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# v-*rel* Induces Expression of Three Avian Immunoregulatory Surface Receptors More Efficiently than c-*rel*

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**The c-*rel* gene is a member of NF-κB/*rel* family of transcription factors that regulate expression of a variety of immunoregulatory molecules. The viral oncogene, v-*rel*, is a truncated and mutated form of the turkey c-*rel* gene expressed by reticuloendotheliosis virus, strain T. In this study, we demonstrated that three avian immunoregulatory receptors, major histocompatibility (MHC) antigens class I and class II as well as the interleukin-2 receptor (IL-2R), were induced on the surface of splenic tumor cells isolated from chickens infected with reticuloendotheliosis virus, strain T. All cell lines derived from splenic tumors expressed these three proteins. Their expression also correlated with the appearance of endogenous c-*rel* during a graft-versus-host reaction. In vitro, both c-*rel* and v-*rel* induced MHC class I, MHC class II, and IL-2R on an avian B-lymphoid cell line, DT95, and a T-lymphoid cell line, MSB-1. Quantitative kinetic analysis demonstrated both the accumulation of MHC class II mRNA and the appearance of surface MHC class II protein in response to the synthesis of either v-*rel* or c-*rel*. We show that v-*rel* induced the expression of MHC class II in the avian B-cell lines DT40 and DT95 more rapidly than c-*rel* and that, several weeks after infection, v-*rel* induced MHC class II as much as 50-fold more efficiently than c-*rel*. Finally, in vitro infection of splenocytes with retroviruses that express v-*rel* or c-*rel* induced MHC class I, MHC class II, and IL-2R expression. Quantitative analysis confirmed that $p59^{v\text{-}rel}$ was consistently more efficient at inducing expression of all three immunoregulatory receptors than exogenous $p68^{c\text{-}rel}$. These data suggest that during tumor development, v-*rel* functions to induce (or suppress) the expression of genes similarly induced (or suppressed) by c-*rel*. The observations reported in this study are not in agreement with a model in which v-*rel* promotes tumor development by functioning as a dominant negative mutant of c-*rel*. In contrast, these findings support the hypothesis that lymphocyte immortalization and tumor development are the result, at least in part, of the capacity of v-*rel* to function as a dominant positive mutant that induces expression of genes normally regulated by c-*rel*.**

The c-*rel* proto-oncogene (16, 18, 38) belongs to the family of NF-κB/*rel* transcription factors, a family that shares a highly related amino-terminal domain of approximately 300 amino acids required for DNA binding and protein dimerization (for reviews, see references 3, 14, and 37). The members of this family include NFKB1 (p105/p50), NFKB2 (p100/p52), RelA (p65), RelB, and the *Drosophila melanogaster* morphogen *dorsal*. These proteins are present in inactive cytoplasmic complexes associated with inhibitory proteins designated IκBs (4). Stimulation of cells by different extracellular signals activates such complexes, resulting in protein translocation to the nucleus and modulation of gene expression mediated through binding of NF-κB/*rel* heterodimers and/or homodimers to κB DNA elements (for reviews, see references 3 and 37). Rel family proteins have been shown to function during (i) embryogenesis (47, 73), (ii) gene regulation in the immune response and/or acute-phase reactions (for reviews, see references 3 and 37), and (iii) liver regeneration (76). c-*rel* mRNA has been detected in all tissues analyzed, but it is most abundant in hematopoietic organs (15, 58). c-*rel* protein is present in the DNA-binding complexes that react with κB sites in the urokinase promoter (41) as well as in the promoters for interleukin-2 (IL-2) and IL-6 (35, 61). It has also been shown that c-*rel* can transactivate the gamma interferon enhancer (69) and the IL-2 receptor alpha (IL-2Rα) promoter (75).

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Deregulation of such potent and ubiquitous transcriptional regulators has the potential to produce a variety of pathological effects. Disruption of the NFKB2 gene by chromosomal translocation has been observed in some human B-cell lymphomas (62). The rearrangement and amplification of the c-*rel* locus have also been detected in human large-cell lymphomas (54). Transduction of the turkey c-*rel* gene by the avian reticuloendotheliosis retrovirus, strain A (REV-A), produced a replication-defective retrovirus, REV-T, encoding v-*rel*, that is able to induce a variety of hematopoietic tumors in young birds. These tumors, which are highly metastatic, are usually fatal (64).

Several structural alterations distinguish the v-*rel* oncogene from the c-*rel* gene (18, 40, 72, 79). The major alteration is a deletion of 118 amino acids of the C terminus of c-*rel* that are part of a transactivation domain and that can also affect cytoplasmic anchoring (46, 63). A comparison of v-*rel* and the turkey c-*rel* gene reveals the presence of 14 amino acid alterations, as well as 3 small deletions in the oncogene. Despite these numerous structural differences, v-*rel* shares two essential properties with c-*rel*: (i) the capacity to bind κB sites in DNA and (ii) the ability to form a large multimeric protein complex(es) that includes proteins of 124, 115, and 36 to 40 kDa (for a review, see reference 14).

The ability of the v-*rel* oncogene to induce hematopoietic neoplasia has been examined in order to elucidate the mechanism(s) through which an aberrant NF-κB/*rel* factor can initiate tumor development. On the basis of transient-expression experiments, several studies have suggested that transformation by v-*rel* is associated with repression of NF-κB/*rel*-

mediated activation of gene expression. These data indicate that v-*rel* functions as a dominant-negative mutant (7, 8, 44, 55, 63). However, other reports have indicated that v-*rel* is able to transactivate several viral promoters in avian and mammalian cell lines (34, 40, 77). A hormone-dependent v-*rel*-estrogen fusion protein has been shown to induce major histocompatibility complex (MHC) class I mRNA expression as well as high-mobility-group protein 14b in a transformed bone marrow cell line (13). Lastly, it has been demonstrated that a v-*rel* transactivation domain is necessary for its transformation potential (66). How this ability of v-*rel* to transactivate relates to the normal functions of c-*rel* or other NF-κB/*rel* proteins has not been analyzed.

This study began with the premise that v-*rel* has conserved some properties of c-*rel* that are essential for the induction of transformation. We have compared the abilities of c-*rel* and v-*rel* to induce expression of three genes that are likely to be regulated by c-*rel* in vivo, specifically, MHC class I, MHC class II, and the IL-2R. The results indicate that all three proteins are induced during an acute immune response as well as on chicken splenic cells infected with retroviruses that express v-*rel* or c-*rel*. Furthermore, a detailed quantitative analysis of the expression of these proteins in several types of cells revealed that v-*rel* induced their transcription more rapidly than exogenous c-*rel* does and with significantly greater efficiency.

## MATERIALS AND METHODS

**Plasmids.** All recombinant DNA techniques were performed by using conventional manipulations (65). pREV-0 is a REV-T-based vector designed for expression of exogenous genes in avian cells. Detailed description of the REV-0-based vector system will be presented elsewhere (61a). Briefly, pREV-T3 (24), a clone of REV-T derived from a circular DNA intermediate containing a single long terminal repeat and cloned as a *Sal*I-permuted linear fragment into pBR322, was modified by (i) transfer of the *Sal*I-linearized viral genome into pTZ-18R (Bio-Rad Laboratories, Richmond, Calif.), (ii) completion of the provirus structure by the addition of a 5′ long terminal repeat and leader sequences (obtained from the 3′ end of the REV-T3 clone) to the *Sal*I-linearized viral genome, (iii) oligonucleotide site-directed mutagenesis of v-*rel* flanking sequences to create *Xho*I and *Bss*HII sites in noncoding regions, and (iv) replacement of v-*rel* by a short synthetic *Xho*I-*Bss*HII fragment. pREV-TW was constructed by insertion of an *Xba*I-*Nru*I fragment containing the entire v-*rel* gene (24) into the *Xho*I-*Bss*HII site of pREV-0 by using an adaptor plasmid. pREV-C was created by insertion of the *Hpa*II fragment of chicken c-*rel* (18) into REV-0 by using the same strategy.

pCSV11S3 is an infectious genomic clone of chicken syncytial virus (CSV) and was isolated in this laboratory from the 11S3 cell line (11) transformed by REV-T with CSV as a helper virus. pSW253 contains an infectious genomic clone of REV-A in pBR322 (23). pREVA6 is the REV-A genomic clone from pSW253 recloned as an *Eco*RI fragment into pUC19.

**Chickens, cell lines, and tissue culture.** Embryonated SC eggs were purchased from Hyline International Hatcheries (Dallas Center, Iowa). DT40 is a B-cell line established from an avian leukosis virus (ALV)-infected chicken bearing a bursa-derived lymphoid tumor. Both the primary tumor and DT40 contain an integrated ALV provirus within the c-*myc* locus (2). DT95, another B-cell line expressing elevated levels of *myc* protein, is also derived from a chicken with ALV-induced lymphoid leukosis and is similar to DT40 except that it exhibits a more mature phenotype (2). MSB-1 is a chicken T-cell line derived from a Marek's disease virus-induced lymphoma (1). CSV infection of S2A3, a REV-T nonproducer cell line, was used to rescue REV-T(CSV) (10). Chicken embryonic fibroblasts (CEF) were prepared from 10-day-old embryos.

All in vitro cell cultures used Dulbecco's modified Eagle's medium (DMEM) 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).

**Transfection, viruses, and viral assays.** Secondary cultures of CEF were plated at $5 \times 10^5$ cells per 90-mm culture dish and cotransfected by a modified calcium phosphate method as described elsewhere (21). CEF were cotransfected with 30 μg of a plasmid, pREV-C or pREV-TW, containing the replication-defective viruses REV-C or REV-TW, respectively, and 1 μg of a plasmid containing a genomic clone of a replication-competent helper virus, pCSV11S3 or pREVA6, or with 1 μg of pCSV11S3 alone. Viruses were harvested after 6 days of cultivation.

The infectious titers for CSV helper virus and for the replication-defective virus, REV-C or REV-TW, were determined by a modified quantitative immunocytochemical assay (74). Two-week-old CEF were plated at $7 \times 10^5$ cells per 60-mm culture dish. Twenty-four hours later, they were infected with 0.4 ml of virus diluted in DMEM containing 2% bovine calf serum and incubated at 37°C for 1 h. For assays of CSV, infected cells were overlaid with DMEM containing 10% tryptose phosphate broth, 4.5% calf serum, 0.5% chicken serum, and 0.7% agar. For assays of REV-C or REV-TW, following the 1-h infection at 37°C, the cells were superinfected for 1 h with $10^6$ infectious units of REV-A, and then 4 ml of growth medium was added to each dish. Six days after infection with CSV, the agar was removed from dishes and the cells were washed in phosphate-buffered saline (PBS), fixed for 20 min in 2% paraformaldehyde, and stained for the presence of CSV antigens. Two days after infection with REV-C or REV-TW, monolayers were similarly washed, fixed, and stained for the presence of v-*rel* or c-*rel* protein. After fixation, all plates were washed three times in PBS. Monolayers to be stained for the presence of *rel* protein were further treated with methanol-acetone (1:1) for 2 min and washed again. All plates were preincubated with PBS–0.1% bovine serum albumin (BSA) for 30 min and then incubated for 1 h with 1 ml of either monoclonal antibody HY87 (anti-v-*rel*) or monoclonal antibody HY83 (anti-CSV). After three washes with PBS, the plates were incubated for 1 h with biotinylated goat anti-mouse immunoglobulin (Ig) antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala.) diluted 1:1,000 in PBS–1% BSA. After a second series of washes in PBS, the monolayers were incubated for 1 h with streptavidin-linked alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, Ind.) diluted 1:2,000 in a solution containing PBS–0.1% BSA, 5 mM EDTA, and 0.5% Triton X-100. The plates were washed twice in washing buffer (10 mM $KH_2PO_4$ [pH 6.5], 0.5 M NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% BSA) for 30 min each and equilibrated with predetection buffer (100 mM Tris-HCl [pH 8.8], 100 mM NaCl, 5 mM $MgCl_2$) with two 2-min washes. One milliliter of the detection solution (100 μg of 5-bromo-4-chloro-3-indolyl phosphate [BCIP]–1 mg of 4-nitroblue tetrazolium chloride per 1 ml of predetection buffer [Boehringer Mannheim Corp.]) was added, and the stain was allowed to develop for 10 to 20 min. Color development was stopped by two washes in 10 mM Tris-HCl (pH 8.0)–20 mM EDTA. Foci

TABLE 1. Monoclonal antibodies used in this study

| Antibody | Antigen or specificity | Isotype | Source or reference |
|---|---|---|---|
| HY32 | Chicken MHC class II | IgG2b[a] | 9 |
| CIa-I | Chicken MHC class II | IgM | 33 |
| F21-2 | Chicken MHC class I | IgG1 | 28, 57 |
| B337 | Chicken IL-2R | IgG2a[a] | 51 |
| INN-CH 16 | Chicken IL-2R | IgM | 39, 67 |
| 3C1 | Chicken v-*rel* and c-*rel* | IgG3 | 43 |
| HY87 | Chicken v-*rel* and c-*rel* | IgG1[a] | This study[b] |
| HY19 | Chicken IgM | IgG1[a] | 10 |
| TCR-1 | Recognizes T-cell receptors in chickens | | 22, 71 |
| TCR-2 | Recognizes T-cell receptors in chickens | IgG1 | 22, 26 |
| TCR-3 | Recognizes T-cell receptors in chickens | IgG1 | 20 |
| CT-4 | Avian homolog of CD4 | IgG1 | 19 |
| CT-8 | Avian homolog of CD8 | IgG1 | 19 |
| CMTDI and CMTDII | Recognize activated chicken macrophages | IgM | 32 |
| CVI-CHNL-68.1 | Recognizes cells of monocyte-macrophage lineage in chickens | IgG1 | 45 |
| HY83 | Recognizes CSV-infected cells | IgG1[a] | This study[b] |

[a] Subclass isotype determined in our laboratory.
[b] Monoclonal antibodies HY87 and HY83 were developed in our laboratory.

of anti-CSV antibody immunoreactive cells were scored by eye, and foci of anti-v-*rel* antibody immunoreactive cells were counted with a microscope.

**Antibodies.** The monoclonal antibodies used in this investigation are listed in Table 1. Monoclonal antibody HY87 was developed against a bacterial fusion protein trpE–v-*rel* (amino acid residues 115 to 292 of v-*rel*) (68) by using the Sp2/0 cell line and standard protocols for hybridoma production (42). This monoclonal antibody specifically recognizes $p59^{v\text{-}rel}$ and $p68^{c\text{-}rel}$ by immunoprecipitation and by Western analysis (immunoblotting) (data not shown). The following pairs of monoclonal antibodies have been shown to react with the same antigen by a combined immunoprecipitation and Western analysis: HY87 and 3C1, HY32 and CIa-I, and B337 and INN-CH 16 (data not shown). Monoclonal antibodies B337 and INN-CH 16 recognize a 45- to 50-kDa antigen present on the surface of activated avian T lymphocytes and analyzed functionally as the chicken IL-2R (39, 51, 67). In agreement with others (27), we will call this antigen chicken IL-2R. Biotin- or fluorochrome-labeled antibodies used in this study were purchased from Southern Biotechnology Associates, Inc., unless otherwise indicated. Isotype analysis of monoclonal antibodies was done by using the SBA Clonotyping System III (Southern Biotechnology Associates).

**Indirect immunofluorescence.** Indirect immunofluorescence staining was performed as follows. Cells were fixed and permeabilized for 5 min with methanol-acetone (1:1), washed twice with PBS, and incubated for 30 min with PBS–0.1% BSA. Cells were then incubated for 1 h with hybridoma supernatant containing monoclonal antibody, washed with PBS, and incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies directed against specific subclasses. Cells were washed two times with PBS, with a final wash overnight, and mounted with Fluoromount-C (Southern Biotechnology Associates). Intensity of staining was analyzed under a microscope.

**Flow cytometry analysis.** Splenic cell suspensions for flow cytometric analysis were prepared by passing the organ through nylon mesh into DMEM supplemented with 1% chicken serum. Mononuclear white splenocytes were separated by centrifugation of the cell suspension on Isolymph (Gallard-Schlesinger Industries, Inc., Carle Place, N.Y.) for 40 min at 25°C and 400 × *g*. After being washed twice with a 50-ml volume of PBS, cells were fixed with cold 100% methanol for 15 min on ice (52). Fixed cells were washed three times with PBS, preincubated with PBS–1% BSA, and then incubated with one or more monoclonal antibodies for 30 min on ice. After being washed with PBS–1% BSA, cells were incubated with a mixture of FITC- and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgGs of the appropriate subclasses, each diluted 1:80, washed, and analyzed by FACScan (Becton-Dickinson). Data were analyzed by using the LYSIS program (Becton-Dickinson).

Induction of cell surface antigen expression on lymphoid cell lines was quantified by staining of live cells. Cells ($10^6$) were washed and incubated for 30 min at 4°C with a monoclonal antibody diluted in PBS–1% BSA–2 mM $NaN_3$. After being washed, cells were incubated with FITC-conjugated goat anti-mouse IgGs of the appropriate subclasses for 1 h at 4°C, washed, and analyzed by immunofluorescence flow cytometry. Dead cells were eliminated by staining with propidium iodide (10 μg/ml).

**Western analysis.** Cells ($10^7$) were collected, washed with PBS, resuspended in 25 μl of suspension buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl–1 μg of aprotinin per ml and 100 μg of phenylmethanesulfonyl fluoride per ml, and then 25 μl of 2× sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) was added, boiled for 10 min, and centrifuged for 10 min, after which the supernatant was removed and stored at −20°C. Lysates were analyzed by the Laemmli discontinuous system. Ten microliters of sample was electrophoresed in 10% polyacrylamide slab gels with a 5% polyacrylamide stacking gel on Mini-PROTEAN II (Bio-Rad Laboratories). Molecular mass marker proteins ranging from 14 to 97 kDa were loaded on each gel (Bio-Rad Laboratories). Following electrophoresis, proteins were electroblotted to 0.45-μm-pore-size nitrocellulose filters (Schleicher and Schuell, Keene, N.H.) for 3 h at 4°C and 1.2 A in transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, 20% methanol). Membranes were stained with Ponceau S (0.2% Ponceau S, 3% trichloroacetic acid, 3% sulfosalicylic acid), cut, destained with water, and dried. For comparison of protein loading, a gel with a parallel set of samples was stained with Coomassie blue, destained, and dried. For immunostaining, membranes were wet with TBST

(10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) and then saturated in blocking solution (TBST–1% BSA) for 1 h. Membranes were incubated with monoclonal antibody diluted in blocking solution for 1 h. Membranes were washed three times with TBST and incubated with goat anti-mouse IgG biotinylated antibody diluted 1:1,000 in blocking solution for 1 h. After three washes, membranes were incubated with streptavidin-linked alkaline phosphatase at 1:2,000 for 1 h. The membranes were thoroughly washed, and staining was developed in detection buffer (100 μg of BCIP, 1 mg of 4-nitroblue tetrazolium chloride per ml of 100 mM Tris-HCl [pH 8.8], 100 mM NaCl, 5 mM $MgCl_2$). Development was stopped with 10 mM Tris-HCl (pH 8.0)–20 mM EDTA. The relative amount of protein was determined by densitometric analysis with a Bioscan Optimas Imaging System (Bioscan, Edmonds, Wash.).

**Northern analysis and probes.** Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (25). For Northern (RNA) analysis, 5 to 15 μg of RNA was heated in 15 μl of denaturation buffer {1.5 M deionized glyoxal [pH 5.0], 66% dimethyl sulfoxide, 20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer [pH 7.0]} for 1 h at 55°C. Samples were cooled and kept on ice until loading. Five microliters of loading buffer (50% glycerol, 0.25% bromophenol blue) was added. RNA was separated by electrophoresis in a 1% agarose gel with circulating, cooled 20 mM MOPS buffer. RNA was transferred to Immobilon N membrane (Millipore Corporation, Bedford, Mass.) in 10× SSC (1× SSC is 15 mM sodium citrate plus 150 mM NaCl [pH 7.2]) and fixed by air drying. Samples were electrophoresed with 1 μg of ethidium bromide per lane, after which the gel was transferred, and the membrane was photographed after blotting. Recombinant plasmids or DNA fragments were labeled with [α-$^{32}$P]dCTP by nick translation (Bethesda Research Laboratories, Gaithersburg, Md.). Hybridization was in 0.5 M $Na_2HPO_4 \cdot NaH_2PO_4$ (pH 7.2)–5 mM EDTA–0.1% BSA–0.1% Ficoll (molecular weight, 400,000)–tRNA (0.1 mg/ml) at 68°C. Filters were washed twice briefly in 2× SSC–0.5% SDS at 20°C, once for 30 min at 68°C, and briefly in 0.1× SSC at 20°C. RNA samples for slot blot analysis were denaturated as described above, serially diluted in denaturation buffer at 4°C, and transferred to Immobilon N membranes with a Hybri-slot manifold (Bethesda Research Laboratories). After being dried, membranes were hybridized as described above.

The pCCII-4-$\beta_2$ probe is a subclone of a chicken MHC class II β-chain gene (80). pCCII-4-$\beta_2$ contains almost the whole $\beta_2$ exon of the chicken MHC class II chain gene as a 260-bp *Pst*I-*Hae*III fragment. pc-rel2 contains the *Hpa*II fragment of the chicken c-*rel* gene (18) in a modified phagemid pTZ-18R (Bio-Rad Laboratories).

## RESULTS

**A comparative analysis of MHC class I, MHC class II, and IL-2R proteins expressed on *rel*-transformed cells and on GVHD splenocytes.** Changes in the expression of MHC class I, MHC class II, and IL-2R proteins on splenocytes during v-*rel*-induced tumor development were examined and compared with changes in the same proteins during an immunological reaction. One-day-old chickens were (i) infected with REV-TW(CSV), a retrovirus that expresses v-*rel* and that replicates with the helper virus CSV, or with CSV alone or (ii) injected with a splenic allograft in order to induce graft-versus-host disease (GVHD). The autopsy data from animals sacrificed 7 days after infection are presented in Table 2. All birds infected by REV-TW(CSV) exhibited macroscopically recognizable tumor lesions in the spleen and liver. Groups of CSV-infected or REV-TW(CSV)-infected birds, as well as birds with GVHD, exhibited a decrease in body weight but an increase in spleen weight. It was notable that average splenic weights in the CSV-infected and GVHD birds were 185 and 170% that of control birds, respectively, while the REV-TW(CSV)-infected spleen was, on average, seven times larger than that of the uninfected bird and four times larger than that of the CSV-infected bird.

TABLE 2. Autopsy data from animals infected with REV-TW(CSV) or CSV or injected with an allograft

| Animals[a] | Presence of macroscopic tumor[b] | Body wt (g) (mean ± SD) | Spleen wt (mg) (mean ± SD) |
|---|---|---|---|
| Uninfected | – | 81.7 ± 8.0 | 71.0 ± 14.4 |
| CSV infected | – | 75.3 ± 4.0 | 131.0 ± 17.4 |
| REV-TW(CSV) infected | + | 66.0 ± 5.7 | 508.3 ± 325.8[c] |
| GVHD induced | – | 77.0 ± 5.0 | 120.6 ± 3.2 |

[a] Each group contained three birds.
[b] –, no tumor present; +, tumor present.
[c] The actual spleen weights were 215, 451, and 859 mg. The large variation is a result of the rapid and exponential growth of tumorous spleens relative to the time of sacrifice.

The expression of surface antigens and *rel* proteins (exogenous v-*rel* and/or endogenous c-*rel*) was examined by flow cytometric analysis of mononuclear white splenocytes (Fig. 1). Compared with uninfected cells, all REV-TW(CSV)-infected, CSV-infected, and GVHD birds had increased numbers of splenocytes expressing MHC class I, MHC class II, and IL-2R proteins. These data indicate that there was an increase in the number of cells expressing these proteins as well as in the steady-state levels expressed in these cells. In all four groups of birds, there was a positive correlation between *rel* protein expression and the amount of each of the three antigens examined. The quadrant analysis reveals that, when compared with CSV-infected cells, the percentage of REV-TW(CSV)-infected cells positive for MHC class I and *rel* increased by 5%, the percentage positive for MHC class II and *rel* increased by 29%, and the percentage positive for IL-2R and *rel* increased by 49%. Similar results were obtained from the analysis of GVHD birds. Compared with those from uninfected birds, splenocytes from GVHD birds possessed an increased percentage of double-positive cells for each of the immunoregulatory proteins analyzed: 21% for MHC class I and *rel*, 16% for MHC class II and *rel*, and 3% for IL-2R and *rel*. Increased expression of *rel* protein in these cells represents endogenous expression of c-*rel* associated with the GVHD immune response.

These data indicate that v-*rel*-transformed cell lines that emerge from REV-TW-infected birds could express all three of these immunoregulatory surface molecules. In order to determine whether expression of these proteins persists with propagation and continued selection of the cells, over 50 lines representing three different phenotypes were analyzed for expression of these surface markers. The lines analyzed also included several developed from chickens infected with REV-C (a retrovirus that expresses c-*rel*) and that contained deleted forms of c-*rel*. The analysis of nine representative lines (Table 3) revealed that all three phenotypes, represented as (i) IgM positive, (ii) TCR-2 positive, or (iii) IgM, TCR-1, TCR-2, and TCR-3 negative, expressed high levels of MHC class I, MHC class II, and IL-2R. The expression of MHC class II was further confirmed by the Northern analysis of these cell lines (Fig. 2). Some differences between the ratio of MHC class II surface protein to mRNA can be detected among the clones.

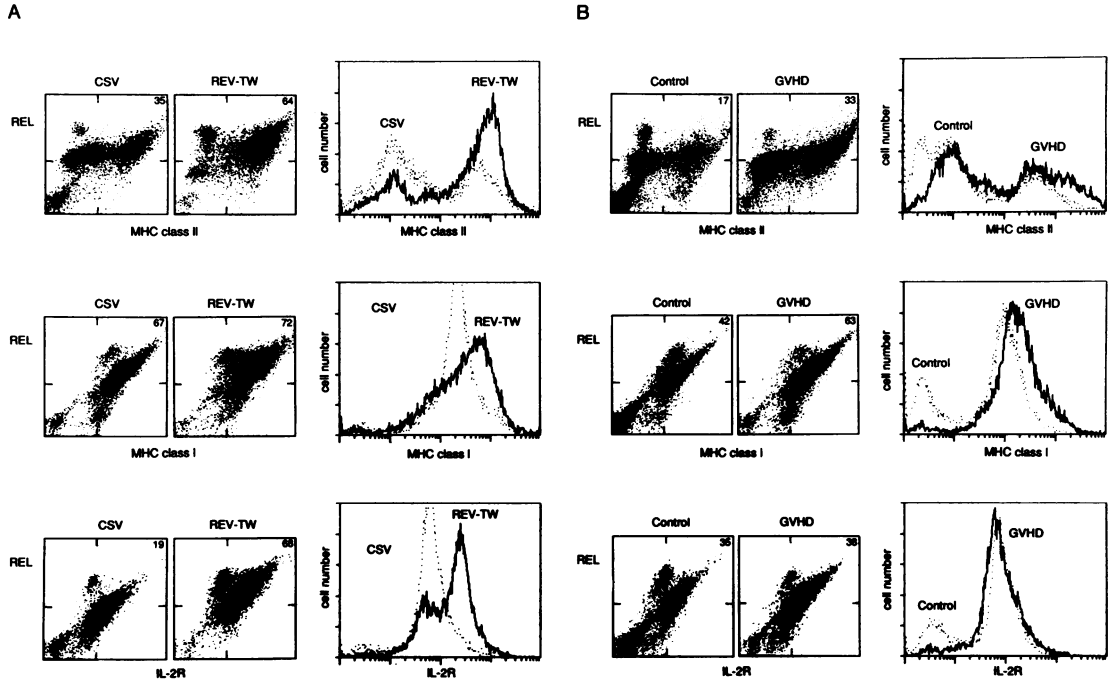


FIG. 1. Expression of MHC class I, MHC class II, and IL-2R proteins on the surface of spleen cells from REV-TW(CSV)-infected chickens and chickens with GVHD. (A) One-day-old chicks were infected intravenously with 0.3 ml of REV-TW(CSV) containing $10^5$ infectious units (IU) of REV-TW and $10^6$ IU of CSV per ml or with 0.3 ml of CSV ($10^6$ IU/ml). (B) In parallel, a second group of 1-day-old chicks was injected intravenously with 0.3 ml of mononuclear white splenocytes ($3 \times 10^8$ cells per ml) isolated by Isolymph centrifugation from the spleen of an adult allogeneic chicken. Chickens from both groups, with controls, were killed 7 days after injection. The autopsy data are summarized in Table 2. Mononuclear white splenocytes were analyzed by flow cytometry. Each sample was double-stained with 3C1 (anti-*rel*) antibody and a monoclonal antibody against the surface antigen indicated. Dot plots present relative immunofluorescence intensity on two log axes. Experimental samples are compared with negative controls stained only with a mixture of the appropriate second antibodies. Numbers in the upper right corners represent the percentage of double-positive cells. Immunofluorescence intensity data from the two populations are also presented as histograms showing relative immunofluorescence intensity (log scale) versus cell number (linear scale). The scales are the same for all histograms. Dashed lines represent cells from CSV-infected (A) and uninfected (B) chickens. Solid lines represent splenocytes from REV-TW(CSV)-infected chickens (A) and from chickens with GVHD (B).

This variation is most likely related to differences in the type of the target cell transformed by *rel*, differences in regulation of the amount of mRNA transcribed per cell and in the efficiency of its translation, and differences in the amount of protein correctly modified for presentation on the cell surface. The data presented in Fig. 2, therefore, are consistent with those presented in Fig. 1 and suggest that *rel*-induced expression persists on tumor-derived cell lines. The extent to which any antigen is induced by different *rel* proteins, however, is most likely clone specific. The inability to examine the original target cell, as well as to culture CSV-infected cells for extended periods of time, makes it impossible to quantify this induction.

**Induction of MHC class I, MHC class II, and IL-2R expression on DT95 and MSB-1 lymphoid cell lines following infection with REV-C(CSV) or REV-TW(CSV).** The in vivo studies indicate that infection with a retrovirus that expresses v-*rel* is associated with induction of MHC class I, MHC class II, and IL-2R proteins. The analysis indicates that expression of endogenous c-*rel* also induces expression of these proteins in splenic cells. As presented above, however, heterogeneity of the target cell population in the spleen, as well as variation in selective pressures that influence cell line evolution, complicates a quantitative analysis of induction. In order to continue this analysis, therefore, an in vitro system suited to this purpose was established. Two different chicken lymphoid cell lines, DT95 and MSB-1, were infected with REV-TW(CSV) (v-*rel*), REV-C(CSV) (c-*rel*), or CSV (Fig. 3). DT95 has the phenotype of a mature B-cell line expressing surface IgM that is secreted into the culture medium (2). The level of expression of MHC class I, MHC class II, and IL-2R antigens on this cell line is at the threshold of detection for immunofluorescence procedures. The MSB-1 cell line possesses the phenotype of a mature T cell, expressing both T-cell receptor 2 (TCR-2) and CT-4, low levels of MHC class II, and high levels of both MHC class I and IL-2R antigens. Infection of DT95 or MSB-1 with either REV-TW(CSV) or REV-C(CSV) induced expression of all three immunoregulatory surface proteins (Fig. 3A). Expression of the proteins was dependent upon the synthesis of an exogenous *rel* protein. Five days after infection, both cell lines expressed elevated amounts of either $p68^{c\text{-}rel}$ or $p59^{v\text{-}rel}$ (Fig. 3B). In all cases, induction following REV-TW(CSV) infection was higher than with REV-C(CSV). The largest difference observed was for the induction of MHC class II protein (Tables 4 and 5).

**Kinetic analysis of MHC class II induction following infection of DT40 and DT95 with REV-C(CSV) or REV-TW(CSV).** REV-C(CSV) and REV-TW(CSV) infection of DT95 and MSB-1 suggested differences in the efficiency with which c-*rel* and v-*rel* induced MHC class I, MHC class II, and IL-2R proteins. In order to better characterize these differences, the appearance of MHC class II was examined as a function of

TABLE 3. Expression of MHC class I, MHC class II, and IL-2R antigens on REV-T(CSV)-, REV-TW(CSV)-, and REV-C(CSV)-derived cell lines

| Cell line[a] | Virus | Staining intensity with monoclonal antibody against designated antigen[b]: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IgM | TCR-1 and TCR-3 | TCR-2 | CT-4 | CT-8 | CVI-CHNL-68.1[c] | CMTD I | CMTD II | MHC class I | MHC class II | IL-2R |
| SS.1 | REV-T(CSV)[d] | ++ | NT | − | NT | NT | +++ | − | − | ++++ | +++ | ++ |
| 123/12 | REV-TW(REV-A) | ++ | NT | − | NT | NT | ++ | − | − | +++ | ++ | ++ |
| 160/2 | REV-TW(CSV) | − | NT | + | + | + | ++ | − | − | +++ | +++ | +++ |
| 160/8 | REV-TW(CSV) | − | NT | + | − | + | ++ | ++ | − | ++++ | ++ | ++ |
| 189/5[e] | REV-C(CSV) | − | NT | + | ++++ | − | ++ | + | − | ++++ | ++++ | ++++ |
| 189/1[e] | REV-C(CSV) | − | NT | + | + | − | + | − | − | ++++ | ++ | + |
| 123/6 | REV-TW(CSV) | − | − | − | − | − | ++ | − | − | ++++ | +++ | +++ |
| 123/6T | REV-TW(REV-A) | − | − | − | − | − | ++ | ++ | + | +++ | +++ | ++ |
| 123/8T | REV-TW(REV-A) | − | − | − | − | − | ++ | ++ | + | +++ | +++ | ++ |

[a] Eight cell lines were derived from splenic tumors isolated from chickens infected 1 day after hatching by REV-TW(CSV) or REV-C(CSV). Cell line SS.1 was isolated from an in vitro transformation assay in which splenic cells were infected with REV-T(CSV).

[b] Cell lines were analyzed by indirect immunofluorescence staining with the indicated antibodies. Cells were classified visually into five groups by staining intensity as follows: ++++, very high; +++, high; ++, medium; +, low; −, negative. NT indicates not tested. Cell lines stained homogenously (90 to 100% of the population) for each antigen tested. About $10^5$ cells were analyzed for reactivity with a specific monoclonal antibody.

[c] The induction of a monocyte-macrophage lineage-specific antigen, CVI-CHNL-68.1, was detected on DT95 following infection with REV-TW(CSV) or REV-C(CSV) (data not shown).

[d] REV-T(CSV) was rescued from a nonproducer cell line, S2A3, by infection with CSV and differs from REV-TW(CSV) (64a).

[e] These cell lines contained altered proviral structures that express c-*rel* with C-terminal deletions in addition to the original REV-C provirus.

exogenous *rel* expression in the DT40 and DT95 cell lines. These chicken B-cell lines expressed viral *rel* proteins for several months after infection (data not shown). This observation indicates that the cells received an in vitro proliferative advantage from the expression of $p59^{v\text{-}rel}$ and $p68^{c\text{-}rel}$. Flow cytometric analysis demonstrated that MHC class II expression gradually increased over a period of one month (Fig. 4). The increase in MHC class II protein was more rapid after infection with REV-TW(CSV) than after infection with REV-C(CSV), and the difference in induced expression was evident in both B-cell lines. Infection of DT40 and DT95 with CSV did not significantly alter expression of MHC class II (data not shown).

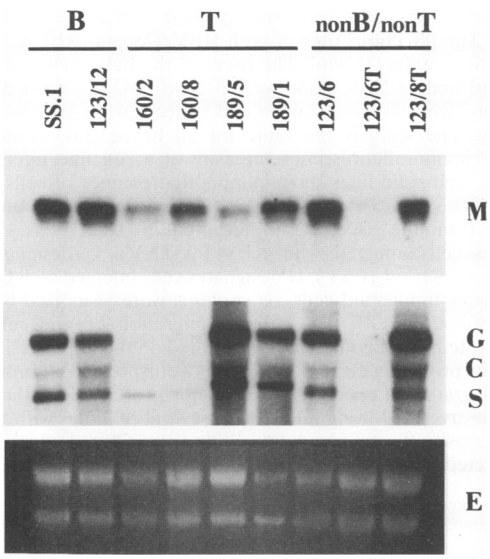


FIG. 2. Northern analysis of MHC class II and *rel* expression in *rel*-transformed cell lines of three different phenotypes. The *rel*-transformed cell lines expressed IgM (B) or TCR-2 (T), or they were negative for IgM and TCR-1, TCR-2, and TCR-3 (nonB/nonT). (Detailed immunofluorescence analysis of the same cell lines is presented in Table 3). Each lane contains 10 μg of total cellular RNA. The upper filter (M) was hybridized with a pCCII-4-$\beta_2$ probe, and the middle filter (G, C, S) was hybridized with a pc-rel2 probe. The viral genomic (G) and spliced (S) RNAs, the endogenous c-*rel* (C) mRNA, and MHC class II mRNA (M) are indicated. Ethidium bromide (E) staining of RNA in a parallel gel is also shown.

Northern analysis revealed that the relative expression of v-*rel* and c-*rel* RNA also changed with time (Fig. 5A). Six days after infection, both *rel* RNAs were equally abundant in DT95, reflecting the fact that comparable amounts of virus were used to establish the infection. Two weeks later, however, both cell lines expressed significantly more c-*rel* than v-*rel* RNA. MHC class II mRNA was more abundant in cell lines expressing v-*rel*. Measurement of the amount of *rel* and MHC class II RNA by dilution analysis (Fig. 5B) indicated that v-*rel* was 50-fold more efficient than c-*rel* at inducing the expression of MHC class II in DT40 several weeks after infection. Similar results were obtained from an analysis of *rel*-induced expression of MHC class II in DT95. Additional experiments demonstrated that the ratios of c-*rel*/v-*rel* protein and c-*rel*/v-*rel* RNA differ by less than twofold in these cell lines (data not shown). It is possible to conclude, therefore, that $p59^{v\text{-}rel}$ is significantly more efficient than $p68^{c\text{-}rel}$ at inducing the expression of MHC class II.

**Expression of MHC class I, MHC class II, and IL-2R protein on the splenic cells infected by retroviruses expressing c-*rel* and v-*rel*.** In order to confirm this conclusion and extend it to the process of v-*rel*-mediated transformation, splenic mononuclear cells were infected in vitro with CSV, REV-C(CSV), or REV-TW(CSV). The infected cells were analyzed for *rel* protein expression, cellular proliferation, and induction of MHC class I, MHC class II, and IL-2R proteins (Fig. 6). As described above, within a few days of infection there was more $p68^{c\text{-}rel}$ than $p59^{v\text{-}rel}$ (Fig. 6A). Immunofluorescence analysis demonstrated that nearly 100% of the cells expressed c-*rel* or v-*rel* and that the level of expression increased with time (data

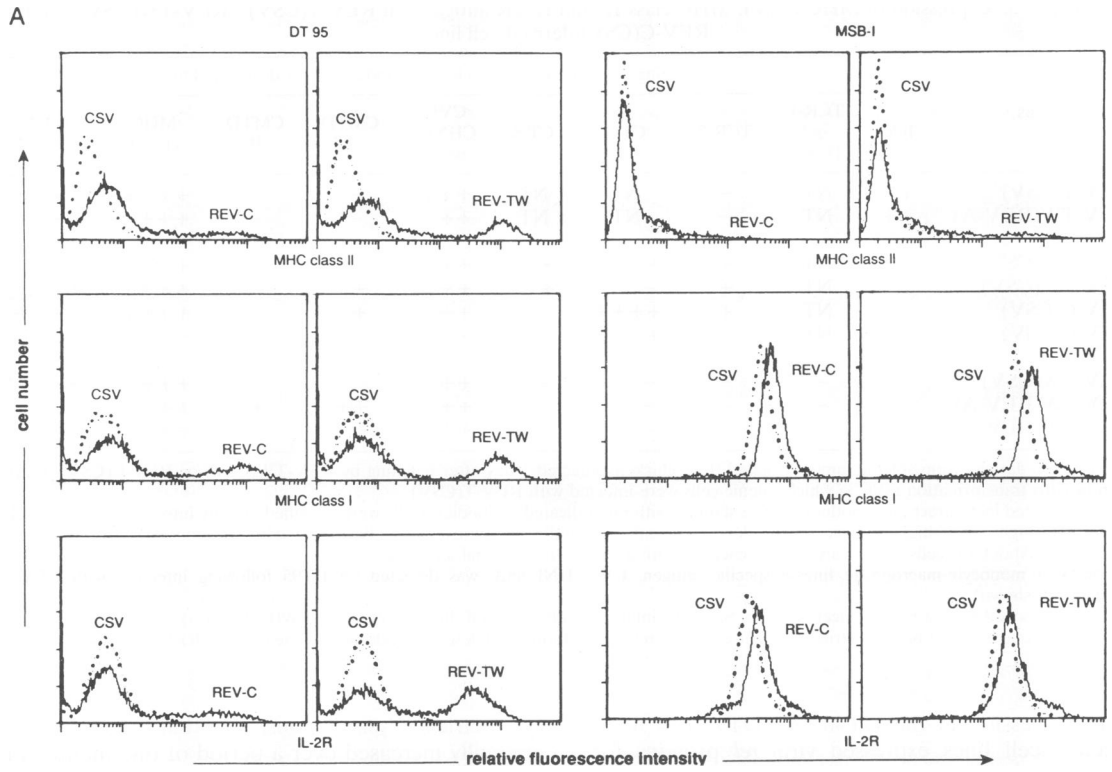


FIG. 3. Expression of MHC class I, MHC class II, and IL-2R proteins on the surface of REV-C(CSV)- and REV-TW(CSV)-infected chicken lymphoid cell lines. (A) DT95 and MSB-1 were infected by the indicated virus at a multiplicity of infection of >3. Cells ($5 \times 10^5$) were incubated with 10 ml of virus stock for 1 h at 37°C. After centrifugation, the cells were resuspended in 5 ml of fresh growth medium. The infectious titer of both REV-TW and REV-C was $4 \times 10^5$ infectious units (IU)/ml. The titers of the helper viruses in these stocks and that of CSV alone were $3 \times 10^6$ IU/ml. After 5 days of cultivation, live cells were stained and $10^4$ cells were analyzed by flow cytometry. The scale is the same for all histograms. Dashed lines represent immunofluorescence intensity of a cell line infected only with CSV, and solid lines show immunofluorescence of cells infected by either REV-C(CSV) or REV-TW(CSV). (B) Western analysis of $10^4$ cells from the experiment presented in panel A is shown. T designates cells infected with REV-TW(CSV), C designates cells infected with REV-C(CSV), H designates cells infected with CSV, and U designates uninfected cells. *rel* proteins were visualized by monoclonal antibody HY87. The relative migration of molecular mass marker proteins (in kilodaltons) is indicated on the left. It is likely that the smaller proteins detected below the overexpressed *rel* proteins are specific degradation products of the major protein, since the protein species observed following infection of several cell lines with the same *rel* virus construct were the same, while they differed when the cells were infected by different *rel* virus constructs (data not shown).

not shown). Two days after infection, splenocyte cultures infected with either REV-C or REV-TW contained more cells than splenic cultures infected with only helper virus (CSV). Four days later, cultures infected with REV-C or REV-TW contained 5-fold and 18-fold more cells, respectively. Ten days after infection, cells expressing v-*rel* were 10-fold more abundant than cells expressing c-*rel* (Fig. 6B). These data demonstrated that both c-*rel* and v-*rel* expression induce a proliferative response by splenic cells but that v-*rel* was significantly more potent in this regard. Measurement of the three immunoregulatory receptors was normalized to the levels of expressed *rel* protein. Six days after infection, $p59^{v\text{-}rel}$ protein was 8-fold more efficient in inducing MHC class I, 10-fold more efficient at inducing MHC class II, and 2-fold more efficient at inducing IL-2R (Fig. 6C). This induction pattern is very similar to that observed in our studies on DT95 and MSB-1 (Tables 4 and 5). Taken together, these data indicate that analysis of *rel* protein induction of target gene expression in DT95 and DT40 represents a viable model system in which to examine aberrant gene expression induced during transformation of avian hematopoietic cells.

## DISCUSSION

**Induction of MHC class I, MHC class II, and IL-2R on splenic tumor cells and splenocytes from animals with GVHD.** Mussman and Twiehaus noticed a striking similarity between the pathogenesis of REV-T(REV-A) infection and GVHD (60), and they initially proposed calling REV-T-induced disease GVH-like or acute runting syndrome. We have observed that chickens infected by REV-TW(CSV) and chickens injected with an allogeneic graft were similar not only histopathologically but also in their *rel*-associated elevated expression of three immunoregulatory molecules, MHC class I,

TABLE 4. Expression of MHC class I, MHC class II, and IL-2R proteins on the surface of DT95 after infection by REV-C(CSV), REV-TW(CSV), or CSV

| Virus | SFI[a] (relative units) of: | | | | | |
|---|---|---|---|---|---|---|
| | MHC class I | | MHC class II | | IL-2R | |
| | CSV (+)[b] | CSV (−) | CSV (+) | CSV (−) | CSV (+) | CSV (−) |
| None | 621 | | 15 | | 459 | |
| CSV | 666 | 0 | 205 | 0 | 667 | 0 |
| REV-C(CSV) | 29,400 | 28,800 | 10,300 | 10,100 | 12,700 | 12,000 |
| REV-TW(CSV) | 31,300 | 30,600 | 29,400 | 29,200 | 21,800 | 21,200 |

[a] Summary fluorescence intensity (SFI) was calculated by integration of the immunofluorescence intensity of $10^4$ cells and correction by subtraction of the immunofluorescence intensity of $10^4$ cells stained only with second antibody. The analysis was performed on cells harvested 5 days after infection (Fig. 3). The values were rounded off to 3 significant numbers. This experiment has been repeated five times with similar results. Data from one typical experiment are presented.

[b] CSV (+) represents the summary fluorescence intensity (SFI) value for cells infected with REV-TW(CSV), REV-C(CSV), or CSV or for uninfected cells. CSV (−) is the same SFI value corrected by subtraction of the SFI value for cells infected with CSV alone.

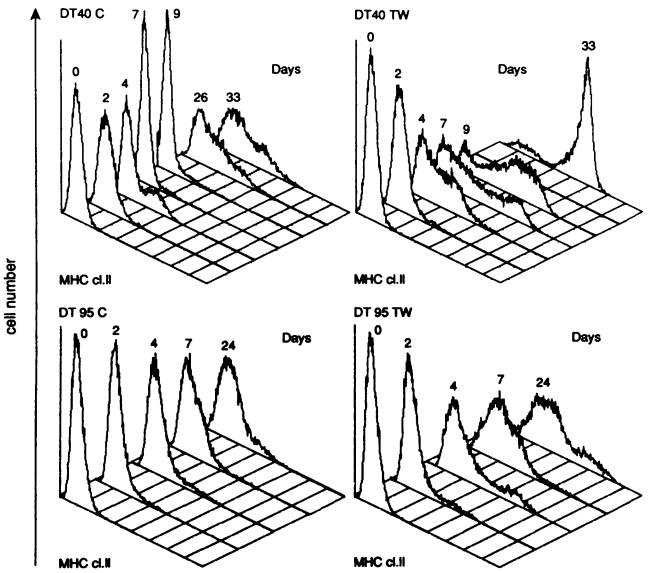


FIG. 4. Kinetic analysis of MHC class II antigen expression on DT40 and DT95 infected with REV-C(CSV) or REV-TW(CSV). DT40 and DT95 cell lines were infected with REV-C(CSV) (DT40 C and DT 95 C), with REV-TW(CSV) (DT40 TW and DT 95 TW), or with CSV (not shown). The infectious titer of both REV-C and REV-TW was $6 \times 10^4$ infectious units (IU)/ml. Titers of CSV in these stocks were $6 \times 10^4$ and $5 \times 10^5$ IU/ml, respectively, and the titer of CSV alone was $3 \times 10^5$ IU/ml. Cells ($5 \times 10^5$) were cultivated with 10 ml of virus stock and harvested on the indicated days after infection for analysis by flow cytometry. Immunofluorescence data were collected from $10^4$ cells in each sample. The *x* axis represents relative fluorescence intensity of MHC class II protein (MHC cl. II) (log scale), and the *y* axis represents cell number. *rel* overexpression was verified by staining with monoclonal antibody HY87 at each time point. Analysis by immunofluorescence microscopy demonstrated that greater than 90% of the cells expressed *rel* proteins at all times after infection.

MHC class II, and IL-2R. While there is now no doubt regarding the neoplastic nature of the principal pathogenic response to REV-T infection, our results indicate that there remains a basis for describing the tumorous spleen and one that is undergoing GVHD as similar. Immune cells in an allograft are stimulated by histocompatibility antigens expressed on cells of the recipient host. This stimulation is acute and continuous and results in immune reactions that are severe and include proliferation of blasting lymphocytes, enlargement of the spleen and lymph nodes, weight reduction, and possibly death (49). It has been proposed that members of the NF-κB/*rel* family play an important role in the regulation of gene expression that accompanies activation of various immune reactions (3). Our data demonstrate that the expression of three proteins is modulated in a similar fashion during both v-*rel*-mediated tumorigenesis and an acute immune response. These data indicate that v-*rel* might act as a polyclonal, constitutive activator of immune response reactions in vivo.

Our analysis of cell surface markers expressed on v-*rel*-transformed cell lines demonstrates the presence of MHC class I, MHC class II, and IL-2R proteins on three phenotypic groups of v-*rel*-transformed cell lines: B-cell lines, T-cell lines, and lines with neither B- nor T-cell markers. High levels of MHC class II mRNA, particularly on cell lines with T-cell markers, suggest an activated cell (27). Consistent with the results reported in this study, several previous studies reported the presence of MHC class I and class II antigens on the surface of cell lines transformed by v-*rel* (9, 11, 81). Our data suggest, for the first time, that v-*rel*-induced expression of these genes contributes to the phenotype of the tumor cell.

**Induction of MHC class I, MHC class II, and IL-2R in avian lymphoid cell lines.** By infecting avian lymphoid cell lines with retroviruses encoding v-*rel* or c-*rel*, we have been able to demonstrate induction of MHC class I, MHC class II, and IL-2R proteins on the B-cell line DT95 and the T-cell line MSB-1. The expression of all three proteins induced by v-*rel* was consistently higher than that induced by c-*rel*. A kinetic analysis of MHC class II expression in two B-cell lines revealed that on the basis of RNA levels, v-*rel* induced MHC class II expression more efficiently than c-*rel* and that this difference increased with time. We have examined a number of additional experimental models. While the difference in efficiency varied with different protocols, $p59^{v\text{-}rel}$ was consistently more efficient than $p68^{c\text{-}rel}$ (Fig. 6 and data not shown). For example, v-*rel* induced MHC class II expression 50-fold more efficiently 45 days after REV-TW infection of DT40 (Fig. 5), while it induced IL-2R expression 4-fold more efficiently 5 days after infection of DT95 (data not shown).

Our major conclusion is in apparent contradiction to recent data that support a model proposing that transformation is the result of v-*rel* acting as a dominant negative repressor of transcriptional regulation by NF-κB/*rel* proteins (7, 8, 44, 55,

TABLE 5. Expression of MHC class I, MHC class II, and IL-2R proteins on the surface of MSB-1 after infection by REV-C(CSV), REV-TW(CSV), or CSV

| Virus | SFI[a] (relative units) of: | | | | | |
|---|---|---|---|---|---|---|
| | MHC class I | | MHC class II | | IL-2R | |
| | CSV (+)[b] | CSV (−) | CSV (+) | CSV (−) | CSV (+) | CSV (−) |
| None | 51,100 | | 403 | | 37,900 | |
| CSV | 42,400 | 0 | 343 | 0 | 27,000 | 0 |
| REV-C(CSV) | 65,700 | 23,200 | 911 | 568 | 43,400 | 16,400 |
| REV-TW(CSV) | 83,600 | 41,200 | 8,080 | 7,740 | 43,900 | 16,900 |

[a] For an explanation, see Table 4, footnote *a*.

[b] For an explanation, see Table 4, footnote *b*.

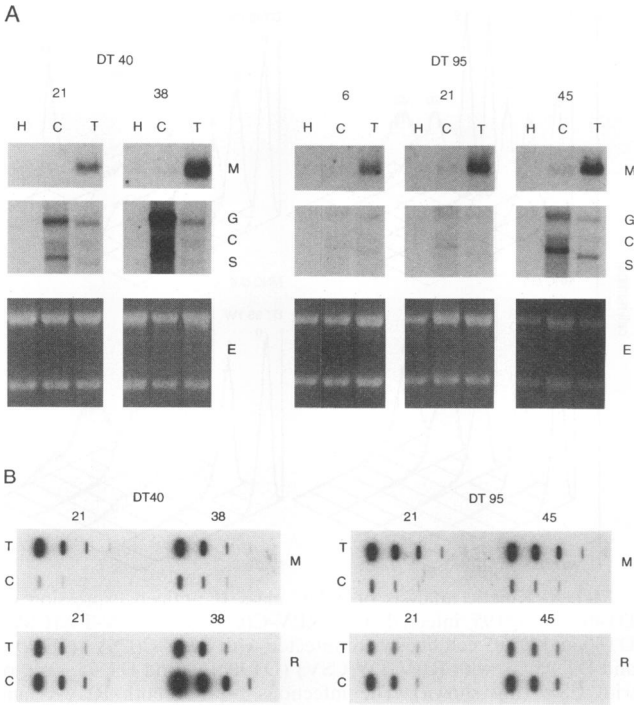


FIG. 5. Kinetic analysis of MHC class II and *rel* RNA expression after infection of DT95 and DT40 with REV-C(CSV) or REV-TW(CSV). RNA was isolated on the indicated days (numbers above panels) after infection from the cells analyzed in the experiment described in Fig. 4. (A) Total RNA from cells infected with CSV helper virus (H), REV-C(CSV) (C), or REV-TW(CSV) (T) was subjected to Northern analysis with a pCCII-4-$\beta_2$ probe (upper panels; 15 μg of RNA) and a pc-rel2 probe (middle panels; 5 μg of RNA). Expression of MHC class II mRNA (M), the viral genomic (G) and spliced (S) RNAs, and the endogenous c-*rel* (C) mRNA is indicated. Ethidium bromide (E)-stained RNA is shown in the lower panels. (B) Total cellular RNA from the experiment described in panel A was analyzed by slot blot hybridization of 10 μg of RNA prepared by fivefold serial dilution. Samples were transferred as described in Materials and Methods, and the membranes hybridized with specific fragments from pCC-II-4-$\beta_2$ (M) or pc-rel2 (R). C and T indicate infection by REV-C(CSV) and REV-TW(CSV), respectively.

63). Several experimental differences suggest a basis for these discrepancies. The most important consideration is that our analysis measured expression from an endogenous promoter in an avian lymphoid cell expressing avian *rel* proteins. Since truncation of v-*rel* substantially damaged its transactivation domain (63), p59$^{v\text{-}rel}$ may require adequate levels of NF-κB/*rel* proteins with which it can cooperate to induce transcription. These proteins are most abundant in lymphoid cells (15, 58) where they may facilitate v-*rel*-mediated transcription, making them preferred targets for transformation. Such a relationship is suggested by the synergistic effect produced in Jurkat cells by a full-length human c-*rel* protein and a truncated form lacking the transactivation domain (75). Another factor that needs to be considered is the duration of v-*rel* expression prior to the measurement of induction. In transient transfection assays, expression is most commonly measured 48 h after transfection. In our assays, only low levels of the three proteins examined were induced at that time.

Kinetic analysis prompted a second observation. The amounts of v-*rel* and c-*rel* expressed shortly after in vitro infection of DT40, DT95, and splenic cultures were approxi-

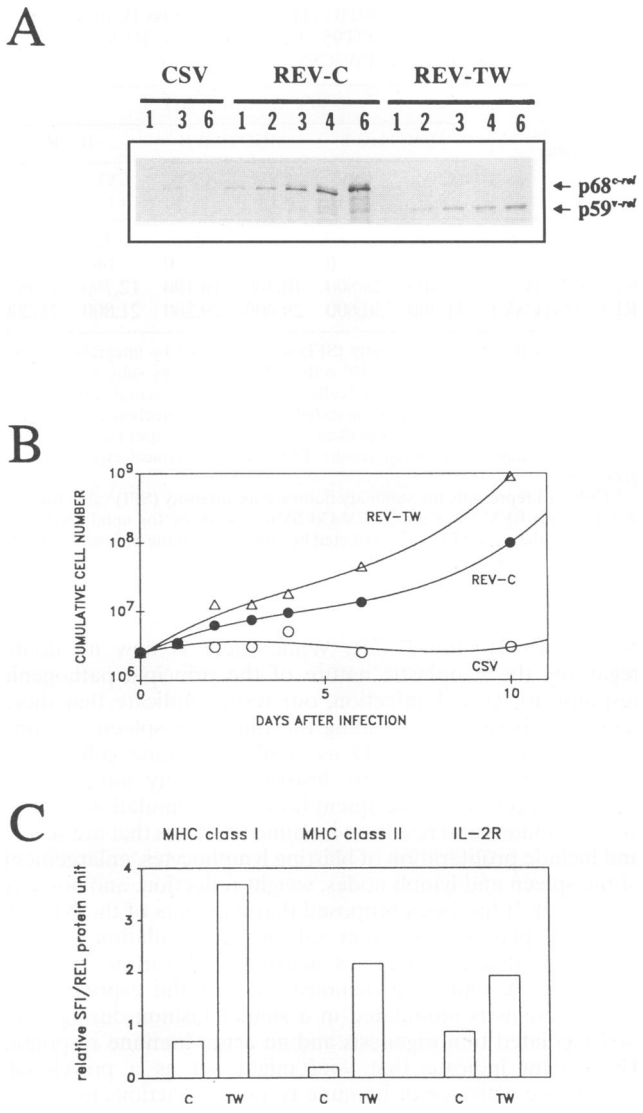


FIG. 6. Induction of MHC class I, MHC class II, and IL-2R proteins on the surface of avian splenocytes in vitro. The spleen from a 4-week-old chicken was passed through nylon mesh, and the cell suspension was placed in culture ($2 \times 10^6$ cells per ml). Four days later, the mononuclear white splenocytes were separated by centrifugation of the cell suspension on Isolymph. Adherent cells in the tissue culture flask were discarded. The cells were infected by REV-TW(CSV), REV-C(CSV), or CSV at a multiplicity of infection of 1. The cells were harvested at the indicated days, counted with a hemocytometer, and analyzed by Western transfer and flow cytometry. (A) Expression of *rel* proteins by Western transfer was analyzed by using monoclonal antibody HY87. Each lane contains the equivalent of $5 \times 10^5$ cells. Numbers indicate the days after infection when the cells were analyzed. Arrows indicate the migration of c-*rel* and v-*rel* proteins. REV-C, REV-C(CSV); REV-TW, REV-TW(CSV). (B) Growth of splenic cells in vitro after infection with CSV (○), REV-C(CSV) (REV-C) (●), or REV-TW(CSV) (REV-TW) (△). (C) Induction of MHC class I, MHC class II, and IL-2R proteins on the surface of splenic cells 6 days after infection with REV-C(CSV) (C) or REV-TW(CSV) (TW). Six days after infection, $10^4$ splenic cells infected with CSV, REV-C(CSV), or REV-TW(CSV) were analyzed for expression of the indicated proteins by flow cytometry and summary immunofluorescence intensity (SFI) was determined. SFI of CSV-infected cells was subtracted. The corrected values were then normalized to the amount of *rel* protein expressed in the sample as determined by densitometry of the Western blot.

mately equal. Proliferation of the infected cells was associated with a gradual increase in the difference between the amount of v-*rel* and c-*rel* expression (Fig. 5 and 6). Levels of c-*rel* that are higher than those of v-*rel* are consistent with the observation that v-*rel* induces expression of c-*rel* target genes more efficiently than exogenous c-*rel*. This observation is a general phenomenon that was observed in several different types of infected cultures. This change in *rel* expression most likely represents a selection for cells that express optimal levels of proteins that promote proliferation. The basis for this selection is variability within the cell population regarding the level of *rel* protein expression. Major sources of this variability are likely to include (i) the stochastic nature of retroviral integration, (ii) transcriptional activation of the long terminal repeat promoter by cellular proteins, and (iii) acquisition of additional proviruses.

**Induction, repression, and transformation by *rel* proteins.** NF-κB/*rel* regulation of gene expression mediated directly through κB sites has been demonstrated, but transactivation independent of κB sites and produced via interaction with other transcription factors is also a possibility (for a review, see reference 53). In the case of the three immunoregulatory receptors analyzed in this study, v-*rel*- or c-*rel*-induced expression, direct or indirect, is likely to occur through κB sites. κB sites have been identified within the promoter of mouse MHC class II (three different sites) (12, 31, 36), human MHC class I (*H2-K*$^b$) (5), and human IL-2Rα genes (6). Similar sites can be found in the chicken MHC class II promoter (80) and in the chicken MHC class I promoter (50). The potential κB binding site of chicken MHC class I gene promoter referred to as the class I-responsive element was shifted in an electrophoretic mobility assay with a v-*rel*–estrogen receptor fusion protein (13). The majority of v-*rel* protein is localized in the cytoplasmic compartment of transformed lines; however, significant levels can be detected in the nucleus (29, 59, 78). For these reasons, both direct and indirect mechanisms can effect v-*rel*-mediated gene expression. v-*rel* may induce transcription by binding to κB sites as a homodimer or a heterodimer with other members of the NF-κB/*rel* family, or v-*rel* may compete for and bind inhibitory molecules that would otherwise negatively regulate other DNA-binding complexes, thereby inducing expression indirectly. We have not distinguished between these two possibilities, which could occur simultaneously. The transactivation capability of v-*rel* indicates that it can induce the expression of MHC class I, MHC class II, and IL-2R proteins by direct interaction with DNA κB sites (34, 40, 66, 77). On the other hand, the presence of a majority of v-*rel* protein in cytoplasmic complexes with IκBα, NFKB1, NFKB2, and c-*rel* suggests that an indirect mechanism may also contribute to the v-*rel*-mediated induction of gene expression (17, 29, 30, 70).

The goal of this study was to address the relationship between aberrant modulation of transcription in v-*rel*-transformed cells and the biological function of members of the NF-κB/*rel* family in the target cells for transformation. We have demonstrated a correlation between induction of endogenous c-*rel* protein and the expression of three avian immunoregulatory proteins, MHC class I, MHC class II, and the IL-2R, during an acute immune response, GVHD. We have shown that v-*rel*, in an analogous fashion, induces these proteins on tumor splenic cells. Furthermore, our experiments revealed that v-*rel* induces expression of these three immunoregulatory receptors more efficiently than exogenous c-*rel*. We propose that the transcription induced by v-*rel* mimics that associated with activation of endogenous c-*rel* and other NF-κB/*rel* members. Therefore, v-*rel* expression may activate biochemical pathways that evolved primarily for host defense and in which c-*rel* plays a regulatory role. In so doing, v-*rel* would induce extended cell proliferation and immortalization. In what may be a related observation, activation of NF-κB/*rel*-regulated genes in v-*rel* tumor cells resembles the process of activation of NF-κB/*rel*-mediated transcription during oncogenesis associated with infection by human T-cell leukemia virus I and hepatitis B virus (48, 56).

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