

Chromatographic and Electrophoretic Analysis of Viral Proteins from Hamster and Chicken Cells Transformed by Rous Sarcoma Virus

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Several methods have been explored for the detection and characterization of viral proteins from soluble extracts of cells transformed by Rous sarcoma virus (RSV). Viral antigens have been analyzed after gel filtration in several solvents. In addition, immune complexes formed with virus-specific sera have been isolated by agarose gel filtration and by high- or low-speed centrifugation through sucrose solutions. Radioactive proteins from these immune complexes have been analyzed by gel filtration in 6 M guanidine hydrochloride or by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Comparison with proteins from purified virus indicates the presence of two viral core proteins (gs1 and gs2) in the soluble fraction from virus-producing chicken cells. In the same fraction from RSV-transformed hamster cells (which do not produce virus), three gs proteins (gs1, gs2, and gs3) could be identified. The soluble viral gs proteins are strongly bound to at least two larger polypeptides in cell extracts. These polypeptides do not appear to be viral in origin and have the property of undergoing a time-dependent aggregation in the extracts. One of these cell-derived proteins, which is present in a variety of uninfected cell types, closely resembles actin.

The investigation of biochemical events in the replication of oncogenic ribonucleic acid (RNA) viruses (oncornaviruses) has been complicated by the problem of distinguishing viral synthetic processes from cellular processes. Because viral RNA synthesis is sensitive to agents such as actinomycin D and because viral protein synthesis is only a small fraction of total cellular protein synthesis (which proceeds undiminished after virus infection), specific reagents are needed to identify virus-specific macromolecules within infected cells. For viral RNA, such reagents are now available in the form of deoxyribonucleic acid (DNA) copies of viral genetic material made *in vitro* by the viral reverse transcriptase (6, 14, 18). For viral proteins, reagents of comparable specificity are antibodies directed against virus-coded proteins.

Classical immunological techniques which have been used to study virus-cell interactions include fluorescent-antibody staining, complement fixation, and immunodiffusion (sometimes combined with autoradiography [22]). These techniques offer considerable sensitivity but are not as easily quantifiable as methods

employing determination of radioactivity by direct counting procedures. Among the latter methods, direct precipitation by addition of antibody to radiolabeled extracts offers the advantage that the radioactive antigens thus purified are available for further analysis.

Detection of virus-coded proteins in radiolabeled cell extracts by means of antibody precipitation has been successfully applied for viruses such as polio and adenoviruses (23, 26). The problem of background due to non-virus-specific precipitation is greater in analysis of oncornavirus-infected cells than in such cytopathic systems because of the low percentage of total protein synthesis represented by oncornavirus-directed synthesis. However, if the radioactive proteins in immune precipitates are further analyzed by techniques such as gel filtration in 6 M guanidine hydrochloride (GuHCl) or polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), the discrimination of radioactivity in viral proteins from nonspecific background can be sharpened.

In this report we present the application of such immunological and biochemical methods

to the detection of virus-specific proteins in avian and mammalian cells infected with avian oncornaviruses. Besides productively infected cells, the avian cells which have been studied include cells not overtly infected which nevertheless express group-specific (gs) antigens of these viruses as a Mendelian trait (15, 21). The mammalian cells are hamster cells transformed by Rous sarcoma virus (RSV), which likewise express gs antigens and do not produce RSV (12). In these initial findings the emphasis is on the isolation and identification of individual viral proteins and the description of factors which complicate such an analysis. Among the latter is the fact that much of the soluble viral protein in cell extracts is strongly bound to protein species which appear to be nonviral in origin; one of these species is a protein resembling actin.

MATERIALS AND METHODS

Cells and viruses. The procedures for growing chicken embryo fibroblasts and hamster cells transformed by the Bryan high-titer strain of RSV [H-RSV(BH) cells] have been described (12, 13). Viscera from individual "COFAL-negative" embryos (SPAFAS, Inc., Norwich, Conn.) were typed for gs antigens (15) before their cells were put into culture. Embryos from this flock are almost entirely of C/O or C/O' type (H. Hanafusa, *personal communication*). The RAV-1 pseudotype of BH-RSV was obtained from H. Hanafusa. Avian myeloblastosis virus (AMV) was generously supplied by J. W. Beard as plasma from viremic chicks, and avian leukosis virus, strain MC29, was grown in tissue culture (13); these viruses were purified as previously described (12, 13). Methods for labeling cell cultures with radioactive amino acids were similar to those already reported for labeling virus-infected cultures (13).

Immunological techniques. Preparations of COFAL sera from hamsters bearing tumors induced by the Schmidt-Ruppin strain of RSV and rabbit hyperimmune serum against disrupted AMV have been detailed elsewhere (12, 13), as have the procedures used for complement fixation (CF) and Ouchterlony tests. Hamster COFAL sera contain antibodies against viral gs antigens but not against type-specific determinants. The rabbit anti-AMV serum contains both gs and type-specific antibodies. In one experiment, rabbit anti-AMV serum was absorbed with an equal volume of supernatant fluid (centrifuged at $17,000 \times g$ for 5 min) from C/O' cells and then centrifuged at $10^5 \times g$ for 80 min before use. Sera from hamsters bearing tumors induced by Moloney sarcoma virus (MSV) were a gift from G. Geering and L. J. Old.

Preparation of cell extracts. This was a modification of an already published procedure (12) in that DTT was not added to the reticulocyte standard buffer (RSB) in which the cells were broken, and the extract of homogenized cells was centrifuged directly at $40,000 \times g$ for 5 min. After a second centrifugation

for $40,000 \times g$ for 5 min, ribosomes and endoplasmic reticulum were removed by an 80-min centrifugation period at 49,000 rpm in the Spinco no. 50 angle rotor. The high-speed supernatant fraction was used as a source of antigens in various studies detailed in this report. When smaller volumes were used, an adapted angle tube with a 2-ml capacity (diameter 8 mm) was used, and the time of centrifugation at 49,000 rpm was shortened to 40 min.

Preparation of immune complexes. The high-speed supernatant fraction from cells labeled for 16 hr in vivo with radioactive amino acids was mixed at 0 C with the volume of antiserum required to titrate the available antigen (usually 1/5 to 1/10 volume of serum, prespun at 49,000 rpm for 80 min). Without further incubation (see results in Fig. 7) the mixture was subjected to one of three procedures, all at 0 to 4 C.

(i) The mixture was subjected to gel filtration through Sepharose 2B or 4B (Pharmacia) equilibrated with phosphate-buffered saline (PBS) (10). The eluate was monitored for radioactivity and ability to fix complement without use of additional antiserum. Typical results are shown in Fig. 1, which compares the radioactivity and complement-binding patterns

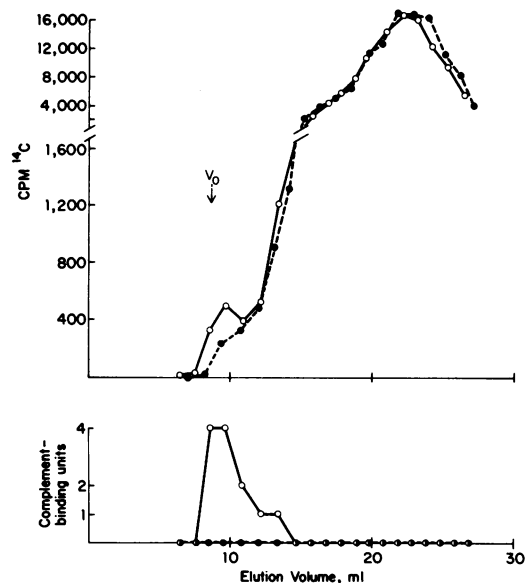


FIG. 1. Chromatography on Sepharose 2B of serum-treated soluble fractions from H-RSV(BH) cells labeled for 16 hr with ^{14}C -amino acid mixture. Top, Superimposed radioactivity profiles of eluates from extract treated with hamster COFAL serum (open circles) and normal hamster serum (closed circles). Bottom, Titers of complement-binding capacity for the same eluates, determined without addition of antiserum to the eluted fractions. Two identical columns, each of total included volume of 25 ml, were used; samples applied were 1 ml each. Similar results were obtained if sera were preabsorbed with an extract of BHK cells.

obtained with COFAL and normal hamster sera when applied to the soluble fraction from H-RSV(BH) cells. About twice as much radioactivity is obtained in the void-volume fraction with COFAL serum as with normal hamster serum, and this increment in radioactivity coincides with a definite peak of complement-binding capacity, which is absent in the experiment with the normal serum. The void volume fractions from the two eluates were pooled separately, and the immune complexes were subjected to indirect precipitation by the addition of 5 μ liters of 1% hamster immunoglobulin G (IgG) and 100 μ liters of goat anti-hamster IgG (Hyland Laboratories, Costa Mesa, Calif.). The precipitates were collected by low-speed centrifugation (2,000 rpm, 20 min), washed with isotonic NaCl, and counted. Of the radioactivity originally in the void-volume fractions, 40% was indirectly precipitated in this manner from the experiment with COFAL serum, whereas only 5% was precipitated if normal hamster serum had been used.

(ii) Immune complexes could also be separated from the great majority of soluble proteins in the cell by high-speed centrifugation through sucrose containing an intermediate zone of anti-IgG. In the bottom of a Spinco SW41 tube was placed 4 ml of 30% sucrose in RSB, followed by 0.25 ml of 22% sucrose containing a 1:1.5 dilution of anti-IgG serum in RSB; over this was layered 4 ml of 15% sucrose in RSB, followed by 4 ml of the high-speed supernatant fraction mixed with antiserum. After centrifugation at 36,000 rpm for 2 hr, the fluid was carefully removed from the tube, and the pellet was rinsed with 1/10 isotonic NaCl before analysis. In experiments with RSV-specific serum, it could be shown that 75 to 95% of the CF activity (assayed as complement-binding units) was centrifuged out of the supernatant zones and collected in the pellet (assayed after sonic treatment). If normal serum or serum from a hamster bearing an MSV-induced tumor was used, little sedimentation of CF activity occurred. In this procedure, as in procedure (i), less than 1% of the total soluble radioactive protein was recovered in the immune precipitate.

(iii) The third procedure for recovery of immune complexes from soluble cell extracts was more orthodox. The mixture of extract and antiserum was allowed to incubate at 0 C for 2 hr, and then a volume of anti-IgG serum was added, equivalent to 20 times the volume of immune serum added in the first step (in this procedure the volume of immune serum was usually less than 1/10 that of the cell extract to avoid having too bulky a precipitate). Of the slightly turbid preparation, 0.5 ml was layered on 1 ml of 5% sucrose in RSB (in a 15-ml conical centrifuge tube), and centrifugation was performed at 2,000 rpm for 20 min. The precipitate was washed twice with 1/10 isotonic saline.

Protein analysis. Soluble cell extracts were subjected to gel filtration on Sephadex G-200 (Pharmacia) (10 ml of high-speed supernatant fluid applied to a column 2.5 by 90 cm) in the cold. The column buffer was 0.1 M NaCl with 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4. Effluent fractions were concen-

trated 5- to 10-fold with Lyphogel (Gelman Instrument Co., Ann Arbor, Mich.) before assaying for CF activity. The same buffer system, with the addition of 8 M urea, was used in some experiments with G-200 (cf. Fig. 2b); in these cases the soluble fraction was dialyzed against the column buffer (with 1% β -mercaptoethanol) at room temperature before loading, and the chromatography was carried out at room temperature.

Gel filtration in 6 M GuHCl was carried out as previously described (13, 20). Immune precipitates were dissolved directly in the 8 M GuHCl solvent, while cell extracts were dialyzed into the solvent at room temperature after fourfold concentration (with no loss of CF activity) in Centriflo filter cones (permeability \leq 50,000 mol wt., Amicon Corp., Lexington, Mass.). For CF analysis of eluates, dialysis was performed as described previously (13).

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was as described in published procedures (13); immune precipitates were dissolved in the same solvent mixture used for virus.

Peptide analysis. The analysis of lysine-labeled tryptic peptides on Dowex 50 (Spherix resin XX907-10, Phoenix Precision Instrument Co., Philadelphia Pa.) was carried out as described by Cooper et al. (7). Peptides labeled with 35 S-methionine were separated by thin-layer electrophoresis according to the method of Lazarowitz et al. (17).

Radiolabeling procedures. 14 C- and 3 H-labeled amino acid mixtures (reconstituted algal hydrolysates) were obtained from New England Nuclear Corp., as were 3 H-L-lysine (2.8 mCi/ μ mole), 3 H-L-arginine (3.5 mCi/ μ mole), 3 H-L-leucine (36 mCi/ μ mole), 14 C-L-phenylalanine (450 μ Ci/ μ mole), and 14 C-L-valine (250 μ Ci/ μ mole). 35 S-L-methionine (20 mCi/ μ mole) was obtained from Amersham-Searle. Levels of radioactivity used for labeling cultures were 2 to 5 μ Ci/ml for 14 C-amino acids and 20 to 50 μ Ci/ml for 3 H-amino acids; labeling periods were 16 hr. Radioactivity determinations were made in a scintillation counter using toluene-Triton X-100 (2:1) scintillation mixture (Packard Instruments).

RESULTS

Gel filtration of viral antigens from RSV-transformed hamster cells. In a previous report (12) it was demonstrated that, in cytoplasmic extracts of RSV-transformed hamster cells, viral gs antigens are both soluble and membrane-bound, with the same antigenic specificities in both fractions. By sucrose gradient centrifugation, the soluble antigens (monitored by CF activity) were found to have sedimentation coefficients of up to 12s, and very little activity was found in the size range of gs protein monomers (E. Fleissner, unpublished data). It was therefore of interest to examine more closely the physical characteristics of gs antigens in the soluble fraction, especially if this fraction were to be used as a source of radiolabeled antigens for analysis by direct precipitation with antisera.

When soluble gs antigens from H-RSV(BH) cells were chromatographed on a column of Sephadex G-200, the CF pattern was complex, but all of the antigenic activity appeared in elution positions equivalent to molecular weights from 100,000 to more than 400,000 (Fig. 2a). The same antigenic specificities were found in the earlier and later eluting fractions by Ouchterlony analysis. It was considered possible that the CF pattern was due to association of viral proteins with small fragments of membrane or other lipid-containing structures or to some relatively weak protein-protein interactions. However, the CF pattern on G-200 was substantially unaltered by the following treatments: (i) exposure of the soluble antigens to 0.7% deoxycholate \pm 0.7% Tween 40 and including these detergents in the elution buffer, (ii) use of 0.6 M KCl in the elution buffer (a procedure known to dissociate some viral antigens from membranes in these cells [*unpublished data*]), and (iii) adjustment of the antigen preparation to pH 11 in 0.6 M salt and elution with either glycine buffer at pH 10 or with buffer at pH 7.4. Therefore, stronger dissociating conditions were employed.

In Fig. 2b is shown the result of chromatography of the soluble cell-derived gs antigens on G-200 in the presence of 8 M urea and a buffer of moderate ionic strength. All of the CF activity is recovered after dialysis in the region of molecular weight 90,000, still well in excess of the molecular weights expected for viral gs protein monomers (10,000 to 27,000). In Fig. 2c is shown the recovery of gs antigens after gel filtration in the presence of 6 M GuHCl and a reducing agent, a procedure which has proved effective for separation and recovery of gs antigens from RSV (13). With this treatment, for the first time, there is detectable gs antigen at the elution positions expected of separated viral polypeptides gs1, gs2, and gs3, but the bulk of the CF activity still elutes in positions corresponding to molecular weights of 50,000 to 100,000. The CF elution pattern of virus is shown for comparison (Fig. 2d).

The demonstration of CF activity with sera from hamsters bearing tumors induced by RSV does not establish a relationship to viral structural proteins, since the hamster sera may be reacting against some other virus-induced proteins in the tumors. However, when a rabbit serum, prepared against Tween-ether-disrupted AMV, was used to assay the renatured fractions in an experiment like that in Fig. 2c, a similar CF pattern was obtained. Since the hyperimmune rabbit serum was prepared against AMV rather than RSV-0 [the resident

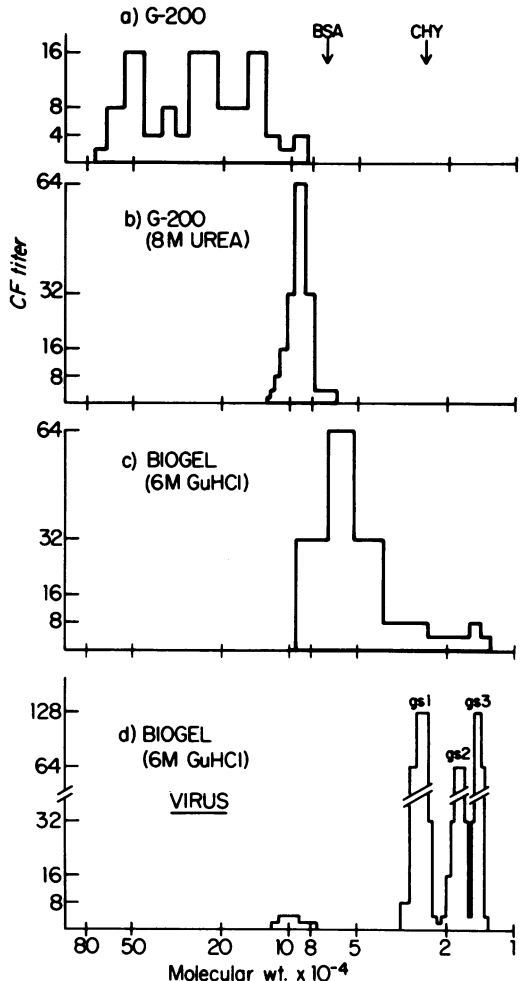


FIG. 2. Gel filtration of the soluble fraction from H-RSV(BH) cells in three solvents. Column conditions: (a) G-200 in 0.1 M NaCl with 0.1 M Tris-hydrochloride, pH 7.4; (b) G-200 in the same buffer containing 8 M urea; and (c) BioGel A-5m in buffer containing 6 M GuHCl and a reducing agent (see Materials and Methods). In (d) is shown the pattern for virus (AMV) under the same conditions as were used for the cell material in (c). For (b), (c), and (d), CF activity was titered after dialysis; hamster CO-FAL serum was used in all CF tests. Columns were calibrated with chymotrypsinogen (molecular weight 25,700) and bovine serum albumin (molecular weight 69,000) with an extrapolation for higher molecular weights according to published data (3). Due to the logarithmic relationship of elution volume to molecular weight in all these systems, the abscissa used here closely approximates actual elution volumes on a linear scale.

genome of H-RSV(BH) cells], the CF activity in the higher-molecular-weight range presumably represents antigens with gs specificity. In confirmation of this interpretation, a combina-

tion of proteins gs1, gs2, and gs3 (isolated by gel filtration of AMV proteins [13]) was capable of absorbing out the antibodies in hamster COFAL serum which were reactive with the higher-molecular-weight antigens from H-RSV(BH) cells (cf. Fig. 2c).

The interaction of RSV with hamster cells is defective in that no virus is produced although the cells are transformed (12, 25). It could therefore be asked whether the presence of higher-molecular-weight antigens resistant to 6 M GuHCl is characteristic only of such a defective interaction, rather than typical of RSV infection in general. This is not the case, for when RSV-infected chicken tumor cells, which are producing virus in large amounts, are analyzed by the method of Fig. 2c, they are found to contain species of higher-molecular-weight antigens similar to those from nonproducing hamster cells. The remainder of this report is concerned with the further characterization of these anomalously large gs antigens in serological and biochemical terms.

Gel filtration in 6 M GuHCl of radioactive immunoprecipitates from RSV-transformed hamster cells. An alternative method of analyzing viral proteins in cell extracts is to precipitate radiolabeled proteins with specific antisera. The immune precipitates can then be dissociated, and the radioactive proteins can be characterized. The extracts used were high-speed supernatant fluids from tissue culture cells grown in the presence of radioactive amino acids. Antigen-antibody complexes were recovered by three methods: (i) chromatography on Sepharose gels, (ii) high-speed centrifugation through sucrose, or (iii) low-speed centrifugation through sucrose (see Materials and Methods). The complexes were then subjected to gel filtration in the presence of 6 M GuHCl. The use of gel filtration in 6 M GuHCl in the dissection of immune complexes is helpful, for it permits a differential analysis of CF activity due to gs antigens in the immune precipitate as well as radioactivity representing total cell-derived protein.

In Fig. 3 is presented such a differential serological and radiochemical analysis of immune complexes obtained from H-RSV(BH) cells using hamster COFAL serum. The profile of radioactive proteins is compared with that of coeluted viral proteins (top), and at the bottom is shown the characteristic profile of CF activity recovered from the immune precipitate after dialysis of individual fractions in the eluate. It is apparent that the bulk of the CF activity from the immune complex is not coincident with the major peaks of radioactive

proteins from the complex. The CF activity is in the molecular-weight range of 50,000 to 90,000, the same range in which the major CF activity was found in gel filtration of the whole soluble fraction from H-RSV(BH) cells (Fig. 2c). The radioactive peaks, by contrast, are at the void volume (equivalent molecular weight $\geq 100,000$) and at $V_e/V_o = 1.3$ to 1.4 (molecular weight about 40,000). It is striking that there is no CF activity recoverable in the eluate at the position corresponding to the radioactive species of molecular weight 40,000 (c). The possibility must not be overlooked that this species might not renature well after exposure to 6 M GuHCl and β -mercaptoethanol. However the evidence presented below (Fig. 6-9) indicates that the presence of this species in immune precipitates is not due to a direct interaction with virus-specific antibodies in the sera employed.

In the gel filtration patterns of proteins from H-RSV(BH) cells shown in Fig. 3 there are no distinct peaks at the positions of viral gs protein monomers. However, as shown in Fig. 4a, if the immune precipitate is exposed to SDS before being subjected to gel filtration in 6 M GuHCl, protein species of the sizes of gs1, gs2, and gs3 can be detected, as well as two major radioactive peaks similar to those depicted in Fig. 3. When the immune precipitate, together with marker virus, is analyzed by PAGE in the presence of SDS (Fig. 4b), gs1 and gs3 monomers are discernible, as well as three larger species designated *a*, *b*, and *c* with molecular weights of approximately 90,000, 65,000, and 40,000, respectively. Of the latter, species *c* is the most prominent. (There is also some material with a mobility greater than gs3 derived from both cells and virus. This material does not represent gs4 and p5 [13] and has occasionally been seen by other workers in PAGE profiles of viral proteins [24].) Much less labeled material is recovered near the top of the gel in Fig. 4b than in the equivalent molecular weight range of the elution pattern in Fig. 4a (at or near the void volume). This shows that much of the early eluting material in the gel filtration pattern represents aggregates of smaller components, such as component *c* and gs proteins.

To summarize briefly the results obtained with proteins from RSV-transformed hamster cells, it appears that at least some of the antigenically active species which are larger than gs monomers may represent aggregates of gs proteins with antigenically inactive species (e.g., component *c* in Fig. 4). Such aggregates appear to be stable under some conditions in 6

M GuHCl but tend to dissociate in the presence of SDS.

Gel filtration and PAGE analysis of radioactive immunoprecipitates from RSV-transformed chicken cells. To extend the analysis of viral proteins in RSV-infected cells, chicken cells which were producing virus were examined. When radioactive proteins, precipitated with hamster COFAL serum from extracts of MC29-infected chicken cells, were analyzed by gel filtration in 6 M GuHCl and PAGE in the presence of SDS, the results shown in Fig. 5 were obtained. Proteins from purified virus were chromatographed under the same conditions (Fig. 5a) or subjected to co-electrophoresis (Fig. 5b) to establish mobilities

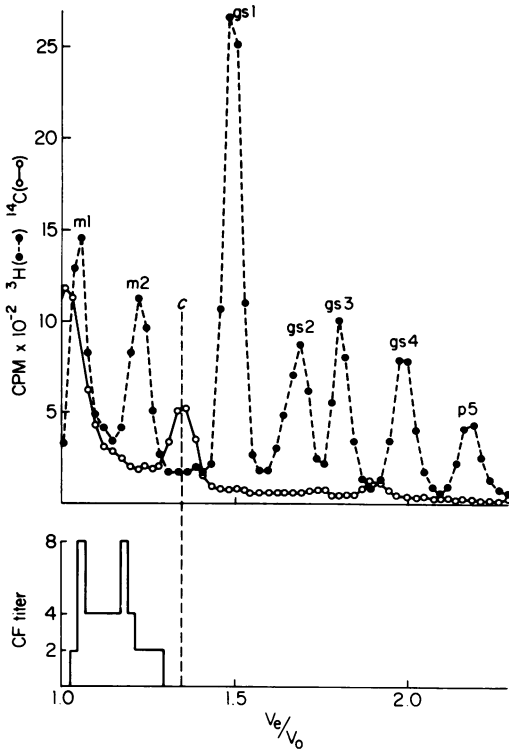


FIG. 3. Gel filtration in 6 M GuHCl of proteins in immune precipitates prepared from radiolabeled H-RSV(BH) cells (method ii [see Materials and Methods], with hamster COFAL serum). Comparison of results from radioactivity determination (top) and recovery of CF activity (bottom). Open circles represent ^{14}C -amino acid-labeled proteins from the immune complex; closed circles show the pattern for ^3H -amino acid-labeled proteins from MC29 virus which were co-run. Marker virus was not included for the run in which CF activity was recovered. Hamster COFAL serum was used to titer CF activity after dialysis of individual fractions in the eluate.

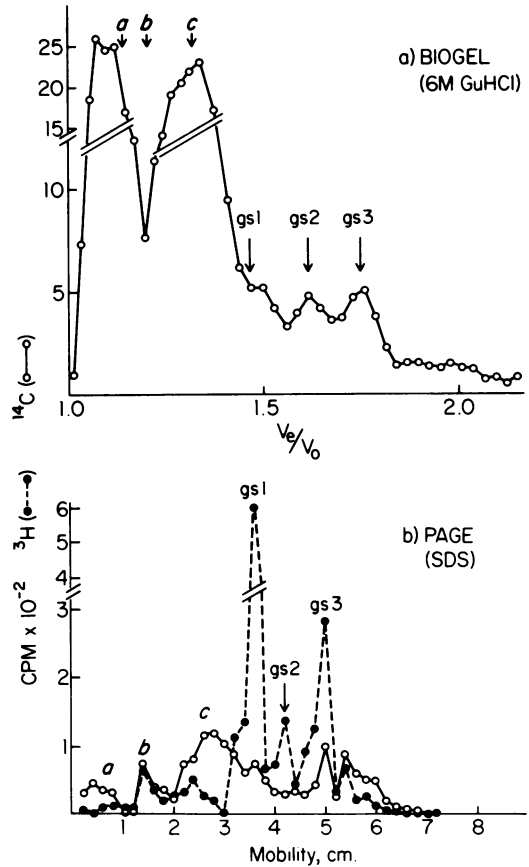


FIG. 4. Proteins in immune precipitates prepared from H-RSV(BH) cells with hamster COFAL serum. Comparison of results by (a) gel filtration in 6 M GuHCl and (b) PAGE in the presence of SDS. Open circles represent ^{14}C -amino acid-labeled proteins from immune complexes (Method i for a, ii for b [see Materials and Methods]); closed circles show the pattern for ^3H -amino acid-labeled proteins from MC29 virus which were subjected to co-electrophoresis in (b). In (a) the precipitate was taken up in a small volume of SDS-containing PAGE buffer (13) before exposure to GuHCl; this is in contrast to the procedure in Fig. 3 (top), where the precipitate was dissolved directly in the GuHCl solvent. The profiles from gel filtration and PAGE as plotted here have abscissas with approximately equivalent positions for the same molecular weights, as indicated by the arrows showing expected positions of proteins a to c and gs1 to gs3 in gel filtration.

of viral proteins gs1, gs2, and gs3. The gel filtration pattern in Fig. 5a has major species near the void volume and at $V_e/V_0 = 1.3$ to 1.4, and a distinct peak at the position of gs2. The species of lowest molecular weight in both Fig. 5a and b does not coincide precisely with gs3 or any other major viral protein, making the iden-

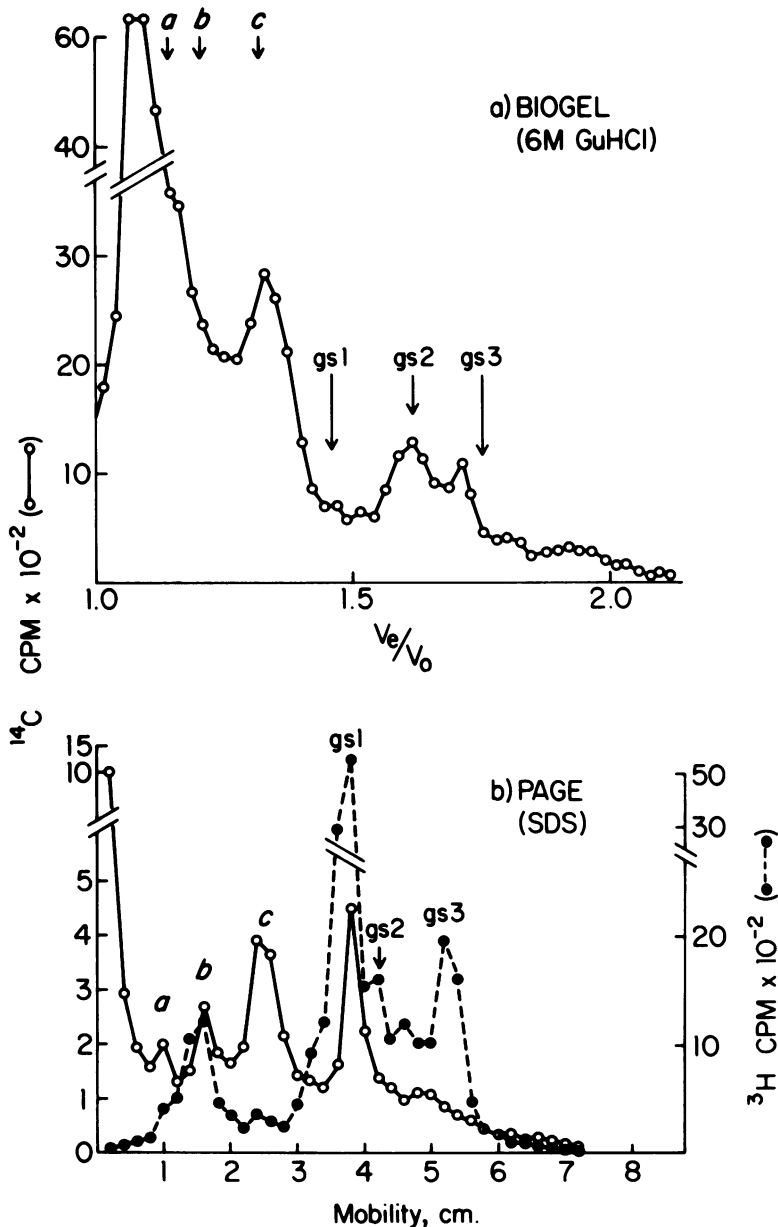


FIG. 5. Proteins in immune precipitates prepared from MC29-infected chicken cells (method ii [see Materials and Methods], with hamster COFAL serum). Comparison of results by (a) gel filtration in 6 M GuHCl and (b) PAGE in the presence of SDS. Open circles represent ^{14}C -amino acid-labeled proteins from immune complexes; closed circles show the pattern for ^3H -amino acid-labeled proteins from MC29 virus which were subjected to co-electrophoresis in (b). As in Fig. 4, the profiles from gel filtration and PAGE as plotted here have abscissas with approximately equivalent positions for the same molecular weights, as indicated by the arrows showing expected positions of proteins a to c and gs1 to gs3 in gel filtration.

tification of gs3 in these chicken cell extracts uncertain.

The ^{14}C -labeled, cell-derived proteins in Fig. 5b display five peaks of radioactive proteins which have entered the gel. The best defined of

these coincides with the major viral structural protein (gs1). A comparison with Fig. 5a confirms that the protein having the mobility of gs1 in PAGE elutes in an aggregate of higher molecular weight in gel filtration. Like the hamster

cell profile shown in Fig. 4b, the PAGE profile from infected chick cells also contains protein species of higher molecular weight (*a*, *b*, and *c* in Fig. 5b). The molecular weights of these three species again are approximately 90,000, 65,000, and 40,000 for *a*, *b*, and *c*, respectively. As before, the species of molecular weights 65,000 and 40,000 (*b* and *c*) are most reproducibly obtained, and *c* has the same molecular weight by PAGE as the molecular weight in gel filtration by PAGE as the molecular weight in gel filtration of material eluting at $V_e/V_o = 1.3$ to 1.4.

That the presence of large protein species such as *a*, *b*, and *c* in immune precipitates is not due to the presence in the serum of antibodies specific for these proteins is shown by the experiment in Fig. 6. Here an immune precipitate was prepared from MC29-infected chicken cells by using rabbit anti-AMV serum which had been absorbed with an extract of C/O' chicken cells. (As shown below [Fig. 7] C/O' cells lack *gs* proteins but contain species *a*, *b*, and *c*.) In gel filtration (Fig. 6a) a pattern similar to that in Fig. 5a was obtained, i.e., having *gs2* but lacking *gs1* or a *gs3* peak; in PAGE (Fig. 6b), both *gs1* and *gs2*, but not *gs3*, were resolved. The point to be emphasized is that, despite the use of preabsorbed serum, the usual species of proteins with higher molecular weights are well represented in both Fig. 6a and b. (In Fig. 6a the peak expected for protein *c* has eluted somewhat early, perhaps due to a high proportion being complexed with *gs* proteins.)

Comparisons of proteins in C/O and C/O' chicken cells. Because of the complexity of the PAGE profiles obtained from radioactive immunoprecipitates, the relationship of these proteins to virus was investigated by a genetic approach. The expression of oncornavirus *gs* proteins in non-virus-producing chicken embryo cells is a Mendelian character (15, 21). In the Hanafusas' work, the particular cell type expressing *gs* antigens is designated C/O; the cells not expressing *gs* antigens are called C/O' cells. When radioactive immunoprecipitates are prepared from C/O and C/O' cells by using hamster COFAL serum or a control serum from hamsters bearing tumors induced by MSV, protein profiles in PAGE such as those shown in Fig. 7 are obtained. The pattern from C/O cells is similar to that from MC29-infected chicken cells, and the pattern has little dependence on the time of incubation with serum (Fig. 7a and b). By contrast, even if more concentrated extracts from C/O cells are incubated with MSV-hamster serum, or if C/O' cell extracts are incubated with

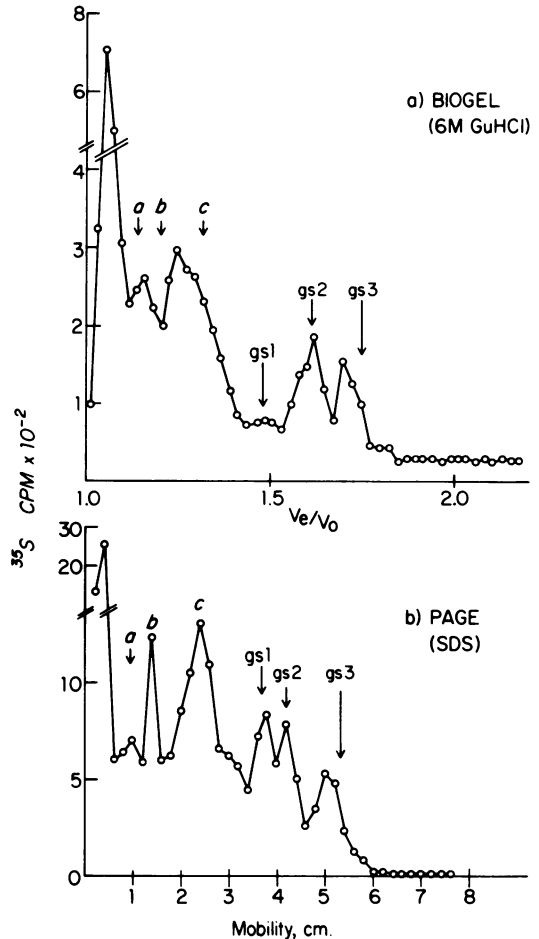


FIG. 6. The same preparation of ³⁵S-methionine-labeled immune complexes from MC29-infected chicken cells analyzed by (a) gel filtration and (b) PAGE. Immune complexes were collected by method ii (see Materials and Methods) with rabbit antiserum prepared against disrupted AMV and preabsorbed with an extract of unlabeled C/O' cells. Molecular weights corresponding to equivalent positions on the abscissas for (a) and (b) are approximately the same, as shown by the relative positions of proteins *a* to *c* and *gs1* to *gs3*.

COFAL serum, essentially identical profiles are obtained (Fig. 7c and d) which lack any peak in the position of *gs1*, but retain higher-molecular-weight species *a*, *b*, and *c*. It is clear, therefore, that a gene locus controlling the expression of *gs* antigen detectable by CF activity also controls the appearance of a protein in cell extracts with the mobility of *gs1* by PAGE. This is consistent with published work identifying *gs1* in leukosis-free cells (1) and with the finding that both *gs1* and *gs2* are pres-

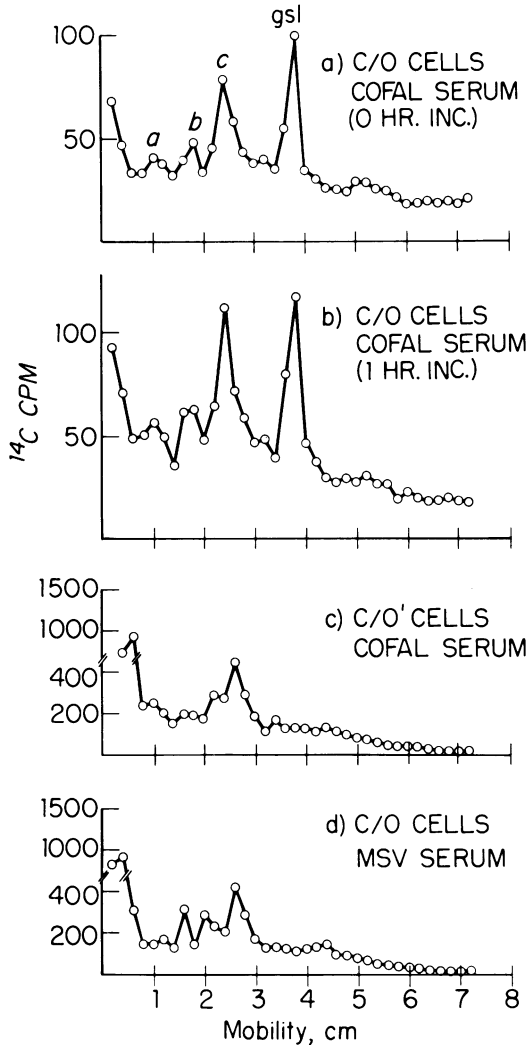


FIG. 7. PAGE of radioactive proteins precipitated from extracts of C/O or C/O' chicken cells using hamster COFAL serum or serum from hamsters bearing tumors induced by MSV. Immune complexes were collected by method ii (see Materials and Methods), except that in (b) a 1-hr incubation period with serum at 0 C was performed before centrifugation through sucrose. In (c) and (d) the volumes of packed cells which were extracted were three times those that were used in (a) and (b); labeling conditions with ^{14}C -amino acids were similar throughout.

ent in C/O but not in C/O' cells by a radioimmunoassay procedure utilizing sera monospecific for gs1 and gs2 (E. Fleissner, unpublished data). In quantitative comparisons of radioactivity associated with the gs1 peak in PAGE, under similar labeling conditions, C/O cells have about 25% the amount of (soluble) gs1 which is found in MC29-infected chick cells.

That gs proteins are directly associated with proteins a, b, and c is also shown by PAGE analysis of immunoprecipitates collected by low-speed centrifugation. In Fig. 8 is shown an experiment in which an extract of C/O cells was exposed to either COFAL or MSV-hamster serum, followed by anti-hamster IgG, before low-speed centrifugation through sucrose and analysis by PAGE. Not only is there a well-defined gs1 peak (with a shoulder at the gs2 position) in the presence of COFAL serum, there is correspondingly more of proteins a, b, and c precipitated along with gs1 and gs2 in the presence of COFAL serum than in the presence of MSV-hamster serum. The inference that gs1 and gs2 are physically associated with the larger proteins in the cell extract is difficult to avoid.

Spontaneous aggregation of components a, b, and c and similarity of c to actin. The question arises as to why substantial amounts of components a, b, and c are present in pellets obtained from cell extracts lacking CF activity (Fig. 7c) or treated with control sera (Figs. 7d and 8). The explanation is that these protein species are capable of self-aggregation. In fact, if radiolabeled extracts of a variety of cell types are prepared by high-speed centrifugation (sufficient to pellet ribosomes) and allowed to age at 0 C for 4 to 5 hr, centrifugation through sucrose by the high-speed procedure described in Materials and Methods or a simple repeti-

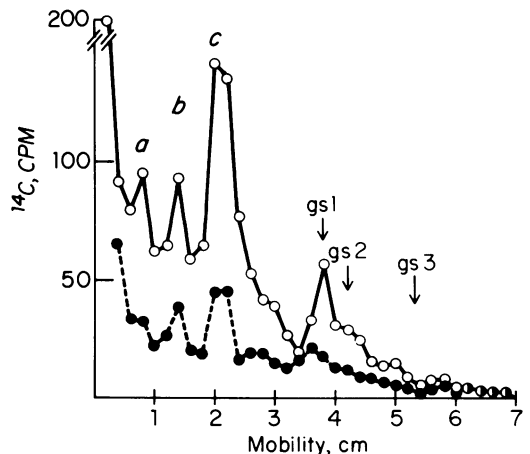


FIG. 8. PAGE of radioactive proteins precipitated from an extract of ^{14}C -amino acid-labeled C/O chicken cells. Method iii (see Materials and Methods) was used, with either hamster COFAL serum (open circles) or serum from hamsters bearing MSV-induced tumors (closed circles). The results are plotted as the superposition of the radioactivity profiles from two gels run in parallel, under the same conditions used for the experiment in Fig. 7.

tion of the original high-speed centrifugation, will produce a fresh pellet. This pellet, on analysis by PAGE, contains largely protein *c*, with traces of *a* and *b*. The result of such an experiment for hamster (BHK) cells not infected by RSV is shown in Fig. 9. Cell types from which such a spontaneous protein aggregate have been obtained include, besides BHK cells, a bovine cell line as well as RSV-transformed chicken and hamster cells. For the latter, the spontaneous precipitates carry with them *gs* antigens; the aggregation of *gs* antigens proceeds as a function of time, with about 50% of the CF activity becoming sedimentable after 4 hr at 0 C. The patterns of CF activity obtained by gel filtration in 6 M GuHCl of such spontaneous precipitates from H-RSV(BH) cells, RSV-infected chicken cells, or C/O cells resembles that shown for cell-derived material in Fig. 2 and 3. Again, in PAGE, the dominant protein species is component *c*.

This laboratory has carried out analyses of tryptic peptides from protein *c* isolated from extracts of hamster or chicken cells after labeling *in vivo* with ^{35}S -methionine or ^3H -lysine. The peptides have been analyzed by thin-layer electrophoresis and autoradiography (for ^{35}S -methionine) or chromatography on Dowex-50 (for lysine-labeled peptides). Peptides derived from purified viral proteins have been subjected to co-electrophoresis and co-chromatography in all cases. The results show that the major lysine and methionine peptides of component *c* are not the same as peptides from viral proteins *gs1*, *gs2*, *gs3*, and *gs4*. Components *a* and *b* could not be obtained in pure enough form by gel filtration for an analysis of tryptic peptides. However the molecular weight of component *a* suggests that it may be a dimer of *c*.

In recent reports a protein resembling actin has been found to be a major constituent of cultured cells from several species (2, 11, 28). Evidence that component *c* is also actin-like is provided by double-label experiments in MC29-infected chicken cells employing pairs of ^{14}C and ^3H -amino acids. The ratios of isotopic incorporation into *b* and *c* as well as virus-derived *gs1* were determined by PAGE and normalized to published data for *gs1*. The amino acid ratios for *c* resemble those for chicken actin within the accuracy of the method (Table 1); this is consistent with the molecular weight (40,000 to 45,000) reported for actin (2).

In summary, the analysis of viral proteins in extracts of RSV-transformed cells is complicated by the association of *gs* proteins with at least two species of cell proteins which have the

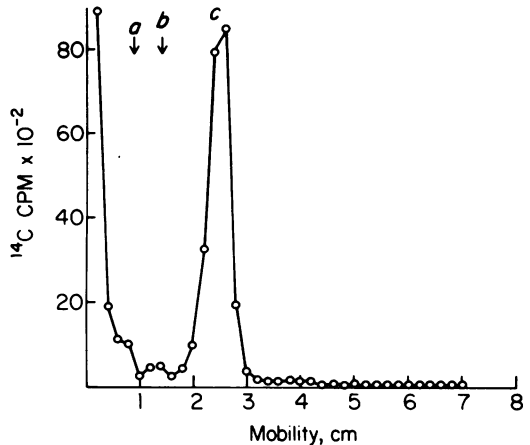


FIG. 9. PAGE of radioactive proteins spontaneously aggregated from an extract of ^{14}C -amino acid-labeled BHK cells. High-speed supernatant was allowed to stand at 0 C for 3 hr without serum addition before centrifuging as in method ii (see Materials and Methods). The resulting pellet was dissolved in the usual SDS solvent for analysis by PAGE.

TABLE 1. Amino acid ratios in proteins *b* and *c*

$^3\text{H}/^{14}\text{C}$ amino acid pair ^a	Protein species			
	<i>b</i>	<i>c</i>	Actin ^b	<i>gs1</i>
Lys/Val	1.4	1.2	1.1	0.65
Arg/Phe	1.9	1.3	1.5	4.0
Leu/Phe	2.1	2.2	2.2	6.4

^aRatios were determined by polyacrylamide gel electrophoresis of immune precipitates prepared from MC29-infected chicken cells labeled with the indicated pairs of ^3H - and ^{14}C -amino acids. The ratios of ^3H and ^{14}C in protein species *b* and *c* were converted to ratios of mole-percents for each amino acid pair by comparison with the $^3\text{H}/^{14}\text{C}$ ratios of *gs1* from virus produced in the same cells, utilizing published data for the amino acid composition of *gs1* (19).

^bThe amino acid ratios are those of chicken actin (5).

property of undergoing a rapid, spontaneous aggregation in cell extracts. This aggregation occurs with or without the presence of *gs* proteins; when these are present, they may become precipitable even in the absence of serum addition. One of the proteins with the property of self-aggregation appears to be actin-like.

DISCUSSION

Mammalian cells infected by RSV can undergo neoplastic transformation without concomitant virus production. This laboratory has a continuing interest in the identification of

viral gene products in such cells and the elucidation of how their synthesis is controlled. As a first approach, methods have been developed for the detection of viral structural proteins in cell extracts. Preliminary experiments had revealed that, when classical procedures such as ammonium sulfate fractionation and ion exchange chromatography were applied to cell extracts, the yields of viral proteins, as monitored by CF activity, decreased sharply as purification progressed. Gel filtration analysis of viral proteins in the high-speed supernatant fraction from cells showed a preponderance of aggregated structures (Fig. 2). The use of strongly dissociating solvents was therefore indicated. Two methods were selected, employing solvents which have been successfully applied to the resolution of proteins from preparations of purified oncornaviruses (13, 20). These are gel filtration in 6 M GuHCl and PAGE in the presence of SDS. The gel filtration method has the advantage that antigenic activity can be recovered in high yield by removal of GuHCl through dialysis.

The profiles of CF activity recovered after gel filtration in 6 M GuHCl of both the high-speed supernatant fraction and immune precipitates derived from this fraction of H-RSV (BH) cells were monitored with serum from hamsters bearing Rous sarcomas. There was found a high proportion of antigens with gs specificity but of a size considerably larger than gs proteins from virus (Fig. 2 and 3). This is not a unique feature of RSV-transformed cells not producing virus, since the same anomalously large antigens can be demonstrated in RSV-producing chicken cells.

There are at least five possible explanations for the existence of viral antigens stable in 6 M GuHCl and displaying higher molecular weights than those of the major viral structural proteins. (i) The CF activity in the higher-molecular-weight range may be a serological phenomenon (e.g., hamster alloantigens or chicken heteroantigens) unrelated to virus infection at all. (ii) The antigens may represent large virus-coded proteins which are unrelated to the major proteins incorporated into virus. (iii) They may represent precursor proteins of large size which must be cleaved before being included in virus but which have some antigenic sites already identical to those of viral structural proteins. (iv) The large antigens may be very stable aggregates of structural protein monomers. (v) They may be aggregates of structural protein monomers with some other virus- or cell-specified protein(s) for which the monomers have a very high affinity.

Explanations (i) and (ii) are ruled out by the finding that the large antigens from H-RSV(BH) cells react with antiserum prepared against virus from chicken cells, as well as by the fact that antibodies to the large antigens can be absorbed by purified gs proteins from virus. Explanation (iii) is not compatible with findings that gel filtration of cell extracts in 8 M urea yields only large antigens (Fig. 2b), whereas the same extracts in 6 M GuHCl (Fig. 2c) yield some gs monomers (as does the material recovered from 8 M urea if it is rechromatographed in 6 M GuHCl [E. Fleissner, unpublished data]). In addition, gel filtration of immune precipitates exhibits smaller amounts of gs monomers than does analysis by PAGE (Fig. 4, 5, and 6); this argues against a covalent association of gs protein in the larger antigenic structures.

Since the viral membrane glycoprotein m1 does not elute as a monomer in 6 M GuHCl (13) and viral structural proteins are notoriously apt to aggregate in solution, strong consideration was given to (iv) as an explanation for the elution profiles of CF activity, as well as the recovery of higher-molecular-weight species from immune precipitates. Numerous attempts have been made to demonstrate aggregates of purified viral gs proteins which would remain stable in strong protein solvents, particularly under the conditions used for PAGE. These have included dialyses of mixtures of gs proteins into and out of various denaturing solvents at high concentrations. Neither the native nor the denatured viral proteins have shown a tendency to form very stable aggregates on their own (E. Fleissner, unpublished data). (These proteins will precipitate spontaneously from physiological buffers, but the precipitates can easily be dissociated in 6 M GuHCl or SDS-containing buffers.)

The explanation which fits the data best is (v), specifically, that the large antigens in question represent aggregates of viral structural proteins with cell-specified proteins, notably a protein (c) of about 40,000 molecular weight resembling actin. This protein is recovered as a principal component of immune precipitates analyzed by either gel filtration or PAGE (Fig. 4, 5, and 6). It does not have CF activity (Fig. 3), nor does it resemble viral structural proteins in amino acid ratios (Table 1) or in peptide composition. It is present in C/O' chicken cells, which lack gs antigens (Fig. 7). The association of this protein with viral gs proteins in cell extracts is demonstrated by the fact that its presence in immune precipitates is gs antibody-dependent under controlled conditions (Fig. 8).

Like actin, this protein is capable of self-aggregation under conditions of moderate ionic strength (Fig. 9). When such self-aggregation occurs in extracts from cells containing gs proteins, the aggregates recovered by centrifugation are rich in gs antigens which retain their primary association to nonvirion protein even in 6 M GuHCl. This association (in either spontaneous or immune precipitates) is largely if not completely disrupted, however, by PAGE analysis in SDS-containing buffers (Fig. 4, 5, and 6). Thus, when antiserum is used to precipitate viral proteins from cell extracts, the analysis of the proteins in the precipitates is complicated by two factors: (i) the presence of nonvirion proteins, especially protein *c*, in the precipitates due to their association with the antigens, and (ii) the spontaneous aggregation of these nonvirion proteins, with and without associated antigens, which inflates the recoveries of labeled proteins even when control sera are used (Fig. 7 and 8).

The gs proteins detected by gel filtration and PAGE are, for H-RSV (BH) cells, gs1, gs2, and gs3 (Fig. 4), and for virus-infected chicken cells, gs1 and gs2 (Fig. 5 and 6). Although gs3 is of course produced in the infected chicken cells, this protein could not be unambiguously identified in the immune precipitates from these cells; it thus appears to be in lower amount or more firmly associated with membranes than in H-RSV(BH). Direct demonstration of individual antigens in higher-molecular-weight fractions from gel filtration by Ouchterlony analysis was complicated by the expected strong aggregation of protein complexes after dialysis and further concentration of the antigenic material. Only gs3 from H-RSV(BH) cells could be demonstrated by this method in the elution region of molecular weights 80,000 to 100,000. The corresponding radioactive material from this elution position yielded a considerable amount of gs3 monomer in PAGE, again only using immune precipitates from H-RSV(BH) cells and not when precipitates from infected chicken cells were used. Material with the mobilities of gs1 and gs2 could also be recovered by PAGE analysis of high-molecular-weight fractions from gel filtration, confirming evidence for gs1 and gs2 specificities in these fractions by absorption of monospecific antisera in radioimmunoassays (*unpublished data*).

No unequivocal evidence could be found for the presence of gs4 in the soluble fraction from either H-RSV(BH) cells or infected chicken cells, but in chicken cells this protein might be expected to be one of the first associated with nascent viral cores (9, 13). The gs4 protein is

clearly present under some circumstances in RSV-transformed hamster cells, since sera from hamsters bearing RSV-induced tumors contain antibodies against gs4. A search for viral glycoproteins and DNA-polymerase in H-RSV(BH) cells will require more sensitive techniques with monospecific antisera. Preliminary tests with the related Schmidt-Ruppin transformant, H-RSV(SR) cells (12), utilizing monospecific sera against viral proteins m1, m2, and viral DNA-polymerase and indirect fluorescent-antibody staining, revealed staining too weak for positive interpretation (R. Nowinski, *personal communication*).

The identification of protein *c* as actin is supported by its molecular weight (ca. 40,000), its composition in terms of certain amino acid ratios (Table 1), and its capacity for self-aggregation. In addition, autoradiographs of ³⁵S-methionine-labeled tryptic peptides from *c* subjected to electrophoresis at pH 3.5 resemble those of an actin-like protein from a monkey cell line (reference 2; E. Fleissner and C. W. Anderson, *unpublished data*). The significance of the association between viral gs proteins and the actin-like, host-cell protein *c* is at present unknown, as is the nature of the other host proteins *a* and *b* which appear in immune precipitates of the gs proteins. Complex formation between a basic (13, 16) viral protein such as gs1 and a highly acidic protein like actin is not surprising; what is surprising is the stability of the complex in conditions of high ionic strength (6 M GuHCl). This implies a strong hydrophobic contribution to the binding. In vivo association has been reported between reovirus and microtubule protein, which is closely related to actin in amino acid composition (8, 27). The evidence presented here for an interaction between structural proteins of an oncogenic virus and a protein which is implicated in cell movement (11), while suggestive, obviously indicates the need for further study of a possible in vivo role for this phenomenon.

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ADDENDUM IN PROOF

Protein components, apparently of host origin and resembling components *a*, *b*, and *c* by their electrophoretic mobilities relative to viral gs proteins, have been found in "rapid harvest" oncornavirus preparations by Shanmugam et al. (J. Virol., 1971, 10:447-455) and by Cheung et al. (Virology, 1971, 50:851-864). Whether these results reflect the high affinities between such components and viral gs proteins is at present unclear.

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