JOURNAL OF VIROLOGY, Feb. 1990, p. 584–591
0022-538X/90/020584-08$02.00/0

Vol. 64, No. 2

Avian Reticuloendotheliosis Virus-Transformed Lymphoid Cells Contain Multiple pp59$^{v\text{-}rel}$ Complexes

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Received 21 August 1989/Accepted 16 October 1989

The v-*rel* oncogene of avian reticuloendotheliosis virus type T (REV-T) encodes a 59-kilodalton (kDa) phosphoprotein located principally in the cytosol of transformed lymphoid cells. All of the detectable pp59$^{v\text{-}rel}$ was present in high-molecular-weight complexes containing at least five cellular proteins (p124, p115, p75$^{c\text{-}rel}$, p70^{hsc}, and pp40). Antiserum was developed against the 40-kDa protein, the most abundant cellular protein associated with the complex. The 40-kDa phosphoprotein was complexed with pp59$^{v\text{-}rel}$ in REV-T-transformed lymphoid cell lines arrested at different stages of B-cell development as well as in lymphoid tumor cells and in fibrosarcomas. The half-life (8 h) of pp40 in REV-T-transformed lymphoid cells was the same as that of pp59$^{v\text{-}rel}$. Antiserum against pp40 permitted the identification of two pp59$^{v\text{-}rel}$ complexes. The most abundant cytoplasmic complex contained approximately 75% of the pp59$^{v\text{-}rel}$ and all of the detectable pp40 in REV-T-transformed lymphoid cells. Twenty-five percent of the pp59$^{v\text{-}rel}$ was present in a minor complex that contained the majority of p75$^{c\text{-}rel}$ along with p115 and p124. In nuclear extracts of REV-T-transformed lymphoid cells, pp59$^{v\text{-}rel}$ was complexed with pp40. The two high-molecular-weight proteins (p115 and p124) and p75$^{c\text{-}rel}$ were not detected in the nuclear complex. In the cytosolic complexes, pp40 was heavily phosphorylated, whereas the nuclear form was much less extensively phosphorylated.

Reticuloendotheliosis virus type T (REV-T) is the most virulent of all retroviruses and induces an invariably fatal lymphoma with a latent period of 7 to 10 days in experimentally infected chickens (30). It transforms and prevents the subsequent differentiation of lymphoid cells at phenotypically different stages of B-cell development (4, 21, 31). REV-T is the only avian retrovirus that converts a normal cell to a tumorigenic state in a helper virus-independent fashion (21). REV-T also induces solid tumors of connective tissue origin and transforms cultured avian fibroblasts (12, 24). Like other acutely transforming retroviruses, the genome of REV-T contains deletions in its structural genes and has acquired a helper virus-unrelated sequence (7, 16). This 1.4-kilobase sequence, designated v-*rel*, is inserted within envelope sequences at the 3′ end of the virus genome (7, 27). The v-*rel* oncogene is distinct from other known oncogenes (7, 8, 39) and is transcribed into a 3.0-kilobase subgenomic RNA that is found at modest levels in REV-T-transformed lymphoid cells (8, 17).

Antisera generated against either bacterial fusion proteins containing portions of the v-*rel* gene (13, 15, 18) or synthetic peptides derived from the predicted protein (13, 26) have identified a 59-kilodalton (kDa) phosphoprotein. This protein, designated pp59$^{v\text{-}rel}$, has a half-life of 6 to 8 h (15, 37) and represents only 0.003% to 0.004% of the total methionine-containing polypeptides in REV-T-transformed lymphoid cells (15, 26, 37). The vast majority of pp59$^{v\text{-}rel}$ is cytosolic in REV-T-transformed cells (15, 34, 37), although 10% of pp59$^{v\text{-}rel}$ is nuclear (15, 37). The v-*rel* transforming protein has been purified by sequential gel filtration and immunoaffinity chromatography, using a monoclonal antibody against pp59$^{v\text{-}rel}$ from cytosolic extracts of REV-T-transformed lymphoid cells (35, 36). The protein, eluted from the immunoaffinity column, has a mass of approximately 350 to 400 kDa, as determined by gel filtration and fast protein liquid chromatography (35). All of the detectable pp59$^{v\text{-}rel}$ in cytosolic extracts from REV-T-transformed cells is present in this high-molecular-weight complex (35). This complex contains a number of cellular proteins in addition to pp59$^{v\text{-}rel}$. The most abundant cellular protein associated with the complex is a 40-kDa protein (35) that is extensively phosphorylated on serine residues (36). At least five cellular proteins (p124, p115, p75, p70, and pp40) coprecipitate with pp59$^{v\text{-}rel}$ from [^{35}S]methionine-labeled extracts of REV-T-transformed lymphoid cells, using antisera against different regions of pp59$^{v\text{-}rel}$, and copurify with pp59$^{v\text{-}rel}$ on immunoaffinity columns (33, 35, 36). The 75-kDa protein is the product of the c-*rel* proto-oncogene, and p70 is the constitutive form of avian heat shock protein 70 (25, 33; M. Y. Lim, N. Davis, J. Zhang, and H. R. Bose, Jr., submitted for publication).

This report describes the production of antiserum against pp40, the most abundant cellular protein associated with pp59$^{v\text{-}rel}$. Antiserum specific for pp40 has permitted the identification of two pp59$^{v\text{-}rel}$-containing complexes in the cytosol of v-*rel*-transformed lymphoid cells. In the nucleus, pp59$^{v\text{-}rel}$ is also complexed with pp40.

MATERIALS AND METHODS

Cell culture. REV-T-transformed cell lines and MSB-1 cells were grown in RPMI 1640 medium (Hazelton, Lenexa, Kans.) supplemented with 5% bovine calf serum (Hazelton), penicillin (200 U/ml), and streptomycin (100 μg/ml). Cultures were maintained at 37°C in an atmosphere of 5% CO_2. The principal cell line analyzed in these studies was RECC-UTC4-1. This is a non-virus-producing cell line that contains a single integrated REV-T provirus and does not contain REV-A provirus sequences (41).

Metabolic labeling. Logarithmically growing cells (5×10^6 cells per ml) were incubated in methionine-free RPMI 1640 medium for 1 h at 37°C, and [^{35}S]methionine (110 Ci/mmol; ICN Biomedicals, Inc., Irvine, Calif.) was added to a concentration of 100 μCi/ml. The cells were incubated for 3 h at 37°C and then washed three times in cold phosphate-buffered

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saline before analysis. To radioactively label the phosphoproteins in REV-T-transformed cells, the cells (10^7/ml) were suspended in phosphate-free RPMI 1640 medium supplemented with dialyzed fetal calf serum (Hazelton). After 4 h, the cells were incubated with medium containing 1 mCi of $^{32}P_i$ (ICN Biomedicals) per ml for 2 h at 37°C. The cells were then washed three times in Tris-saline buffer (10 mm Tris hydrochloride [pH 7.5], 0.9% NaCl) before analysis.

Antiserum production. The purified pp59$^{v\text{-}rel}$-pp40 complexes were isolated from REV-T-transformed cells as previously described (35). Briefly, large-scale cultures (12 to 15 liters) of the REV-T-transformed lymphoid cell line, RECC-UTC4-1 were prepared in roller bottles, and the cells were collected by centrifugation (1,500 × *g* in a GSA rotor [Ivan Sorvall, Inc., Norwalk, Conn.]). The cells were lysed by mechanical shearing, using a Virtishear tissue homogenizer (The Virtis Co., Gardiner, N.Y.), in 25 mM Tris (pH 7.5–5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)–2 mM EDTA–0.2 mM phenylmethylsulfonyl fluoride–0.05% β-mercaptoethanol. The resulting lysate was centrifuged at 250,000 × *g* for 30 min to prepare a cytosolic extract. The extract was then subjected to gel filtration on a Sephacryl S200 or S300 column (26 by 70 cm). The pp59$^{v\text{-}rel}$-containing fractions were subjected to immunoaffinity chromatography, using a monoclonal antibody directed against the carboxy terminus of pp59$^{v\text{-}rel}$ (35).

The highly purified protein complex was disrupted, and the protein was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by the procedure of Laemmli (20). The proteins were electroeluted onto nitrocellulose and visualized by staining in 0.5% Ponceau-S in 1% acetic acid. The 40-kDa protein was excised, solubilized in dimethyl sulfoxide, and injected subcutaneously into New Zealand White rabbits. Rabbits were boosted with antigen every 2 weeks.

Radioimmunoprecipitations. Labeled cells were suspended in immunoprecipitation buffer (20 mM Tris [pH 7.5], 0.5% Triton X-100, 100 mM NaCl, 100 Kallikrein inhibitor units of aprotinin per ml, 0.2 mM phenylmethylsulfonyl fluoride). The cells were allowed to swell on ice for 30 min and then lysed by 100 strokes in a tight-fitting Dounce homogenizer. Cell lysates were clarified by centrifugation for 15 min in a microcentrifuge. Immunoprecipitation reactions were performed by using 50 μg of protein and 4 μg of immunoglobulin G-enriched antiserum in a total volume of 500 μl. The reaction mixture was incubated with constant mixing at 4°C for 1 to 2 h. A sample (200 μl) of a 2.5% slurry of protein A-Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) in immunoprecipitation buffer was added and mixed for 1 h. The sample was centrifuged for 2 min in a microcentrifuge and washed three times in 1 ml of buffer. High-detergent immunoprecipitations were performed identically, with addition of SDS to 0.1% and sodium deoxycholate to 0.5%.

Western immunoblotting. Samples were subjected to SDS-PAGE analysis, and proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Dassel, Federal Republic of Germany) (6). The filter was stained after transfer in Ponceau-S, and molecular weight standards were marked. The membranes were blocked in Tris-saline buffer (TSB; 0.5% NaCl in 10 mM Tris hydrochloride [pH 7.5]) containing 10% nonfat dry milk. The filter was incubated with antiserum (1:250 dilution) for 1 h at 22°C. Filters were washed twice for 10 min in TSB and TSB plus 0.5% Triton X-100. The filter was then incubated with ^{125}I-labeled *Staphylococcus aureus* protein A prepared by the method of Markwell (23) or with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Filters were washed as described above and developed accordingly.

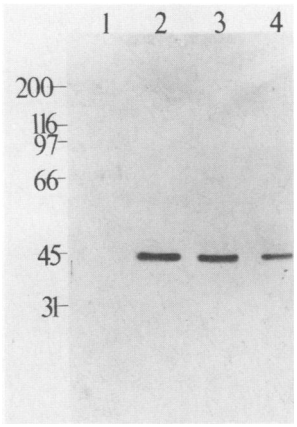

FIG. 1. Expression of pp40 in REV-T-transformed lymphoid cells. Western analysis of whole-cell extracts was performed with pp40-specific antiserum. Lanes: 1, 25 μg of whole-cell lysate of MSB-1 cells; 2 to 4, 20, 10, and 5 μg, respectively, of whole-cell lysate of RECC-UTC4-1. Positions of molecular size markers (in kilodaltons) are shown on the left.

Immunological reagents. Antisera specific for the carboxy terminus, amino terminus, and midregion of the v-*rel* protein have been previously described (8, 26). Antiserum directed against full-length v-*rel* protein expressed in plasmid pOTS in *Escherichia coli* is described by Lim et al. (submitted). Preparation and specificity of the monoclonal antibody to the carboxyl terminus of pp59$^{v\text{-}rel}$ were previously described (35).

Protein determinations. Quantitation of the protein content of each sample was assayed by using the Bio-Rad protein microassay (Bio-Rad Laboratories, Richmond, Calif.) (5).

RESULTS

Generation of antiserum specific for pp40. To isolate pp59$^{v\text{-}rel}$ complexes, cytosolic extracts were obtained from the large-scale culture of RECC-UTC4-1, a non-virus-producing REV-T-transformed lymphoid cell line. The extracts were subjected to sequential gel filtration on Sephacryl S200 and immunoaffinity chromatography, using a monoclonal antibody directed against the carboxy-terminal region of pp59$^{v\text{-}rel}$ (35). The highly purified pp59$^{v\text{-}rel}$-pp40 complex that eluted from the immunoaffinity column was disrupted, and the protein was separated by SDS-PAGE. The 40-kDa protein was injected into New Zealand White rabbits to obtain an antiserum.

The specificity of the pp40 antiserum was determined by Western analysis, using extracts obtained from an REV-T-transformed cell line (RECC-UTC4-1) and a cell line transformed by Marek's disease virus (MSB-1). The antiserum prepared against pp40 recognized a single protein in the REV-T-transformed cell extracts (Fig. 1, lanes 2 to 4) and failed to recognize any protein in extracts obtained from MSB-1 cells (lane 1). This antiserum detected pp40 when as little as 5 μg of total protein was analyzed. REV-T-transformed lymphoid cells arrested in different stages of B-cell differentiation expressed pp40 (data not shown). Antisera specific for pp40 did not recognize REV-A structural proteins, nor did antiviral antiserum react with pp40 (data not shown). Furthermore, the nucleotide sequence of REV-T does not reveal an open reading frame that would encode a 40-kDa polypeptide; therefore, pp40 is not a viral protein.

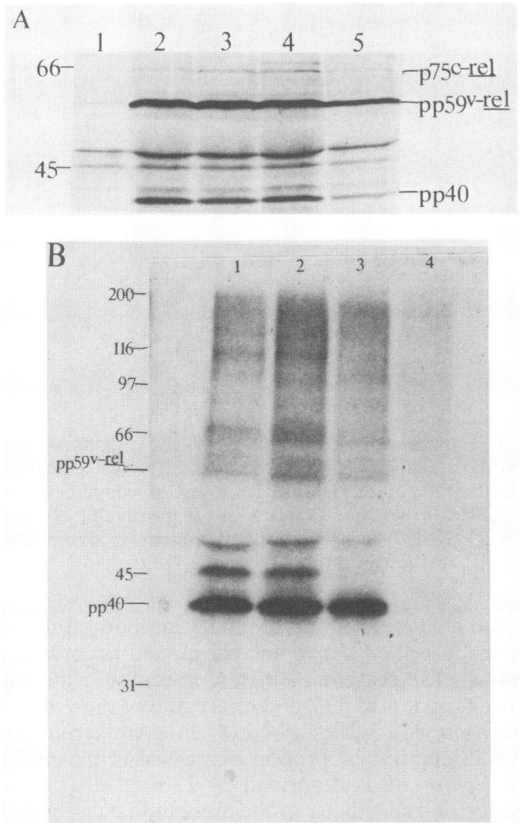

FIG. 2. Immunoprecipitation analysis of metabolically labeled RECC-UTC4-1 cell extracts. (A) Immunoprecipitation of proteins from extracts of [^{35}S]methionine-labeled RECC-UTC4-1 cells. Lanes: 1, preimmune serum; 2, anti-pp40 serum; 3, antiserum directed against full-length pp59$^{v\text{-}rel}$; 4, antiserum specific for the carboxy-terminal region of pp59$^{v\text{-}rel}$; 5, antiserum specific for the amino-terminal region of pp59$^{v\text{-}rel}$. (B) Immunoprecipitation of proteins from extracts of $^{32}P_i$-labeled RECC-UTC4-1 cells. Lanes: 1, antiserum directed against full-length pp59$^{v\text{-}rel}$; 2, antiserum specific for the carboxy-terminal region of pp59$^{v\text{-}rel}$; 3, pp40-specific antiserum; lane 4, preimmune serum.

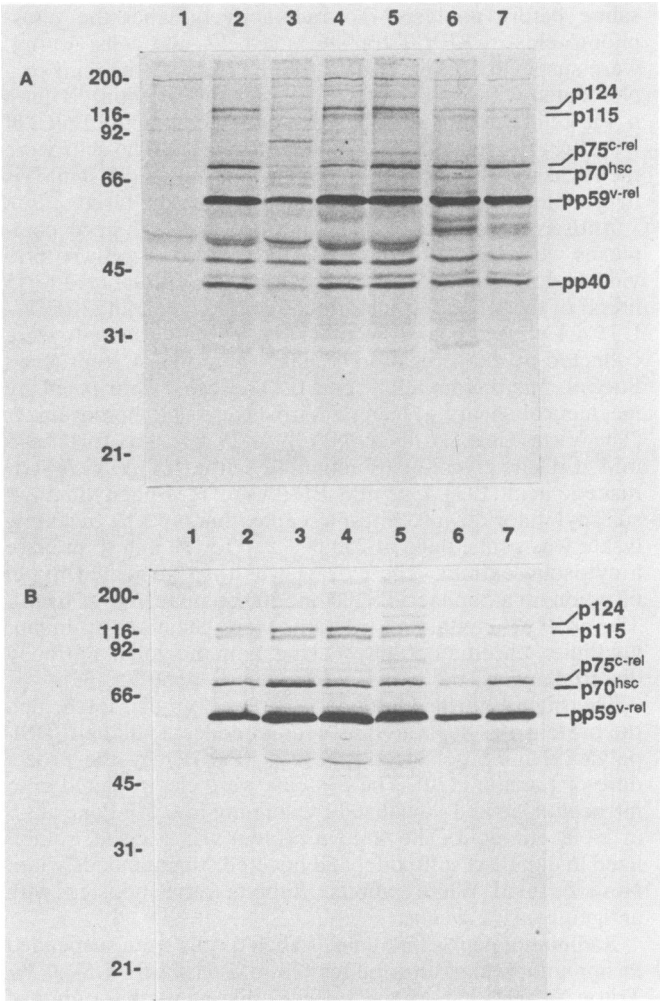

FIG. 3. Inhibition of the immunoprecipitation of pp59$^{v\text{-}rel}$-pp40 complexes by antisera specific for the amino-terminal region of pp59$^{v\text{-}rel}$. Immunoprecipitations were performed on extracts of [^{35}S]methionine-labeled RECC-UTC4-1 cells in the presence of nonionic (A) and ionic (B) detergents. Lanes: 1, preimmune serum; 2, antiserum against full-length pp59$^{v\text{-}rel}$; 3 to 5, antisera specific for the amino, middle, and carboxy regions, respectively, of pp59$^{v\text{-}rel}$; 6 and 7, monoclonal antibodies specific for the middle and carboxy regions, respectively, of pp59$^{v\text{-}rel}$.

Immunoprecipitation experiments using the 40-kDa antiserum on extracts of [^{35}S]methionine- and $^{32}P_i$-labeled cells demonstrated that the 40-kDa protein recognized in Western analysis corresponded to the 40-kDa protein associated with pp59$^{v\text{-}rel}$. The pp40-specific antisera precipitated pp59$^{v\text{-}rel}$ and p75$^{c\text{-}rel}$ in addition to pp40 (Fig. 2A, lane 2). These proteins were also immunoprecipitated from extracts of REV-T-transformed cells with use of antisera made against full-length protein (lane 3) and against the carboxy-terminal (lane 4) and amino-terminal (lane 5) regions of pp59$^{v\text{-}rel}$. Both pp40 and pp59$^{v\text{-}rel}$ are phosphorylated on serine residues in REV-T-transformed lymphoid cells (36). pp40 and pp59$^{v\text{-}rel}$ were immunoprecipitated by both the pp40-specific (Fig. 2B, lane 3) and pp59$^{v\text{-}rel}$-specific (lanes 1 and 2) antisera.

Antiserum against the amino-terminal region of pp59$^{v\text{-}rel}$ fails to immunoprecipitate pp59$^{v\text{-}rel}$-pp40 complexes. We have previously established that pp40 is a detergent-sensitive member of the pp59$^{v\text{-}rel}$ complex and dissociates in the presence of ionic detergents (35). When pp59$^{v\text{-}rel}$ complexes were immunoprecipitated from extracts of [^{35}S]methionine-labeled REV-T-transformed cells by using pp59$^{v\text{-}rel}$ region-specific sera in the presence of ionic detergents, these antisera immunoprecipitated essentially equivalent amounts of pp59$^{v\text{-}rel}$, p75$^{c\text{-}rel}$, p115, and p124 (Fig. 3B). When the immunoprecipitation experiments were performed in nonionic detergent buffer, the antiserum directed against the amino-terminal region of pp59$^{v\text{-}rel}$ immunoprecipitated significantly less of the pp59$^{v\text{-}rel}$ complex from [^{35}S]methionine-labeled cell extracts (Fig. 3A, lane 3). Antiserum specific for the amino-terminal region of pp59$^{v\text{-}rel}$ recognizes bacterially expressed pp59$^{v\text{-}rel}$ as efficiently as do sera specific for the midregion and carboxy-terminal region (37). These results suggest that pp40 may be bound to the amino-terminal region of pp59$^{v\text{-}rel}$, thereby reducing the ability of the antiserum specific for the amino-terminal region to immunoprecipitate the pp59$^{v\text{-}rel}$ complex. Alternatively, the binding of pp40 to pp59$^{v\text{-}rel}$ could cause a conformational change in pp59$^{v\text{-}rel}$ that would make the amino terminus inaccessible to the region-specific antisera.

Metabolic stability of the cellular proteins in the pp59$^{v\text{-}rel}$ cytosolic complex. To determine the half-life of the cellular

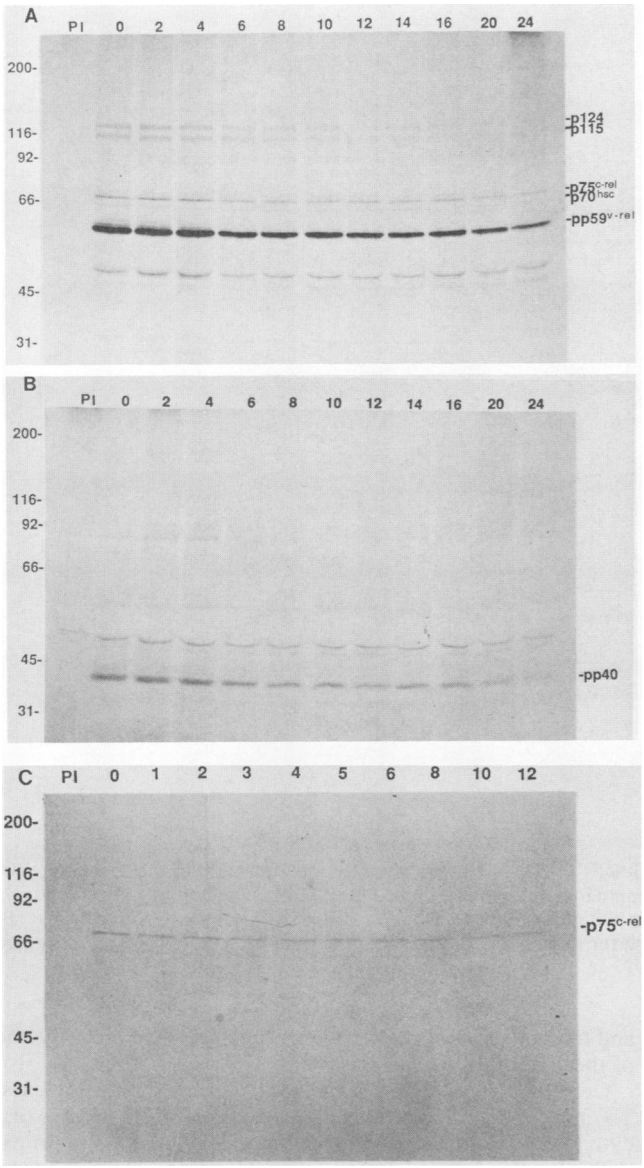

FIG. 4. Metabolic stability of proteins complexed with pp59$^{v\text{-}rel}$. Immunoprecipitations were performed on extracts obtained from pulse-labeled RECC-UTC4-1 or MSB-1 cells at the time intervals (in hours) indicated above the lanes. Preimmune samples were taken from time zero extracts. Immunoprecipitations were performed with RECC-UTC4-1 extracts and antiserum specific for full-length pp59$^{v\text{-}rel}$ in buffer containing ionic detergents (A), with RECC-UTC4-1 cell extracts and antiserum specific for the 40-kDa protein (B), and with MSB-1 cell extracts and antiserum specific for full-length pp59$^{v\text{-}rel}$ (C).

proteins associated with pp59$^{v\text{-}rel}$, RECC-UTC4-1 and MSB-1 cells were pulse-labeled with [^{35}S]methionine and chased in unlabeled medium. At various time intervals during the chase period, cytosolic extracts were prepared, and the [^{35}S]methionine-labeled proteins were immunoprecipitated with either pp40-specific antiserum or antiserum directed against full-length pp59$^{v\text{-}rel}$. The metabolic stabilities of pp40, pp59$^{v\text{-}rel}$, p70^{hsc}, p75$^{c\text{-}rel}$, p115, and p124 were determined by densitometer tracings of the autoradiograms generated after SDS-PAGE analysis. Alternatively, the labeled proteins were excised from the SDS-PAGE gels and eluted, and the radioactivity of each protein in the complex was

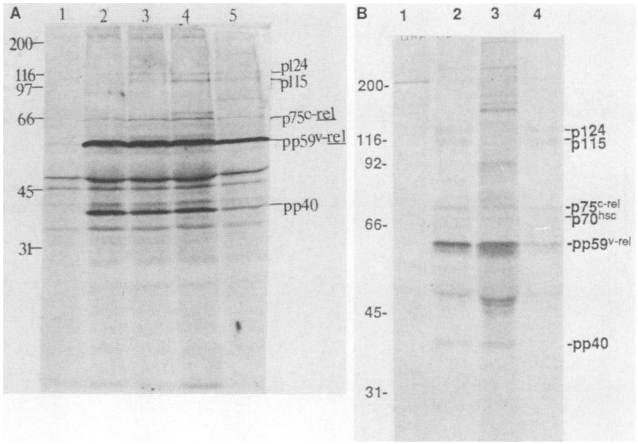

FIG. 5. Identification of two pp59$^{v\text{-}rel}$-containing complexes in the cytosol of REV-T-transformed cells. (A) Immunoprecipitations performed with [^{35}S]methionine-labeled protein from RECC-UTC4-1 lysates. Lanes: 1, preimmune serum; 2, antiserum specific for pp40; 3 to 5, pp59$^{v\text{-}rel}$ full-length-, carboxy-region-, and amino-region-specific antisera, respectively. (B) Immunoprecipitations performed on [^{35}S]methionine-labeled RECC-UTC4-1 extracts that had been cleared with the pp40-specific antiserm. Lanes: 1, preimmune serum; 2, full-length anti-pp59$^{v\text{-}rel}$-specific serum; 3, clearing with the 40-kDa specific antiserum; 4, immunoprecipitation of the pp40-cleared extract with antiserum specific for full-length pp59$^{v\text{-}rel}$.

determined. The results of a pulse-chase experiment in which the immunoprecipitations were performed in ionic detergent with anti-pp59$^{v\text{-}rel}$ are shown in Fig. 4A. The half-life of pp59$^{v\text{-}rel}$ was approximately 8 h, as previously reported (37). The half-life of p75$^{c\text{-}rel}$ in the REV-T-transformed cell line was approximately 11 h. The half-lives of p124, p115, and p70^{hsc} in the pp59$^{v\text{-}rel}$ complex were 6 to 8, 6 to 8, and 14 h, respectively. The half-life of pp40 was determined by immunoprecipitating pp40 from extracts of [^{35}S]methionine-labeled cells at various time intervals during the chase period with antiserum specific for pp40. The half-life of pp40 was approximately 8 h (Fig. 4B), essentially the same as that of pp59$^{v\text{-}rel}$.

The product of the c-*rel* proto-oncogene is expressed in lymphoid cells transformed by Marek's disease virus but does not appear to be complexed with cellular proteins (32; Lim et al., submitted). Since REV-T-transformed cells also express p75$^{c\text{-}rel}$ at 20-fold-higher levels, we determined the half-life of p75$^{c\text{-}rel}$ in MSB-1 cells. Antiserum directed against full-length pp59$^{v\text{-}rel}$ was used to immunoprecipitate p75$^{c\text{-}rel}$ from extracts of [^{35}S]methionine-pulse-labeled MSB-1 cells at different time intervals after the cultures were incubated in unlabeled medium. The half-life of p75$^{c\text{-}rel}$ in MSB-1 cells was 11 h and essentially the same as that of p75$^{c\text{-}rel}$ that was complexed with pp59$^{v\text{-}rel}$ in REV-T-transformed lymphoid cells (Fig. 4C). The presence of p75$^{c\text{-}rel}$ in the pp59$^{v\text{-}rel}$ complex therefore did not appear to stabilize this protein.

REV-T-transformed lymphoid cells contain two distinct cytosolic pp59$^{v\text{-}rel}$ complexes. At least five cellular proteins (p124, p115, p75$^{c\text{-}rel}$, p70^{hsc}, and pp40) copurified on immunoaffinity columns and coprecipitated with pp59$^{v\text{-}rel}$ from cytosolic extracts of REV-T-transformed cells, using antisera directed against different regions of pp59$^{v\text{-}rel}$ (Fig. 5A, lane 3 to 5) (25, 33). To determine whether cellular proteins with the same apparent molecular mass coprecipitate with pp59$^{v\text{-}rel}$ by using antisera specific for pp40, proteins were

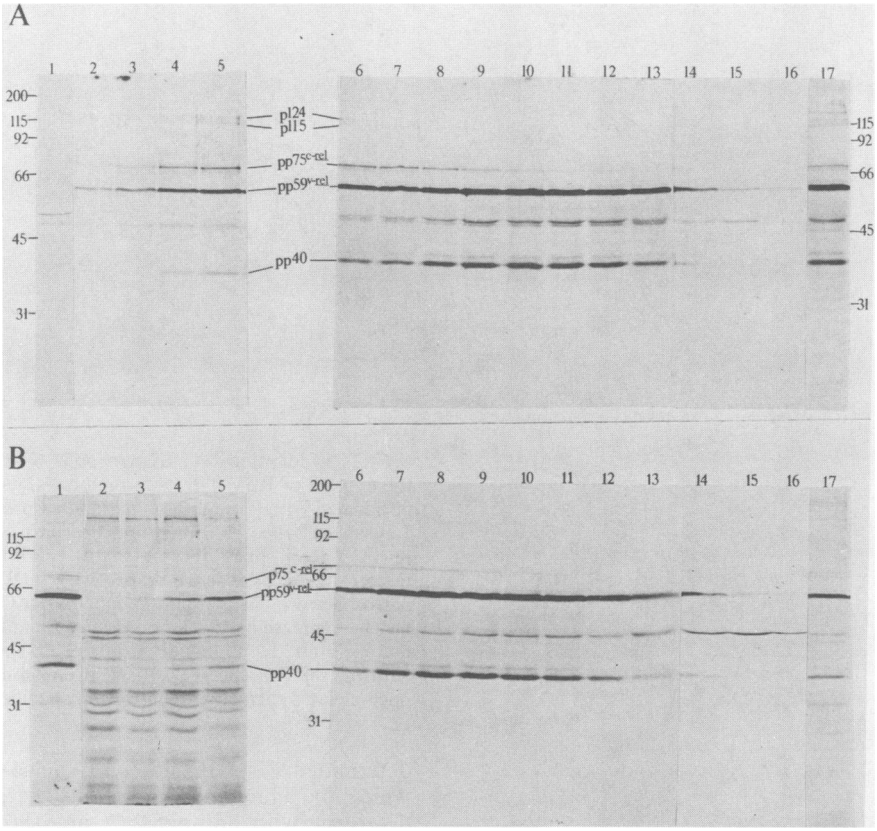

FIG. 6. Immunoprecipitation performed on [^{35}S]methionine-labeled RECC-UTC4-1 extracts after gel filtration on Sephacryl S300 columns. (A) Immunoprecipitations performed on the consecutive odd-numbered column fractions (lanes 2 to 16), beginning with fraction 95 and using antiserum specific for the carboxy region of pp59$^{v\text{-}rel}$. Lane 1, Preimmune serum; lane 17, RECC-UTC4-1 cytosolic extracts. (B) Immunoprecipitations performed as for panel A, using antiserum specific for the 40-kDa protein. Lanes 1 and 17, Precipitations performed on unfractioned RECC-UTC4-1 cytosolic extracts.

immunoprecipitated from cytosolic extracts obtained from [^{35}S]methionine-labeled REV-T-transformed lymphoid cells with antisera directed against pp59$^{v\text{-}rel}$ and pp40 (Fig. 5). The pp40 antisera immunoprecipitated p75$^{c\text{-}rel}$, pp59$^{v\text{-}rel}$, and pp40 (Fig. 5A, lane 2). The two high-molecular-weight proteins (p115 and p124) did not coprecipitate with antiserum specific for pp40, suggesting that the cytosol may have contained two distinct pp59$^{v\text{-}rel}$ complexes. The amounts of pp59$^{v\text{-}rel}$ immunoprecipitated from extracts of REV-T-transfromed cells by antisera specific for pp40 and pp59$^{v\text{-}rel}$ were essentially equivalent, suggesting that the complex containing p115 and p124 that lacks pp40 represents a minor pp59$^{v\text{-}rel}$-containing complex in REV-T-transformed cells. In addition, the anti-pp40 serum coimmunoprecipitated less p75$^{c\text{-}rel}$ than did the pp59$^{v\text{-}rel}$-specific serum.

To verify the existence of a pp59$^{v\text{-}rel}$ complex that does not contain pp40, sequential immunoprecipitations were performed. Cytosolic extracts of [^{35}S]methionine-labeled REV-T-transformed cells were immunoprecipitated with the pp40-specific serum (Fig. 5B, lane 3). This serum precipitated all of the detectable pp40 (data not shown) and most of the pp59$^{v\text{-}rel}$ from extracts of transformed cells. However, when the pp40 cleared extract was immunoprecipitated with pp59$^{v\text{-}rel}$-specific serum, the remaining pp59$^{v\text{-}rel}$ was immunoprecipitated as well as p75$^{c\text{-}rel}$, p115, and p124 (Fig. 5B, lane 4). This complex contained the p115 and p124 associated with pp59$^{v\text{-}rel}$ and lacked detectable pp40. Therefore, pp59$^{v\text{-}rel}$ was present in two different cytosolic complexes, and the antiserum directed against pp40 recognized only one of these complexes.

Gel filtration analysis of the pp59$^{v\text{-}rel}$ cytosolic complexes. To more fully characterize the two pp59$^{v\text{-}rel}$ complexes, cytosolic extracts of [^{35}S]methionine-labeled REV-T-transformed cells were subjected to gel filtration on a Sephacryl S300 column. The column fractions containing labeled proteins were used in immunoprecipitation reactions, using antiserum specific for pp40 or pp59$^{v\text{-}rel}$. Antiserum directed against pp40 identified a single complex containing the majority of the pp59$^{v\text{-}rel}$ and pp40 and trace amounts of p75$^{c\text{-}rel}$ (Fig. 6B, lanes 3 to 15). Two pp59$^{v\text{-}rel}$-containing complexes were detected when the proteins eluted from the Sephacryl column were immunoprecipitated with antiserum against pp59$^{v\text{-}rel}$. The larger molecular weight complex contained pp59$^{v\text{-}rel}$, p124, p115, and p75$^{c\text{-}rel}$ (Fig. 6A, lanes 4 to 7).

Signal strength for each protein in the complex was determined by densitometer analysis of the proteins immunoprecipitated from the column fractions by using antiserum against either pp59$^{v\text{-}rel}$ or pp40. The minor pp59$^{v\text{-}rel}$ cytosolic complex had an apparent molecular mass of approximately 800 kDa (Fig. 7A). The minor complex contained all of the detectable p124 and p115 as well as most of the p75$^{c\text{-}rel}$ expressed in this REV-T-transformed cell line. Approximately 25% of the pp59$^{v\text{-}rel}$ was associated with this complex. The majority of pp59$^{v\text{-}rel}$ was complexed with pp40 and had a mass of approximately 400 kDa. When the proteins in

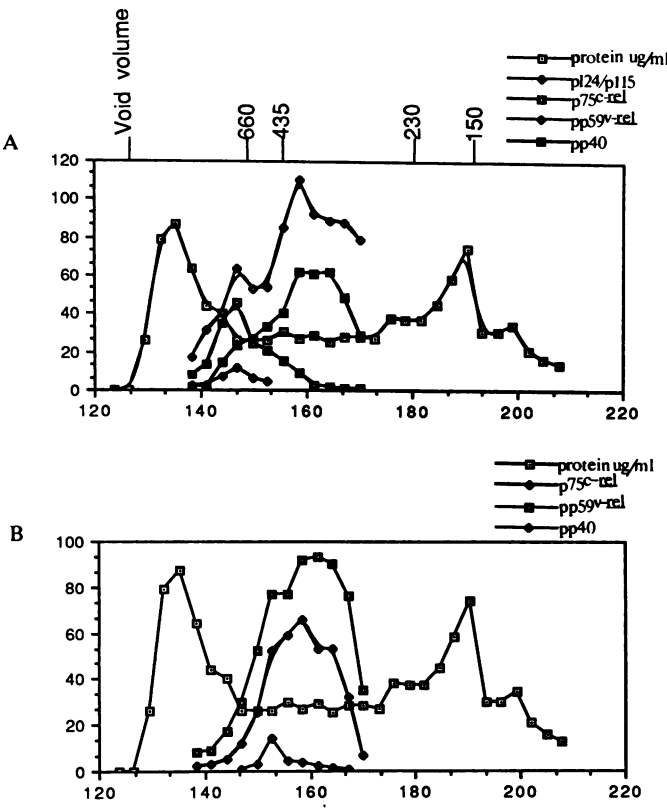

FIG. 7. Relative amounts of proteins associated with pp59$^{v\text{-}rel}$ as determined by elution volume of a Sephacryl S300 sizing column. Vertical axis, Signal strength as determined by densitometric analysis; horizontal axis, elution volume (in milliliters). Molecular size standards (in kilodaltons) are indicated above panel A (void volume, dextran blue; 660 kDa, thyroglobulin; 435 kDa, ferritin; 230 kDa, catalase; 158 kDa, aldolase). (A) Immunoprecipitations performed by using antiserum to the carboxy terminus of pp59$^{v\text{-}rel}$. All proteins complexed to pp59$^{v\text{-}rel}$ were analyzed. (B) Immunoprecipitations performed by using antiserum to pp40.

the cytosolic complexes were identified with anti-pp40 serum, the only proteins detected were pp59$^{v\text{-}rel}$, p75$^{c\text{-}rel}$, and pp40. The two higher-molecular-weight proteins were not detected in immunoprecipitates with anti-40-kDa-specific serum (Fig. 7B). The mass of the major pp59$^{v\text{-}rel}$-pp40 complex identified with anti-pp40 serum corresponded to the mass of this complex immunoprecipitated by anti-pp59$^{v\text{-}rel}$. The majority of the pp59$^{v\text{-}rel}$-pp40 complex did not contain detectable levels of p75$^{c\text{-}rel}$, possibly because of the limiting amounts of p75$^{c\text{-}rel}$ available to complex with pp40. The level of p75$^{c\text{-}rel}$ expressed in REV-T-transformed cells was substantially less than the level of either pp59$^{v\text{-}rel}$ or pp40.

pp59$^{v\text{-}rel}$ complex in the nucleus of REV-T-transformed lymphoid cells. The majority of pp59$^{v\text{-}rel}$ is located in the cytosol in REV-T-transformed lymphoid cell lines; less than 10% is detected in the nuclear fraction (15, 34, 37). To determine whether the nuclear form of pp59$^{v\text{-}rel}$ is complexed with the same or different cellular proteins, nuclear and cytosolic fractions were prepared from logarithmically growing REV-T-transformed lymphoid cells labeled with [^{35}S]methionine. Nuclear extracts were tested for lactic dehydrogenase activity, an indicator of cytosolic contamination, and nuclear extracts did not contain lactic dehydrogenase. Proteins in cytosolic and nuclear extracts were immunoprecipitated, using anti-pp59$^{v\text{-}rel}$ and anti-pp40 sera. Antisera against both pp59$^{v\text{-}rel}$ (Fig. 8A, lane 7) and pp40 (lane 6) coimmunoprecipitated pp59$^{v\text{-}rel}$ and pp40 from the nuclear fraction of [^{35}S]methionine-labeled cell extracts. The other cellular proteins present in the pp59$^{v\text{-}rel}$-pp40 cytosolic complexes (p115, p124, p75$^{c\text{-}rel}$, and p70^{hsc}) were not detected in the nuclear extracts. When sequential immunoprecipitations were performed with the pp40-specific antiserum to preclear the nuclear extract, pp59$^{v\text{-}rel}$-specific antiserum precipitated pp59$^{v\text{-}rel}$ that did not appear to be complexed with any cellular proteins (data not shown). Whether this apparently free pp59$^{v\text{-}rel}$ actually exists in the nucleus or represents the labile nature of the nuclear pp59$^{v\text{-}rel}$-pp40 complex has not been determined.

Three proteins in pp59$^{v\text{-}rel}$ complexes in the cytosol are phosphorylated (pp40, pp59$^{v\text{-}rel}$, and p75$^{c\text{-}rel}$). The most extensively phosphorylated protein is pp40. To determine the extent of phosphorylation of pp40 and pp59$^{v\text{-}rel}$ in the nucleus, proteins in cytosolic and nuclear extracts from $^{32}P_i$-labeled cells were immunoprecipitated with pp59$^{v\text{-}rel}$- and pp40-specific sera. pp59$^{v\text{-}rel}$ was phosphorylated in both the nuclear (Fig. 8B, lanes 8 and 9) and cytoplasmic (lanes 2 to 6) locations. The 40-kDa protein in the nucleus, however, was significantly less phosphorylated (lanes 8 and 9) than the pp40 found in the pp59$^{v\text{-}rel}$ complex in the cytosol (lanes 2 to 6). Although pp40 is not apparent in this autoradiogram, weakly phosphorylated pp40 has been detected in the nucleus (data not shown).

DISCUSSION

The ability of a specific antiserum to precipitate an antigenically unrelated protein is suggestive but not conclusive evidence that the proteins associate to form a heterocomplex. It was previously reported that the protein product of the v-*rel* oncogene, pp59$^{v\text{-}rel}$, is associated with at least four cellular proteins (pp40, p75$^{c\text{-}rel}$, p115, and p124) (25, 33–36; Lim et al., submitted). This report describes the preparation of an antiserum specific for the most abundant cellular protein associated with pp59$^{v\text{-}rel}$. The antiserum reacts specifically with one protein at 40 kDa by Western analysis of REV-T-transformed whole cell lysates and immunoprecipitates the 40-kDa phosphoprotein, pp59$^{v\text{-}rel}$, and p75$^{c\text{-}rel}$ from lysates of metabolically labeled REV-T-transformed cells. The ability of the 40-kDa-specific antiserum to coprecipitate pp59$^{v\text{-}rel}$ and p75$^{c\text{-}rel}$ provides conclusive evidence that pp40 and pp59$^{v\text{-}rel}$ associate to form a heterocomplex and strengthens the argument for the presence of p75$^{c\text{-}rel}$ in the complex.

The antiserum generated to the 40-kDa cellular protein has permitted the identification of two pp59$^{v\text{-}rel}$ protein complexes in the cytoplasm of REV-T-transformed cells. Since the minor pp59$^{v\text{-}rel}$ complex contains p124, p115, and p75$^{c\text{-}rel}$ but lacks pp40, the 40-kDa-specific antiserum selectively immunoprecipitates the major complex that contains p75$^{c\text{-}rel}$, pp59$^{v\text{-}rel}$, and pp40. The product of the cellular homolog, p75$^{c\text{-}rel}$, is predominantly found in the minor complex. It is unclear what function(s) these complexes serve in transformation or how they interact. The presence of the proto-oncogene protein product suggests that these complexes may be part of a normal regulatory mechanism that has been disrupted by pp59$^{v\text{-}rel}$ participation.

A third pp59$^{v\text{-}rel}$ complex has been identified in the nucleus of REV-T-transformed lymphoid cells that is composed of pp59$^{v\text{-}rel}$ and pp40. Whereas pp40 in the cytosol is heavily phosphorylated on serine residues, phosphorylation of pp40 is much reduced when the protein is precipitated from nuclear extracts. Whether this change in phosphoryla-

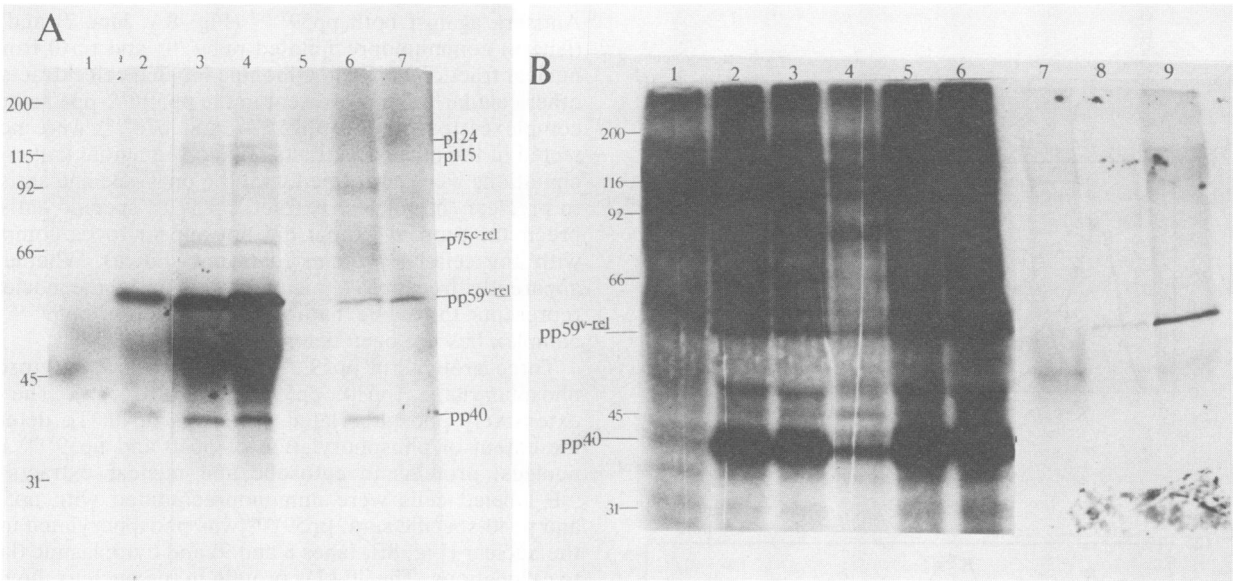

FIG. 8. Immunoprecipitations of cytosolic and nuclear extracts of metabolically labeled RECC-UTC4-1 cells. Nuclei were prepared by lysing cells in 20 mM Tris (pH 7.5)–20 mM KCl in a tight-fitting Dounce homogenizer. A crude nuclear pellet was obtained by centrifugation at 10,000 × *g* for 10 min. Whole cells were removed by suspending the crude nuclear pellet in 20 mM Tris buffer containing 40% sucrose and pelleting the nuclei through a 50% sucrose cushion at 50,000 × *g*. Purity was determined by assaying for lactic dehydrogenase activity (40). (A) Immunoprecipitations of [^{35}S]methionine-labeled proteins prepared from the cytosol and nuclear extracts of RECC-UTC4-1 cells. Lanes: 1 to 4, cytosolic extracts and preimmune, pp40-specific, and pp59$^{v\text{-}rel}$ full-length- and carboxy-region-specific antisera, respectively; 5 to 7, nuclear extracts and preimmune, pp40-specific, and pp59$^{v\text{-}rel}$ carboxy-region-specific antisera, respectively. (B) Immunoprecipitations of $^{32}P_i$-labeled proteins prepared from the cytosol and nuclear extracts of RECC-UTC4-1 cells. Lanes: 1 to 6, cytosolic proteins precipitated with preimmune, pp40-specific, pp59$^{v\text{-}rel}$ amino-terminus-specific, pp59$^{v\text{-}rel}$ carboxy-terminus-specific, and pp59$^{v\text{-}rel}$ full-length-specific antisera, respectively; 7 to 9, nuclear extracts and preimmune, pp40-specific, and pp59$^{v\text{-}rel}$ full-length-specific antisera, respectively.

tion level is important in targeting the complex to the nucleus or is involved in the regulation of v-*rel* function is unknown.

Recently, Morrison and co-workers (25) reported that the nuclear form of pp59$^{v\text{-}rel}$ is associated with a 200-kDa protein, p115, p124, and p75$^{c\text{-}rel}$. They failed to detect pp40 in the nucleus. We have never detected nuclear pp59$^{v\text{-}rel}$ complexes containing p115, p124, or p75$^{c\text{-}rel}$. Morrison et al. (25) did not use techniques to remove whole cells from the nuclear preparations, nor were assays for cytosolic contamination reported. The proteins that they identified would be present in immunoprecipitations of cytosolic pp59$^{v\text{-}rel}$ complexes performed in the presence of ionic detergents. We and others have shown that pp40 is a detergent-sensitive member of the pp59$^{v\text{-}rel}$ complexes, and pp40 would have been removed under the conditions used to rupture the nuclei (33, 35).

The mechanism of transformation by v-*rel* is not understood. The presence of two cytosolic pp59$^{v\text{-}rel}$ complexes and a nuclear complex hinders identification of the active form of pp59$^{v\text{-}rel}$. It has recently been suggested that pp59$^{v\text{-}rel}$ encodes a transactivating factor, suggesting that the active form would reside in the nucleus. In the nucleus, pp59$^{v\text{-}rel}$ is complexed with pp40, suggesting that pp40 is likely to play a critical role in lymphoid cell transformation by v-*rel*. Ironically, although pp59$^{v\text{-}rel}$ resides in a cytoplasmic complex containing p75$^{c\text{-}rel}$, we have been unable to detect p75$^{c\text{-}rel}$ in the nuclei of REV-T-transformed cells. If pp59$^{v\text{-}rel}$ acts in the nucleus, perhaps p75$^{c\text{-}rel}$ in the minor cytoplasmic complex is inactive.

One level of transcriptional control involves sequestering transactivators in the cytosol. Nuclear factor NF-κB is a lymphoid-specific transactivating factor that binds enhancer elements in the J-C intron of the κ light-chain gene (3, 28). NF-κB is largely cytosolic in unstimulated cells, and the cytosolic form fails to bind DNA in vitro (29). Exposure of pre-B cells to activators of protein kinase C results in nuclear NF-κB activity (29). The phosphorylation of an inhibitor in the cytosolic complex results in the rapid translocation of NF-κB to the nucleus and its activation. It is possible that pp59$^{v\text{-}rel}$ acts by interfering with the proper function of a transcription complex that regulates lymphoid cell differentiation or activation.

Alternatively, the pp59$^{v\text{-}rel}$ cytosolic complex might function in cell cycle regulation. Protein complexes can act to regulate cell division without directly activating the transcription of cellular genes. The maturation-promoting factor (MPF) in *Xenopus laevis* embryos is a complex that mediates the G2-to-M transition in the cell cycle (2, 11; for a review, see reference 22). Similar cell cycle regulatory factors have been described in several eucaryotic systems (9, 10, 14, 19). The MPF complex exists in two forms: an inactive 400-kDa complex and a 260-kDa active form. A protein kinase activity has been associated with the MPF complex, and phosphorylation leads to activation of the complex (1, 2, 9–11). The pp59$^{v\text{-}rel}$-containing complexes present in REV-T-transformed lymphoid cells have a number of features in common with MPF. At present, characterization of the roles of the pp59$^{v\text{-}rel}$ complexes is too preliminary to determine whether these complexes function in transactivation or other aspects involved in the regulation of cell proliferation.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants CA 33192 and CA 26169 from the National Cancer Institute.

LITERATURE CITED

1. **Arien, D., L. Maijer, L. Brizuela, and D. Beach.** 1988. *cdc2* is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. Cell **55**:371–378.
2. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**:211–217.
3. **Bergmeyer, H. U., and F. Bernt.** 1974. p. 574–579. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 2, 2nd ed. Verlag Chemie/Academic Press, Inc., New York.
4. **Beug, H., H. Muller, S. Drieser, G. Doederlein, and T. Graf.** 1981. Hematopoietic cells transformed *in vitro* by REV-T avian reticuloendotheliosis virus express characteristics of very immature lymphoid cells. Virology **115**:295–309.
5. **Bradford, M. M.** 1984. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and protein-A. Anal. Biochem. **112**:195–203.
7. **Chen, I. S. Y., T. W. Mak, J. J. O'Rear, and H. M. Temin.** 1981. Characterization of reticuloendotheliosis virus stain T DNA and isolation of a novel variant of reticuloendotheliosis virus strain T by molecular cloning. J. Virol. **40**:800–811.
8. **Chen, I. S. Y., K. C. Wilhelmsen, and H. M. Temin.** 1983. Structure and expression of c-*rel*, the cellular homolog to the oncogene of reticuloendotheliosis virus strain T. J. Virol. **45**: 104–113.
9. **Draetta, G., L. Brizuela, J. Potashkin, and D. Beach.** 1987. Identification of p34 and p13, human homologs of the cell cycle regulators of fission yeast encoded by *cdc2*$^+$ and *suc1*$^+$. Cell **50**:319–325.
10. **Dunphy, W. G., and J. W. Newport.** 1988. Unraveling of mitotic control mechanisms. Cell **55**:925–928.
11. **Dyert, M. S., and M. W. Kirschner.** 1988. Regulation of MPF activity *in vitro*. Cell **53**:211–217.
12. **Franklin, R. B., C. Y. Kang, K. M. M. Wan, and H. R. Bose, Jr.** 1977. Transformation of chick embryo fibroblasts by reticuloendotheliosis virus. Virology **83**:313–321.
13. **Garson, K., and C. Y. Kang.** 1986. Identification of the v-*rel* protein in REV-T transformed chicken bone marrow cells and expression in COS1 cells. Biochem. Biophys. Res. Commun. **134**:716–722.
14. **Gautier, J., C. Norbury, M. Lohka, P. Nurse, and J. Maller.** 1988. Purified maturation-promoting factor contains the product of a Xenopus homology of the fission yeast cell cycle control gene cdc2$^+$. Cell **54**:433–439.
15. **Gilmore, T. D., and H. M. Temin.** 1986. Different localization of the product of the v-*rel* oncogene in chicken fibroblasts and spleen cells correlates with transformation by REV-T. Cell **44**:791–800.
16. **Gonda, M. A., N. R. Rice, and R. V. Gilden.** 1980. Avian reticuloendotheliosis virus: characterization of the high molecular weight viral RNA in transforming and helper virus populations. J. Virol. **34**:743–751.
17. **Herzog, N. K., W. J. Bargmann, and H. R. Bose, Jr.** 1986. Oncogene expression in reticuloendotheliosis virus-transformed lymphoid cell lines and avian tissues. J. Virol. **57**:371–375.
18. **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.
19. **Labbe, J. C., M. G. Lee, P. Nurse, A. Picard, and M. Doree.** 1988. Activation of M-phase of a protein kinase encoded by a starfish homolog of the cell cycle control gene *cdc2*$^+$. Nature (London) **335**:251–254.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lewis, R. B., J. McClure, B. Rup, D. W. Niesel, R. F. Garry, J. D. Hoelzer, K. Nazarian, and H. R. Bose, Jr.** 1981. Avian reticuloendoetheliosis virus: identification of the hematopoietic target cell for transformation. Cell **25**:421–431.
22. **Lohka, M. J., M. K. Hayes, and J. L. Miller.** 1988. Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. Proc. Natl. Acad. Sci. USA **85**:3009–3013.
23. **Markwell, M. A. K.** 1982. A new solid-state reagent to iodinate proteins. Anal. Biochem. **125**:427–432.
24. **Moore, B. E., and H. R. Bose, Jr.** 1988. Expression of the v-*rel* oncogene in reticuloendotheliosis virus-transformed fibroblasts. Virology **162**:377–387.
25. **Morrison, L. E., N. Kabrun, S. Mudri, M. J. Hayman, and P. J. Enrietto.** 1989. Viral *rel* and cellular rel associate with oncogene proteins in transformed and normal cells. Oncogene Res. **4**: 677–683.
26. **Rice, N. R., T. D. Copeland, S. Simek, S. Oroszlan, and R. V. Gilden.** 1986. Detection and characterization of the protein encoded by the v-*rel* oncogene. Virology **149**:217–229.
27. **Rice, N. R., R. R. Hiebsch, M. A. Gonda, H. R. Bose, Jr., and R. V. Gilden.** 1982. Genome of reticuloendotheliosis virus: characterization by use of cloned proviral DNA. J. Virol. **42**:237–252.
28. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**:705–716.
29. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47**:921–928.
30. **Sevoian, M., R. N. Larose, and D. M. Chamberlain.** 1964. Avian lymphomatosis. VI. A virus of unusual potency and pathogenicity. Avian Dis. **8**:336–347.
31. **Shibuya, J., I. Chen, A. Howatson, and T. W. Mak.** 1982. Morphological, immunological, and biochemical analyses of chicken spleen cells transformed *in vitro* by reticuloendotheliosis virus strain T. Cancer Res. **42**:2722–2728.
32. **Simek, S., and N. R. Rice.** 1988. Detection and characterization of the protein encoded by the chicken c-*rel* proto-oncogene. Oncogene Res. **2**:103–119.
33. **Simek, S., and N. R. Rice.** 1988. pp59$^{v\text{-}rel}$, the transforming protein of reticuloendotheliosis virus, is complexed with at least four other proteins in transformed chicken lymphoid cells. J. Virol. **62**:4730–4736.
34. **Simek, S. L., R. M. Stephens, and N. R. Rice.** 1986. Localization of the v-*rel* protein in reticuloendotheliosis virus strain T-transformed lymphoid cells. J. Virol. **59**:120–126.
35. **Tung, H. Y. L., W. J. Bargmann, M. Y. Lim, and H. R. Bose, Jr.** 1988. The v-*rel* oncogene product is complexed to a 40 kDa phosphoprotein in transformed lymphoid cells. Proc. Natl. Acad. Sci. USA **85**:2479–2483.
36. **Tung, H. Y. L., W. J. Bargmann, and H. R. Bose, Jr.** 1988. Serine phosphorylation of the v-*rel* oncogene product/pp40 complex. Biochem. Biophys. Res. Commun. **152**:441–448.
37. **Walro, D. S., N. K. Herzog, J. Zhang, M. Y. Lim, and H. R. Bose, Jr.** 1987. The transforming protein of avian reticuloendotheliosis virus is a soluble cytoplasmic protein which is associated with a protein kinase activity. Virology **160**:433–444.
38. **Wittenberg, C., and S. J. Reed.** 1988. Control of the yeast cell cycle is associated with assembly/disassembly of the Cdc 28 protein kinase complex. Cell **54**:1061–1072.
39. **Wong, T. C., and M. M. C. Lai.** 1981. Avian reticuloendotheliosis virus contains a new class of oncogene of turkey origin. Virology **111**:289–293.
40. **Zhang, J., W. Bargmann, and H. R. Bose, Jr.** 1989. Rearrangement and diversification of immunoglobulin light-chain genes in lymphoid cells transformed by reticuloendotheliosis virus. Mol. Cell. Biol. **9**:4970–4976.
41. **Zhang, J., and H. R. Bose, Jr.** 1989. Acquisition of new proviral copies in avian lymphoid cells transformed by reticuloendotheliosis virus. J. Virol. **63**:1107–1115.