Biochemical and Immunological Characterization of the Major Envelope Glycoprotein gp69/71 and Degradation Fragments from Rauscher Leukemia Virus

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Analysis of the proteins of Rauscher murine oncornavirus by immunoprecipitation showed that antiserum to the purified envelope glycoprotein of approximately 69,000 and 71,000 daltons (gp69/71) reacted as well with a number of other components of several murine oncornaviruses of approximately 45,000, 32,000, and 15,000 daltons. Polypeptides of similar size were also produced by limited proteolysis of purified gp69/71; these degradation fragments were shown to contain carbohydrate by the incorporation of ^3H from sodium boro[^3H]hydride after neuraminidase and galactose oxidase treatment. Each of these glycoproteins was isolated by preparative polyacrylamide gel electrophoresis and was analyzed by tryptic peptide mapping. The major virion components of 69,000 and 71,000 daltons were nearly identical, as were the primary degradation fragments. Analysis of the immunological properties of the glycoproteins showed that the 71,000-, 69,000-, and 32,000-dalton glycoproteins behaved similarly with respect to type and group-specific antigenic determinants. In contrast, the 45,000-dalton glycoprotein lacked detectable interspecies and some of the group-specific reactivity. Components of about 45,000 and 32,000 daltons isolated directly from virions were also identified as constituents of the major envelope glycoprotein by immune precipitation and tryptic peptide mapping. These results indicate that all of the examined virion glycoproteins of approximately 71,000, 69,000, 45,000, and 32,000 daltons are derived from the same viral gene and that these lower-molecular-weight glycoproteins can readily be produced from the major envelope glycoprotein.

A number of glycoproteins of purified oncornavirus virions have been described. In the case of Rauscher virus, two major components of approximately 69,000 and 71,000 daltons (gp69/71) have been resolved (37), and, for a number of viruses, minor glycoprotein components of lower molecular weight, most frequently about 45,000, have been described (6, 8, 20, 21, 26). It was not known whether these additional components reflected a greater genomic complexity of the virus, were degradation products of a single high-molecular-weight compound, or were in part derived from the cell or tissue culture media.

The major glycoprotein of murine oncornaviruses, of apparent molecular weight about 70,000, has been purified and characterized (15, 19, 24, 28, 37). It exhibits type, group, and interspecies antigenic determinants (15, 17, 19, 38), and monospecific antisera to the glycoprotein show strong virus-neutralizing activity (9, 17, 22, 36). It is the major structural protein localized on the surface of virions (22, 23, 32, 44) and is also synthesized by cells of many strains of mice, often in the absence of virus production, and apparently under the control of the host cell (7, 16, 40).

In the present study, we have sought to further characterize the genetic complexity and protein structure of the oncornavirus by chemical and immunological studies of several of the glycoproteins of Rauscher murine virus. The results show that the Rauscher virus glycoproteins of about 71,000 and 69,000 daltons are indistinguishable by analysis of the peptide products of tryptic digestion and that other minor glycoproteins of virions are fragments of these components.

MATERIALS AND METHODS

Materials. Neuraminidase (*Vibrio chlolera*) was obtained from Calbiochem and was used without further purification. Galactose oxidase (*Dactylium*

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dendroides), from Worthington Biochemicals Corp., was purified by affinity chromatography on Sepharose 4B. The enzyme, recovered after elution with two column volumes of 0.1 M potassium phosphate, pH 7.0, had a specific activity of 1,600 U/mg, a purification of about 15-fold. The neuraminidase and galactose oxidase had no detectable proteolytic activity on the glycoprotein substrate. Bovine pancreatic trypsin, treated with diphenyl carbamyl chloride to inhibit chymotrypsin, was obtained from Sigma Chemical Co. The enzyme, 1 mg/ml in 1 mM HCl, showed no loss of activity with tosyl arginine methyl ester when stored at 0°C for several months. α-Chymotrypsin and papain coupled to carboxymethyl (CM)-cellulose, and concanavalin A coupled to agarose, were from Sigma Chemical Co. Bovine gamma globulin (fraction II) was from Miles Laboratories, Inc. Sodium [^{125}I]iodide (11 to 17 mCi/μg of I) and sodium boro[^{3}H]hydride (8.6 mCi/mg) were purchased from Amersham/Searle. Ethyleneimine was from ICN Life Sciences (K and K). Cellulose thin-layer plastic sheets (polygram Cel 300, 20 by 20 cm) were obtained from Brinkmann Instruments, Inc.

Type C oncornaviruses. Rauscher murine virus (30), propagated in a BALB/c mouse bone marrow cell line (JLS-V9) (45), was provided by G. Shibley, Frederick Cancer Research Center. Moloney murine virus (25) and plaque-purified (14) Gross murine virus (passage A) (13), both propagated in an NIH 3T3 cell line from NIH Swiss mouse embryo cultures, as well as BALB/c xenotropic virus 2 (1) propagated in a human cell line A673 (12), were all provided by J. Lemp, Electro-Nucleonics Laboratories, Inc. Friend murine virus (10), propagated in STU "Eveline" mouse cells (33), was a gift from D. Bolognesi, Duke University.

Antisera. Rabbit anti-Kirsten murine virus serum, rabbit anti-feline virus (Theilen strain) serum, goat anti-woolly monkey virus serum, and goat anti-Rauscher virus gp69/71 were prepared as described previously (39, 41). All other anti-type C virus sera, anti-Moloney murine virus (1S-166), anti-Gross murine virus (3S-296), anti-xenotropic NZB virus (5S-29), anti-xenotropic NIH virus (5S-652), and anti-xenotropic BALB virus 2 (5S-169) were obtained from goats immunized with purified virus particles disrupted by treatment with Tween-ether; these sera were kindly provided by R. Wilsnack, Huntingdon Research Center, Brooklandville, Md.

Purification of Rauscher virus gp69/71. The Rauscher murine virus envelope glycoprotein gp69/71 was purified as described previously (39).

Limited proteolytic cleavage of envelope glycoprotein. CM-cellulose (5 mg), containing 3 U of covalently bound chymotrypsin, was washed with 20 mM Tris-hydrochloride (pH 7.6)-1.0 M NaCl and was equilibrated with the same buffer containing 0.1 M NaCl. Envelope glycoprotein (10 to 50 μg) was added, and after 5 to 10 min of digestion at room temperature with shaking the cellulose-bound chymotrypsin was removed by centrifugation. For partial papain digestion, 0.7 U of papain covalently bound to 5 mg of CM-cellulose was activated for 15 min at room temperature in 0.5 M sodium acetate, pH 5.2, containing 5 mM cysteine and 2 mM EDTA.

The supernatant fluid was removed, and digestion was carried out for 2 to 5 min as described above for chymotrypsin. Limited tryptic cleavage was carried out in 50 mM NH$_4$HCO$_3$ for 20 min, with an enzyme-to-substrate ratio of 1:20. With all enzymes, the cleavage products and enzyme were either denatured immediately by boiling for 1 min in 0.1% sodium dodecyl sulfate (SDS), or phenylmethylsulfonyl fluoride was added to 1 mM to inhibit any residual protease activity.

Iodination. Several different procedures were utilized, depending upon the subsequent analysis of the protein and whether the procedure utilized pure protein, intact or disrupted virions, or proteins labeled for mapping of tryptic peptides. Purified Rauscher virus glycoprotein was iodinated by the chloramine-T method (18) as previously described (41). Iodination of viral proteins was carried out in the same manner with virions disrupted by incubation at 37°C for 10 min in 0.4% (final concentration) Triton X-100. After iodination of the virus, 400 mM KCl was added, the Triton X-100 concentration was adjusted to 1%, and the labeled proteins were separated from free [^{125}I]iodide by gel filtration on Sephadex G-25 (fine). The excluded fraction was centrifuged at 100,000 $\times$ g for 60 min, and the resulting supernatant fluid was used for immunoprecipitation. Proteins labeled for tryptic peptide mapping, except in those cases when retention of immune activity was required (as below), were denatured by boiling at 100°C for 1 min in 0.5 M sodium phosphate, pH 7.5, containing 0.2% SDS; after iodination, 5 μmol of NaI was added as carrier (in place of KI, so as to avoid precipitation of potassium dodecyl sulfate), and protein was recovered from the reaction mixture by chromatography on Sephadex G-25 using 1.0 M Tris-hydrochloride buffer, pH 8.6, containing 0.1% SDS.

Immunoprecipitation. All reactions were carried out in siliconized glass tubes. The reaction mixture for immunoprecipitation of purified protein contained the following: 1.4 to 2.5 ng of ^{125}I-labeled glycoprotein (6 $\times$ 10^4 to 7 $\times$ 10^4 cpm per ng), 3 μg of unlabeled, Triton X-100-disrupted Rauscher murine virus as carrier, and a pretitrated amount of immune serum. The final volume was adjusted to 0.15 ml with TEN buffer (20 mM Tris-hydrochloride, pH 7.6, 1 mM EDTA, 100 mM NaCl). The reaction mixture was incubated at 4°C for 18 h. Cold TEN buffer (0.5 ml) was added, and the precipitate was collected by centrifugation at 4°C. The pellet was washed twice with 0.5 ml of TEN buffer, followed by a final wash with 0.5 ml of cold acetone. The precipitate was solubilized in 0.056 ml of buffer containing 62.5 mM Tris-hydrochloride, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol. The samples were heated at 100°C for 1 min and then were analyzed by electrophoresis in a high-resolution, 5 to 20% gradient polyacrylamide gel in the presence of 0.1% SDS, as described by Baum et al. (3).

When ^{125}I-labeled virus was used for immunoprecipitation, the conditions were the same as described above except that the total volume of the reaction mixture was 0.2 ml. The reaction mixture contained 80 to 140 ng of ^{125}I-labeled viral protein (1.9 $\times$ 10^3 to 3.3 $\times$ 10^3 cpm per ng) and 3 μg of protein of unla-

beled, Triton X-100-disrupted virus corresponding to the labeled virus.

Tritium labeling of glycoproteins. Galactosyl residues of glycoproteins were labeled with tritium by methods similar to those previously described (11, 23). Glycoprotein (5 to 15 μg) was dissolved in 50 μl of 20 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), pH 6.3, 2 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride. Neuraminidase (0.5 U) was added, and, after incubation at 37°C for 30 min, galactose oxidase (1.2 U) was added and the incubation was continued for 3.5 h. A 10-μl amount of 0.05 N NaOH containing 0.3 mCi of sodium boro[^{3}H]hydride was added, and, after 30 min at room temperature, the reaction was stopped by the addition of 0.2 ml of 50 mM acetic acid. The solution was evaporated to dryness in vacuo, 0.25 ml of acetic acid was added to the residue, and after 30 min the solvent and residual [^{3}H]hydrogen were removed in vacuo. Proteins were separated by polyacrylamide slab gel electrophoresis as described above and stained with Coomassie brilliant blue R-250. After the gel was photographed, it was impregnated with scintillator and dried, and tritium-containing bands were detected by scintillation autoradiography using Kodak RP Royal X-Omat film (4).

Separation of proteins. Polyacrylamide gel electrophoresis was carried out as described above. Protein bands were stained with Coomassie brilliant blue R-250, cut from the dried gel, and cut into pieces about 1 mm³. Protein was recovered, and the stain was removed by a modification of the procedure of Bray and Brownlee (5). Gels were eluted overnight at 37°C in 1.5 ml of 50 mM sodium phosphate, pH 7.5, containing 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and a trace of chloroform. The supernatant fluid was filtered through glass wool, KCl was added to a final concentration of 0.2 M, and the samples were left on ice for 15 min. Precipitates were collected by centrifugation at 10,000 × g for 20 min, washed twice with 1 ml of cold acetone, and dried under vacuum.

Reduction and aminoethylation. Sulfhydryl groups were blocked prior to tryptic digestion by reduction and aminoethylation (35). The advantages of this method compared with performic acid oxidation include quantitative reaction with great specificity under mild conditions (29) and rapid recovery of the modified protein in good yield. An amount of 5 μg or less of ^{125}I-labeled protein in 0.5 ml of 1.0 M Tris-hydrochloride, pH 8.6, containing 0.1% SDS, was reduced by the addition of dithiothreitol to a final concentration of 0.1 M. The tube was flushed with nitrogen, capped, and incubated at 37°C for 3 h. The tube was again flushed with nitrogen at room temperature and, while flushing, 10-, 5-, and 5-μl amounts of ethyleneimine were added at 10-min intervals. At 10 min after the final addition, the entire reaction mixture was applied to a column (0.8 by 20 cm) of Sephadex G-25 (fine), which was equilibrated and eluted with 50 mM NH$_4$HCO$_3$. The void volume fraction (0.5 ml) containing the peak of radioactivity was used for peptide mapping.

Peptide mapping. Bovine gamma globulin (50 μg), trypsin (1 μg), and a trace of chloroform were added to the column fraction containing the S-ami-

noethylated ^{125}I-labeled protein. After several hours at room temperature, a second 1 μg of trypsin was added, and digestion was continued overnight. A sample containing about 10⁶ cpm (usually 5 to 20 μl) was spotted on a cellulose thin-layer sheet, and electrophoresis was carried out at 1,000 V in acetic acid-pyridine-water (10:1:89) for 30 to 40 min, until a spot of acid fuchsin tracking dye had moved 16.5 cm. Ascending chromatography was then carried out in the second dimension in butanol-acetic acid-pyridine-water (5:1:4:4). Autoradiography of the chromatograms was carried out with Kodak No Screen X-Ray film (Kodak NS-54T).

Viral protein labeling, immunoprecipitation, and peptide mapping. Triton X-100-disrupted Rauscher virus (11.3 μg of protein) was iodinated as described above, by use of 1.5 mCi of [^{125}I]iodide. Labeled proteins were recovered from the reaction mixture by gel filtration on Sephadex G-25 in TEN buffer containing 0.1% Triton X-100, followed by centrifugation at 100,000 × g for 60 min as described above. Pretitrated goat anti-Rauscher gp69/71 was added to the ^{125}I-labeled viral proteins (about 7 μg of protein, 3 × 10⁴ cpm per ng), and the immunoprecipitate was collected and analyzed by polyacrylamide gel electrophoresis as described above. The bulk of the immunoprecipitate was distributed among four gel lanes (5 × 10⁶ cpm per lane) for preparative isolation of the components, and a small amount (3 × 10⁴ cpm) was loaded on the same gel for analytical comparison with ^{125}I-labeled virions (5 × 10⁵ cpm). Radioactive proteins were detected by autoradiography, cut from the dried gel, eluted, and precipitated with KCl as described above, except that 30 μg of bovine gamma globulin was added before precipitation. The precipitates were dissolved in 0.5 ml of 1.0 M Tris-hydrochloride, pH 8.6, containing 0.1% SDS for reduction and aminoethylation, as described above. So as to recover the protein in a small volume, ice-cold 50% trichloroacetic acid (0.4 ml) was added, and after 15 min on ice the precipitate was collected by centrifugation at 20,000 × g and washed with cold 10% trichloroacetic acid and acetone. The washed pellet was taken up in 50 μl of 0.05 M NH$_4$HCO$_3$. The protein was digested with trypsin, and the peptides were analyzed as described above.

RESULTS

Components of murine oncornaviruses reactive with anti-Rauscher gp69/71 serum. Several murine oncornaviruses, Rauscher, Friend, BALB xenotropic virus 2, Moloney, and Gross, were analyzed for components that reacted with antiserum against the purified Rauscher virus gp69/71 glycoprotein. The viruses, ^{125}I-labeled and disrupted with Triton X-100, were treated with the antiserum, and the immunoprecipitates, as well as the labeled viruses, were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Of the many iodinated proteins, several were immunoprecipitated by anti-Rauscher gp69/71 serum (Fig. 1). With Rauscher virus, four major components were precipitated, a doublet of 69,000
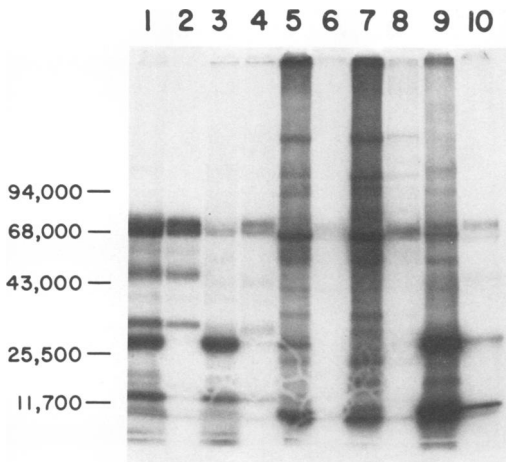
FIG. 1. *Autoradiographic analysis of* ^{125}I-labeled *murine viruses and of proteins immunoprecipitated by anti-Rauscher virus gp69/71 serum.* ^{125}I-labeling *of virions, immunoprecipitation, electrophoresis in a linear 5 to 20% polyacrylamide gel gradient in the presence of 0.1% sodium dodecyl sulfate, and autoradiography were as described in Materials and Methods. (1)* ^{125}I-labeled *Rauscher murine virus (2.3 × 10^5 cpm of trichloroacetic acid-precipitable* ^{125}I*); (2) proteins of* ^{125}I-labeled *Rauscher virus immunoprecipitated by anti-Rauscher gp69/71 serum (3.1 × 10^4 cpm); (3)* ^{125}I-labeled *Friend murine virus (2.6 × 10^5 cpm of trichloroacetic acid-precipitable* ^{125}I*); (4) proteins of* ^{125}I-labeled *Friend virus immunoprecipitated by anti-Rauscher gp69/71 serum (2.6 × 10^4 cpm); (5)* ^{125}I-labeled *xenotropic BALB virus 2 (4.5 × 10^5 cpm of trichloroacetic acid-precipitable* ^{125}I*); (6) proteins of* ^{125}I-labeled *xenotropic BALB virus 2 immunoprecipitated by anti-Rauscher gp69/71 serum (1.4 × 10^4 cpm); (7)* ^{125}I-labeled *Moloney murine virus (6.6 × 10^5 cpm of trichloroacetic acid-precipitable* ^{125}I*); (8) proteins of* ^{125}I-labeled *Moloney virus immunoprecipitated by anti-Rauscher gp69/71 serum (2.5 × 10^4 cpm); (9)* ^{125}I-labeled *Gross murine virus (8.6 × 10^5 cpm of trichloroacetic acid-precipitable* ^{125}I*); (10) proteins of* ^{125}I-labeled *Gross virus immunoprecipitated by anti-Rauscher gp69/71 serum (2.6 × 10^4 cpm). The standard proteins were cytochrome c (2.5 μg; mol wt, 11,700), chymotrypsinogen (4.3 μg; mol wt, 25,500), ovalbumin (4.3 μg; mol wt, 43,000), bovine serum albumin (1.7 μg; mol wt, 68,000), and phosphorylase a (7.2 μg; mol wt, 94,000).*

and 71,000 daltons as expected, and also components with apparent molecular weights of 45,000 and 32,000. There also were other minor components of about 15,000 and 10,000 daltons. The immunoprecipitate of Friend virus also contained a doublet at 69,000 and 71,000 daltons, plus polypeptides of 31,000 and 15,000 daltons and a minor polypeptide at 42,000 daltons. The immunoprecipitate of BALB virus 2 showed only very faint peptides at 69,000 and 71,000 daltons. Moloney virus showed a major component at 69,000 daltons, and minor high-molecular-weight and 10,000-dalton components. Gross virus, in contrast to the others, showed chiefly a low-molecular-weight peptide of about 14,000 daltons, with additional components of 10,000, 30,000, 67,000, and 70,000 daltons. This 14,000-dalton Gross virus protein has been preparatively purified (unpublished data), and its properties in relationship to the higher-molecular-weight glycoproteins are being extensively studied; these results will be presented in detail elsewhere.

As the anti-gp69/71 serum does not cross-react with the other major viral proteins, p30, p15, or p12 (38, 39) and p10 (unpublished data), the components efficiently immunoprecipitated by anti-Rauscher gp69/71 serum must be suspected to contain antigenic determinants shared with the major envelope glycoprotein of 69,000 and 71,000 daltons. As shown below, this was confirmed by tryptic peptide mapping of the fragments. The specificity of the immune precipitation of the minor fragments was not further characterized because of the relatively low proportion of the corresponding protein that was precipitated.

Immunoprecipitation of purified Rauscher gp69/71 by various antisera. One necessary control in these experiments was to determine whether the lower-molecular-weight components observed were degradation products of gp69/71 resulting from the immunoprecipitation reaction method. This was examined by applying the same procedure (^{125}I-labeling, immunoprecipitation, polyacrylamide gel electrophoresis, and autoradiography) to purified Rauscher virus envelope glycoprotein. All of the various antisera used in this study were so tested (Fig. 2). In no case did the immunoprecipitate contain polypeptides other than the initial starting material, gp69/71; thus, the procedures themselves did not appear to give rise to degradation products.

Proteolytic degradation of gp69/71. Another explanation for the presence of these low-molecular-weight virion peptides reactive with anti-gp69/71 serum is that they arose from proteolytic activity prior to immunoprecipitation. For this reason, controlled enzymatic digestion of purified Rauscher virus glycoprotein gp69/71 was carried out to determine whether this would give rise to specific fragments.

When intact gp69/71 was treated with chymotrypsin, papain, or trypsin, two major cleavage fragments with apparent molecular weights of 45,000 and 32,000 were produced which, by gel electrophoresis, were indistinguishable from the polypeptides immunoprecipitated from
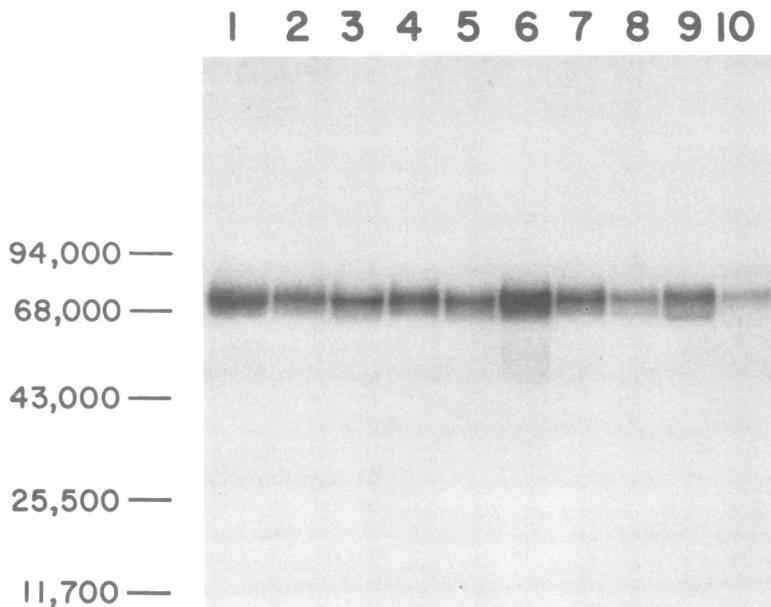
Fig. 2. *Autoradiographic analysis of purified, ^{125}I-labeled Rauscher virus gp69/71 immunoprecipitated by various antisera. Polyacrylamide gel electrophoresis, autoradiography, and immunoprecipitation were carried out as described in Materials and Methods. Approximately 3×10^4 cpm was applied to each lane: (1) purified, ^{125}I-labeled Rauscher virus gp69/71; (2) purified, ^{125}I-labeled Rauscher gp69/71 immunoprecipitated by anti-Rauscher virus gp69/71 serum; (3) anti-Gross virus serum; (4) anti-Moloney virus serum; (5) anti-xenotropic NZB virus serum; (6) anti-xenotropic NIH virus serum; (7) anti-xenotropic BALB virus 2 serum; (8) anti-Kirsten virus serum; (9) anti-feline virus (Theilen strain) serum; and (10) anti-woolly monkey virus serum. Standard proteins were as described in the legend of Fig. 1.*

Rauscher virus (Fig. 3). The generation of these two similar fragments by a variety of proteolytic conditions suggested that these fragments are protected cores. Quantitative conversion of the glycoprotein to these components was not possible; conditions necessary to complete the cleavage of intact glycoprotein resulted in loss of most of the protein as small peptides.

Spontaneous cleavage fragments from Rauscher virus gp69/71. Spontaneous cleavage fragments from purified Rauscher virus glycoprotein gp69/71 were also observed. In this case, a gel filtration step using Sepharose 4B was carried out on one preparation of purified gp69/71. The column (1.5 by 100 cm) of Sepharose 4B was packed and eluted in 10 mM BES, pH 6.5, and 1 mM EDTA. A single symmetrical peak containing homogeneous gp69/71 was obtained. However, after storage of individual column fractions at 0°C for several weeks, partial breakdown of the glycoprotein had occurred in all fractions, producing two major fragments with apparent molecular weights of 45,000 and 32,000 and the residual high-molecular-weight component (Fig. 3). These fragments will be referred to as spontaneous cleavage products to distinguish them from fragments produced by

chymotrypsin. Subsequent storage of partially degraded glycoprotein at 20°C for several weeks in the presence of chloroform did not result in further breakdown.

Immunoprecipitation of purified Rauscher gp69/71 and spontaneous fragments. The antigenicity of the spontaneously fragmented envelope glycoprotein was analyzed with several antisera (Fig. 4). The residual gp69/71 and the 32,000-dalton fragment behaved in a similar manner with all the antisera employed, but the 45,000-dalton fragment appeared to lack certain interspecies and group determinants. With anti-NZB virus serum and anti-feline virus serum, very little of the 45,000-dalton component was precipitated, whereas with the other murine virus antisera, including two other xenotropic murine viruses (NIH and BALB virus 2), the same pattern as with anti-Rauscher gp69/71 serum was observed. Only minor immunoprecipitation was obtained with anti-woolly monkey virus serum. These results demonstrate that the fragments of 45,000 and 32,000 daltons contain type-specific antigenic determinants and a spectrum of group-specific determinants shared with the intact envelope glycoprotein gp69/71. The apparent absence of interspecies
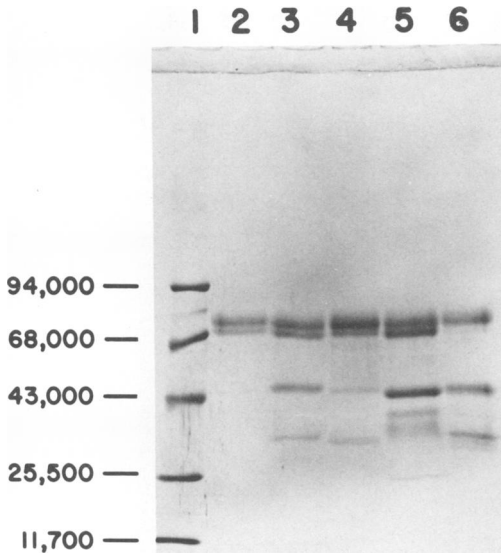
FIG. 3. *Polyacrylamide gel electrophoresis of Rauscher murine virus envelope glycoprotein gp69/71 and products of proteolytic digestion. Electrophoresis was carried out as described in Materials and Methods. (1) Standard proteins: cytochrome c (1.5 μg; mol wt, 11,700), chymotrypsinogen (3.0 μg; mol wt, 25,500), ovalbumin (2.5 μg; mol wt, 43,000), bovine serum albumin (1.4 μg; mol wt, 68,000), and phosphorylase a (3.0 μg; mol wt, 94,000); (2) purified gp69/71 (4.5 μg); (3) purified gp69/71 (18 μg) treated with chymotrypsin; (4) purified gp69/71 (18 μg) treated with papain; (5) purified gp69/71 (18 μg) treated with trypsin; (6) purified gp69/71 (13.5 μg) which was spontaneously degraded. The conditions for enzymatic digestion were as described in Materials and Methods.*

determinants in the 45,000-dalton component may reflect localization of such determinants in the 32,000-dalton component, but such a conclusion would require more extensive analysis with additional sera.

Carbohydrate content of fragments. Two methods of analysis suggest that the 45,000- and 32,000-dalton fragments both contain oligosaccharide chains. When the fragmented glycoproteins were treated with neuraminidase, galactose oxidase, and sodium boro[^{3}H]hydride, tritium was incorporated into all the major glycoprotein-derived bands detected by Coomassie brilliant blue staining (Fig. 5). In another experiment, all of the major bands of a chymotrypsin-treated sample of glycoprotein were adsorbed by concanavalin A covalently linked to agarose. When the concanavalin A-agarose was eluted with a shallow (100 volumes) gradient of α-methyl mannoside (0 to 100 mM), both major fragments co-eluted with the intact glycoprotein at about 20 mM α-methyl man-

noside (data not shown), again indicating the presence of carbohydrates.

Separation of glycoproteins and tryptic peptide analysis. Preparative separation of gp69 from gp71, as well as separation of spontaneous and chymotryptic degradation fragments, was carried out by polyacrylamide gel electrophoresis as described in Materials and Methods. About 75 μg of degraded gp69/71 was applied to the gel, divided among five gel lanes. The relative amount of gp69 in different virus preparations was variable; in some cases the content was comparable to that of gp71, but other preparations contained relatively less gp69. The preparation of glycoprotein which degraded spontaneously contained relatively little gp69, but it was possible to cut out separate stained bands corresponding to gp71, gp69, gp45, and gp32 from both preparations which were examined. The homogeneity of each polypeptide after elution from the gel and iodination was analyzed by polyacrylamide gel electrophoresis. Autoradiograms of these gels showed that the initial separation was effective, but some degradation of the individual polypeptides was detected (Fig. 6). The results were the same with fragments derived by digestion with chymotrypsin (data not shown). Control experiments with intact gp69/71 (*not* recovered from a gel) showed that no degradation occurred during iodination or during any other step prior to tryptic digestion (data not shown).

Tryptic peptide analysis of these polypeptides isolated from a sample of spontaneously degraded gp69/71 showed that the maps of gp71 and gp69 were identical (Fig. 7). The peptide maps of gp45 and gp32 were also nearly identical to those of gp71 and gp69, despite the apparently lower molecular weights of these polypeptides.

Corresponding peptide maps of polypeptides isolated after limited chymotryptic cleavage of gp69/71 were comparable (Fig. 8). In this case, maps of gp71 and gp69 were nearly, but not completely, identical. There was an apparent slight difference in the mobility of one peptide. Both spots were visible on a peptide map of the unseparated gp69/71 complex (data not shown), confirming the difference in mobility. A number of major peptides were either lacking, or present only in small amounts in the maps of gp45 and gp32, which had been produced by chymotryptic cleavage. Similar results were obtained when gp45 and gp32 were isolated from chymotrypsin-cleaved gp69/71 in three separate experiments, using three samples of the same gp69/71 preparation.

The streak from the origin and one spot of the peptide maps (noted in the legends of Fig. 7 and
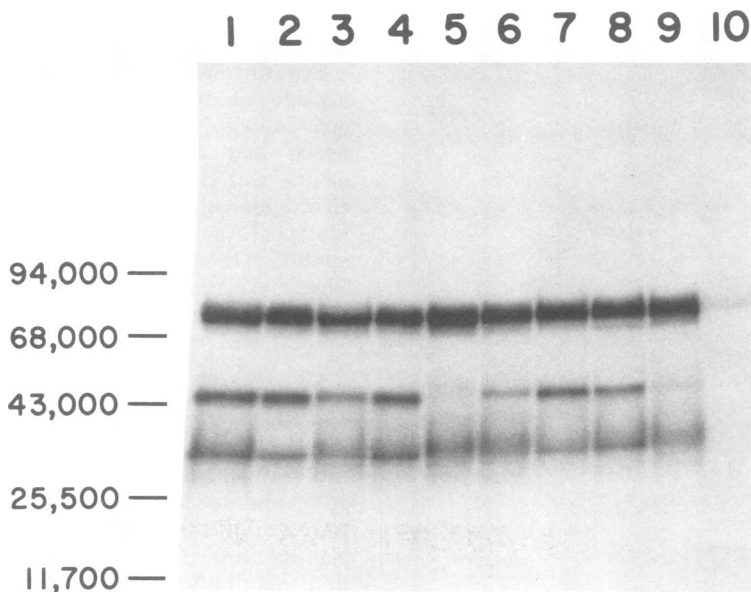
FIG. 4. *Autoradiographic analysis of purified, spontaneously fragmented, ^{125}I-labeled Rauscher virus envelope glycoprotein immunoprecipitated by various antisera. Polyacrylamide gel electrophoresis, autoradiography, and immunoprecipitation were carried out as described in Materials and Methods. Approximately 5×10^4 cpm was applied to each lane: (1) purified, fragmented ^{125}I-labeled envelope glycoprotein; (2) purified fragmented, ^{125}I-labeled envelope glycoprotein immunoprecipitated by anti-Rauscher virus gp69/71 serum; (3) anti-Gross virus serum; (4) anti-Moloney virus serum; (5) anti-xenotropic NZB virus serum; (6) anti-xenotropic NIH virus serum; (7) anti-xenotropic BALB virus 2 serum; (8) anti-Kirsten virus serum; (9) anti-feline virus (Theilen strain) serum; and (10) anti-woolly monkey virus serum. Standard proteins were as described in the legend of Fig. 1.*

8) were observed when blank areas of the polyacrylamide gel were carried through the same elution, labeling, and mapping procedure. However, gp69/71 which was mapped without exposure to polyacrylamide gave a similar streak from the origin, indicating that some of the radioactivity in this area is peptide in nature. Lysozyme peptides did not streak when prepared by the usual procedure, but gross overdigestion with 200-fold more enzyme did not significantly reduce streaking in the gp69/71 map.

Peptide analysis of virion components reactive with anti-gp69/71 serum. Tryptic peptide analysis was also carried out with the lower-molecular-weight components reactive with anti-gp69/71 serum (as shown in Fig. 1), which were isolated directly from virions by gel electrophoresis. A principal question was whether or not the glycoprotein fragment of 45,000 daltons described here corresponds to the minor glycoprotein gp45 which has been observed by metabolic labeling of viruses with glucosamine.

Disrupted virions were iodinated, and the solubilized proteins were reacted with anti-Rauscher gp69/71 serum on a preparative scale as described in Materials and Methods. The

individual components of 45,000 and 32,000 daltons as well as gp69/71 were isolated by preparative polyacrylamide gel electrophoresis, and peptide maps were obtained as described in Materials and Methods (Fig. 9). The three maps were nearly identical, suggesting that the polypeptides of 45,000 and 32,000 daltons were derived from gp69/71. This experiment was repeated with a different preparation of Rauscher virus which had been ^{125}I-labeled prior to disruption. Again, the maps were nearly identical and were almost indistinguishable from those shown above (data not shown). All of the peptide maps of immunoprecipitated virion components were similar, but not identical, to those of corresponding glycoproteins and fragments which had been iodinated after denaturation with SDS. Since the conformation of the protein is likely to affect the labeling pattern, some differences can be expected in such a comparison.

It should be noted that, with three additional preparations of Rauscher virus that were examined, the amount of immunoreactive material observed in the regions of 45,000 and 32,000 daltons was quite low and differed for each virus preparation (data not shown). Also, for
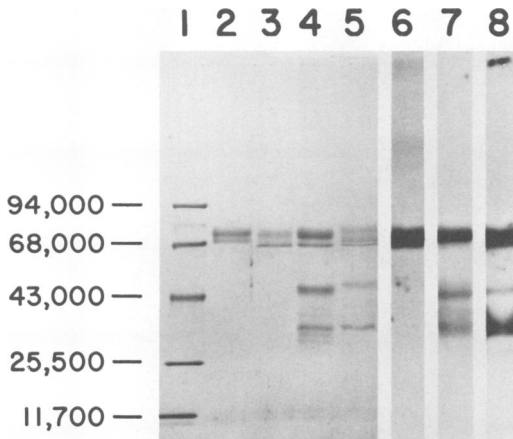
FIG. 5. *Carbohydrate content of fragments derived from Rauscher murine virus glycoprotein gp69/71. Intact or fragmented gp69/71 was labeled in the carbohydrate moiety with ³H and was analyzed by polyacrylamide gel electrophoresis followed by protein staining (lanes 1 through 5) or autoradiography (lanes 6 through 8) as described in Materials and Methods. (1) Standard proteins as described in Fig. 3; (2) purified gp69/71 (5 μg); (3 and 6) purified gp69/71 (5 μg), tritium labeled; (4 and 7) purified gp69/71 (15 μg), spontaneously degraded and tritium labeled; (5 and 8) purified gp69/71 (15 μg), partially cleaved by chymotrypsin and tritium labeled. Scintillation autoradiograms were exposed for 2 days (lane 6), 1 day (land 7), and 9 days (lane 8). Tritium incorporation could not be determined except by autoradiography because polyacrylamide gel electrophoresis was required to eliminate residual sodium boro[³H]hydride. The stained band of 67,000 daltons in lanes 3, 4, and 5 was derived from the enzyme preparation used for tritium labeling.*

several virus preparations, a protein band in the region of 45,000 daltons was observed by staining of the analytical polyacrylamide gel with Coomassie brilliant blue R-250. This stained band was also isolated from one virus preparation by excision from the polyacrylamide gel, labeled with [¹²⁵I]iodide, and subjected to peptide mapping as described in Materials and Methods. In contrast to the results obtained with iodinated protein recovered by specific immunoprecipitation, this additional component of similar molecular weight showed no relationship to gp69/71 (data not shown). Thus, for some virus preparations, selective purification such as immunoprecipitation is required to demonstrate a component bearing sequence homology with the major glycoprotein gp69/71.

DISCUSSION

Our principal conclusion from these studies utilizing immunoprecipitation with monospecific antiserum and tryptic peptide analysis is that each of the observed glycoproteins of Rauscher virus of approximately 71,000, 69,000, 45,000 and 32,000 daltons contains common amino acid sequences and therefore is very likely derived from the same viral gene. It must be suspected that the glycoprotein gp45 which has been detected in virions by incorporation of radioactive glucosamine (8, 26) corresponds to the 45,000-dalton degradation fragment which we have characterized. It has previously been reported to cross-react with antisera to the major glycoprotein (6, 21; R. A. Lerner, personal communication). The other glycoprotein of about 32,000 daltons has not previously been reported. Thus, from current knowledge, the complexity of the oncornaviruses genome in respect to glycoproteins is limited to a component of about 70,000 daltons, derived from a glycosylated precursor of about 90,000 daltons (27, 34, 42).

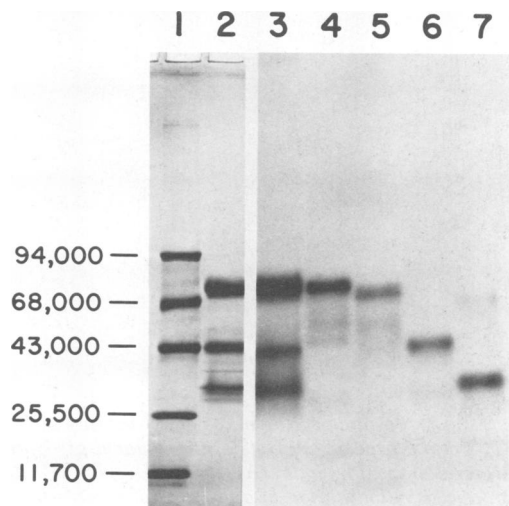The fragments described in this study could

FIG. 6. *Preparative separation of gp71, gp69, gp45, and gp32 by polyacrylamide gel electrophoresis of spontaneously degraded gp69/71. Isolation of the individual components of purified, spontaneously fragmented Rauscher murine virus gp69/71 was carried out by polyacrylamide gel electrophoresis as described in the text. The isolated proteins were labeled with [¹²⁵I]iodide as described in Materials and Methods and were analyzed by polyacrylamide gel electrophoresis, as depicted. Lanes 1 and 2 were stained by Coomassie brilliant blue; lanes 3 through 7 were autoradiograms of the ¹²⁵I-labeled polypeptides. (1) Standard proteins as described in Fig. 3; (2) purified spontaneously degraded gp69/71 (15 μg); (3) ¹²⁵I-labeled, purified spontaneously degraded gp69/71; (4) ¹²⁵I-labeled gp71; (5) ¹²⁵I-labeled gp69; (6) ¹²⁵I-labeled gp45; (7) ¹²⁵I-labeled gp32. Similar results were obtained with chymotrypsin-cleaved gp69/71.*
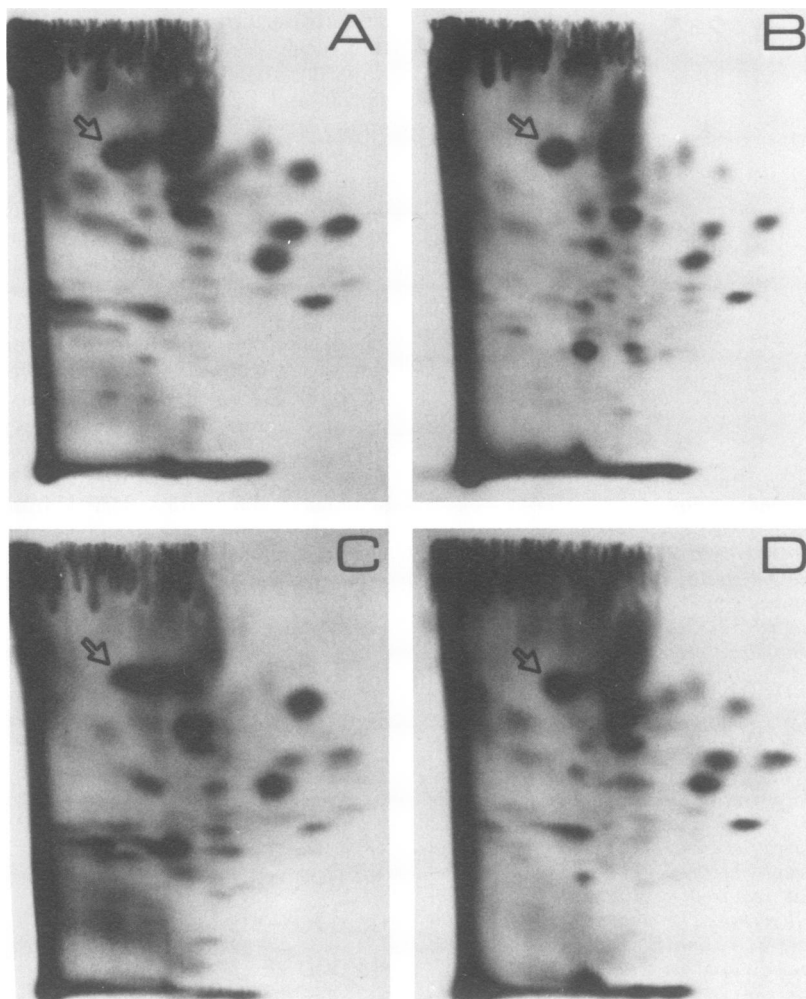
FIG. 7. *Tryptic peptide maps of* ^{125}*I-labeled gp71, gp69, and fragments spontaneously derived from gp69/ 71. Tryptic peptide analysis of the isolated glycoproteins depicted in Fig. 6 was carried out as described in Materials and Methods. (A) gp71; (B) gp69; (C) gp45; (D) gp32. The spot identified by an arrow was also produced by iodination of an extract of protein-free polyacrylamide gel.*

theoretically have resulted from either specific transcriptional, translational, or post-translational processes, or from nonspecific proteolysis at susceptible sites. Cleavage of purified envelope glycoprotein to discrete forms of lower molecular weight during storage in the absence of any known proteolytic agent presumably was the result of contamination by protease. Similar fragments could be produced in reasonable yield by controlled proteolytic digestion with several enzymes. These results suggest that specific regions of the polypeptide chain of gp69/ 71 have conformations which render them susceptible to a number of proteases of diverse specificity. Such regions of facile cleavage could account for the frequent presence of minor glycoprotein components in virus preparations.

However, although such nonspecific fragmentation may be the most likely explanation for the presence of the different molecules, this is conjecture, and it also is possible that one or more of the smaller components provide an essential biological function. As yet, such a function is not recognized, but, with the complex structure and function of these viruses, it would be difficult to rule out this possibility.

There are several possible explanations for the similarity of the tryptic maps of the different components. Possibly, the regions of the molecule which were removed in generating the 45,000- and 32,000-dalton fragments were not subject to iodination. Also, the apparent difference in size possibly could be attributed largely to the removal of very carbohydrate-
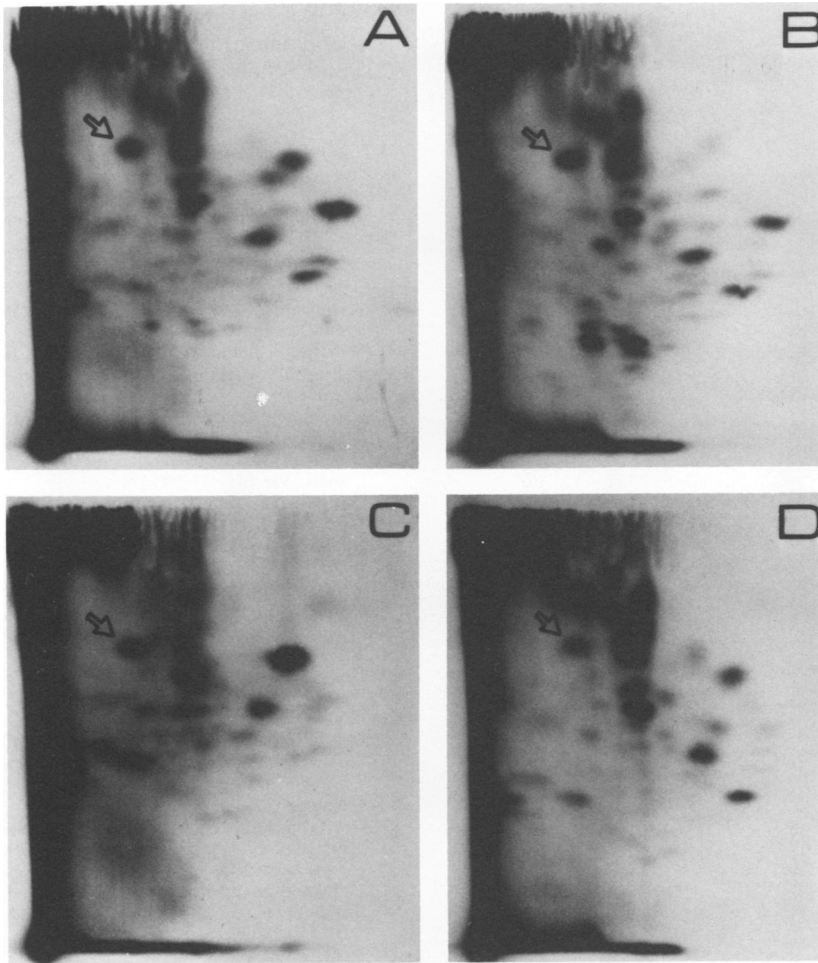
FIG. 8. *Tryptic peptide maps of* ^{125}I-*labeled gp71, gp69, and fragments produced by cleavage of gp69/71 with chymotrypsin. Glycoproteins were isolated as described in the legend of Fig. 6, and tryptic peptide analysis of the isolated proteins was carried out as described in the text. (A) gp71; (B) gp69; (C) gp45; (D) gp32. The arrow is explained in the legend of Fig. 7.*

rich peptide(s) so that there actually was little difference in the polypeptide chains of the different molecules. Another explanation is that the 45,000- and 32,000-dalton components are each composed of two or more different segments of the glycoprotein, which coincidentally have the same electrophoretic mobility and which, in sum, represent all of the sequences of the intact molecule. Such fragment heterogeneity could explain why the peptide map of the chymotryptic 54,000-dalton fragment, in contrast to that of the corresponding spontaneous fragment, had only trace amounts of some peptides which were major components of the gp69/71 from which it was derived.

Anti-Rauscher gp69/71 serum also precipitated other lower-molecular-weight polypeptides from the different viruses tested. Of principal interest is one such small component of about 15,000 to 17,000 daltons. This component does not appear to be a degradation product of the 70,000-dalton glycoprotein. It was never observed as a degradation product of gp69/71, either by controlled enzymatic digestion or as a result of spontaneous degradation of purified glycoprotein. Rather, there is considerable evidence that it is another component derived from the 90,000-dalton precursor glycoprotein and that it bears some antigenic determinants shared or overlapping with the 70,000-dalton product. Such a component of Gross virus has been preparatively purified and characterized (Strand and August, unpublished data). A polypeptide of similar size was also reported by Witte and Weissman (43) to be precipitated from Moloney sarcoma-leukemia virus by the
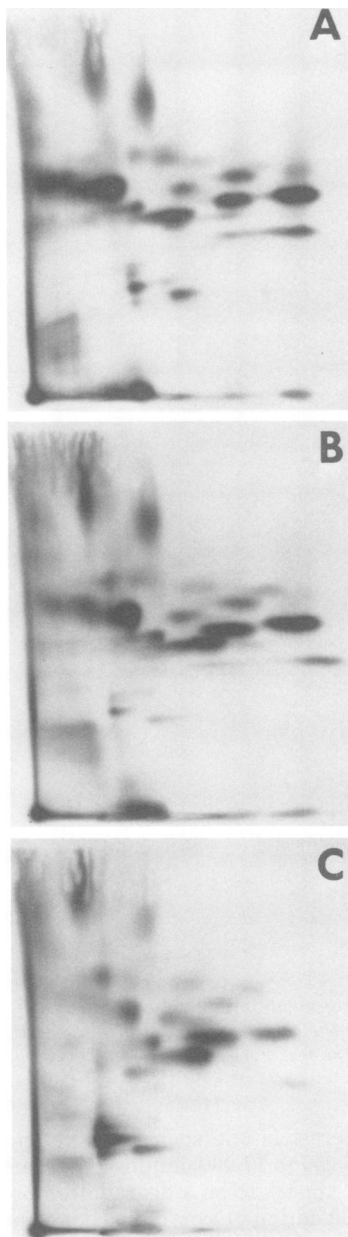
FIG. 9. *Tryptic peptide maps of* [125]*I-labeled virion components reactive with anti-gp69/71 serum. Isolation and tryptic peptide analysis of the immunoprecipitated components were carried out as described in the text. (A) gp69/71; (B) gp45; (C) gp32.*

anti-Rauscher gp69/71 serum. Moreover, studies by Arcement et al. (2) and Van Zaane et al. (42) have suggested that a polypeptide from Rauscher leukemia virus of approximately 17,000 daltons may be derived from the same precursor as gp69/71, and these workers pro-

posed a correspondence between this polypeptide and the p15(E) identified by Ikeda et al. (21) and Schafer et al. (31).

LITERATURE CITED

1. **Aaronson, S. A., and J. R. Stephenson.** 1973. Independent segregation of loci for activation of biologically distinguishable RNA C-type viruses in mouse cells. Proc. Natl. Acad. Sci. U.S.A. 70:2055-2058.

2. **Arcement, L. J., W. L. Karshin, R. B. Naso, G. Jamjoom, and R. B. Arlinghaus.** 1976. Biosynthesis of Rauscher leukemia viral proteins: presence of p30 and envelope p15 sequences in precursor polypeptides. Virology 69:763-774.

3. **Baum, S. G., M. S. Horwitz, and J. V. Maizel, Jr.** 1972. Studies of the mechanism of enhancement of human adenovirus infection in monkey cells by simian virus 40. J. Virol. 10:211-219.

4. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.

5. **Bray, D., and S. M. Brownlee.** 1973. Peptide mapping of proteins from acrylamide gels. Anal. Biochem. 55:213-221.

6. **Del Villano, B. C., and R. A. Lerner.** 1976. Relationship between the oncornavirus gene product gp70 and a major protein secretion of the mouse genital tract. Nature (London) 259:497-499.

7. **Del Villano, B. C., B. Nave, B. P. Croker, R. A. Lerner, and F. J. Dixon.** 1975. The oncornavirus glycoprotein gp69/71: a constituent of the surface of normal and malignant thymocytes. J. Exp. Med. 141:172-187.

8. **Duesberg, P. H., G. S. Martin, and P. K. Vogt.** 1970. Glycoprotein components of avian and murine RNA tumor viruses. Vriology 41:631-646.

9. **Fischinger, P. J., W. Schafer, and D. P. Bolognesi.** 1976. Neutralization of homologous and heterologous oncornaviruses by antisera against the p15(E) and gp71 polypeptides of Friend murine leukemia virus. Virology 71:169-184.

10. **Friend, C., and J. Haddad.** 1959. Local tumor formation with transplants of spleen or liver of mice with a virus-induced leukemia. Proc. Am. Assoc. Cancer Res. 3:1.

11. **Gahmberg, C. G., and S.-I. Hakomori.** 1973. External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. J. Biol. Chem. 248:4311-4317.

12. **Giard, D. J., S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik, and W. P. Parks.** 1973. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51:1417-1423.

13. **Gross, L.** 1957. Development and serial cell-free passage of a highly potent strain of mouse leukemia virus. Proc. Soc. Biol. Med. 94:767-771.

14. **Hartley, J. W., W. P. Rowe, and R. J. Huebner.** 1970. Host-range restrictions of murine leukemia viruses in mouse embryo cell cultures. J. Virol. 5:221-225.

15. **Hino, S., J. R. Stephenson, and S. A. Aaronson.** 1975. Antigenic determinants of the 70,000 molecular weight glycoprotein of woolly monkey type C RNA virus. J. Immunol. 115:922-927.

16. **Hino, S., J. R. Stephenson, and S. A. Aaronson.** 1976.

Radioimmunoassays for the 70,000-molecular-weight glycoproteins of endogenous mouse type C viruses: viral antigen expression in normal mouse tissues and sera. J. Virol. 18:933-941.

17. Hunsmann, G., V. Moennig, L. Pister, E. Seifert, and W. Schafer. 1974. Properties of mouse leukemia viruses. VIII. The major viral glycoprotein of Friend leukemia virus. Seroimmunological, interfering and hemagglutinating capacities. Virology 62:307-318.

18. Hunter, W. M. 1967. The preparation of radioiodinated proteins of high activity, their reaction with antibody *in vitro*: the radioimmunoassay, p. 608-642. *In* D. M. Weir (ed.), Handbook of experimental immunology. F. A. Davis Co., Philadelphia.

19. Ihle, J. N., T. P. Denny, and D. P. Bolognesi. 1976. Purification and serological characterization of the major envelope glycoprotein from AKR murine leukemia virus and its reactivity with autogenous immune sera from mice. J. Virol. 17:727-736.

20. Ihle, J. N., M. G. Hanna, L. E. Roberson, and F. T. Kenney. 1974. Autogenous immunity to endogenous RNA tumor virus. Identification of antibody reactivity to select viral antigens. J. Exp. Med. 139:1568-1581.

21. Ikeda, H., W. Dardy, Jr., E. Tress, and E. Fleissner. 1975. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. V. Identification of a new murine viral protein, p15(E). J. Virol. 16:53-61.

22. Ikeda, H., T. Pincus, T. Yoshiki, M. Strand, J. T. August, E. A. Boyse, and R. C. Mellors. 1974. Biological expression of antigenic determinants of murine leukemia virus proteins gp69/71 and p30. J. Virol. 14:1274-1280.

23. McLellan, W. L., and J. T. August. 1976. Analysis of the envelope of Rauscher murine oncornavirus: in vitro labeling of glycopeptides. J. Virol. 20:627-636.

24. Moennig, V., H. Frank, G. Hunsmann, I. Schneider, and W. Schafer. 1974. Properties of mouse leukemia viruses. VII. The major viral glycoprotein of Friend leukemia virus. Isolation and physicochemical properties. Virology 61:100-111.

25. Moloney, J. B. 1960. Biological studies on a lymphoid-leukemia virus extracted from sarcoma 37. I. Origin and introductory investigations. J. Natl. Cancer Inst. 24:933-951.

26. Moroni, C. 1972. Structural proteins of Rauscher leukemia virus and Harvey sarcoma virus. Virology 47:1-7.

27. Naso, R. B., L. J. Arcement, and R. B. Arlinghaus. 1975. Biosynthesis of Rauscher leukemia viral proteins. Cell 4:31-36.

28. Nowinski, R. C., E. Fleissner, N. H. Sarkar, and T. Aoki. 1972. Chromatographic separation and antigenic analysis of the oncornaviruses. II. Mammalian leukemia-sarcoma viruses. J. Virol. 9:359-366.

29. Raftery, M. A., and R. D. Cole. 1963. Tryptic cleavage at cysteinyl peptide bonds. Biochem. Biophys. Res. Commun. 6:467-472.

30. Rauscher, F. J. 1962. A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J. Natl. Cancer Inst. 29:515-543.

31. Schafer, W., G. Hunsmann, V. Moennig, F. Ne-Noronha, D. P. Bolognesi, R. W. Green, and G. Huper. 1975. Polypeptides of mammalian oncornavi-

ruses. II. Characterization of a murine leukemia virus polypeptide (p15) bearing interspecies reactivity. Virology 63:48-59.

32. Schwarz, H., G. Hunsmann, V. Moennig, and W. Schafer. 1976. Properties of mouse leukemia viruses. XI. Immunoelectron microscopic studies on viral structural antigens on the cell surface. Virology 69:169-178.

33. Seifert, E., M. Claviez, H. Frank, G. Hunsmann, H. Schwarz, and W. Schafer. 1975. Properties of mouse leukemia viruses. XII. Production of substantial amounts of Friend leukemia virus by a suspension tissue culture line (Eveline suspension cells). Z. Naturforsch. 30c:698-700.

34. Shapiro, S. Z., M. Strand, and J. T. August. 1976. High molecular weight precursor polypeptides to structural proteins of Rauscher murine leukemia virus. J. Mol. Biol. 107:459-477.

35. Slobin, L. I., and S. J. Singer. 1968. The specific cleavage of immunoglobulin polypeptide chains at cysteinyl residues. J. Biol. Chem. 243:1777-1786.

36. Steeves, R. A., M. Strand, and J. T. August. 1974. Structural proteins of mammalian oncogenic RNA viruses: murine leukemia virus neutralization by antisera prepared against purified envelope glycoprotein. J. Virol. 14:187-189.

37. Strand, M., and J. T. August. 1973. Structural proteins of oncogenic ribonucleic acid viruses. Interspec II, a new interspecies antigen. J. Biol. Chem. 248:5627-5633.

38. Strand, M., and J. T. August. 1974. Structural proteins of mammalian oncogenic RNA viruses: multiple antigenic determinants of the major internal protein and envelope glycoprotein. J. Virol. 13:171-180.

39. Strand, M., and J. T. August. 1976. Structural proteins of ribonucleic acid tumor viruses. Purification of envelope, core, and internal components. J. Biol. Chem. 251:559-564.

40. Strand, M., F. Lilly, and J. T. August. 1974. Host control of endogenous murine leukemia virus gene expression: concentrations of viral proteins in high and low leukemia mouse strains. Proc. Natl. Acad. Sci. U.S.A. 71:3682-3686.

41. Strand, M., R. Wilsnack, and J. T. August. 1974. Structural proteins of mammalian oncogenic RNA viruses: immunological characterization of the p15 polypeptide of Rauscher murine virus. J. Virol. 14:1575-1583.

42. Van Zaane, D., M. J. A. Dekker-Michielsen, and H. P. J. Bloemers. 1976. Virus-specific precursor polypeptides in cells infected with Rauscher leukemia virus: synthesis, identification and processing. Virology 75:113-119.

43. Witte, O. N., and I. L. Weissman. 1976. Oncornavirus budding: kinetics of formation and utilization of viral membrane glycoprotein. Virology 69:464-473.

44. Witte, O. N., I. L. Weissman, and H. S. Kaplan. 1973. Structural characteristics of some murine RNA tumor viruses studied by lactoperoxidase iodination. Proc. Natl. Acad. Sci. U.S.A. 70:36-40.

45. Wright, B. S., P. A. O'Brien, G. P. Shibley, S. A. Mayyasi, and J. C. Lasfargues. 1967. Infection of an established mouse bone marrow cell line (JLS-V9) with Rauscher and Moloney murine leukemia viruses. Cancer Res. 27:1672-1677.