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Structural Components of Mouse Mammary Tumor Virus
II. Isolation and Purification of Virion Polypeptides

MARY JANE YAGI,†* ROLAND E. STUTZMAN, BETTY H. ROBERTSON,†† AND RICHARD W. COMPANS

Department of Microbiology, University of Alabama Medical Center, Birmingham, Alabama 35294

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Mouse mammary tumor virus (MMTV) glycoproteins and nonglycosylated polypeptides were purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Primary amino groups were labeled with fluorescamine to enable visualization of MMTV polypeptides in the gels. Protein bands were sliced from the gels and eluted with 90 to 95% recovery. Eight MMTV polypeptides, including three of the major viral components as well as five minor proteins, were routinely obtained. Double diffusion assays and immunoelectrophoresis confirmed the retention of antigenicity identical to that of untreated MMTV virions. Antisera obtained from MMTV-free BALB/c mice immunized with these purified proteins reacted with the polypeptide immunogen as well as with detergent-disrupted MMTV virions from mouse milk or cell culture. Double diffusion assays using the specific mouse antisera failed to detect any cross-reactivity among the isolated polypeptides. A hemagglutination-inhibition assay demonstrated that the ability of MMTV virions to inhibit the hemagglutinating properties of influenza virus resides in the glycosylated polypeptides gp52, gp37.7, and gp33.

We have demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that mouse mammary tumor virus (MMTV) virions isolated from the MJY-alpha cell line were similar to MMTV derived from other in vivo and in vitro sources (21). The virions characteristically are composed of four major polypeptides, gp60, gp52, gp37.7, and p24, as well as six to eight of the minor components gp83, p46, gp33, p30, gp22, p17, p13, and p8. Biochemical and immunological analyses of individual MMTV virion polypeptides have previously utilized proteins isolated by a variety of ion-exchange and affinity chromatographic procedures (3, 9, 12) or polyacrylamide gel electrophoresis (16). These purification techniques have enabled isolation of the major internal polypeptide (p24), two major glycoproteins (gp52 and gp37.7), and one minor, nonglycosylated component (p15). However, these procedures have yielded only small amounts of these proteins, and purified preparations of other MMTV polypeptides have not been obtained. It has also been difficult to obtain preparations that are free of contaminating proteins and polypeptides that are unaltered in their antigenic specificities.

We have developed a procedure using fluorescamine to label MMTV polypeptides that enables direct isolation of viral proteins. MMTV polypeptides purified by this procedure were analyzed for their antigenicity, immunogenicity, and ability to inhibit the hemagglutination of chicken erythrocytes by influenza virus. The results of these analyses indicate that this procedure is reproducible and probably applicable for the isolation of other viral and nonviral polypeptides.

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† Present address: Department of Neoplastic Diseases, Mount Sinai School of Medicine, New York, NY 10029.

†† Present address: Department of Microbiology, University of Utah Medical Center, Salt Lake City, UT 84132.

MATERIALS AND METHODS

Virus and cells. MMTV virions were obtained from the chronically infected BALB/cfC3H/Crgl mouse mammary tumor cell line, MJY-alpha (18, 19). The cell culture conditions and procedures for isotopic labeling, viral harvests, and purification were carried out as previously described (18, 20, 21).

SDS-PAGE. Virus samples in 1% SDS and 1% β-mercaptoethanol were boiled for 1 min at 100°C. Samples were subjected to electrophoresis on slab gels having a 10 to 20% gradient of polyacrylamide with 0.27% *N,N'*-methylene-bisacrylamide and SDS, and a discontinuous buffer system as described by Laemmli (5).

Gels were stained with 0.25% Coomassie blue, scanned at 590 nm using a Schoeffel spectrodensitometer (model SD3000), and/or processed for determination of radioactivity as previously described (21). Molecular weights of MMTV polypeptides were determined with standard molecular-weight markers, ly-

sozyme (14,000), chymotrypsinogen (25,000), carbonic anhydrase (31,000), and pyruvate kinase (57,000).

Fluorescamine labeling and isolation of polypeptides. MMTV virions in 1% NH_4HCO_3 (pH 8.3) were labeled with 2 to 2.5 μg of fluorescamine per μg of viral protein as described by Udenfriend et al. (17). The labeling of the polypeptides was accomplished within a few seconds. Labeling of the ammonia in the buffer did not interfere with the analysis of the polypeptides. Labeled viral polypeptides were separated by preparative gel electrophoresis as described above. The thickness of the preparative gels varied from 1 to 3 mm depending on the amount of protein applied. Routinely, 3 to 5 mg of viral protein was applied to 1-mm-thick gels and 8 to 10 mg of protein was applied to 3-mm-thick gels. Fluorescent-labeled protein bands were visualized with a short-wave UV light source, sliced from the gel, crushed, and eluted with 0.01 M phosphate buffer (pH 7.2) or deionized water by incubation for 48 h at 37°C. The polypeptides were separated from the acrylamide gel suspension by filtration through glass wool.

MMTV polypeptides free of fluorescamine were also isolated by parallel electrophoresis of unlabeled MMTV and fluorescamine-treated, marker MMTV virions in adjacent wells. This procedure was carried out to determine whether the fluorescamine and/or the labeling procedure altered the antigenicity of hemagglutination inhibition (HI) activity of the isolated polypeptides.

HI. Assay of the ability of MMTV virions and isolated polypeptides to inhibit the hemagglutination of influenza virus was carried out as previously described (2, 21). Polypeptides eluted from preparative gels were first chromatographed on Bio-Gel P2 (100 to 200 mesh) to remove salts and SDS before use in the assay. SDS was also removed by the method of Boeyé (1) using precipitation of the detergent with saturated KCl. Protein concentration was determined by the Lowry method (7) and by optical density readings at 206 nm using a Beckman model 25 spectrophotometer.

Antisera. Anti-MMTV polypeptide antisera were raised in isogenic, MMTV-free BALB/c/Crgl female mice. Purified polypeptides (2 to 5 μg) in 0.05 ml were mixed with an equal volume of complete Freund adjuvant and injected at multiple sites subcutaneously. A second inoculation of 0.5 to 1 μg of protein was given in incomplete Freund adjuvant 2 weeks after the initial immunization. Mice were bled from the tail at weeks 2, 4, 5, and 6 and exsanguinated at week 7. Rabbit antisera against MMTV from BALB/cfC3H milk was kindly provided by Phyllis B. Blair, Department of Bacteriology and Immunology, and the Cancer Research Laboratory, University of California, Berkeley. Rabbit antisera against the endogenous, FUKU murine leukemia virus (MuLV) was obtained as previously described (18). All sera were heat inactivated at 60°C for 20 min prior to use in the immunological assays.

Immunological assays. Two-dimensional, double diffusion assays (IDA) were performed with 0.65% Noble agar as previously described (20). Single-dimension, double diffusion assays (Preer tests) were carried out in 2-mm capillaries as described by Preer (11) using 0.3% agarose in 0.05 M barbitol-sodium buffer (pH 8.6) containing 0.035% EDTA. The IDA and Preer tests were read for 3 to 10 days.

Immunoelectrophoresis (IEP) as described by Grabar and Williams (4) was carried out using Corning ACI universal agarose electrophoresis film and barbitol-sodium buffer. Electrophoresis of antigens (3 μl) was carried out at 10 V/cm for 1.5 h. After application of 10 μl of antiserum, the plates were incubated in a humidified atmosphere at 37°C for 24 to 48 h for the development of precipitin lines. The plates were washed with 0.85% NaCl (pH 7.2) for 24 h, followed by washing with deionized water for 0.5 to 1 h. The gels were stained with Coomassie blue, destained, and air dried as previously described (21).

Electroimmunoassay (EI) as described by Laurell (6) was carried out using 1% agarose in barbitol-sodium buffer containing 2.0 or 2.5% (vol/vol) rabbit anti-MMTV antiserum. Antigens in 5-μl volume were applied to 2.5-mm wells just before electrophoresis at 8.5 to 10 V/cm for 4 to 16 h. The gels were washed for 24 to 48 h in 0.85% NaCl (pH 7.2), followed by a 1- to 2-h wash in deionized water. Staining with Coomassie blue was carried out as described above.

Control antigens such as BALB/c milk and sera, endogenous MuLV (FUKU strain; Yagi, 1973), and fetal calf serum were included in each test.

Chemicals and isotopes. ^3H- and ^{14}C-labeled reconstituted protein hydrolysates and [^3H]glucosamine were obtained from Schwarz/Mann, Orangeburg, N.Y. Components for polyacrylamide gels and IEP were obtained from BioRad Laboratories, Rockville Centre, N.Y. Molecular-weight markers were obtained from Worthington Biochemical Corp., Freehold, N.J., and neuraminidase was from Behring Diagnostics, Somerville, N.J. Fluorescamine was manufactured by Roche Diagnostics, Nutley, N.J.

RESULTS

Isolation of MMTV polypeptides. MMTV virions were labeled with fluorescamine and analyzed by SDS-PAGE. Nine fluorescent bands were routinely detected in the gels. Untreated and fluorescamine-treated MMTV virions were compared by densitometer scans of Coomassie blue-stained gels or by determination of ^{14}C-amino acid and [^3H]glucosamine radioactivity to ascertain the identity of the fluorescent bands. No detectable change was observed in the migration of fluorescamine-labeled polypeptides when compared with untreated controls (Fig. 1). Eight of the fluorescent bands corresponded to MMTV proteins gp52, p46, gp37.7, gp33, p24, p17, p13, and p8. The ninth band, with a molecular weight of approximately 6,000, migrated similarly to a band thought to be glycolipid which is usually observed in this region (21). Coelectrophoresis of isolated, fluorescamine-treated ^3H-amino acid-radiolabeled polypeptides with ^{14}C-amino acid-labeled marker MMTV virus confirmed the identity of the fluorescent-stained protein bands.

The isolated fractions were analyzed by electrophoresis on a second discontinuous gradient

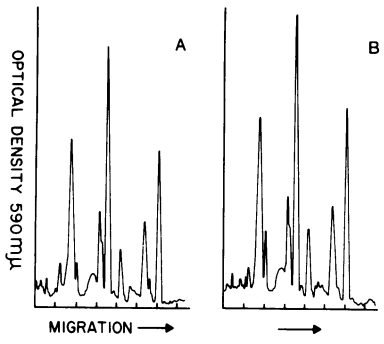

FIG. 1. *Densitometer scans of SDS-PAGE of MMTV polypeptides stained with Coomassie blue. (A) Control MMTV virions; (B) fluorescamine-labeled MMTV virions.*

gel to ascertain that the preparations contained only one protein band. Densitometer scans of a typical Coomassie blue-stained gel (Fig. 2) demonstrated the homogeneity of the fluorescamine-free preparations. Similar results were obtained when fluorescamine-labeled and radiolabeled preparations were analyzed. The degree of separation of the polypeptides on preparative gels was dependent on the linearity of the 10 to 20% acrylamide gradient as well as the length of the gel and the duration of electrophoresis. Complete separation of gp52 from p46 and gp37.7 from gp33 was initially difficult, as shown by their close migration in Fig. 1. This technical problem was solved by lengthening preparative gels from 14 to 21 cm and increasing electrophoresis time from 16 to 26 h. Continuous buffer exchange during electrophoresis also increased resolution of the protein bands and was found to be necessary for the separation of these polypeptides.

Recovery of isolated polypeptides from the gels was determined using ^{14}C-amino acid- and [^{3}H]glucosamine-labeled MMTV virions. The total counts applied to the gels and the levels of radioactivity found to be associated with the polypeptide bands by counting sliced gels were compared. Routinely, 65 to 70% of the counts applied were accounted for as MMTV proteins. Approximately 15 to 20% of the remaining radioactivity was associated with the band believed to be glycolipid at 6,000 daltons and also with several minor components of low electrophoretic mobility. The amount of radioactivity that could be eluted from the gel was compared with that remaining in the gel fragments and with the level of radioactivity in parallel gels directly processed for isotopic determination. These results revealed that 90 to 95% of the radioactivity associated with each band was recovered from the polyacrylamide gel.

Antigenicity and immunogenicity of isolated MMTV polypeptides. MMTV virions from BALB/cfC3H milk or the MJY-alpha cultures were analyzed by double diffusion (IDA, Preer tests) and immunoelectrophoretic (IEP, EI) techniques following disruption with 0.5% Triton X-100, 1% SDS, and 1% SDS–1% β-mercaptoethanol to determine the effects of the

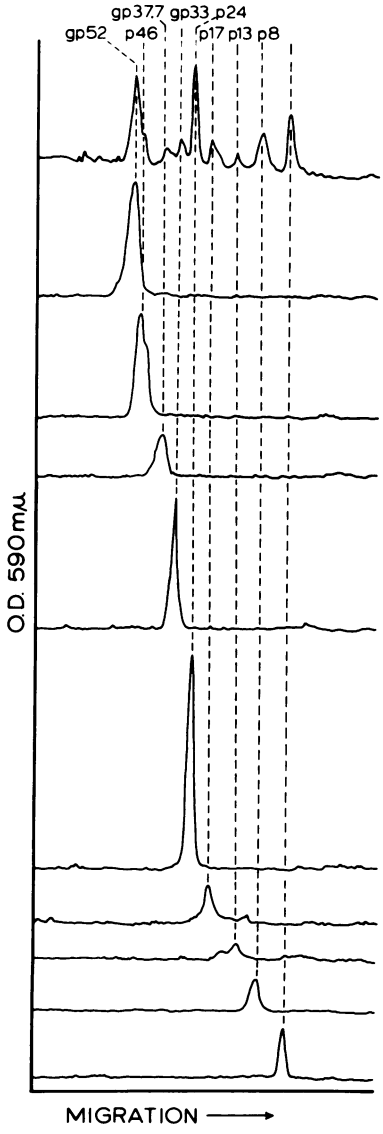

FIG. 2. *Densitometer scans of MMTV virion polypeptides and isolated, fluorescamine-free MMTV polypeptides subjected to electrophoresis in parallel on an SDS-polyacrylamide slab gel. The gel was stained with Coomassie blue, and the individual lanes containing polypeptides of intact virions (top) and the isolated polypeptides were scanned for comparison.*

detergents used in SDS-PAGE on the antigenicity of the viral proteins. Three broad bands were present in the one-dimensional Preer tests when rabbit anti-MMTV antiserum was reacted against MMTV disrupted by the three procedures. The bands were not sharply demarcated and often appeared as a broad smear with regions of greater opacity. Four precipitin lines were routinely observed in two-dimensional diffusion assays when utilizing the same antiserum and MMTV preparations. In either of these tests there was no observable change in the number and the migration rates of the precipitin lines, and in IDA lines of identity were formed between the three detergent-disrupted virion preparations.

When the same reagents were assayed by IEP and EI, differences in the anodal electrophoretic mobility and number of antigens detected were observed. Triton X-100-disrupted MMTV yielded five precipitin lines close to the antigen well in IEP (Fig. 3). In contrast, MMTV virion preparations disrupted for SDS-PAGE with SDS or SDS-mercaptoethanol yielded four and five precipitin lines, respectively, whch migrated rapidly toward the anode.

The number and height of the rockets formed in EI assays by disrupted MMTV also varied with the detergent used (Fig. 4). Five rockets were detected with Triton X-100-disrupted MMTV, whereas only two to three rockets were observed for SDS- and SDS-mercaptoethanol-disrupted virus. Both SDS- and SDS-mercaptoethanol-treated MMTV preparations yielded a slowly migrating, rocket-like smear within the other sharply defined rockets when the antigen was in excess. This smear was removed and an increase in the amount of precipitate associated with another rocket was observed when the antigen was diluted (Fig. 4, wells 4 to 6). Similar changes in anodal mobility and differences in the number of immunoprecipitates observed in EI assays with SDS-treated antigens have been previously reported by investigators studying other antigen-antibody systems (8, 13).

Detergent-disrupted, fluorescamine-treated

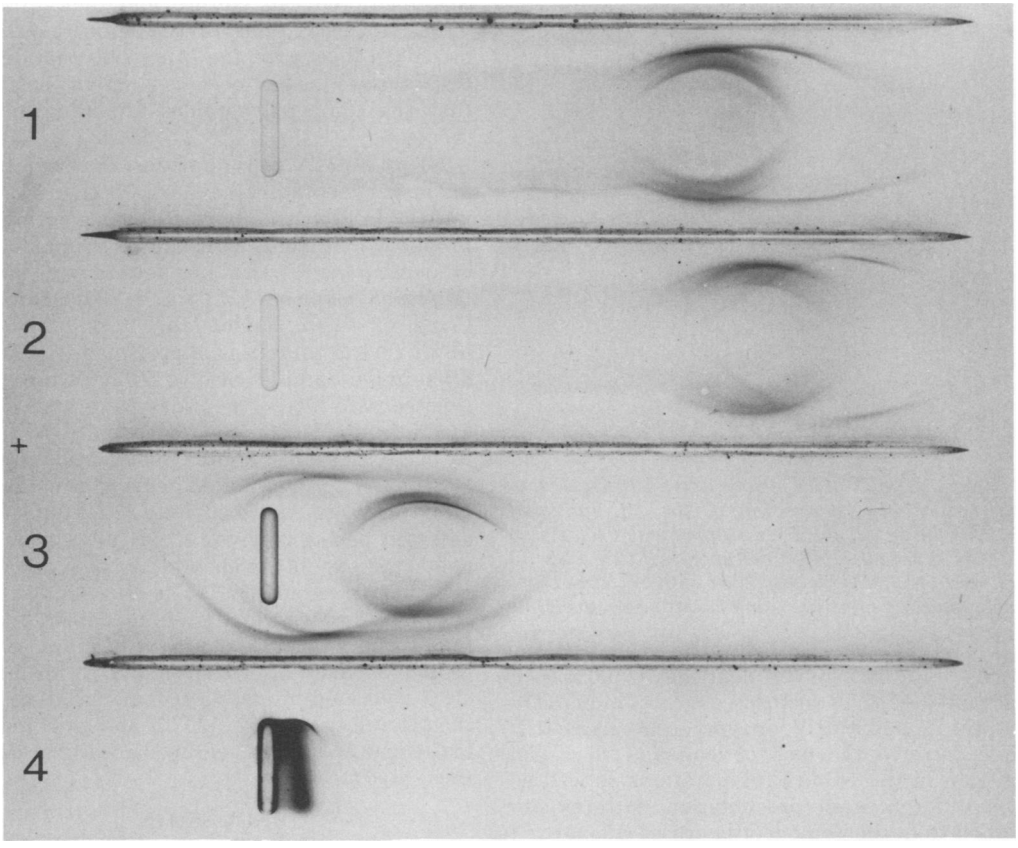

FIG. 3. *IEP of MMTV virions (10 μg) reacted with rabbit antiserum against MMTV from BALB/cfC3H milk. Wells: (1) MMTV disrupted with 1% SDS–1% β-mercaptoethanol; (2) MMTV disrupted with 1% SDS; (3) MMTV disrupted with 0.5% Triton X-100; and (4) untreated MMTV.*

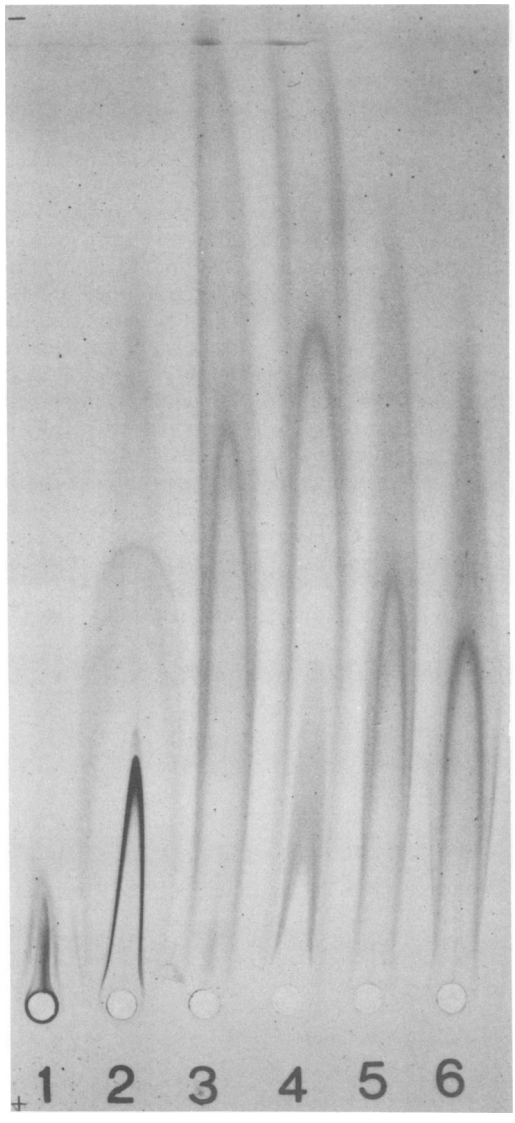

FIG. 4. *EI of MMTV virions and 2% (vol/vol) rabbit anti-MMTV antiserum. Wells: (1) untreated MMTV, 10 μg; (2) MMTV disrupted with 0.5% Triton X-100, 10 μg; (3) MMTV disrupted with 1% SDS, 10 μg; and (4–6) MMTV disrupted with 1% SDS–1% β-mercaptoethanol at 10, 5, and 2.5 μg, respectively.*

MMTV virions behaved identically to untreated, disrupted MMTV in these assays. Comparison of MMTV and MuLV virus preparations by IEP demonstrated the lack of leukemia virus p30 antigen in the MMTV preparations as well as the differences in precipitation patterns obtained from the two oncornaviruses (Fig. 5).

Rabbit anti-MMTV antisera reacted against both fluorescamine-labeled and unlabeled, isolated polypeptides (2 to 5 μg) in Preer tests and IDA. A single precipitin line was formed with each isolated polypeptide in either test. In IDA, these precipitin lines all formed lines of identity with one of the precipitin lines obtained from disrupted MMTV virions. No lines of identity were observed among the eight polypeptides (either labeled or unlabeled with fluorescamine) using 5 to 10 μg of MMTV protein and the rabbit antisera. EI of the fractions using rabbit anti-MMTV antiserum further demonstrated the retention of antigenicity in the polypeptide preparations. Typical rocket formation was obtained for each polypeptide against the rabbit antiserum (Fig. 6). Control antigens such as MMTV-free BALB/c milk and normal sera, disrupted MuLV, and fetal calf serum were negative in these tests.

Antisera were elicited against the eight isolated, fluorescamine-free polypeptides by immunization of isogeneic mice. Antibody reactivity was detected within 4 weeks after the initial injection by one-dimensional Preer tests. The mouse antisera precipitated the autologous, isolated polypeptide as well as Triton X-100- and SDS-disrupted cell culture and milk MMTV virus. However, none of the antisera formed precipitin lines with the other polypeptides in Preer tests. This lack of cross-reactivity suggests that the eight polypeptides are antigenically unique.

HI by MMTV polypeptides. Previous studies in our laboratory have shown that MMTV virions inhibit the normal hemagglutinating property of influenza virus due to the binding of influenza particles with the neuraminic acid-containing envelope of MMTV (21). The HI titer of 1 mg of fluorescamine-labeled or unlabeled MMTV viral protein is approximately 8,000 to 8,500 units. Samples of 5 to 50 μg of the eight isolated MMTV polypeptides were assayed to ascertain which components possessed HI activity. Only the glycosylated polypeptides, gp52, gp37.7, and gp33, inhibited hemagglutination by influenza virus. Approximately 22 HI units were detected per μg of gp52, 18 HI units were detected per μg of gp33, and 9 HI units were detected per μg of gp37.7. The HI activity of the glycoproteins was unaltered by labeling with fluorescamine. Prior treatment of either glycoprotein with 4.0 U of neuraminidase for 2 h at 37°C abolished HI activity, demonstrating that the HI ability of gp52, gp37.7, and gp33 resides in the neuraminic acid of the oligosaccharides of these glycoproteins.

DISCUSSION

The availability of isolated MMTV polypeptides and their respective antisera should greatly facilitate further studies of MMTV structure

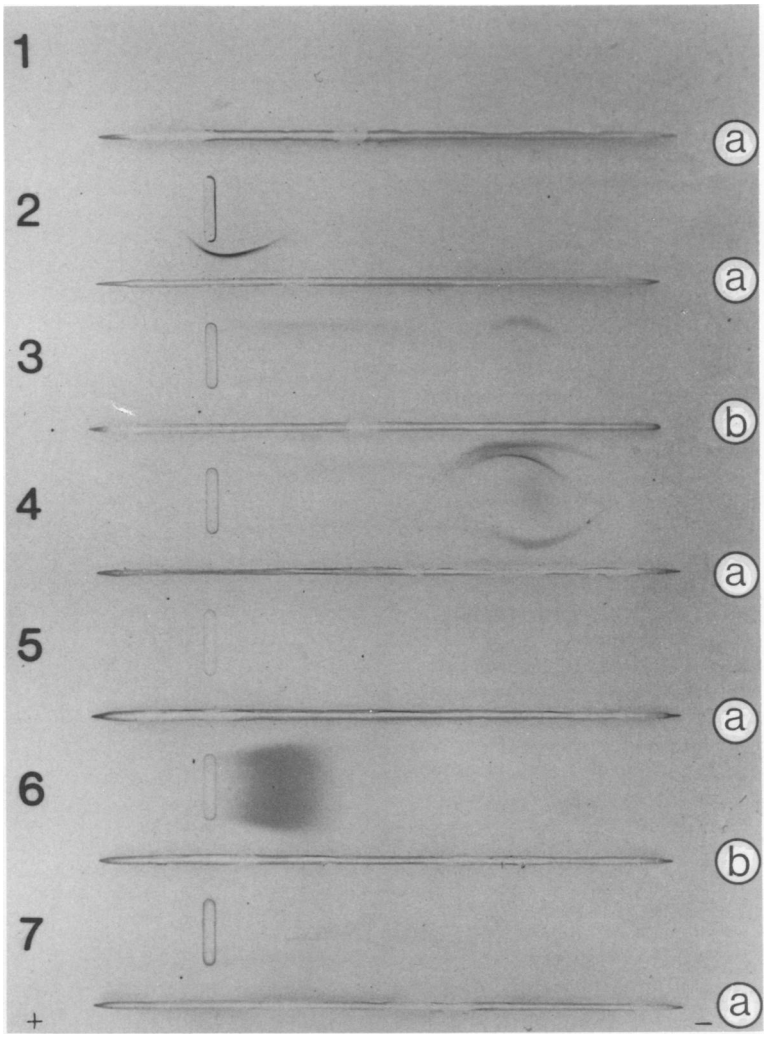

FIG. 5. *IEP of MMTV virions and control antigens with (a) rabbit anti-MuLV and (b) rabbit anti-MMTV antisera. Wells: (1) 1% SDS–1% β-mercaptoethanol; (2) MuLV disrupted with 0.5% Triton X-100; (3) MuLV disrupted with 1% SDS–1% β-mercaptoethanol; (4) MMTV and MuLV disrupted with 1% SDS–1% β-mercaptoethanol; (5) MMTV disrupted with 1% SDS–1% β-mercaptoethanol; (6) BALB/c serum; and (7) BALB/c milk.*

and replication. The procedure described in this report is rapid and yields polypeptides free of detectable contaminating proteins with efficient recovery. Unlike most of the procedures used previously, which enable isolation of one or two proteins, at least eight MMTV virion polypeptides can be obtained from a single preparative gel.

The double diffusion and EI assays demonstrated the retention of MMTV antigenicity of the isolated polypeptides. The use of rabbit anti-MMTV antisera elicited against untreated MMTV from mouse milk confirmed the antigenic identity of the virions from the MJY-alpha cell line as well as the eight polypeptides isolated from these virions. The inability of the rabbit anti-MMTV antisera to form more than five distinct precipitin lines against disrupted MMTV virions even though it does detect all eight isolated polypeptides is probably due to the relative concentration of the proteins in the virions and/or to the inability to obtain good separation of the proteins in Noble agar and agarose gels. The percentage of antigenically reactive protein in each polypeptide preparation has not been established. However, precipitin lines were observed in our IDA tests only when the antigen concentration was greater than 1 μg.

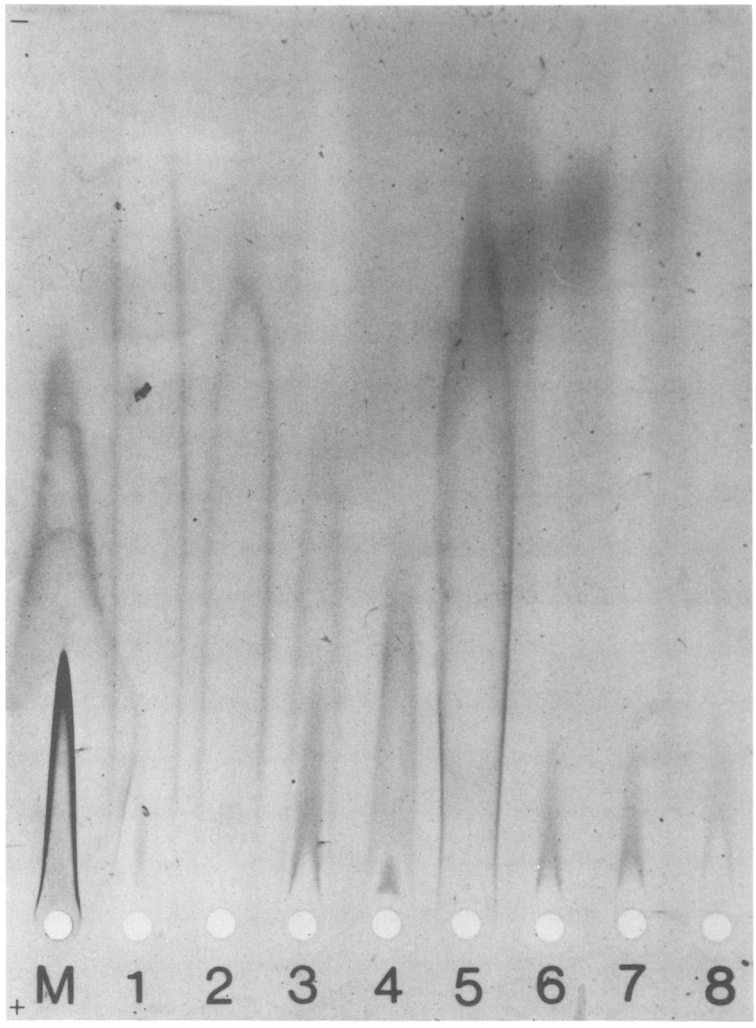

FIG. 6. *EI of isolated, fluorescamine-free MMTV polypeptides and 2% (vol/vol) rabbit anti-MMTV antiserum. Wells: (M) 10 μg of MMTV disrupted with 0.5% Triton X-100; (1-8) 2 to 5 μg of MMTV polypeptides gp52, p46, gp37.7, gp33, p24, p17, p13, and p8, respectively.*

For the results presented here, we utilized 2 to 5 μg of protein, which suggests that at least 20 to 50% of the polypeptides were antigenically active. Further assessment of the immunological integrity of the isolated polypeptides using radioimmunoassays is in progress. Parks et al. (10) reported that their gp52 preparations isolated by sequential ion-exchange and gel chromatography contained denatured polypeptides with altered antigenicity as discerned by radioimmunoprecipitation-inhibition assays. Native gp52 only partially inhibited precipitation of their isolated gp52 polypeptide; heating of the native gp52 resulted in complete displacement of the anti-gp52 antibody.

Preliminary immunization tests carried out in isogeneic mice indicated that the isolated polypeptides were immunogenic. The ability of the mouse antisera to precipitate the autologous polypeptides and antigens of disrupted MMTV virions not subjected to biochemical isolation procedures is additional evidence for the immunological integrity of the isolated proteins. Utilization of the isolated polypeptides and specific mouse antisera in IDA and Preer tests failed to demonstrate any cross-reactivity among the eight isolated MMTV proteins. This lack of reactivity could be due to the sensitivity of the assays, and studies are now in progress to ascertain whether this finding will be supported. Other investigators (9, 10; G. Schochetman, C. Long, and L. O. Arthur, Abstr. Annu. Meet. Am.

Soc. Microbiol. 1977, S21, p. 282) have reported that MMTV polypeptides gp52, gp37.7, p24, and p13 all possess distinct antigenic specificities which do not cross-react in radioimmunoassays. These studies must be extended to include the other glycoprotein, gp60, as well as all of the minor components associated with MMTV virions.

Our results indicating the lack of cross-reactivity of the eight polypeptides suggests that these proteins are unrelated and that none of the smaller components is a cleavage product of gp52. We have previously shown that gp60 and gp52 are readily cleaved when MMTV virions are exposed to proteases in cell cultures or under defined in vitro conditions (21). The cleavage products are both glycosylated and have molecular weights of 33,000 and 22,000. The molecular weights of these two components were based on SDS-PAGE analyses using 10% acrylamide gels in a continuous buffer system. We have repeated the analyses using the discontinuous-gradient gel system outlined here and have observed that the cleavage products have molecular weights of 37,000 and 22,000. This is in agreement with the molecular weights reported by Sheffield et al. (15) for gp52 cleavage products in a similar gel system. The lack of reactivity of mouse anti-gp52 to either gp33 or gp37.7 or of anti-gp33 and anti-gp37.7 to gp52 suggests that the 37,000-dalton cleavage product observed after protease treatment of MMTV is not identical to gp37.7 found in untreated virions. Further analyses of the relationship of the cleavage products of the glycoproteins to other MMTV polypeptides must be carried out using tryptic peptide analyses and antisera against specific polypeptides.

We have been unable to isolate the other polypeptides, gp83, gp60, p30, and p22, probably due to their low level in the virus or low reactivity with fluorescamine. The amounts of gp83, p30, and gp22 in our MMTV preparations vary, and it is possible that gp83 and p30 are contaminants and that gp22 is a cleavage product of gp60 and/or gp52 (21). The ^{14}C-amino acid radioactivity associated with these polypeptides ranges from 2.3 to 4.2% of the total viral radioactivity; therefore, the amount of fluorescence associated with these polypeptides should be small relative to the other virion polypeptides. For this reason their isolation will probably require identification of the protein bands by determination of radioactivity.

The HI assays demonstrated that the MMTV virion glycoproteins possessed HI activity. Our results (21) and those of Schloemer et al. (14) strongly indicate that HI activity is due to the presence of neuraminic acid residues in specific MMTV glycoproteins or glycolipids in the preparations. Comparison of the HI titers of gp52, gp33, and gp37.7 reveals that gp52 and gp33 have 2.4- and 2.0-fold-higher HI activity than gp37.7 per microgram of protein, suggesting that the neuraminic acid content of these two glycoproteins is greater than that of gp37.7.

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