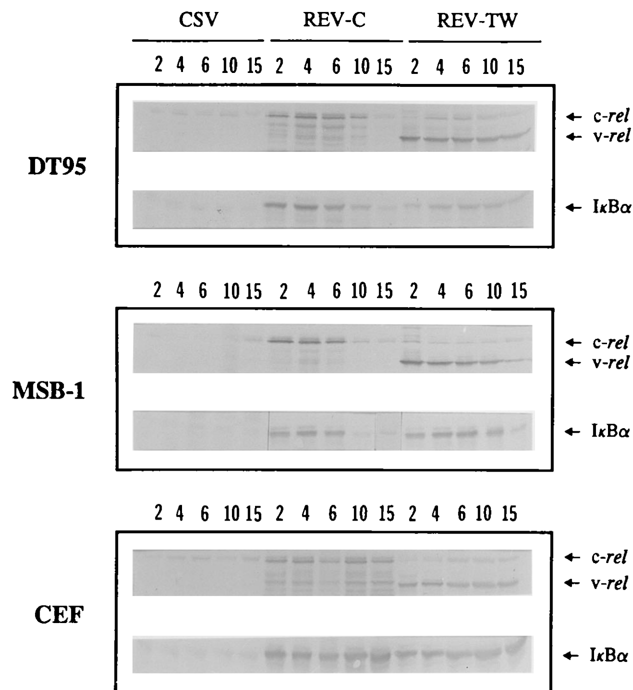

FIG. 5. Expression of IκB-α and Rel proteins in DT95 and MSB-1 cells and CEF following the infection with REV-C(CSV) or REV-TW(CSV). Cells (3 × 10^5 per lane) from the experiment described in the legend to Fig. 4 and similarly infected CEF were analyzed on 8% gels by Western transfer. Monoclonal antibodies HY87 and HY95 were used for the detection of Rel and IκB-α, respectively. The locations of c-Rel, v-Rel, and IκB-α proteins are indicated on the right-hand side of the panel. Numbers above each panel represent time (days) after infection with CSV, REV-C(CSV), or REV-TW(CSV).

NF-κB1 may play an important role in determining the subcellular location of v-Rel and c-Rel (reviewed in reference 7).

To examine this possibility, the expression of *nfkb1*, *Ikba*, and MHC class II mRNA and the level of IκB-α protein (Fig. 4 and 5) were analyzed. Northern analysis of DT95 cells infected with REV-C(CSV) or REV-TW(CSV) revealed that the amount of *nfkb1*, *Ikba*, and MHC class II mRNA increased with expression of either exogenous c-*rel* or v-*rel*. However, there was at least 10-fold-less *Ikba* mRNA in cells expressing v-*rel* than in cells expressing c-*rel*. Using the endogenous level of expression of *Ikba* in CSV-infected cells as background, we first detected the induction of *Ikba* mRNA in REV-TW-infected cells 6 days after infection. Detection required a longer exposure than that shown in Fig. 4 (data not shown). At 6 days after REV-TW(CSV) infection, the abundance of MHC class II mRNA increased, while cells infected with REV-C(CSV) were characterized by only low levels of MHC class II RNA. At the same time, the expression of *nfkb1* RNA increased significantly in REV-TW(CSV)-infected cells.

Western analysis performed on extracts obtained from DT95, MSB-1, and CEF cultures also indicated that IκB-α levels were increased. In cells infected with REV-C(CSV) and REV-TW(CSV), the steady-state level of IκB-α increased dramatically (Fig. 5). In both CEF and MSB-1 cells, comparable amounts of IκB-α appeared following infection with either REV-C(CSV) or REV-TW(CSV). In DT95 cells expressing v-Rel, however, the IκB-α protein was less abundant than in cells expressing c-Rel. In contrast, the results of the Northern analysis (Fig. 4), in which only very low levels of *Ikba* RNA were detected in DT95 cells expressing v-Rel, IκB-α was significantly more abundant and reduced only two- or threefold from that detected in DT95 cells expressing c-Rel (Fig. 5). Moreover, the level of IκB-α had increased by 2 days after infection and had reached its maximum level 48 h later. It should be noted that the appearance of IκB-α occurred prior to detection of an increase in the level of mRNA. These results suggest that IκB-α is stabilized through an association with Rel proteins, in a manner similar to that described for RelA (81). This possibility is also supported by the observation that the half-life of IκB-α and v-Rel in a v-Rel-transformed cell line is the same (approximately 7 h) (21). To test this hypothesis directly, the half-life of IκB-α was measured 4 days after infection of DT95 cells infected with a retrovirus expressing IκB-α and either c-Rel or v-Rel (Fig. 6). The results indicate that IκB-α expressed alone in these cells has a short half-life (1 to 2 h). In DT95 cells expressing v-Rel, the v-Rel in the transformed cells is complexed with IκB-α (data not shown), as previously reported for other lymphoid cells lines and avian fibroblasts (21, 22, 67). In the cells expressing c-Rel or v-Rel, the half-life of IκB-α corresponded to the half-life of the associated Rel protein (about 12 h in DT95 cells expressing c-Rel and IκB-α and approximately 8 h in DT95 cells expressing v-Rel and IκB-α). These results suggested that both Rel proteins stabilized IκB-α and that this interaction played a significant role in increasing the levels of IκB-α in Rel-expressing cells. In cells overexpressing IκB-α, the half-life did

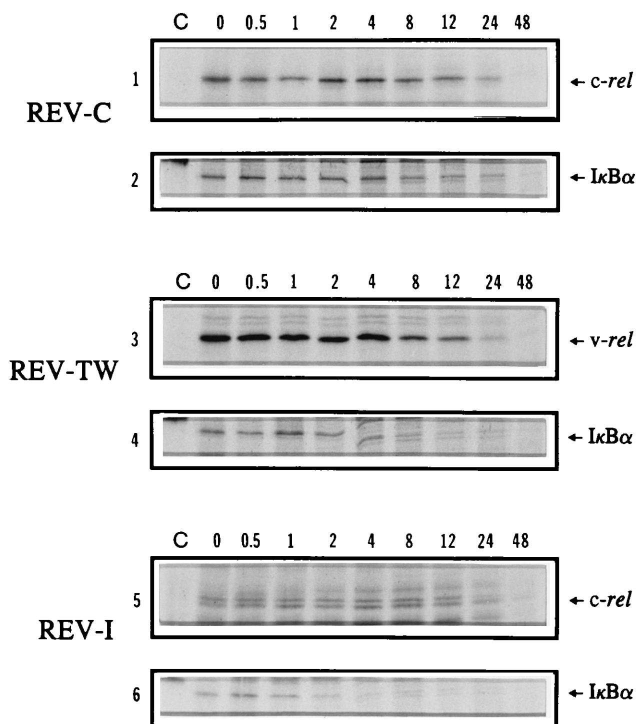

FIG. 6. Pulse-chase analysis of Rel and IκB-α proteins in DT95 cells infected with retroviral vectors expressing *Ikba*, c-*rel*, or v-*rel*. DT95 cells (10^7) were infected with retroviruses expressing c-*rel* (REV-C), v-*rel* (REV-TW), or *Ikba* (REV-I) at a multiplicity of infection of 1. At 4 days after infection, 4×10^7 cells were washed with methionine-free medium, starved for 30 min in methionine-free medium, and pulse-labeled with [^{35}S]methionine for another 30 min. The cells were refed with complete Dulbecco's modified Eagle's medium and harvested at the indicated times (numbers above the panels are time in hours). Cells (4×10^6) were lysed and immunoprecipitated as described in Materials and Methods. Half of the lysate was immunoprecipitated with rabbit anti-chicken Rel antiserum IV and analyzed on an 8% polyacrylamide gel. The second half was immunoprecipitated with rabbit anti-chicken IκB-α antisera (1275 and 1276). The migration of c-Rel, v-Rel, and IκB-α is shown on the right-hand side. C represents the same lysate treated with nonspecific rabbit serum. Panels 1, 3, and 6 (numbers on the left-hand side) were exposed for 2 days, while panels 2, 4, and 5 were exposed for 5 days. c-Rel in panel 5 represents endogenous c-Rel protein, and the higher of the two visible bands corresponds in migration to the protein expressed from the retroviral vector (panel 1).

not correspond to the half-life of endogenous c-Rel, which is approximately 12 h (Fig. 6, panels 5 and 6). The low level of expression of endogenous c-Rel is unable to efficiently stabilize the large amount of IκB-α made as the result of expression from a retroviral vector. Therefore, in cells expressing only endogenous Rel/NF-κB proteins, the average half-life of IκB-α is not determined by the half-life of Rel/NF-κB partners (73). A smaller species of IκB-α was detected during the process of IκB-α degradation (Fig. 6, panels 2 and 4, 8 to 24 h). This protein most probably corresponds to the N-terminally deleted IκB-α proteolytic intermediate recently described in the degradation of IκB-α following destabilization of a *ts* mutant of v-Rel at the nonpermissive temperature (91).

Expression of IκB-α or NF-κB1 is associated with retention of v-Rel in the cytoplasm. v-Rel induction and stabilization of IκB-α and/or NF-κB1 (Fig. 4 and 5) may explain why the majority of v-Rel initially detected in the nucleus accumulates in the cytoplasm (Fig. 3). To test this hypothesis, DT95, MSB-1, and CEF cultures were coinfected with REV-TW (CSV) and an excess of either REV-I(CSV) or 214p105(CSV), retroviral vectors expressing avian IκB-α or NF-κB1 proteins, respectively (Fig. 7). Immunofluorescence analysis of all three cell lines 2 days after coinfection revealed a significant shift of v-Rel to the cytoplasm compared with the cells infected with REV-TW(CSV) alone. The demonstration that both IκB-α and NF-κB1 are able to retain v-Rel in the cytoplasm is consistent with the hypothesis that the induction and possibly the stabilization of these proteins by v-Rel is responsible for its redistribution, leading to the majority of the v-Rel in the cytoplasm of transformed cells.

Kinetics of IκB-α and Rel protein expression during establishment of the transformed cell line. The studies described above, in which the interactions of IκB-α, NF-κB1, c-Rel, and v-Rel have been examined, have relied on the use of both CEF cultures and two transformed avian hematopoietic cell lines. To relate the findings from these studies to the process of in vitro transformation and, by analogy, to tumor development, the relationship between the subcellular localization of v-Rel and the appearence of IκB-α during REV-TW transformation of chicken spleen cells was examined (Fig. 8 and 9). Immunofluorescence analysis of splenic cells infected with REV-TW (CSV) or REV-C(CSV) revealed a distinct nuclear localization of v-Rel immediately after infection followed by a gradual shift to predominantly cytoplasmic expression 3 to 4 days later (Fig. 8). In contrast, c-Rel was observed primarily in the cytoplasm coincident with its first detection. The kinetics of IκB-α induction in these cells correlated with the appearance of the Rel proteins (Fig. 9A). The ratios of c-Rel/IκB-α and v-Rel/IκB-α were very similar. A comparison of the subcellular compartmentalization of c-Rel and v-Rel during the later times after infection demonstrated that a greater proportion of v-Rel retained a nuclear localization in the transformed cell (Fig. 9B). This observation is consistent with the results of our recent study (43).

DISCUSSION

Autoregulatory pathways in the Rel/NF-κB and IκB system of transcription factors. A number of studies have established the central role that the Rel/NF-κB and IκB system of proteins play in modulating the expression of a variety of genes. Both extracellular and intracellular signal transduction pathways are important in the initiation of a rapid response that leads to altered gene expression. Protein complexes sequestered in the cytoplasm are modified by signal transduction, enabling Rel/NF-κB proteins to be translocated to the nucleus, where homo- and heterodimeric complexes bind different κB sites. Generally, Rel/NF-κB complexes modulate the expression of two classes of genes: (i) effector genes such as immunoregulatory receptors and cytokines and (ii) genes encoding proteins of the Rel/NF-κB and IκB system. The induction of this latter class of molecules is likely to be important in both reestablishing the responsiveness of the system and modifying the pattern of target gene expression. Moreover, the induction of IκB proteins may inhibit the transcriptional activity of Rel/NF-κB when the exogenous signal is terminated.

Therefore, retrovirus long terminal repeat-mediated expression of Rel proteins, in contrast to that modulated by signal transduction, ought to result in the subsequent induction of inhibitory molecules able to repress the activity of the retrovirally expressed Rel. Consequently, one might expect limited induction of gene expression from retrovirally expressed transcription factors. Analysis of the three cell lines infected with retroviruses expressing c-Rel demonstrated that c-Rel was generally less efficient than v-Rel in inducing the expression of MHC class I and II proteins and IL-2R with increasing time after infection. By contrast, the level of these immunoregula-

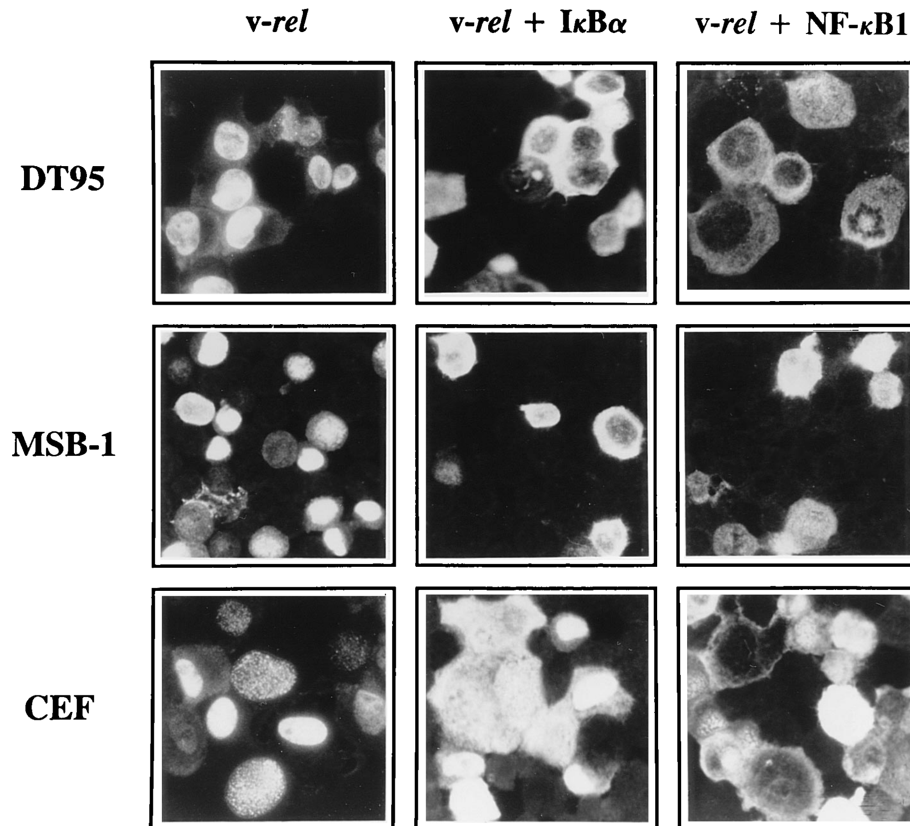

FIG. 7. Expression of IκB-α or NF-κB1 alters the subcellular localization of v-Rel. DT95 and MSB-1 cells and CEF were infected with REV-TW(CSV) (v-*rel*) at a multiplicity of infection of 2 alone or together with 214p105(CSV), a vector expressing NF-κB1, or REV-I(CSV), a vector expressing IκB-α, at a multiplicity of infection of 10. Independent immunofluorescence analysis confirmed that all cells from a population infected with REV-I(CSV) overexpressed IκB-α. The amount of CSV was adjusted with the addition of extra CSV to cells infected with REV-TW(CSV) alone so that the multiplicity of infection of CSV was equal for all infected cells. The expression of Rel proteins was examined by indirect immunofluorescence with monoclonal antibody 3C1 2 days after infection.

tory molecules increased with time after infection by the retrovirus expressing v-Rel. This observation suggests that the autoregulatory mechanisms inactivated more effectively the induction of immunoregulatory receptors mediated by exogenous c-Rel than induction mediated by v-Rel. These results may be explained by impaired induction of and/or impaired responsiveness to autoregulation pathways by v-Rel. Alterations in the structure of v-Rel are probably responsible for these effects.

This work analyzed the changes in the level of expression of *Ikba* and *nfkb1*, two members of the *rel/NF-κB* and *Ikb* families known for their inhibitory functions. It was proposed previously that c-Rel induces the transcription of *Ikba* and *nfkb1* in an autoregulatory fashion (9, 18, 54, 55). However, two other studies that examined overexpression of c-Rel and RelA detected only RelA-mediated induction of *Ikba* (23, 86). The observations reported in this study support the conclusion that c-Rel is able to induce these genes. Northern analysis of *Ikba* and *nfkb1* expression detected the induced expression of these genes 2 days after infection with REV-C (c-Rel). On the other hand, infection with REV-TW (v-Rel) induced *Ikba* mRNA and *nfkb1* only after a significant delay and at reduced levels. These data are consistent with our previous observation that CEF overexpressing c-Rel have a higher level of *Ikba* mRNA than do cells infected with Rous sarcoma virus-based vector expressing v-Rel (54). However, an increased expression of *nfkb1* was found only after overexpression of c-Rel in these cells. This difference between fibroblasts and lymphoid cells may be due to cell specificity and/or to the different levels of Rel being expressed from different retroviral vectors.

However, NF-κB1 and IκB-α are expressed at significantly higher levels in established cell lines transformed by v-*rel* than in nontransformed cells (7). The induction of *rel/NF-κB* and *Ikb* family members in these transformed cell lines may therefore be indirect. As v-Rel accumulates in the transformed cell, it binds to the available IκBs, possibly allowing the inappropriate nuclear translocation of c-Rel and/or other Rel/NF-κB family members, which then turn on the transcription of *Ikba* and *nfkb1*.

The regulation of subcellular localization. Rel/NF-κB autoregulation results in increased levels of cellular IκB, which may lead to inhibition of Rel/NF-κB-mediated transcription. At least two biochemical properties of the IκB molecules could be responsible for its inhibitory ability: (i) the ability to inhibit DNA binding and (ii) the retention of Rel/NF-κB in the cytoplasm. Rel proteins selected for their ability to evade these two interactions with IκB would disrupt transcriptional regulation and have the potential to be oncogenic. It has been demonstrated that the responsiveness to IκB-α-mediated inhibition of DNA binding is substantially decreased with v-Rel (24). The second interaction, the relationship between IκB-α and NF-κB1 and the subcellular localization of v-Rel, is described in this work. Experiments presented here demonstrate that (i) v-Rel-mediated induction of IκB-α as well as *nfkb1* mRNA expression precedes the shift of the majority of v-Rel from the nucleus to the cytoplasm and that (ii) the

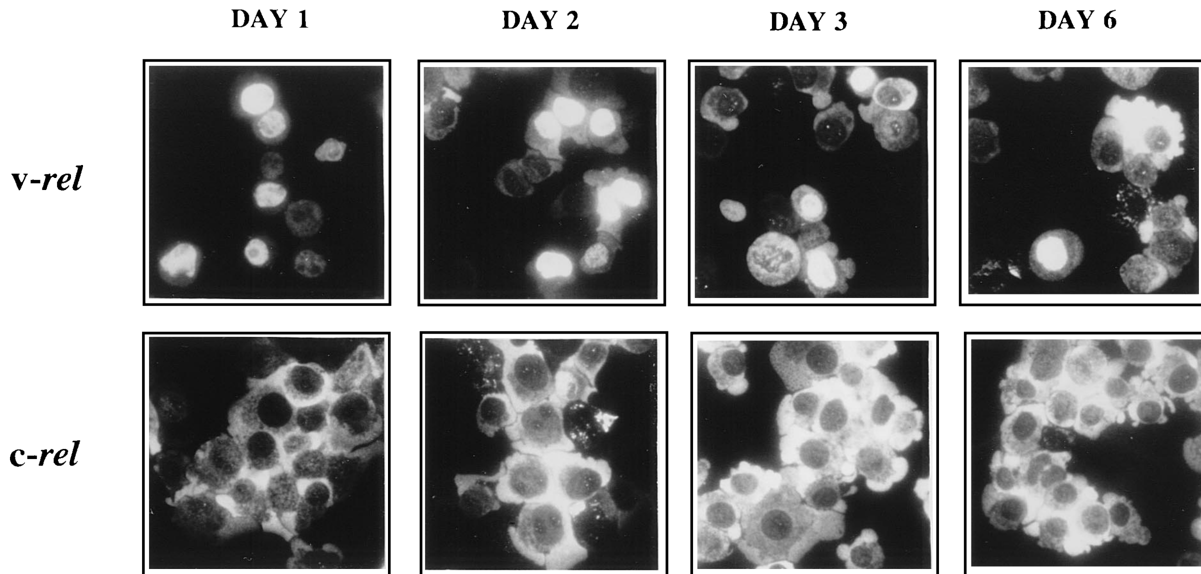

FIG. 8. Kinetic analysis of the subcellular localization of c-Rel and v-Rel following infection of splenic cells with REV-C(CSV) or REV-TW(CSV). The spleens from 4-week-old chickens were passed through nylon mesh and placed in culture in 50 ml. At 4 days later, mononuclear leukocytes were separated by centrifugation of the cell suspension on Isolymph (Gallard-Schlesinger Industries, Inc., New York). The fractionated cells were infected by REV-TW(CSV), REV-C(CSV), or CSV at a multiplicity of infection of 1. The cells were harvested for immunofluorescence analysis and for Western transfer at the indicated times. Rel protein was detected by immunofluorescence staining with monoclonal antibody 3C1.

coexpression of IκB-α or NF-κB1 with v-Rel is capable of mediating this translocation. These results explain the earlier observation that v-Rel was restricted to the nucleus during the early stages of REV-T infection of CEF cultures but became cytoplasmic at later stages (65). The kinetic analysis and coinfection experiments confirm a functional relationship between increased expression of *Ikba* mRNA in CEF infected with Rous sarcoma virus-based vector expressing v-Rel and its cytoplasmic localization, as suggested in our recent work (54). In lymphoid cells expressing either v-Rel or c-Rel, the transcription of NF-κB2 is also upregulated (data not shown). In addition, NF-κB2 is complexed with c-Rel in normal cells and with v-Rel in transformed cells (20, 67, 82). These observations suggest that NF-κB2 may also play a role in the subcellular localization of these Rel proteins. Moreover, the process of relocalization of v-Rel from the nucleus to the cytoplasm is a more general phenomenon. In addition to CEF, the accumulation of v-Rel in the cytoplasm occurs after in vitro infection of avian lymphoid cell lines as well as chicken splenic cells, the targets for v-*rel* transformation. Therefore, the cytoplasmic localization of the large majority of v-Rel in transformed cell lines reported previously (42, 83) is most probably the result of the delay between the time of analysis and the time of infection (reviewed in reference 7).

A striking difference between the translocation of c-Rel and v-Rel to the cytoplasm was observed in all cell types examined, including splenic cells. Translocation of c-Rel was always more rapid and achieved an equilibrium with a higher proportion of c-Rel in the cytoplasm than did v-Rel. As discussed above, IκB-α is able to retain v-Rel in the cytoplasm. For this reason, the differences in the localization of c-Rel and v-Rel in DT95 cells may be the consequence of differences in the ratio of IκB-α/v-Rel and IκB-α/c-Rel resulting from the diminished induction of *Ikba* RNA in cells expressing v-Rel (Fig. 4 and 5). In splenic cultures, however, in which IκB-α/Rel ratios for both Rel proteins were equivalent, a great proportion of v-Rel was still nuclear (Fig. 9). It is likely, therefore, that further functional differences between c-Rel and v-Rel contribute to the different localizations of these two proteins within the cell. In particular, the deletion of 118 C-terminal amino acids from v-Rel which contains a cytoplasmic anchor domain in c-Rel has been shown to be important (13). It was suggested that this alteration contributes to the differences between c-Rel and v-Rel localization in CEF and DT95 cells, as well as in the *rel*-transformed cell lines (43, 49), probably through the exposure of a nuclear localization sequence in the RH domain (33).

Therefore, the reduced ability of v-Rel to induce *Ikba* mRNA, together with deletion of the cytoplasmic anchor, would result in a greater proportion of v-Rel in the nucleus relative to c-Rel. Several lines of evidence suggest that v-Rel must be able to bind κB sites in the nucleus to induce transformation: (i) the disruption of v-Rel DNA binding coincides with the loss of transformation (66, 90a); (ii) the loss of IκB-α-mediated inhibition of DNA binding, together with the C-terminal truncation of c-Rel, is associated with increased transformation potential (24); (iii) the disruption of the transactivation domain present in v-Rel correlates with the loss of the ability of v-Rel to transform splenic cells (78); and (iv) the increased nuclear localization of C-terminally truncated Rel proteins in transformed cells correlates with their tumorigenic potential (43).

Role of stabilization of IκB-α by v-Rel in v-Rel transformation. Both c-Rel and v-Rel are able to stabilize IκB-α, which, in an unassociated form, is highly unstable. The half-life of IκB-α corresponds to the half-life of the overexpressed Rel protein with which it was associated. These data apparently contradict results of reported experiments demonstrating impaired responsiveness of v-Rel to IκB-α-mediated inhibition of DNA binding in comparison with c-Rel (24). However, the IκB-α-mediated inhibition of v-Rel DNA binding involves the interaction of three components: DNA, v-Rel, and IκB-α. If the mutations in v-Rel increased its DNA binding ability, IκB-α would not be able to effectively block v-Rel DNA binding, although the affinity between v-Rel and IκB-α remains unal-

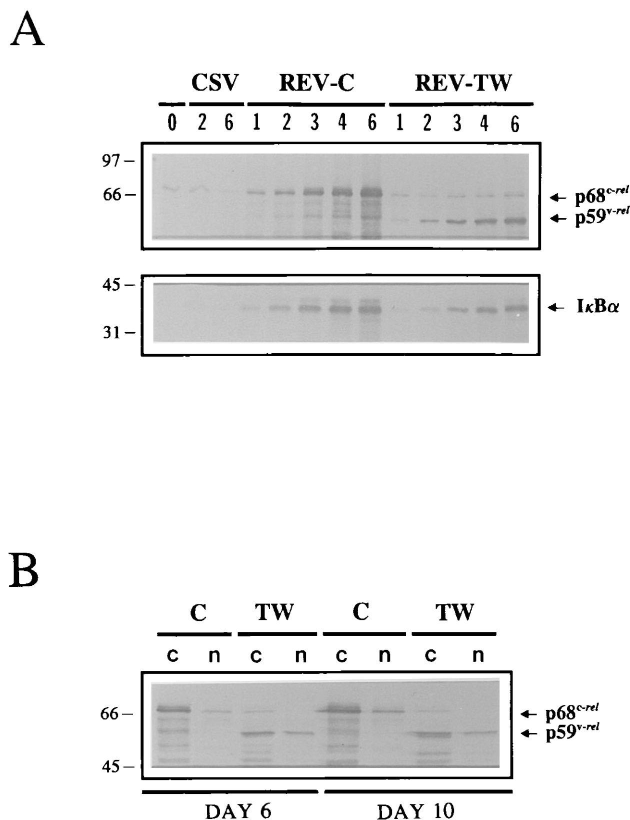

FIG. 9. Western analysis of IκB-α and Rel proteins following infection of splenic cells with REV-C(CSV) or REV-TW(CSV). Western analysis of 10^5 cells was performed on the indicated days with monoclonal antibody HY87 and HY95 for the detection of Rel proteins and IκB-α, respectively. The locations of c-Rel, v-Rel, and IκB-α proteins following electrophoresis are indicated on the right-hand side of the panel. The migration of marker proteins (in kilodaltons) is indicated on the left-hand side of the panel. (A) Kinetic analysis of the expression of Rel and IκB-α proteins. (B) Western analysis of the nuclear (N) and cytoplasmic (C) fractions of the *rel*-transformed cells 6 and 10 days after in vitro cultivation.

tered. In a similar fashion, the inability of IκB-α to inhibit p50 DNA binding is not associated with the inability of these two proteins to bind in vitro (47). Furthermore, like v-Rel, IκB-α is capable of retaining p50 in the cytoplasmic compartment (5).

IκB-α was stabilized by both v-Rel and c-Rel. However, v-Rel, unlike c-Rel (or RelA), was a poor inducer of *Ikba* mRNA early in the transformation process. As v-Rel accumulates in cells transformed early after infection, it may complex with the limited amount of IκB-α in the cytoplasm of the cell, allowing the inappropriate translocation of c-Rel and/or other Rel/NF-κB family members to the nucleus. These endogenous κB DNA binding proteins may heterodimerize with v-Rel and subsequently alter gene transcription, leading to transformation. Indeed, in cells transformed by v-Rel, new endogenous κB DNA binding complexes can be detected in the nucleus approximately 1 week after infection (42a). It is likely, therefore, that v-Rel transformation involves both a direct mechanism whereby it binds DNA and alters gene expression and an indirect mechanism whereby it sequesters IκB-α and the other inhibitory molecules, allowing the inappropriate translocation of other Rel/NF-κB family members to the nucleus. The overexpression of c-Rel also transforms cells but much less efficiently than v-Rel (44, 54, 69). The major difference between c-Rel and v-Rel is acquisition of numerous mutations which enhance the oncogenicity of v-Rel. These mutations allow v-Rel to escape the autoregulatory pathway which controls this family of transcription factors. Since the expression of c-Rel rapidly induces IκB-α transcription after infection, c-Rel fails to induce the release of other Rel/NF-κB family members sequestered in the cytoplasm. This may provide a partial explanation of why c-Rel is less oncogenic than v-Rel.

ACKNOWLEDGMENTS

We thank T. D. Gilmore for the gift of 214p105 and cDNA clone of *nfkb1* and N. R. Rice for gifts of rabbit anti-chicken Rel and IκB-α antisera. We thank K. Skjødt and C. H. Tempelis for gifts of monoclonal antibodies specific for MHC class I and IL-2R, and we thank Debbie Piktel for excellent assistence with flow-cytometric analysis.

This work was supported by Public Health Service grants CA41450 (to E.H.H.) and CA33192 (to H.R.B.) from the National Cancer Institute and by Texas Advanced Research Program 003658-470.

REFERENCES

1. **Abbadie, C., N. Kabrun, F. Bouali, J. Smardova, D. Stéhelin, B. Vandenbunder, and P. J. Enrietto.** 1993. High levels of c-*rel* expression are associated with programmed cell death in the developing avian embryo and in bone marrow cells in vitro. Cell **75:**899–912.
2. **Akiyama, Y., and S. Kato.** 1974. Two cell lines from lymphomas of Marek's disease. Biken J. **17:**105–116.
3. **Baba, T. W., B. P. Giroir, and E. H. Humphries.** 1985. Cell lines derived from avian lymphomas exhibit two distinct phenotypes. Virology **144:**139–151.
4. **Barth, C. F., D. L. Ewert, W. C. Olson, and E. H. Humphries.** 1990. Reticuloendotheliosis virus REV-T (REV-A)-induced neoplasia: development of tumors within the T-lymphoid and myeloid lineages. J. Virol. **64:** 6054–6062.
5. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr.** 1992. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. **6:**1899–1913.
6. **Bhat, G. V., and H. M. Temin.** 1990. Mutational analysis of v-*rel*, the oncogene of reticuloendotheliosis virus strain T. Oncogene **5:**625–634.
7. **Bose, H. R., Jr.** 1992. The Rel family: models for transcriptional regulation and oncogenic transformation. Biochim. Biophys. Acta **1114:**1–17.
8. **Bressler, P., K. Brown, W. Timmer, V. Bours, U. Siebenlist, and A. S. Fauci.** 1993. Mutational analysis of the p50 subunit of NF-κB and inhibition of NF-κB activity by *trans*-dominant p50 mutants. J. Virol. **67:**288–293.
9. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
10. **Bull, P., K. L. Morley, M. F. Hoekstra, T. Hunter, and I. M. Verma.** 1990. The mouse c-*rel* protein has an N-terminal regulatory domain and C-terminal transcriptional transactivation domain. Mol. Cell. Biol. **10:**5473–5485.
11. **Capobianco, A. J., D. Chang, G. Mosialos, and T. D. Gilmore.** 1992. p105, the NF-κB p50 precursor protein, is one of the cellular proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. J. Virol. **66:**3758–3767.
12. **Capobianco, A. J., and T. D. Gilmore.** 1991. Repression of the chicken c-*rel* promoter by v-Rel in chicken embryo fibroblasts is not mediaed through a consensus NF-κB binding site. Oncogene **6:**2203–2210.
13. **Capobianco, A. J., D. L. Simmons, and T. D. Gilmore.** 1990. Cloning and expression of a chicken c-*rel* cDNA: unlike p59$^{v\text{-}rel}$, p68$^{c\text{-}rel}$ is a cytoplasmic protein in chicken embryonic fibroblasts. Oncogene **5:**257–265.
14. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
15. **Chen, I. S. Y., and H. M. Temin.** 1982. Substitution of 5′ helper virus sequences into non-*rel* portion of reticuloendotheliosis virus strain T suppresses transformation of chicken spleen cells. Cell **31:**111–120.
16. **Chiao, P. J., S. Miyamoto, and I. M. Verma.** 1994. Autoregulation of IκB-α activity. Proc. Natl. Acad. Sci. USA **91:**28–32.
17. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
18. **Cogswell, P. C., R. I. Scheinman, and A. S. Baldwin, Jr.** 1985. Promoter of the human NF-κB p50/p105 gene. J. Immunol. **150:**2794–2804.
19. **Crone, M., M. Simonsen, K. Skjødt, K. Linnet, and L. Olson.** 1985. Mouse monoclonal antibodies to class I and class II antigens of the chicken MHC. Evidence for at least two class I products of B complex. Immunogenetics **21:** 181–187.
20. **Davis, J. N., W. Bargmann, and H. R. Bose, Jr.** 1990. Identification of protein complexes containing the c-*rel* proto-oncogene product in avian hematopoi-

etic cells. Oncogene **5:**1109–1115.

21. **Davis, N., W. Bargmann, M.-Y. Lim, and H. Bose, Jr.** 1990. Avian reticuloendotheliosis virus-transformed lymphoid cells contain multiple pp59$^{v\text{-}rel}$ complexes. J. Virol. **64:**584–591.
22. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H. R. Bose, Jr.** 1991. Rel-associated pp40: an inhibitor of the Rel family of transcription factors. Science **253:**1268–1271.
23. **De Martin, R., B. Vanhove, Q. Cheng, E. Hofer, V. Csizmadia, H. Winkler, and F. H. Bach.** 1993. Cytokine-inducible expression in endothelial cells of an IκB-α-like gene is regulated by NF-κB. EMBO J. **12:**2773–2779.
24. **Diehl, J. A., T. A. McKinsey, and M. Hannink.** 1993. Differential pp40$^{I\kappa B\text{-}\beta}$ inhibition of DNA binding by *rel* proteins. Mol. Cell. Biol. **13:**1769–1778.
25. **Dobrzanski, P., R.-P. Ryseck, and R. Bravo.** 1993. Both N- and C-terminal domains of RelB are required for full transactivation: role of the N-terminal leucine zipper-like motif. Mol. Cell. Biol. **13:**1572–1582.
26. **Dougherty, J. P., and H. M. Temin.** 1986. High mutation rate of a spleen necrosis virus-based retrovirus vector. Mol. Cell. Biol. **6:**4387–4395.
27. **Evans, R. B., P. D. Gottlieb, and H. R. Bose, Jr.** 1993. Identification of a Rel-related protein in the nucleus during the S phase of the cell cycle. Mol. Cell. Biol. **13:**6147–6156.
28. **Fan, C.-M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature (London) **354:**395–398.
29. **Franzoso, G., V. Bours, S. Park, M. Tomita-Yamaguchi, K. Kelly, and U. Siebenlist.** 1992. The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. Nature (London) **359:**339–342.
30. **Geisler, R., A. Bergmann, Y. Hiromi, and C. Nüsslein-Volhard.** 1992. *Cactus*, a gene involved in dorsoventral pattern formation of Drosophila, is related to the IκB gene family of vertebrates. Cell **71:**613–621.
31. **Ghosh, S., and D. Baltimore.** 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **344:**678–682.
32. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell **62:**1019–1029.
33. **Gilmore, T. D., and H. M. Temin.** 1988. v-*rel* oncoproteins in the nucleus and in the cytoplasm transform chicken spleen cells. J. Virol. **62:**703–714.
34. **Grilli, M., J. J.-S. Chiu, and M. J. Lenardo.** 1993. NF-κB and Rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143:**1–62.
35. **Hannink, M., and H. M. Temin.** 1990. Structure and autoregulation of c-*rel* promoter. Oncogene **5:**1843–1850.
36. **Hannink, M., and H. M. Temin.** 1989. Transactivation of gene expression by nuclear and cytoplasmic *rel* proteins. Mol. Cell. Biol. **9:**4323–4336.
37. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Haskill, S., A. A. Beg., S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. S. Baldwin, Jr.** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65:**1281–1289.
39. **Hatada, E. N., A. Nieters, F. G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T. W. McKeithan, and C. Scheidereit.** 1992. The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene *bcl-3* act as specific inhibitors of NF-κB. Proc. Natl. Acad. Sci. USA **89:**2489–2493.
40. **Henkel, T., T. Machleidt, I. Alkalay, M. Krönke, Y. Ben-Neriah, and P. A. Baeuerle.** 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature (London) **365:**182–185.
41. **Henkel, T., U. Zabel, K. van Zee, J. M. Müller, E. Fanning, and P. A. Baeuerle.** 1992. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell **68:** 1121–1133.
42. **Herzog, N. K., and H. R. Bose, Jr.** 1986. Expression of the oncogene of avian reticuloendotheliosis virus in Escherichia coli and identification of the transforming protein in reticuloendotheliosis virus T-transformed cells. Proc. Natl. Acad. Sci. USA **83:**812–816.

42a. **Hrdličková, R., J. Nehyba, and H. R. Bose, Jr.** Mutations in the DNA-binding and dimerization domains of v-Rel are responsible for altered κB DNA-binding complexes in transformed cells. Submitted for publication.

43. **Hrdličková, R., J. Nehyba, and E. H. Humphries.** 1994. In vivo evolution of c-*rel* oncogenic potential. J. Virol. **68:**2371–2382.
44. **Hrdličková, R., J. Nehyba, and E. H. Humphries.** 1994. v-*rel* induces expression of three avian immunoregulatory surface receptors more efficiently than c-*rel*. J. Virol. **68:**308–319.
45. **Inoue, J. I., L. D. Kerr, A. Kakizuka, and I. M. Verma.** 1992. IκB-γ, a 70 kD protein identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell **68:**1109–1120.
46. **Inoue, J. I., L. D. Kerr, D. Rashid, N. Davis, H. R. Bose, Jr., and I. M. Verma.** 1992. Direct association of pp40/IκBβ with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA **89:**4333–4337.
47. **Inoue, J. I., T. Takahara, T. Akizawa, and O. Hino.** 1993. Bcl-3, a member of the IκB proteins, has distinct specificity towards the Rel family of proteins. Oncogene **8:**2067–2073.
48. **Isoda, K., S. Roth, and C. Nusslein-Volhard.** 1992. The functional domains of the *Drosophila* morphogen *dorsal*: evidence from the analysis of mutants. Genes Dev. **6:**619–630.
49. **Kamens, J., P. Richardson, G. Mosialos, R. Brent, and T. Gilmore.** 1990. Oncogenic transformation by v-*rel* an amino-terminal activation domain. Mol. Cell. Biol. **10:**2840–2847.
50. **Kerr, L. D., C. S. Duckett, P. Wamsley, Q. Zhang, P. Chiao, G. Nabel, T. W. McKeithan, P. A. Baeuerle, and I. M. Verma.** 1992. The protooncogene *BCL-3* encodes an IκB protein. Genes Dev. **6:**2352–2363.
51. **Kerr, L. D., J. I. Inoue, N. Davis, E. Link, P. A. Baeuerle, H. R. Bose, Jr., and I. M. Verma.** 1991. The Rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. Genes Dev. **5:**1464–1476.
52. **Kidd, S.** 1992. Characterization of the Drosophila *cactus* locus and analysis of interactions between cactus and dorsal proteins. Cell **71:**623–635.
53. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62:**1007–1018.
54. **Králová, J., J. D. Schatzle, W. Bargmann, and H. R. Bose, Jr.** 1994. Transformation of avian fibroblasts overexpressing the c-*rel* and a variant of c-*rel* lacking 40 C-terminal amino acids. J. Virol. **68:**2073–2083.
55. **Le Bail, O., R. Schmidt-Ullrich, and A. Israël.** 1993. Promoter analysis of the gene encoding the IκB-α/MAD3 inhibitor of NF-κB: positive regulation by members of the rel/NF-κB family. EMBO J. **12:**5043–5049.
56. **Lee, T.-H., and C. H. Tempelis.** 1991. Characterization of a receptor on activated chicken T cells. Dev. Comp. Immunol. **15:**329–339.
57. **Link, E., L. D. Kerr, R. Schreck, U. Zabel, I. Verma, and P. A. Baeuerle.** 1992. Purified IκB-β is inactivated upon dephosphorylation. J. Biol. Chem. **267:**239–246.
58. **Liou, H.-C., and D. Baltimore.** 1993. Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. Curr. Opin. Cell Biol. **5:**477–487.
59. **Liou, H.-C., G. P. Nolan, S. Ghosh, T. Fujita, and D. Baltimore.** 1992. The NF-κB p50 precursor, p105, contains an internal IκB-like inhibitor that preferentially inhibits p50. EMBO J. **11:**3003–3009.
60. **Logeat, F., N. Israël, R. Ten, V. Blank, O. Le Bail, P. Kourilsky, and A. Israël.** 1991. Inhibition of transcription factors belonging to the rel/NF-κB family by a transdominant negative mutant. EMBO J. **10:**1827–1832.
61. **Lu, D., J. D. Thompson, G. K. Gorski, N. R. Rice., M. G. Mayer, and J. J. Yunis.** 1991. Alterations at the *rel* locus in human lymphoma. Oncogene **6:** 1235–1241.
62. **Mercurio, F., J. Didonato, C. Rosette, and M. Karin.** 1992. Molecular cloning and characterization of a novel Rel/NF-κB family member displaying structural and functional homology to NF-κB p50/p105. DNA Cell Biol. **11:** 523–537.
63. **Miyamoto, S., P. J. Chiao, and I. M. Verma.** 1994. Enhanced IκBα degradation is responsible for constitutive NF-κB activity in mature murine B-cell lines. Mol. Cell. Biol. **14:**3276–3282.
64. **Moller, L. B., J. Kaufman, S. Verland, J. Salomonsen, D. Avila, J. D. Lambris, and K. Skjødt.** 1991. Variations in the cytoplasmic region account for the heterogeneity of the chicken MHC class I (B–F) molecules. Immunogenetics **34:**110–120.
65. **Moore, B. E., and H. R. Bose, Jr.** 1988. Expression of the v-*rel* oncogene in reticuloendotheliosis virus-transformed fibroblasts. Virology **162:**377–387.
66. **Morrison, L. E., G. Boehmelt, and P. J. Enrietto.** 1992. Mutations in the *rel*-homology domain alter the biochemical properties of v-*rel* and render it transformation defective in chicken embryo fibroblasts. Oncogene **7:**1137–1147.
67. **Morrison, L. E., N. Kabrun, S. Mudri, M. J. Hayman, and P. J. Enrietto.** 1989. Viral *rel* and cellular *rel* associate with cellular proteins in transformed and normal cells. Oncogene **5:**677–683.
68. **Narayanan, R., J. F. Klement, S. M. Ruben, K. A. Higgins, and C. A. Rosen.** 1992. Identification of a naturally occuring transforming variant of the p65 of NF-κB. Science **256:**367–370.
69. **Nehyba, J., R. Hrdličková, and E. H. Humphries.** 1994. Evolution of the oncogenic potential of v-*rel*: *rel*-induced expression of immunoregulatory receptors correlates with tumor development and in vitro transformation. J. Virol. **68:**2039–2050.
70. **Neri, A., C.-C. Chang, L. Lombardi, M. Salina, P. Corradini, A. T. Maiolo, R. S. K. Chaganti, and R. Dalla-Favera.** 1991. B cell lymphoma-associated chromosomal translocation involves candidate oncogene *lyt*-10, homologous to NF-κB p50. Cell **67:**1075–1087.
71. **Neumann, M., K. Tsapos, J. A. Scheppler, J. Ross, and R. Franza, Jr.** 1992. Identification of complex formation between two intracellular tyrosine kinase substrates: Human c-Rel and the p105 precursor of p50 NF-κB. Oncogene **7:**2095–2104.
72. **Ohno, H., G. Takimoto, and T. W. McKeithan.** 1990. The candidate proto-oncogene *bcl*-3 is related to genes implicated in cell lineage determination and cell cycle control. Cell **60:**991–997.
73. **Rice, N. R., and M. K. Ernst.** 1993. *In vivo* control of NF-κB activation by IκBα. EMBO J. **12:**4685–4695.
74. **Rice, N. R., M. L. MacKichan, and A. Israël.** 1992. The precursor of NF-κB

p50 has IκB-like functions. Cell **71:**243–253.
75. **Richardson, M. P., and T. D. Gilmore.** 1991. v-Rel is an inactive member of the Rel family of transcriptional activating proteins. J. Virol. **65:**3122–3130.
76. **Robinson, F. R., and M. J. Twiehaus.** 1974. Isolation of the avian reticuloendotheliosis virus (strain T). Avian Dis. **18:**178–185.
77. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
78. **Sarkar, S., and T. D. Gilmore.** 1993. Transformation by the v-Rel oncoprotein requires sequences carboxy-terminal to the Rel homology domain. Oncogene **8:**2245–2252.
79. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10:**3805–3817.
80. **Schreck, R., P. Rieber, and P. A. Baeuerle.** 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcrtiption factor and HIV-1. EMBO J. **8:**2247–2258.
81. **Scott, M. L., T. Fujita, H.-C. Liou, G. P. Nolan, and D. Baltimore.** 1993. The p65 subunit of NF-κB regulates IκB by two distinct mechanisms. Genes Dev. **7:**1266–1276.
82. **Sif, S., and T. D. Gilmore.** 1993. NF-κB p100 is one of the high-molecular-weight proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. J. Virol. **67:**7612–7617.
83. **Simek, L. S., R. M. Stephens, and N. R. Rice.** 1986. Localization of the v-*rel* protein in reticuloendotheliosis virus strain T-transformed lymphoid cells. J. Virol. **56:**120–126.
84. **Stoker, A. W., and M. J. Bissell.** 1987. Quantitative immunocytochemical assay for infectious avian retroviruses. J. Gen. Virol. **68:**2481–2485.
85. **Storms, R. W., and H. R. Bose, Jr.** 1992. Alterations within pp59$^{v\text{-}rel}$-containing protein complexes following the stimulation of REV-T-transformed lymphoid cells with zinc. Virology **188:**765–777.
86. **Sun, S.-C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκB: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
87. **Ten, R. M., C. V. Paya, N. Israël, O. Le Bail, M.-G. Mattei, J.-L. Virelizier, P. Kourilsky, and A. Israël.** 1992. The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. EMBO J. **11:**195–203.
88. **Tewari, M., P. Dobrzanski, K. L. Mohn, D. E. Cressman, J.-C. Hsu, R. Bravo, and R. Taub.** 1992. Rapid induction in regenerating liver of RL/IF-1 (an IκB that inhibits NF-κB, RelB-p50, and c-Rel–p50) and PHF, a novel κB site-binding complex. Mol. Cell. Biol. **12:**2898–2908.
89. **Thisse, C., F. Perrin-Schmitt, C. Stoetzel, and B. Thisse.** 1991. Sequence-specific transactivation of the Drosophila *twist* gene by the *dorsal* gene product. Cell **65:**1191–1201.
90. **van Krieken, J. H., T. W. McKeithan, S. Raghoebier, L. J. Medeiros, P. M. Kluin, and M. Raffeld.** 1990. Chromosomal translocation t(4; 19) as indicated by bcl-3 rearrangement is a rare phenomenon in non-Hodgkin's lymphoma and chronic lymphocytic leukemia: a molecular genetic analysis of 176 cases. Leukemia **4:**811–812.
90a. **White, D. W., and T. D. Gilmore.** 1993. Temperature-sensitive transforming mutants of the v-*rel* oncogene. J. Virol. **67:**6876–6881.
91. **White, D. W., A. Roy, and T. D. Gilmore.** The v-Rel oncoprotein blocks apoptosis and proteolysis of IκB-α in transformed chicken spleen cells. Oncogene, in press.
92. **Wilhelmsen, K. C., K. Eggleton, and H. M. Temin.** 1984. Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. J. Virol. **52:**172–182.
93. **Xu, Y., J. Pitcovski, L. Peterson, C. Auffray, Y. Bourlet, B. M. Gerndt, A. W. Nordskog, S. J. Lamont, and C. M. Warner.** 1989. Isolation and characterization of three class II MHC genomic clones from the chicken. J. Immunol. **142:**2122–2132.
94. **Zabel, U., and P. A. Baeuerle.** 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell **61:**255–265.
95. **Zabel, U., T. Henkel, M. dos Santos Silva, and P. A. Baeuerle.** 1993. Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. EMBO J. **12:**201–211.