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Structure of the Mouse Mammary Tumor Virus: Characterization of Bald Particles

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The polypeptide, antigenic, and morphological structure of the mouse mammary tumor virus was studied following protease digestion of intact virions. Intact, untreated virions ($\rho = 1.17$ g/ml) had characteristic envelope spikes, five major polypeptides, and were precipitated by antisera against gp52. Two of the major polypeptides, with molecular weights of 52,000 (gp52) and 36,000 (gp36), had carbohydrate moieties. Protease treatment resulted in spikeless, "bald" particles ($\rho = 1.14$ g/ml), which had altered surface antigenicity and which contained neither gp52 nor gp36. These data indicated that gp52 and gp36 were on the viral envelope. Bald particles retained a 28,000 dalton polypeptide (p28) which was proposed as the major internal polypeptide.

The recent development of dependable in vitro sources of the mouse mammary tumor virus (MTV) has made the detailed study of the structure of MTV possible (9, 25). Like other oncorna viruses, MTV has a lipid envelope and an electron-dense nucleoid surrounded by a core shell (18). Unlike the C-type viruses, MTV has stable surface projections, which are referred to as spikes (18). The virion is composed of at least 12 polypeptides, five of which are major polypeptides (9, 25). Three of the major polypeptides have molecular weights of 28,000 (p28), 14,000 (p14), and 10,000 (p10). The other two major polypeptides are glycoproteins with molecular weights of 52,000 (gp52) and 36,000 (gp36) (9, 16, 25). Two of the polypeptides, gp52 and p28, have been purified and characterized (16).

Surface iodination of intact MTV virions by use of the lactoperoxidase method primarily labels gp52 (3, 25, 26). This, together with the fact that glycoproteins of other viruses are usually surface components, (6, 7, 22) suggests an envelope location for gp52, rather than the internal position previously proposed (15, 14).

To further characterize the structure of MTV, surface components of MTV virions were removed by enzymatic digestion. The newly produced "bald" (spikeless) particles were isolated and their physical, immunological, and biochemical characteristics were examined. These studies provide evidence for an envelope location for gp52 and other MTV glycoproteins.

MATERIALS AND METHODS

Tissue culture. Spontaneous mammary gland tumors from multiparous BALB/cfC3H females, obtained from Simonsen Laboratories, Gilroy, Calif. or the Cancer Research Laboratory, University of California, Berkeley, were used for tissue cultures. The methods of trypsin-EDTA dissociation and cell plating used have previously been described (12). Cells were seeded into 75-ml flasks (Falcon) at a density of 1.0×10^6 to 1.2×10^6 cells per ml with 10 ml per flask of Dulbecco modified Eagle medium (Microbiological Associates) containing 15% fetal calf serum (FCS), 10 μg of insulin (Sigma Chemical Co., lot 121C-1350) per ml, 10 μg of hydrocortisone (Sigma Chemical Co., lot 103C-0440) per ml, and antibiotics. After 3 days, the culture fluid was replaced with medium containing 5% FCS, insulin, hydrocortisone, and antibiotics.

Virus purification. Virus-containing tissue culture fluids were clarified by low-speed centrifugation, and an equal volume of cold, saturated ammonium sulfate, previously adjusted to pH 7.4, was added dropwise. The precipitate obtained after centrifugation was resuspended in TEN buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.001 M EDTA, and 0.1 M NaCl) and serially centrifuged in discontinuous sucrose gradients and linear sucrose density gradients (25). All virus preparations monitored for MuLV and other contaminating antigens were negative (25).

Internal labeling of virus. MTV was labeled in tissue culture by adding either ^{3}H-reconstituted protein hydrolysate-algal profile or D-[^{3}H]glucosamine in Earle balanced salt solution using the previously described schedule (25). Counting was performed in aquasol (New England Nuclear Corp.) with or without trichloracetic acid precipitation in a Nuclear Chicago liquid scintillation counter.

External labeling of virus. Gradient purified MTV was externally labeled with ^{125}I (New England Nuclear Corp.) by using the lactoperoxidase technique (3, 26). Intact virions were labeled directly ([^{125}I]MTV). Radioactivity was determined by counting in a gamma spectrometer (Packard).

Immunodiffusion assay. Immunodiffusion tests were done by using 2.0% prepunched Noble agar

plates (Hyland). The plates were incubated at 37 C in a humidified chamber until precipitin lines developed.

The plates were washed in 0.1 M phosphate buffer, pH 7.0, for 24 h, fixed for 1 h in 7% acetic acid, and then stained overnight in 0.25% Coomassie blue dissolved in 7% acetic acid and 50% methanol. The plates were destained by washing in 7% acetic acid and 50% methanol, with a final washing in 7% acetic acid only.

Rabbit antisera against MTV purified from BALB/cfC3H mouse milk were kindly provided by Phyllis Blair, University of California, Berkeley (1). Goat antisera against purified gp52 and p28 were kindly provided by Wade Parks, National Cancer Institute. Chromatographed gp52 and p28 purified from RIII milk were also provided by Wade Parks (16).

RIP. The radioimmunoprecipitation (RIP) for MTV system has been described and characterized (3). Briefly, RIP was accomplished by adding 50 μliters each of [^{125}I]MTV (specific activity: 250,000 counts/min per μg of protein), antisera, buffer, and antiglobulin in sequence. After appropriate incubations, the mixture was centrifuged, and 100 μliters was removed and counted as supernatant. The rest of the mixture was counted as pellet.

For RIP inhibition, the sequential saturation technique was used (27). First, 50 μliters of test antigen was mixed with 50 μliters of antisera (50% end-point dilution) and incubated 2 h at 37 C. Then 50 μliters of [^{125}I]MTV was added followed by another 2-h incubation. Finally, 50 μliters of antiglobulin was added and the mixture was incubated for 1 h at 37 C, then 12 to 18 h at 4 C. The first 100 μliters and the remaining 100 μliters were collected and counted separately. All runs were calculated as percent of the control RIP.

The utilization of goat sera necessitated the development of a separate goat IgG (kindly provided by Wade Parks) and rabbit antiglobulin system. The equivalence zone for this system was identified by the tube precipitation test and all dilutions were performed to keep the secondary system in equivalence (3).

Protease treatment. Purified virus was pelleted at 250,000 × *g* for 20 min, resuspended in TEN buffer, and divided into two samples. An equal volume of *Streptomyces griseus* protease type VI (Sigma) in TEN buffer was added to one sample to a final concentration of 100 mg/ml. The same amount of TEN buffer was added to the other sample, which was used as a control. Both protease-treated and control samples were incubated at 37 C and the morphological results of treatment were monitored by electron microscopy. Samples were then rebanded in 15 to 65% sucrose density gradients for 2 h at 420,000 × *g*, and the resulting light scattering material or appropriate peaks of radioactivity were used for electron microscopic, immunological, and polyacrylamide gel analyses.

PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed with slight modification of previously described procedures (25). Eleven-centimeter gels contained 10 or 12% acrylamide, 0.4% bisacrylamide, and 0.1% sodium dodecyl sulfate (SDS) in 0.1 M PO_4 buffer at pH 6.8. Gel staining and processing for determination of radioactivity have been previously described (25).

Electron microscopy. Samples were pelleted at 250,000 × *g* for 20 min, resuspended in a small volume of TEN buffer, and Formvar-carbon coated grids were floated on a drop of the sample. Alternatively, 5 μliters were removed directly from reaction tubes and placed on grids. After fixation in 2.5% glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.3) and rinsing in distilled water, the grids were stained with 2% uranyl acetate, pH 4.5, containing 0.01% bovine serum albumin.

For thin sectioning, pelleted samples were fixed in glutaraldehyde, resuspended in 2% Noble agar, and postfixed in 1% osmium tetroxide in sodium cacodylate buffer for 1 h at 4 C. After dehydration in a graded series of ethanol and propylene oxide, the agar blocks were embedded in an Araldite-Epon 812 mixture. Thin sections were stained with uranyl acetate and Reynold lead citrate (17). Both negative-stained and thin-sectioned materials were examined with an AEI 6B electron microscope.

RESULTS

Production of bald particles. Initial digestion experiments on MTV with levels of protease used in other animal virus systems (13) met with little success. Thereafter, 100 mg of protease per ml was added and the morphological results of the treatment were monitored at 30- to 60-min intervals by electron microscope examination of negatively stained samples. Particles without spikes started appearing after 30 min of protease treatment. Very few particles with residual spikes were found after 60 min. A 120-min incubation with protease was used for standard preparations. In each experiment, the control samples were incubated in TEN buffer for the same period of time at 37 C.

Isopycnic centrifugation of intact virions of MTV purified from cell culture and incubated at 37 C for 2 h resulted in a light-scattering band at 1.17 g/ml in sucrose (Fig. 1A). This material has previously been characterized as the MTV B-particle (4, 5, 25). In contrast, centrifugation of enzymatically digested virions under the same conditions resulted in a light-scattering band at 1.14 g/ml in sucrose (Fig. 1B).

Distribution of label in gradients. When tritium-labeled B-particles were protease treated and isopycnically centrifuged, radioactive label was found in the 1.14 g/ml region and at the top of the gradient (Fig. 2). Between 80 and 90% of the [^{3}H]uridine label (Fig. 2A) and 40 and 50% of the [^{3}H]amino acid label (Fig. 2B) banded at 1.14 g/ml. In contrast, most of the [^{3}H]glucosamine label was found at the top of the gradient with less than 10% in the 1.14 g/ml region (Fig. 2C). Similarly, digestion of

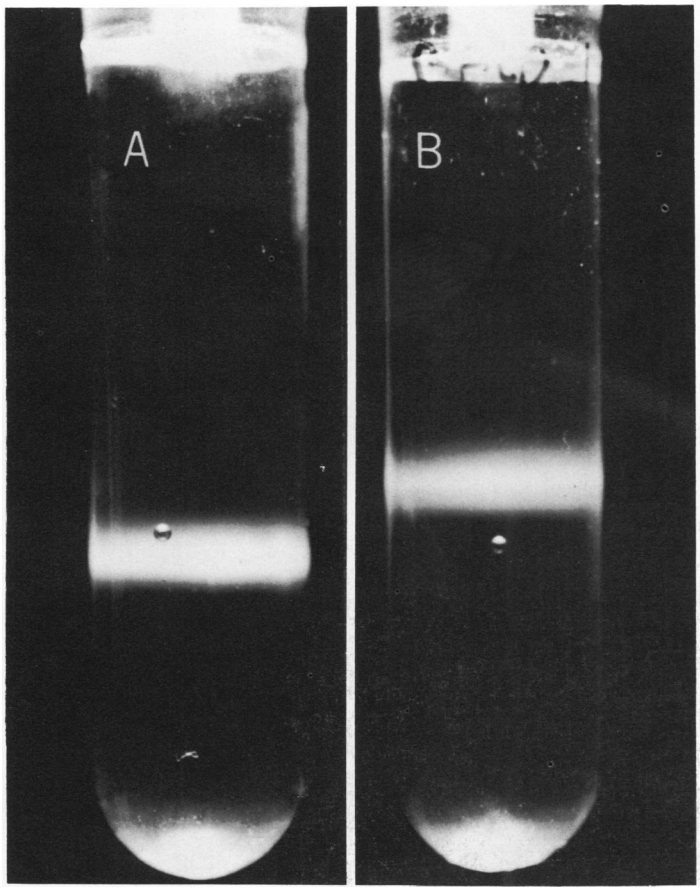

FIG. 1. *Sucrose density gradient centrifugation. Purified MTV was pelleted and resuspended in buffer. Protease (100 mg/ml) was added to one-half of the sample and buffer was added to the other half. Both samples were incubated at 37 C for 2 h, then layered onto 15 to 65% sucrose gradients and centrifuged for 15 h at 420,000 × g in the SW 65 rotor; (A) untreated control; (B) protease-treated sample. Each gradient contains a 1.16 g/ml density micule.*

virions which were iodinated intact resulted in solubilization of the ^{125}I with only a minor portion of the counts sedimenting with the 1.14 g/ml region (Fig. 2D). All the labeled materials found in the 1.14 g/ml region were acid precipitable and could be completely recycled by centrifugation into another sucrose gradient, whereas less than 25% of the label at the top of the gradients was acid precipitable.

Electron microscopy. Light-scattering bands from density gradients of treated and control samples were examined by electron microscopy. The control bands contained B-particles with the typical morphological structure, including spikes, in both negative-stained and thin-sectioned preparations and will be referred to as "intact" particles (Fig. 3A and B). The enzymatically digested particles contained the typical internal structure of MTV, with an eccentric nucleoid structure surrounded by a lipid envelope, but lacked the spike units found on the untreated controls (Fig. 3C and D). Such spikeless virions were dubbed "bald" particles.

Immunological characteristics. When reacted against NP-40 treated control B-particles, anti-MTV formed at least five precipitin lines (Fig. 4A). Three of the reactions were major, whereas two were very faint reactions and closer to the antigen well.

The anti-p28 formed at least two precipitin lines against B-particles (Fig. 4B). One of the reactions formed a line of identity with one of the anti-MTV precipitin lines. Two precipitin lines were formed between anti-p28 and the immunogen p28. These reactions formed lines of identity with bald particles and intact B-particles.

When the bald particles were reacted with anti-MTV, two of the major immunoprecipitin lines were missing (Fig. 4A). The third major

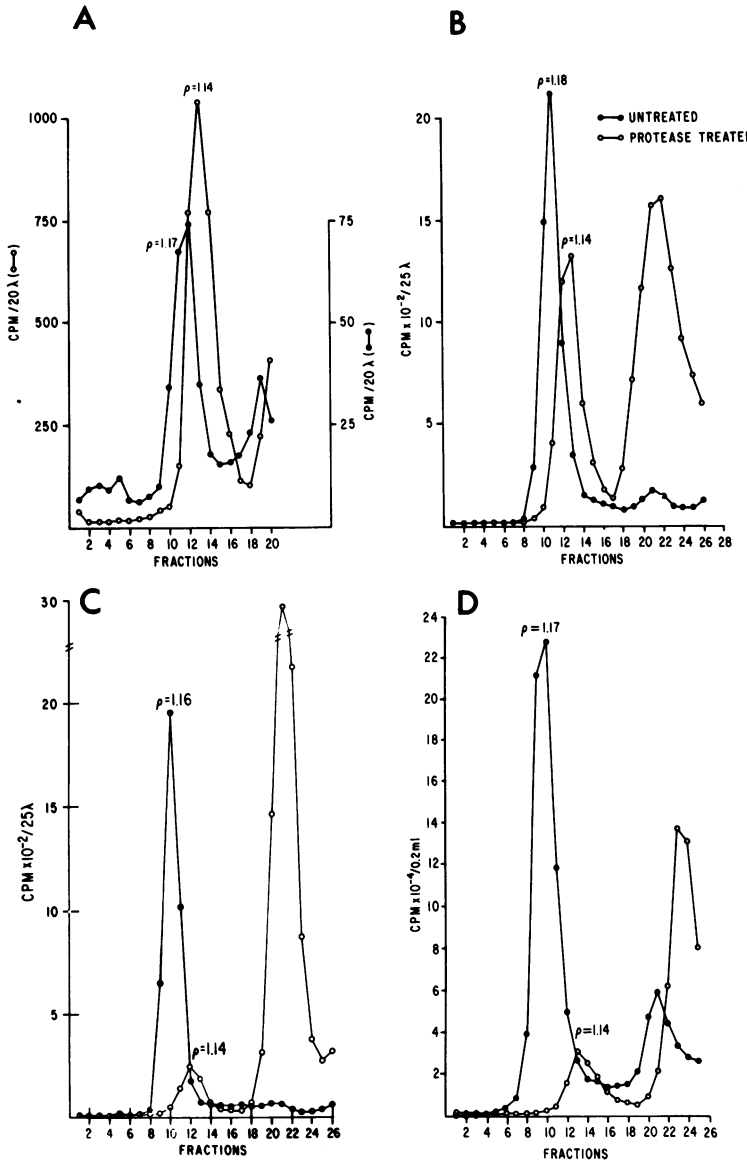

FIG. 2. *Sucrose density gradient centrifugation. Purified radioactive MTV virions were incubated at 37 C with or without protease. Samples were then rebanded in 15 to 65% sucrose density gradients at 420,000 × g for 2 h in the SW 65 rotor. Fractions were collected by bottom puncture and samples were counted. Densities were measured gravimetrically. (A) [³H]uridine-labeled MTV; (B) [³H]amino acid-labeled MTV; (C) [³H]glucosamine-labeled MTV; (D) ¹²⁵I-labeled MTV. Note that the protease-treated* (○) *and the untreated* (●) *samples were frequently not in equal quantities. Generally, much more radioactivity was placed in the protease treatment so that the residual material could be used in PAGE.*

immunoprecipitin line was retained in the bald particles. This line formed a line of identity with chromatographed p28 (Fig. 4A).

The two minor lines formed by anti-MTV were not detected in either the bald particles or the preparation of p28. The inability to detect these minor immunodiffusion lines may reflect the sensitivity of immunodiffusion and should not be considered as substantial evidence of their absence. The anti-MTV reacted with the preparation of p28 with a diffuse split line which may indicate the identification of two antigenic determinants.

RIP was used to further analyze the immuno-

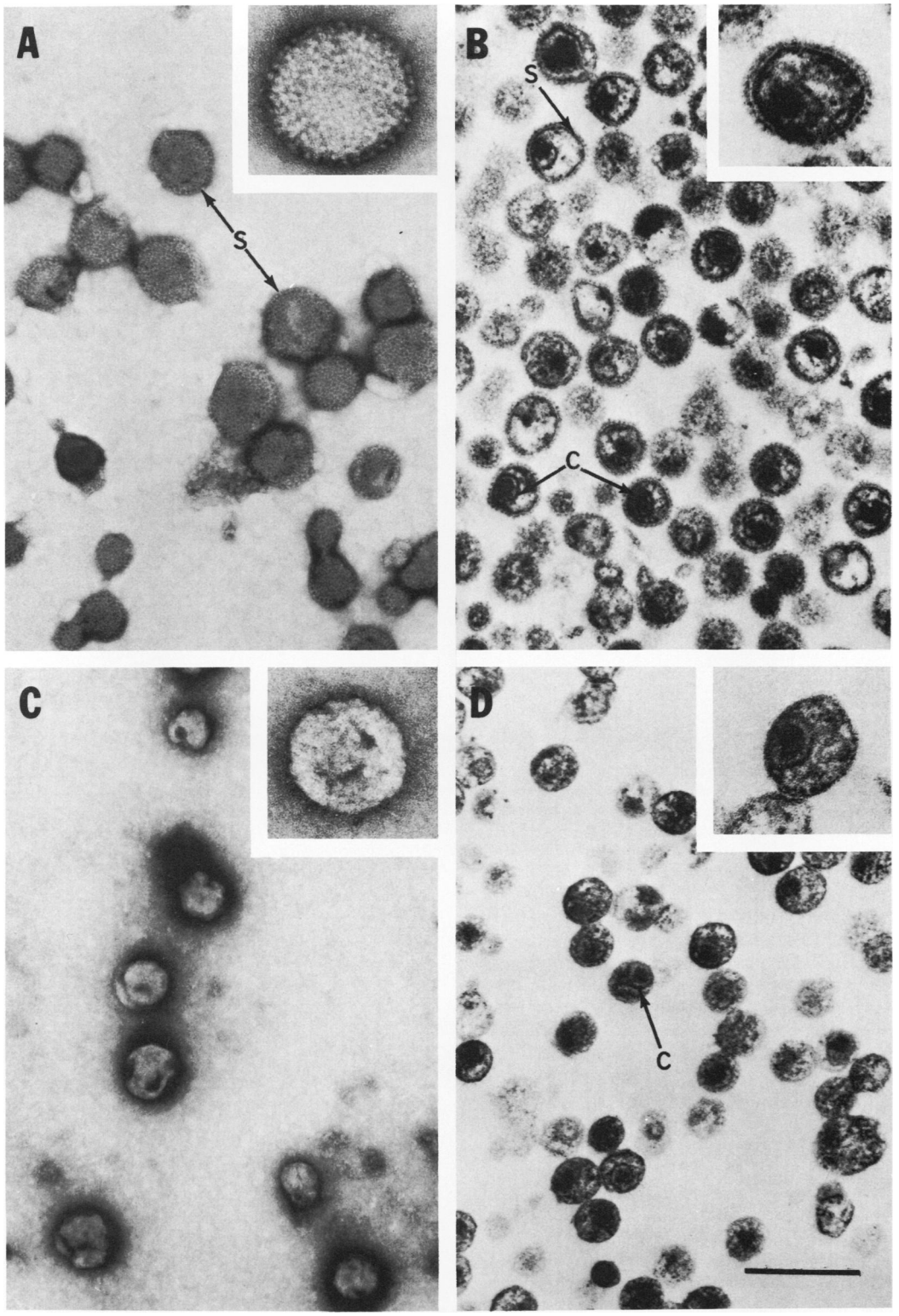

FIG. 3. *Electron micrographs of untreated and protease-treated MTV. Samples were treated as in Fig. 1 and were prepared for electron microscopy. (A) Negative-stained untreated MTV; (B) thin-sectioned untreated MTV; (C) negative-stained protease-treated MTV; (D) thin-sectioned protease treated MTV (×76,500). Spikes (S) and cores (C) are indicated. The insets are higher magnifications of typical particles in each preparation (×153,000). Marker bar is equal to 200 nm at 76,500.*

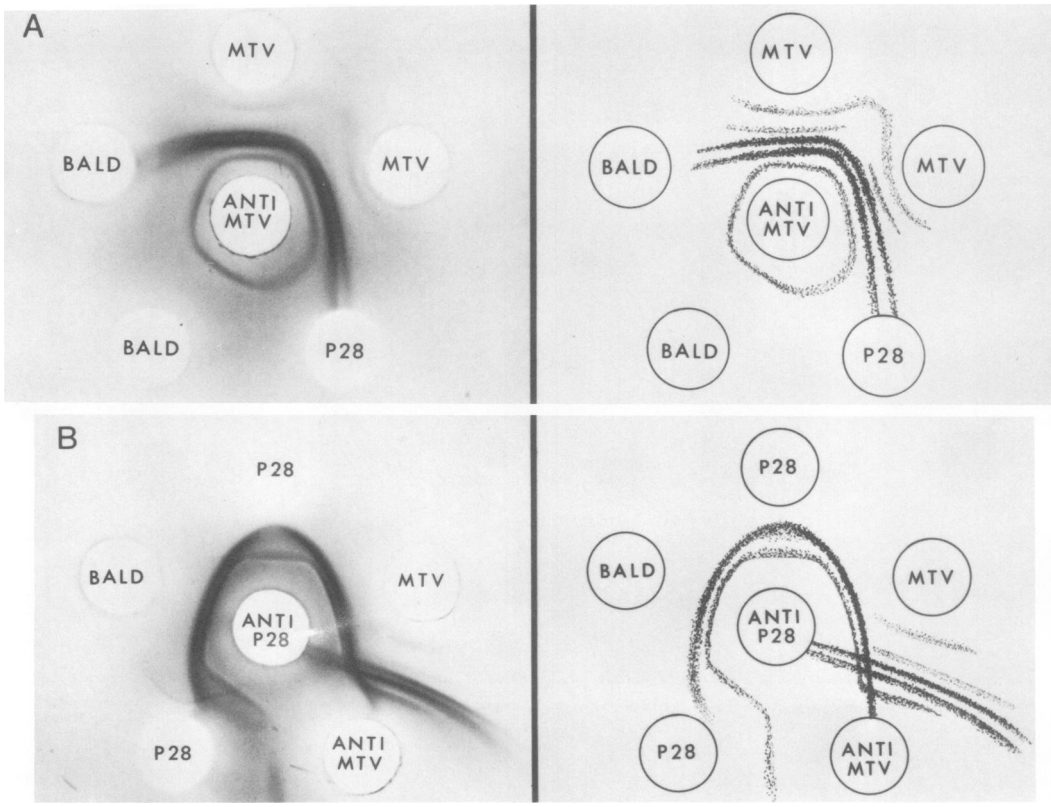

FIG. 4. *Immunodiffusion plates showing the immunological relationships of the various antigens used in these experiments. The bald and intact MTV virions were treated with NP-40 in order to solubilize the antigens before use. The plates were incubated 48 h and then were fixed, washed, and stained with Coomassie blue. (A) Anti-MTV, antisera against MTV virions; MTV, purified MTV (6 μg/well); p28, purified MTV p28 (1.4 μg/well); bald, protease MTV (10 μg/well); (B) anti-p28, antisera against purified p28; remainder as in A. The interpretive diagrams on the right emphasize the different relationships.*

logical characteristics of bald particles. The RIP of intact, iodinated MTV virions by anti-MTV and the inhibition of that RIP have been previously described (3). In the current experiments, RIP of [^{125}I]MTV was also achieved by antiserum against purified MTV gp52 (anti-gp52) (Fig. 5). In contrast, anti-p28 had relatively little ability to precipitate intact [^{125}I]MTV (Fig. 5). The RIP of [^{125}I]MTV with anti-gp52 could be inhibited by the addition of unlabeled MTV but not by BALB/c extract, dog milk, or C-type virus particles. Anti-MTV is also specific for MTV in RIP (3).

Purified gp52 and unlabeled MTV were compared as inhibitors of the RIP of [^{125}I]MTV with both anti-gp52 (Fig. 6A) and anti-MTV (Fig. 6B). Unlabeled MTV inhibited the RIP reactions with both antisera and the displacement curves were roughly parallel (Fig. 6 and 7). Purified gp52 completely inhibited the RIP of [^{125}I]MTV with anti-gp52. The gp52 displacement curve, however, was not totally parallel to

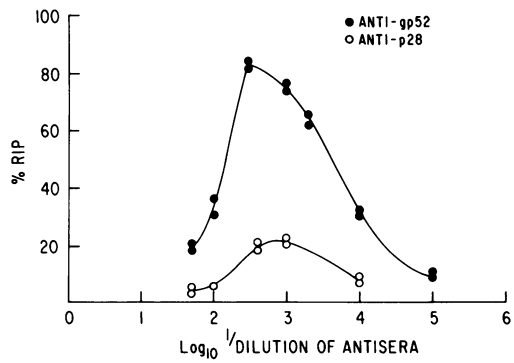

FIG. 5. *Radioimmune precipitation of [^{125}I]MTV. [^{125}I]MTV (40 ng) was reacted with serial dilutions of antisera against purified gp52 (●) or purified p28 (○).*

the MTV curve (Fig. 6A). In contrast, gp52 only partially inhibited the RIP of [^{125}I]MTV with anti-MTV (Fig. 6B). At the highest concentrations of gp52 available, only 60 to 80% inhibition

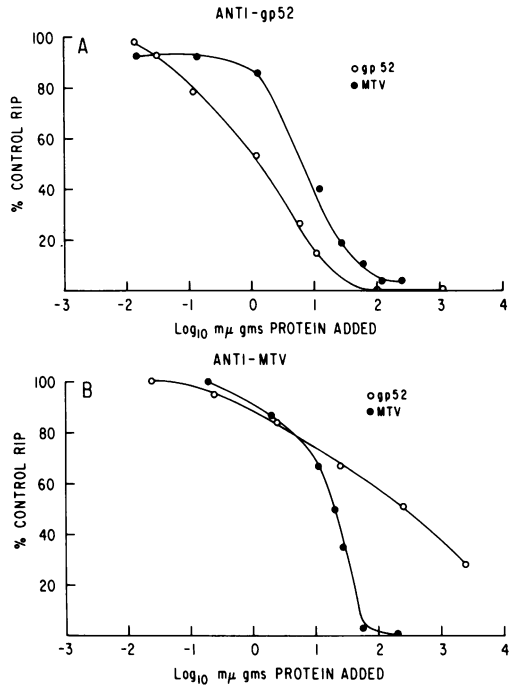

FIG. 6. *Inhibition of the RIP of [^{125}I]MTV. Increasing amounts of purified MTV gp52 (O) or unlabeled MTV (●) were added to tubes containing [^{125}I]MTV (40 ng) and either anti-gp52 at a dilution of 1:3,000 (A) or anti-MTV at a dilution of 1:200,000 (B). Data are expressed as percent of control RIP. The control RIP in each case was 50%.*

was achieved. The displacement curve using gp52 was not at all parallel to the curve generated by using unlabeled MTV as the inhibitor (Fig. 6B).

Although gradient-purified bald particles were able to inhibit the RIP reactions, 100 to 500 times more protein was required to displace [^{125}I]MTV (Fig. 7). This represented a 2.5 log reduction in the antigenicity of bald particles relative to control intact virions.

PAGE analysis. When intact iodinated virus was disrupted and electrophoresed (25), 90% of the ^{125}I appeared in a peak corresponding to gp52 (Fig. 8) (3, 25, 26). Enzymatic digestion of intact iodinated virions resulted in a 90% loss of ^{125}I (Fig. 2D) and a shift of the residual label from a peak corresponding to 52,000 daltons to the middle and lower molecular weight regions of the gel (Fig. 8). Within 0.5 h, almost all the radioactivity had been eliminated from the region of gp52 by protease treatment (Fig. 8). By 1 and 4 h more of the ^{125}I appeared as heterodispersed counts at the lower end of the gel with no readily definable pattern (Fig. 9).

Electropherograms of [^{3}H]amino acid-labeled bald particles (Fig. 9) and densitometer tracings of Coomassie blue-stained gels of bald particles revealed the loss of the higher molecular weight proteins with a retention of the p28 peak. In particular, gp52 and gp36 were missing, and p28 was the major protein of the bald particles. The lower end of such gels contained heterodispersed counts and stain which resembled the pattern observed after the digestion of ^{125}I-labeled intact particles (Fig. 8). The heterodispersed counts obscurred the normal pattern of the lower-molecular-weight polypeptides.

Glucosamine-labeled intact particles contained the major glycoproteins (Fig. 10) but neither gp52 nor gp36 were resolved in either the bald particles or the acid precipitable [^{3}H]glucosamine-labeled material from the top of the gradient (Fig. 2C). In a like manner neither gp52 nor gp36 could be resolved by periodic acid Schiff (PAS) staining of gels after electrophoresis of the bald particles.

DISCUSSION

The experiments reported herein provide immunological and biochemical evidence that the major glycoproteins, in particular, gp52, are on the surface of the MTV virion. This conclusion is based primarily on the characteristics of B-particles after enzymatic digestion.

Protease digestion resulted in a shift in the bouyant density of MTV virions from 1.17 to 1.14 g/ml. This shift would be consistent with a loss of protein and retention of the lipid envelope (22). The density shift was accompanied by more than a 90% loss of glucosamine label and of iodinated gp52 but only 50% loss of total

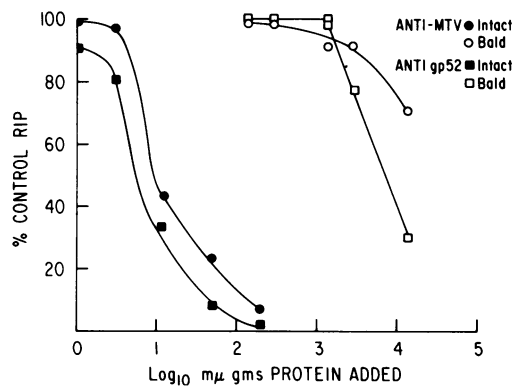

FIG. 7. *Inhibition of the RIP of [^{125}I]MTV by intact or bald particles. Increasing amounts of unlabeled intact MTV (●, ■) or bald particles (O, □) were added to tubes containing ^{125}I-MTV (40 ng) and dilutions of either anti-MTV (●, O) or anti-gp52 (■, □) giving approximately 50% RIP. Data are expressed as percent of control RIP.*

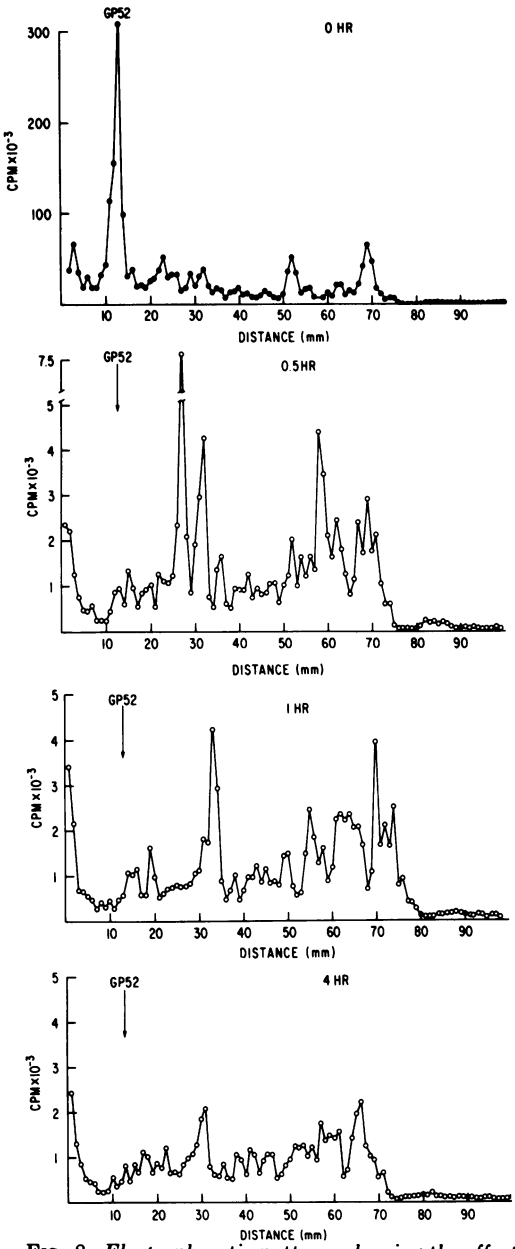

FIG. 8. *Electrophoretic patterns showing the effect of duration of protease treatment on ^{125}I-labeled MTV virions. Iodinated intact virions were treated with protease for the time periods indicated and were centrifuged in separate density gradients. The radioactive peaks from the 1.17 or 1.14 g/ml region from each gradient were analyzed on SDS-polyacrylamide gels. The arrows mark the usual migration distance of gp52.*

protein label and 10% loss of labeled RNA. These observations would be consistent with proteolysis limited to surface components. Electron microscope observations confirmed this hypothesis by revealing bald particles at the 1.14 g/ml density which lacked spikes but retained the envelope and internal core structure.

Bald particles also had a reduced surface antigenicity. Displacement of reference MTV in RIP required 100 to 500 times greater mass of bald particles than control intact MTV. The reduced capacity of the bald particle to inhibit was observed with both anti-MTV and anti-gp52, implying the reduction of antigenicity was due, in part, to loss of gp52. In a like manner, enzymatic treatment resulted in a loss of two of three major antigens identified in immunodiffusion by anti-MTV.

Finally, the major glycoproteins (gp52 and gp36) were missing from the bald particles. This was determined by PAGE comparison of externally labeled, internally labeled, Coomassie blue-stained, and PAS-stained bald particles and intact particles.

The 52,000 dalton glycoprotein of MTV was originally thought to be an internal component (15, 14). The following data would place this polypeptide on the envelope surface: (i) the 52,000 dalton polypeptide is a glycoprotein (9, 11, 16, 25); (ii) gp52 is the only major polypeptide accessible for surface iodination (3, 25, 26); (iii) antisera directed against gp52 precipitates intact virus; (iv) purified gp52 inhibits the RIP of MTV; (v) gp52 is removed by a protease digestion which leaves the internal morphology of MTV intact; (vi) the surface antigenicity of bald particles is drastically reduced. We conclude from these experiments that gp52 is on the envelope surface.

The accessibility of gp52 to iodination and virion-precipitating antibodies suggests that it may be superficial to gp36 and the other glycoproteins (8, 10, 19, 23). Removal of gp52 with a treatment which also removed the characteristic spikes of MTV leads us to suggest that it is the major component of the spike. Although this hypothesis is attractive, other interpretations of the data are also possible. For example, gp52 could be located either between and/or external to the spikes (20, 21). The spike may be composed of other proteins and the enzymatic removal of spikes and gp52 may be merely coincidental. Definite assignment of gp52 must await a more definitive line of experimentation, such as the isolation and characterization of spikes.

Analysis of the RIP displacement curves suggested that gp52 was not the only antigen on the virion surface. The pattern of RIP inhibition by purified gp52 in the presence of anti-MTV differed from that observed with anti-gp52. With anti-gp52, the inhibition was complete.

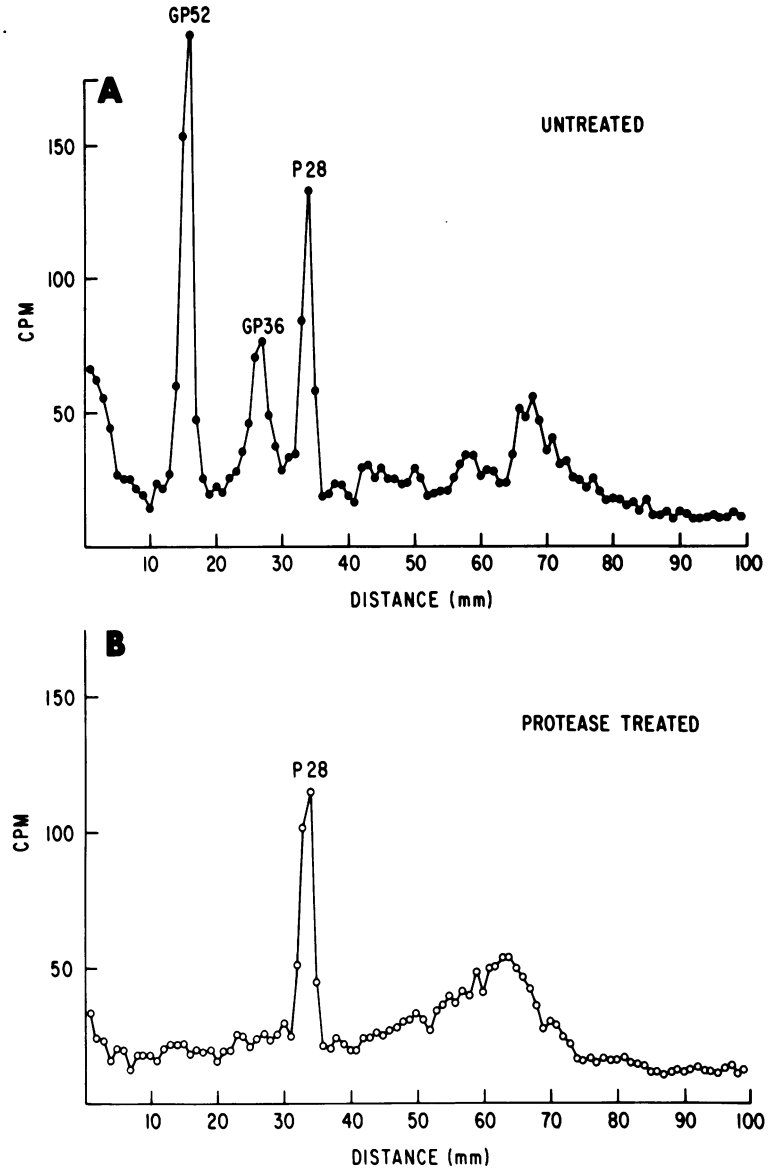

FIG. 9. *Electrophoretic patterns of untreated and protease-treated [^3H]amino acid-labeled MTV. Purified [^3H]amino acid-labeled MTV virions were incubated at 37 C for 2 h with or without protease. Both samples were rebanded in separate sucrose density gradients and the appropriate peaks of radioactivity (Fig. 7A) were examined on SDS-polyacrylamide gels. (A) Untreated; (B) protease treated.*

With anti-MTV, however, the inhibition was incomplete in the ranges of gp52 available, and the displacement curve using gp52 was not parallel to the displacement curve using intact virion as inhibitors. The most likely explanation of these different patterns would be that the two sera identify different antigens. This may be either because (i) gp52 had different configurations in the intact virion, or (ii) entirely separate antigenic proteins were identified by anti-MTV.

The immunodiffusion data favor, but do not prove, the second alternative. Intact MTV had two major antigens which were absent from bald particles. This implies at least two distinct antigenic surface components exist. Glycoproteins other than gp52 could be identified in MTV. These glycoproteins were also removed by protease digestion. The 36,000 dalton moiety was particularly prominent and it, along with the other glycoproteins, may contribute to the virion surface antigenicity.

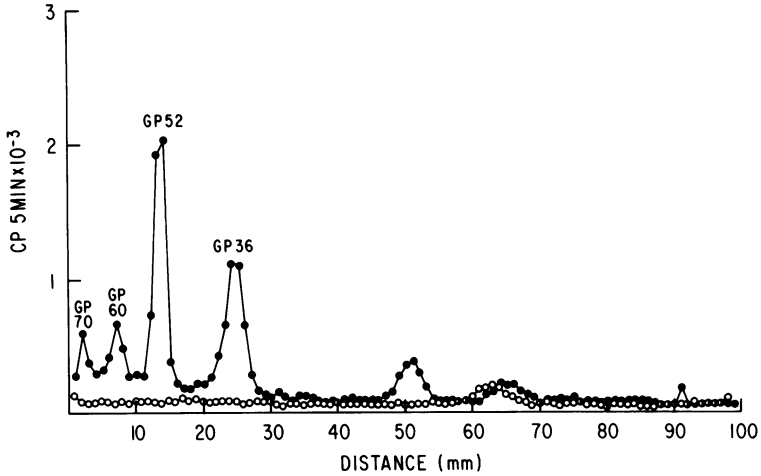

FIG. 10. *Electrophoretic patterns of untreated and protease-treated MTV virions labeled with [^3H]glucosamine. Purified [^3H]glucosamine-labeled MTV virions were incubated at 37 C with or without protease treatment. Both samples were rebanded in separate sucrose density gradients and the appropriate peaks of radioactivity (Fig. 7C) were examined on SDS-polyacrylamide gels. Untreated (●); protease treated (○).*

The major polypeptide detected in the bald particle was p28. This protein was identified in bald particles immunologically and biochemically. Antisera directed against p28 did not RIP intact virions, but anti-p28 did react with detergent disrupted particles in immunodiffusion. The polypeptide, p28, is most likely an internal component of MTV. This hypothesis was reinforced by preliminary identification of p28 in preparations of MTV cores (Teramoto, et al., Bacteriol. Proc. p. 227, 1974). Should these observations be confirmed, p28 would bear a remarkable similarity to the major core shell protein of the type C particles (2, 24).

Without doubt, other polypeptides also contribute to the internal structure of the virion. They were obscurred, however, in the gels by the creation of heterodispersed material by digestion of the surface glycoproteins. Such material reduces resolution and obscures detail at the lower end of the gels, making interpretation very difficult. This was particularly true for p10 and p14 which represent the other two "major" polypeptides of MTV. The data presented here do not provide any information concerning these polypeptides.

The availability of internally labeled MTV from tissue culture, the development of radioimmune assays, and the adaptation of external labeling techniques have led to more thorough studies of the virion structure (3, 9, 16, 25, 26). These and newer approaches to structural studies will stimulate a more comprehensive understanding of the structure and function of MTV.

ACKNOWLEDGMENTS

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