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Localization of the v-*rel* Protein in Reticuloendotheliosis Virus Strain T-Transformed Lymphoid Cells

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The protein (p59^{rel}) encoded by the transforming gene of reticuloendotheliosis virus strain T (REV-T) has been identified in REV-T-transformed avian lymphoid cells by using antisera raised against synthetic peptides whose sequences were derived from three nonoverlapping regions of v-*rel* (N. R. Rice, T. D. Copeland, S. Simek, S. Oroszlan, and R. V. Gilden, Virology 149:217–229, 1986). To obtain polyclonal antibodies directed against a larger number of p59^{rel} epitopes, a 262-amino acid segment was expressed in bacteria. Antisera raised against this fusion protein (v-Δ-*rel*) precipitated p59^{rel} from lysates of [^{35}S]methionine-labeled REV-T-transformed cells, thus confirming previous results obtained with the peptide antisera. We used this new antiserum to localize p59^{rel} in REV-T-transformed cells by subcellular fractionation using differential centrifugation and by indirect immune fluorescent staining. After fractionation and immune precipitation, the majority of p59^{rel} was found in the cytosolic fraction. Indirect immunofluorescence experiments also gave results consistent with the cytoplasmic localization of the v-*rel* protein in transformed lymphoid cells. In previous studies (Rice et al., Virology 149:217–229, 1986) it was shown that immune precipitates formed with one of the three p59^{rel} peptide antisera possessed in vitro protein kinase activity. Immune precipitates formed with the fusion protein antiserum also showed kinase activity in the in vitro assay. Most of this activity was found in the soluble cytoplasmic fraction, indicating that the kinase may be p59^{rel} or a protein closely associated with it.

Reticuloendotheliosis virus strain T (REV-T) is an avian acute leukemia virus. Like most of the transforming retroviruses, REV-T consists of a mixture of a replication-competent helper virus (REV-A) and a defective genome responsible for transformation (16). The defective genome has major deletions in all three viral genes and an insertion (v-*rel*) of about 1.4 kilobases (5, 12, 18, 29, 33, 36) apparently derived from avian (turkey?) DNA (5, 6, 29, 32, 38). REV-T is able to transform avian hematopoietic cells both in vivo and in vitro (11, 17).

The nucleotide sequence of v-*rel* and neighboring viral regions has been determined (33, 36), and these data reveal why antisera to REV-A proteins do not detect the v-*rel* protein. The predicted v-*rel* protein begins with 12 amino acids of the REV-A envelope polyprotein signal sequence and terminates with 19 residues translated out-of-frame with respect to p20E, the REV-A transmembrane envelope protein. To detect the v-*rel* product, therefore, several v-*rel*-encoded peptides were synthesized and antisera were raised against them. The use of these sera in immune precipitation experiments showed that the v-*rel* protein has a molecular weight of about 59,000, that it is not detectably glycosylated or secreted, and that it is a phosphoprotein (28). Furthermore, in immune complexes formed with one of the three peptide antisera, a high level of protein kinase activity was observed. Since v-*rel* has little or no sequence homology with any other known oncogene (33, 36), including those which code for protein kinases, this was an unexpected finding.

Peptide antisera frequently detect not only the protein of interest, but also one or more essentially unrelated proteins which contain regions similar to the peptide (25). To clarify the kinase finding, we considered it desirable to raise polyclonal antisera against a larger segment of p59^{rel} than those encompassed by the peptides. In this paper we describe the expression of a portion of p59^{rel} in bacteria. We have used antiserum raised against this v-*rel* protein segment (designated as v-Δ-*rel*) to determine the subcellular location of p59^{rel}. We also report that immune complexes formed with this antiserum exhibit protein kinase activity.

MATERIALS AND METHODS

Cells and viruses. The transformed chicken spleen cell line S3D6, which produces REV (REV-A), was established by Jacalyn Hoelzer-Pierce (17). These are suspension cells and are grown in RPMI 1640 plus 10% fetal calf serum at 37°C. A herpesvirus-transformed chicken lymphoid cell line (MSB-1) was also grown in suspension in RPMI 1640 plus 10% fetal calf serum at 37°C.

Antisera. Antiserum to peptide III, which corresponds to residues 425 to 437 of p59^{rel}, has been described previously (28). Antiserum to REV-A p20E was a gift of W.-P. Tsai.

Expression of a v-*rel* fusion protein in *Escherichia coli*. The parental plasmid was pBR322 containing a 892-base-pair *Eco*RI fragment of the v-*rel* gene (5). This fragment has an 87-base-pair internal deletion relative to wild type v-*rel* (5; R. M. Stephens and N. R. Rice, unpublished data). When digested with *Cla*I, the plasmid releases an 806-base-pair insert containing the 3′-terminal 785 bases of the v-*rel* insert and 21 bases of the pBR322 backbone. This fragment was isolated after agarose gel electrophoresis and ligated into pJL6 (21), which had been linearized with *Cla*I. After transformation of *E. coli* DC646 (21), DNA from resulting colonies was screened for the presence of inserts after digestion with *Cla*I. Clones containing the insert were checked for orientation by using the restriction enzyme

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*Hin*dIII. Clones containing each of the orientations, as well as pJL6 alone, were then used to transform the heat-inducible expressor cell line MZ-1 (21). After induction at 42°C, MZ-1 lysates contained a new protein which migrated in Coomassie blue-stained gels at the predicted molecular weight of 30,600 (p31). This protein appeared only in cells which had been transformed with DNA carrying the v-*rel* segment in the positive orientation. Both immune precipitation and Western blot analysis using antiserum to peptide III confirmed that this protein was related to $p59^{rel}$.

To prepare p31, plasmid-containing cells were grown at 37°C to A_{660} = 0.3 in YT broth (0.8% N-Z amine, 0.5% yeast extract, 0.5% NaCl) with ampicillin (50 μg/ml). The cells were shifted to 42°C for 90 min, pelleted, and suspended to A_{660} = 14 in lysis buffer (1% sodium dodecyl sulfate [SDS] plus 0.01% β-mercaptoethanol). The lysate was boiled and loaded onto 12.5% Laemmli SDS-polyacrylamide gels (20). After electrophoresis, the gels were stained for 10 min in 0.25 M KCl at 4°C. The p31 band was cut from the gel and crushed in phosphate-buffered saline (PBS). Two New Zealand White rabbits were each immunized with 5 μg of protein. The rabbits were injected subcutaneously at 2-week intervals four times.

Radiolabeling cells and immunoprecipitations. About 20×10^6 S3D6 cells were washed twice in PBS and incubated in methionine-free Eagle minimum essential medium for 10 min at 37°C. The cells were then metabolically labeled for 2 h at 37°C in 3 ml of the same medium supplemented with [^{35}S]methionine (Amersham; 1,000 Ci/mmol) at 50 μCi/ml. Cells were lysed in RIPA buffer (2×10^{-2} M Tris, pH 7.5, 2×10^{-3} M EDTA, 0.15 M NaCl, 0.25% SDS, 1% Triton X-100, 1% sodium deoxycholate, 180 Kallikrein units of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride) and kept on ice for 30 min with frequent vortexing. Lysates were clarified by centrifugation for 10 min at 10,000 × *g*, and supernatants were incubated at 4°C with 3 μl of the respective antiserum per 2×10^6 cells plus protein A-Sepharose for 3 h. Immune complexes were collected by centrifugation, washed several times in RIPA, taken up in loading dye (20% glycerol, 40% β-mercaptoethanol, 0.2 M Tris, pH 7, 8% SDS, and 0.04% bromphenol blue), and loaded onto 10 to 20% SDS-polyacrylamide gels. After electrophoresis, gels were treated with dimethyl sulfoxide and 22% PPO (2,5-diphenyloxazole) as previously described (28).

Cellular fractionation. S3D6 cells were metabolically labeled with [^{35}S]methionine as described above. Cells were swollen in a hypotonic buffer (2.0×10^{-2} M Tris, pH 7.6, 1.0×10^{-2} M KCl) on ice for 20 min at a cell density of approximately 10^7 cells per ml. All subsequent operations were performed at 4°C. Homogenization of swollen cells was accomplished by 50 strokes of a Dounce homogenizer, and lysis was checked using phase-contrast microscopy. Immediately after homogenization the lysate was adjusted to a final concentration of 0.25 M sucrose. We found that this procedure resulted in more complete lysis than when sucrose was included in the homogenizing buffer. Nuclei and large cell fragments (nuclear fraction) were removed by centrifuging the homogenate at 1,000 × *g* for 10 min. The supernatant was then centrifuged at 10,000 × *g* for 30 min to yield a pellet (P10) enriched in plasma membrane fragments and mitochondria (13). The supernatant was centrifuged at 100,000 × *g* for 1 h to obtain a pellet (P100) containing the microsome fraction and a supernatant (S100) cytosol fraction.

The nuclei were separated from large cell fragments by centrifuging through 60% sucrose. This was done by suspending the crude nuclear pellet in 2 ml of 2.0×10^{-2} M Tris plus 1.0×10^{-2} M KCl and adding the supsension to 60% sucrose (20 ml) containing 1 mM Tris. This mixture was shaken gently, underlaid with 10 ml of 60% sucrose, and overlaid with 7 ml of 1.0×10^{-3} M Tris. The resulting gradient was centrifuged in an SW27 rotor (Beckman Instruments) at 50,000 × *g* for 60 min. The pellet was removed and used as the nuclear fraction.

The pellets obtained from the nuclear, P10, and P100 fractions were taken up in RIPA buffer, clarified by centrifugation at 10,000 × *g* for 10 min, and immune precipitated with the respective sera. Salt and detergent were added to the S100 fraction to give final concentrations similar to those in RIPA buffer.

Immunofluorescence. Cells were cytocentrifuged onto clean glass slides at a density of 10^6 cells per 0.2 ml. The cells were fixed in methanol for 20 min at −20°C and then air dried. They were rehydrated in PBS before reacting with primary antibody. Antiserum at a 1:50 dilution in PBS was incubated with the cells at 37°C for 30 min. After three 10-minute washes in PBS, cells were treated for 30 min at 37°C with fluorescein-conjugated goat anti-rabbit immunoglobulin G (Kirkegaard and Perry Laboratories, Inc.) at a 1:20 dilution in PBS. After three more washes in PBS, slides were dried and cover slips were mounted in Permount. Cells were photographed using Kodak Tri-X film.

Kinase assay. Unlabeled S3D6 cells were lysed in TNT buffer (0.2 M NaCl, 2×10^{-2} M Tris, pH 7.5, 1% Triton X-100, containing aprotinin and phenylmethylsulfonyl fluoride as described above) for 30 min on ice with frequent vortexing. The lysates were clarified (10,000 × *g*, 10 min) and immune precipitated with preimmune or *rel*-specific antiserum in the presence of protein A-Sepharose. Precipitates were washed in TNT buffer and then incubated in 30 μl of TNT buffer containing 1.0×10^{-2} M $MnCl_2$ and 10 μCi of [γ-^{32}P]ATP (Amersham; 3,000 Ci/mmol) for 10 min at 30°C. Samples were washed in RIPA buffer and boiled in gel loading buffer. Samples were then electrophoresed on 10% SDS-polyacrylamide gels, dried, and autoradiographed at −70°C with an intensifying screen for 2 to 4 h.

RESULTS

Identification of $p59^{rel}$ in REV-T-transformed cells by using fusion protein antiserum. To obtain antibodies that recognize a larger number of p59 epitopes that those raised against synthetic peptides, a fusion protein (v-Δ-*rel*) was constructed. This protein contains 262 of the total 503 amino acids of the predicted v-*rel* protein (33, 36). The starting material was a 892-base-pair *Eco*RI REV-T fragment that had been inserted into the *Eco*RI site of pBR322 (5). This plasmid was cleaved with *Cla*I, generating a fragment containing 785 base pairs of REV-T and 21 base pairs of pBR322. The *Cla*I fragment was inserted into the plasmid expression vector pJL-6 (21), which uses the PL promoter of λ bacteriophage and the λ *c*II gene initiation site. The final protein product contains 12 N-terminal amino acids of *c*II, 262 amino acids of $p59^{rel}$, and two C-terminal residues derived from pBR322. A schematic diagram of the predicted v-*rel* protein, showing the location of the region expressed in pJL-6 and one of the previously described synthetic peptides (28), is given in Fig. 1.

Rabbit antisera raised against the fusion protein were tested in immune precipitation experiments. In a previous study we used three independent peptide antisera to search

for the protein encoded by v-*rel*. It was found that each peptide serum precipitated a small number of proteins, but that only one protein, of about 59,000 daltons, was precipitated by all three sera. Since p59 was not detected in normal chicken cells and since its size was in good agreement with that predicted by the DNA sequence, we concluded that it is the v-*rel* protein (28). Antisera to the fusion protein raised in two different rabbits recognized a protein of this same size (Fig. 2). This protein was not precipitated by preimmune serum (Fig. 2, lane 1), and immune precipitation could be blocked by incubation of immune serum with purified fusion protein (lane 3). These results demonstrate the specificity of the reaction and confirm the identification of p59 as the protein encoded by v-*rel*.

Subcellular fractionation and localization of p59^{rel} in REV-T-transformed cells. To determine the localization of p59^{rel} in REV-T-transformed cells, cells were metabolically labeled with [^{35}S]methionine and lysates were fractionated using differential centrifugation. The REV-T-transformed cells were homogenized in low salt, 0.25 M sucrose, and the absence of any detergent, conditions which have been shown to preserve the integrity of cellular organelles and their associated proteins (13). The fractions were then immune precipitated with antibodies raised against the fusion protein.

As a preliminary experiment to determine whether p59 is nuclear or non-nuclear, the ^{35}S-labeled cellular homogenate was centrifuged at 1,000 × *g* for 10 min to yield a nuclear pellet and a postnuclear supernatant. Immune precipitation revealed that little or no p59^{rel} was present in the nuclear fraction (Fig. 3A, lane 3), but that it was easily detectable in the supernatant (Fig. 3A, lane 4). To provide an approximation of the total amount of p59^{rel} expected in the separated fractions, an equivalent sample of cells was analyzed before fractionation. The results (Fig. 3A, lane 1 compared with lane 4) showed that p59^{rel} is present in the postnuclear fraction at a level comparable to that seen in the unfractionated lysate. We conclude that p59^{rel} is a cytoplasmic protein.

To determine which cytoplasmic subfraction contained the majority of p59, the postnuclear supernatant was centrifuged at 10,000 × *g*. The pellet (P10) is expected to contain membrane fragments and mitochondria (13). The 10,000 × *g* supernatant was then centrifuged at 100,000 × *g*. The resulting pellet (P100) contains microsomes, and the supernatant (S100) contains nonsedimenting cytoplasmic proteins (13). When these fractions were analyzed by immune precipitation, little or no p59 was found in either P10 or P100 (Fig. 3B, lanes 3 and 6) and the majority was found in the S100 fraction (Fig. 3C, lane 2). The amount of p59 recovered in the S100 fraction (relative to the unfractionated control) varied somewhat from experiment to experiment. Recovery was rather low in the experiment shown in Fig. 3B and 3C, but in other cases the S100 p59 band was nearly as intense as that in the control lane. In all cases little or no p59 was found in the P10 or P100 fraction. These results show that p59^{rel} is localized predominantly in the cytosolic fraction of REV-T-transformed avian lymphoid cells.

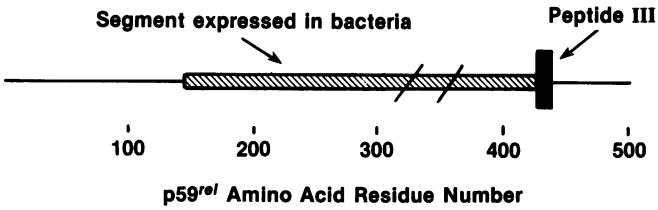

FIG. 1. Segment of v-*rel* expressed in bacteria. Based on the DNA sequence, p59^{rel} has 503 amino acids (33, 36). The v-*rel* segment used for expression has an 87-base-pair deletion (indicated by the slash marks) relative to wild-type transforming v-*rel* (5; Stephens and Rice, unpublished data). The resulting bacterial protein contains 262 v-*rel*-encoded amino acids (p59 residues 148 to 316 and 346 to 438) in addition to 12 N-terminal λ *c*II amino acids and two C-terminal pBR322 residues. The previously described peptide III (28) consists of p59 residues 425 to 437.

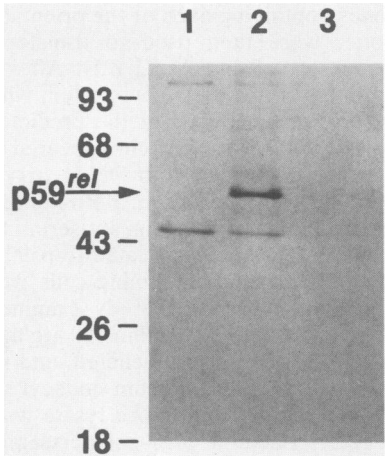

FIG. 2. Detection of p59^{rel} in REV-T-transformed cells using fusion protein antisera. REV-T-transformed avian lymphoid cells were metabolically labeled with [^{35}S]methionine for 2 h, and lysates were immune precipitated with fusion protein antiserum. Each lane contains the precipitate from about 10^6 cells. Samples were analyzed on 10 to 20% SDS-polyacrylamide gels followed by fluorography. Lane 1, Preimmune serum; lane 2, fusion protein antiserum; lane 3, fusion protein antiserum incubated with 5 μg of fusion protein before addition to lysate. The molecular weights (×10^3) of marker proteins are given on the left.

Since the REV-T-transformed cell line used in this experiment is a virus producer, it contains the REV-A helper virus genome in addition to the transforming REV-T genome. To determine whether our fractionation procedure was successful, we took advantage of the presence of REV-A proteins being expressed in these cells. Based on previous studies with cells infected with Friend murine leukemia virus (10, 22), the majority of the envelope precursor polyprotein is expected to be found in the P10 and P100 fractions, consistent with its membrane association. We therefore used antiserum to the REV-A transmembrane protein p20E (34; W.-P. Tsai, T. D. Copeland, and S. Oroszlan, submitted for publication) to localize the REV-A envelope precursor gPr74^{env}. When lysates were immune precipitated with antiserum to p20E, the majority of gPr74^{env} was indeed found in the P10 and P100 fractions (Fig. 3B, lanes 4 and 7), and little or none was present in the S100 fractions (Fig. 3C, lane 3). We conclude from these results that our fractionation procedure gives adequate representation of the localization of p59^{rel} in REV-T-transformed cells.

Immune fluorescence of REV-T-transformed cells using fusion protein antiserum. To confirm the cytoplasmic localization of the v-*rel* protein, indirect immune fluorescent staining experiments were performed using REV-T-transformed cells and antibody raised against the fusion protein. The REV-T-transformed cells are lymphoid in origin and require cytocentrifugation onto glass slides before the immune staining. Since after centrifugation cells appear to consist mainly of nucleus, with only a minor ring of cyto-

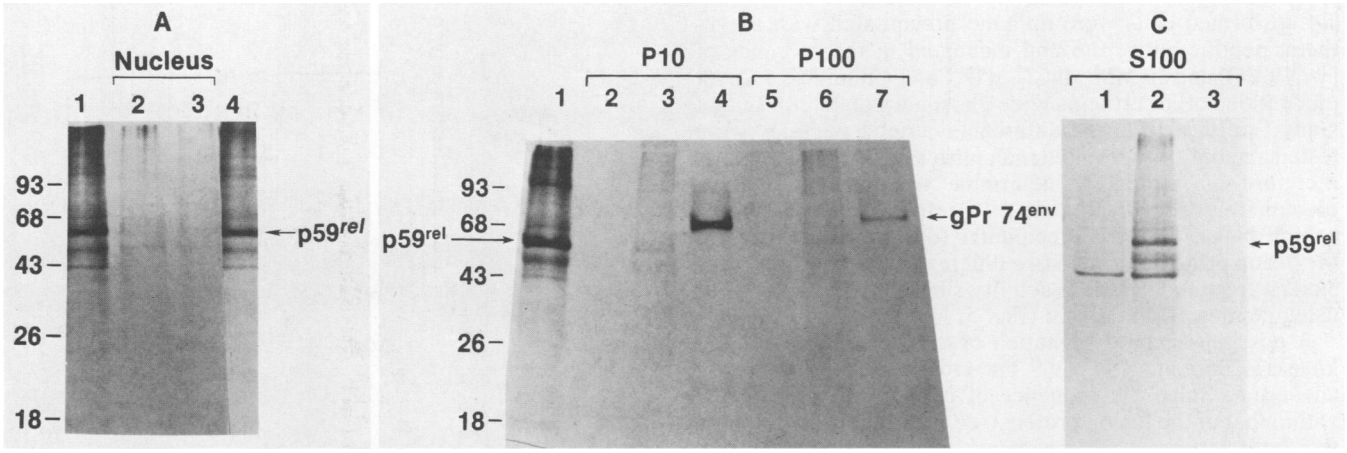

FIG. 3. Subcellular localization of p59^{rel} in REV-T-transformed cells. Virus-producing REV-T-transformed cells were metabolically labeled for 2 h with [^{35}S]methionine. Cells were swollen in hypotonic buffer for 20 min, homogenized, and fractionated by differential centrifugation. A sample of lysate was taken before fractionation; this was analyzed in lane 1 of panels A and B after precipitation with fusion protein antiserum. All lanes contain an equivalent number of cells. (A) Nuclear pellet obtained by centrifuging the lysate at 1,000 × *g*. Lane 2, Preimmune serum; lane 3, fusion protein antiserum. Lane 4 is the postnuclear supernatant precipitated with fusion protein antiserum. (B) P10 fraction was obtained by centrifuging the postnuclear supernatant at 10,000 × *g*. P100 fraction was obtained by centrifuging the P10 supernatant at 100,000 × g. Lanes 2 and 5, Preimmune serum; lanes 3 and 6, fusion protein antisera; lanes 4 and 7, anti-p20E serum. (C) S100 is the supernatant obtained by centrifugation at 100,000 × *g*. Lane 1, Preimmune serum; lane 2, fusion protein antiserum; lane 3, anti-p20E serum. The molecular weights (×10^3) of marker proteins are given at the left of panels A and B.

plasm, the fluorescent staining will only distinguish between a nuclear, cytoplasmic, and plasma membrane location for p59^{rel}.

Indirect fluorescent staining of the REV-T-transformed cells is shown at low and high power in Fig. 4B and C. The staining appeared to be located principally in the cytoplasm, with little detectable fluorescence of the nucleus or surrounding membranes. Pretreatment with Triton X-100 (0.01 to 0.1%) or saponin (0.1 to 0.5%), which increases the permeability of the nuclear membrane, did not affect the results (data not shown). Preimmune serum did not stain these cells (Fig. 4A).

To be sure the cytoplasmic staining we detected was specific for p59^{rel} and not due to a cross-reaction with antibodies made against a bacterial protein, we performed a competition experiment. Antiserum was incubated with gel-purified fusion protein before being added to the cells. The result was a marked reduction in the subsequent fluorescent staining (Fig. 4D), thus confirming the specificity of the reaction.

To determine whether the staining we observed was due to reaction with a normal component of chicken cells, we also tested two other cell types; uninfected chick embryo fibroblasts (data not shown) and a herpesvirus-transformed avian lymphoid line (MSB-1) (Fig. 4E). No staining was detected in either of these cell types. These results indicate that the staining we saw was presumably viral in origin and not due to a normal chicken protein.

Demonstration of kinase activity in REV-T-transformed cells using fusion protein antiserum. When lysates of REV-

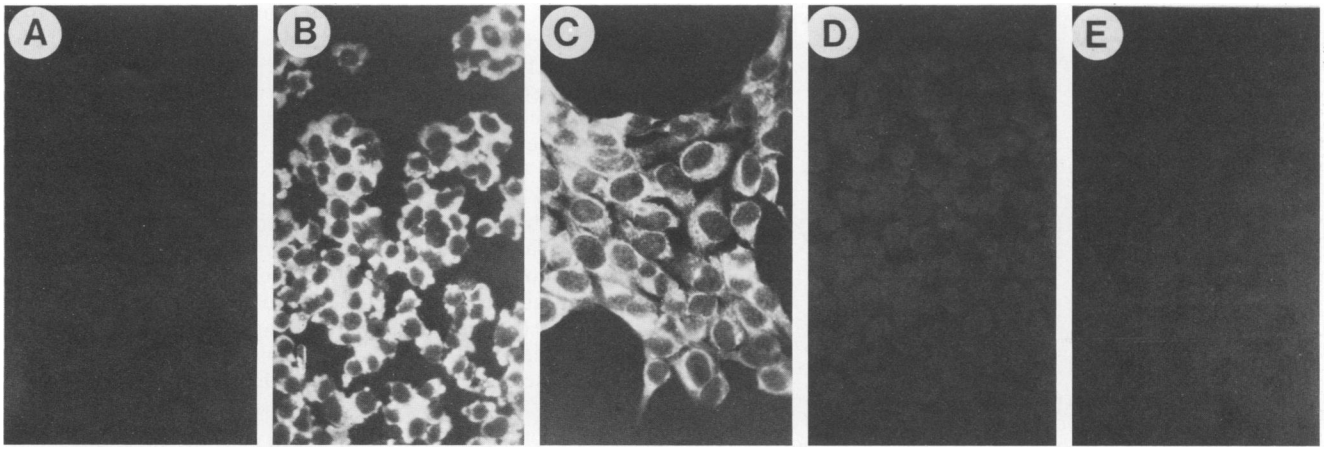

FIG. 4. Immunofluorescent staining of REV-T-transformed cells by using fusion protein antiserum. REV-T-transformed avian lymphoid cells were cytocentrifuged onto glass slides and fixed and stained as described in Materials and Methods. (A) REV-T-transformed cells reacted with preimmune serum. (B) REV-T-transformed cells reacted with fusion protein antiserum, photographed at low magnification (×60). (C) Same cells at high magnification (×100). (D) Cells and conditions as for panel B, except serum was preincubated with approximately 5 μg of purified fusion protein. (E) MSB-1 cells (Marek's disease-transformed chicken lymphoid cells) reacted with fusion protein antiserum (×60).

T-transformed cells were immune precipitated with a synthetic peptide antiserum and incubated in the presence of [γ-^{32}P]ATP and 10 mM $MnCl_2$, p59^{rel} and a number of as yet unidentified other proteins were phosphorylated (28). In that study, antisera to three different synthetic peptides were tested and only one resulted in in vitro kinase activity. It was therefore of interest to determine whether the immune precipitate formed with v-Δ-*rel* protein antiserum would exhibit kinase activity. Precipitates formed with antisera to the fusion protein raised in two different rabbits (Fig. 5, lanes 3 and 4) resulted in kinase activity similar to that seen when using peptide III antiserum (Fig. 5, lane 2).

A possible trivial explanation of these results is that the kinase is unrelated to p59^{rel} but cross-reacts with the p59 antiserum. Since the sequence of peptide III is contained within that of the fusion protein (see Fig. 1), it is conceivable that both antisera could recognize such a hypothetical protein. To explore this possibility, we first tested whether antiserum to the fusion protein was able to recognize peptide III. In an enzyme-linked immunosorbent assay performed by the procedure of Saunders (G. S. Saunders, Am. Soc. Clin. Pathol. workshop, Chicago, Ill., 1977), immune serum reacted strongly with peptide III at a 1:10^4 dilution and continued to bind peptide detectably at a 1:10^5 dilution. In contrast, preimmune serum did not bind peptide III at a 1:10^4 dilution. Thus, the fusion protein antiserum contains antibodies capable of recognizing peptide III. Therefore it is possible that peptide III antiserum and fusion protein antiserum both cross-react with a single non-*rel* protein.

If the kinase were such a cross-reacting protein, its precipitation by v-Δ-*rel* protein antiserum should be eliminated if all antibodies specific for peptide III were removed.

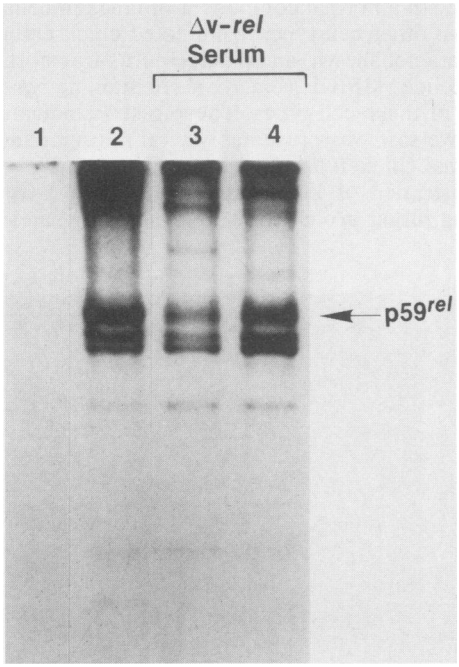

FIG. 5. Kinase activity associated with p59^{rel} in REV-T-transformed cells. After immune precipitation of REV-T-transformed lymphoid cell lysates (1×10^6 to 2×10^6 cells per lane), in vitro kinase assays were performed as described in Materials and Methods. Autoradiography was for 2 h using Kodak XAR-5 film with a Dupont intensifying screen. Lane 1, Preimmune serum; lane 2, peptide III antiserum; lanes 3 and 4, fusion protein (Δ-v-*rel*) antisera raised in two different rabbits.

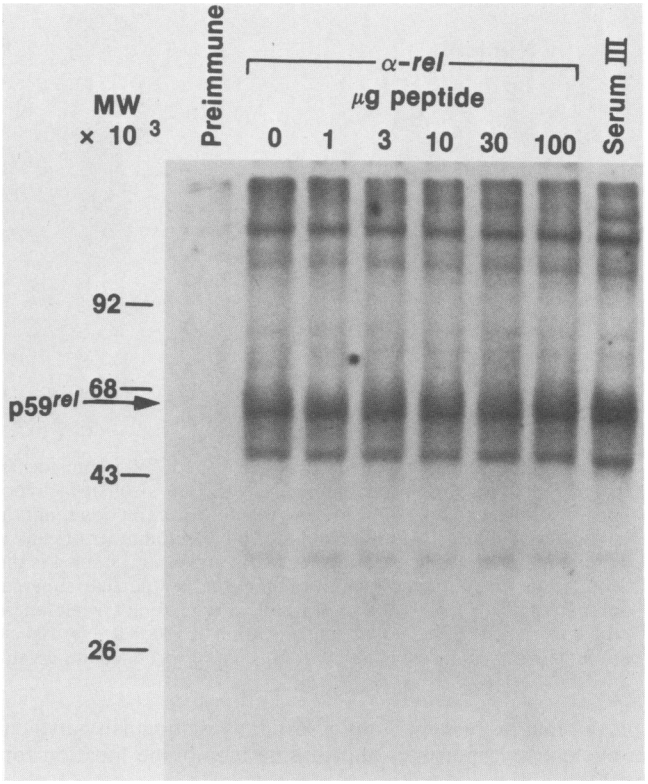

FIG. 6. Precipitation of kinase activity by fusion protein antiserum is unaffected by excess peptide III. Lysates of REV-T-transformed cells were immune precipitated with fusion protein antiserum in the presence of the indicated amount of peptide III. Immune pellets were collected and assayed for kinase activity. Lane 1, Preimmune serum; lane 2, fusion protein antiserum; lanes 3 through 7, fusion protein antisera with increasing amounts of peptide III; lane 8, peptide III antiserum.

This should be accomplished by competing with excess peptide III. We therefore incubated lysates of REV-T-transformed cells with fusion protein antiserum in the presence of increasing amounts of peptide III. The immune precipitates were collected and assayed for kinase activity (Fig. 6). There was no effect of the peptide on kinase activity, even at levels 100 times higher than required to completely block antiserum to peptide III. Precipitation of the kinase by fusion protein antiserum, therefore, is not blockable by peptide III. This suggests that the kinase is not a *rel*-unrelated protein which fortuitously cross-reacts with both peptide III antiserum and fusion protein antiserum.

If the kinase is p59^{rel} itself or a protein closely associated with it, then the kinase activity should be found in the same subcellular fraction as p59^{rel}. Transformed cells were lysed in hypotonic medium in the absence of detergent, as described above, and separated into nuclear, P100, and S100 fractions (a separate P10 fraction was not collected in this experiment). After immune precipitation, most but not all of the kinase activity was found in the S100 fraction (Fig. 7).

DISCUSSION

We have expressed in *E. coli* a 262-amino acid segment of the protein encoded by v-*rel*, the transforming gene of REV. We found that antibodies to this protein, like those raised against three independent v-*rel*-encoded peptides (28), have

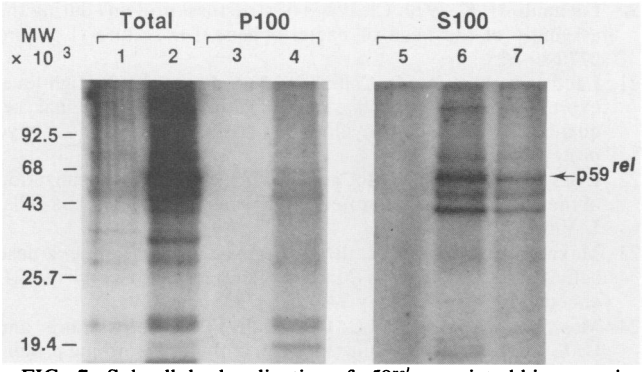

FIG. 7. Subcellular localization of p59^{rel}-associated kinase activity. REV-T-transformed cells were swollen, homogenized, and fractionated as described in Materials and Methods, except that a separate P10 fraction was not collected. After immune precipitation, in vitro kinase assays were performed. To provide a rough indication of the total amount of kinase activity to be expected in the separated fractions, an equivalent sample of unfractionated cells was analyzed after lysis in TNT buffer and clarification at 10,000 × *g* for 10 min (lanes 1 and 2). Lanes 1, 3, and 5, Preimmune serum; lanes 2, 4, and 6, peptide III antiserum; lane 7, affinity-purified peptide III antiserum.

the ability to precipitate a 59,000-dalton protein from REV-transformed avian lymphoid cells. This confirms the identification of p59^{rel} as the v-*rel*-encoded protein.

We also used antiserum to the fusion protein to localize p59^{rel} in the transformed cells. Using subcellular fractionation, we have shown that the majority of p59^{rel} is located in the cytosol of transformed avian lymphoid cells. Since the fractionation was done under hypotonic conditions in 0.25 M sucrose and in the absence of detergent, it is unlikely that p59^{rel} is strongly associated with smaller cellular organelles such as mitochondria and microsomes, or with cellular membranes. The cytosolic location was further substantiated by immunofluorescent staining experiments, which showed diffuse cytoplasmic staining. There was no apparent staining of the nucleus or any concentrated staining along the plasma membrane in REV-T-transformed cells. We cannot, of course, rule out the possibility that a small but functionally significant amount of p59^{rel} is located in one of the particulate fractions.

There are few retroviral transforming proteins whose known location is the cytoplasm (see reference 3 for review). To date, most are found associated with the plasma membrane or nuclear fractions in the transformed cell, examples being P100$^{gag\text{-}myc}$; which is located in the nucleus (1, 8), and p60^{src} (7, 19, 37) and gp65$^{erb\text{-}B}$ (14, 15, 27), which are found associated with the plasma membrane. P130$^{gag\text{-}fps}$ appears in the cytosol when cells are lysed in high salt, but its salt-sensitive association with membrane or cytoskeletal elements may be of functional significance (9, 24, 39). Of the known transforming proteins, only p37^{mos} is clearly cytosolic (26).

The similarity of p59^{rel} to p37^{mos} extends beyond subcellular location. Both are envelope fusion proteins, initiating with a few residues of the envelope polyprotein signal sequence (33, 35, 36). They thus lack the myristate moiety which may play a role in directing *gag*-fusion proteins to the plasma membrane (30). Since they also lack the very hydrophobic membrane-insertion portion of the envelope signal sequence, we would not expect them to be associated with intracellular membranes either.

Like p59^{rel}, p37^{mos} may be closely associated with a kinase activity. Immune precipitates of p37^{mos} from cells infected with strain 124 Moloney murine sarcoma virus exhibit serine-specific kinase activity (23). In addition, the product of the HT1 strain, when expressed in bacteria as a fusion protein containing 14 extra N-terminal amino acids, is able to bind the ATP analog *p*-fluorosulfonylbenzoyl adenosine and exhibits ATPase activity (31). Unlike p37^{mos}, however, p59^{rel} has little or no sequence homology with the *src* family of protein kinases. In p59^{rel} there is no Gly-X-Gly-X-X-Gly sequence typical of many nucleotide binding sites (2) and no Ala-Pro-Glu sequence such as is essential in pp60^{src} for kinase activity and transformation (4).

We are attempting to identify the kinase found in p59^{rel} immune precipitates. In a previous study we showed that immune complexes formed with one, and only one, of three peptide antisera exhibited kinase activity. In the present study we have shown that immune complexes formed with antiserum raised against a 262-amino acid segment of p59^{rel} also possess kinase activity. A trivial explanation of these results would be that both the peptide serum and the fusion protein serum fortuitously cross-react with a cellular kinase unrelated to p59^{rel}. However, a competition experiment involving fusion protein antiserum and excess peptide III failed to provide any evidence for this hypothesis. In addition, we have been unable to separate kinase activity from p59^{rel}. In the previous study, immune complexes were treated with a variety of agents (including 0.5% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, and 2 M NaCl) in an attempt to dislodge a hypothetical loosely associated kinase. None of these treatments affected kinase activity appreciably. In the present study we asked whether the kinase activity is located in the same subcellular compartment as p59^{rel} and found that the majority of it is. These results are consistent with the possibilities that p59^{rel} is itself a protein kinase or is very closely associated with one. To test this, we are raising new antisera against peptides and fusion proteins which do not contain the peptide III sequence, attempting to express the entire p59^{rel} in bacteria, and testing p59^{rel} for the ability to bind ATP. We are also testing the ability of the kinase in the immune precipitates to phosphorylate exogenous substrates.

It has been shown by Wilhelmsen et al. (36) that v-*rel* is very similar to turkey c-*rel*. Of the 262 v-*rel* residues present in the expressed v-Δ-*rel* protein, 97% are identical in turkey c-*rel*. Given the high thermal stability of hybrids formed between v-*rel* and chicken DNA (32, 38), chicken c-*rel* can also be expected to be highly related to v-*rel* and to turkey c-*rel*. Therefore, antiserum to the fusion protein should be capable of recognizing the c-*rel* protein in chicken cells if it is present. To date, immune precipitation experiments with several different chicken cell lines have afforded no clear evidence of the c-*rel* protein. We are currently using Western blot analysis as a more sensitive means of testing avian tissues and cell lines for c-*rel* expression.

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