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Structure of the Mouse Mammary Tumor Virus: Polypeptides and Glycoproteins

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The polypeptide and glycoprotein compositions of the mouse mammary tumor virus virion from primary monolayer cultures of BALB/cfC3H mouse mammary tumor cells were studied by polyacrylamide gel electrophoresis by using internal and external labeling and Coomassie blue and periodic acid Schiff (PAS) staining. Twelve polypeptides were reproducibly resolved by the combined methods. Five major polypeptides were demonstrable with estimated molecular weights of 52,000, 36,000, 28,000, 14,000, and 10,000. Seven minor polypeptides were also consistently detected and had estimated molecular weights of 70,000, 60,000, 46,000, 38,000, 30,000, 22,000, and 17,000. Carbohydrate was associated with five of these polypeptides as measured by PAS stain or [^{3}H]glucosamine labeling, or both. These glycoproteins had estimated molecular weights of 70,000, 60,000, 52,000, 36,000 and 10,000. The majority of the PAS stain and glucosamine was found in the 52,000 and 36,000 dalton peaks.

Although a number of biochemical and biophysical characteristics of mouse mammary tumor virus (MTV) have been described, relatively little is known about the proteins of MTV (1). By using different techniques, several investigators have reported the separation of five (19, 25), seven (11), and possibly eight (23) polypeptides associated with MTV virions purified from milk. Variation in the descriptions and interpretations of MTV polypeptides may be due to the differences in techniques used (23) or in the use of milk as a source of virus, or both, because MTV from milk has been difficult to separate from milk proteins (1).

In order to avoid the purification problem associated with MTV from mouse milk, we purified MTV from primary cultures of mammary tumor cells. Such an in vitro system is relatively free from contaminating proteins, produces adequate quantities of virus, and permits internal labeling. We report here an analysis of the polypeptide and glycoprotein compositions of MTV by use of SDS-polyacrylamide gel electrophoresis. The polypeptide and glycoprotein compositions of MTV as revealed by external [^{125}I]labeling, internal [^{3}H]labeling, and Coomassie blue and periodic acid Schiff (PAS) staining are compared.

MATERIALS AND METHODS

Tissue cultures. Spontaneous mammary gland tumors from multiparous BALB/cfC3H females, obtained from the Cancer Research Laboratory, University of California, Berkeley, were used for all tissue cultures. The method of trypsin-EDTA dissociation and cell plating used was similar to that described previously (13). Cells were seeded into 75-cm^2 flasks (Falcon) at a density of 1.0 to 1.2 $\times$ 10^6 cells/cm^2 with 10 ml per flask of Dulbecco modified Eagle medium (Microbiological Associates) containing 15% fetal calf serum (FCS), 10 μg of insulin per ml, 10 μg of cortisol per ml, and antibiotics. After 3 days, the culture fluid was replaced with medium containing 5% FCS, insulin, cortisol, 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. After centrifugation at 10,400 $\times$ g for 15 min, the precipitate was resuspended in TEN buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.001 M EDTA, and 0.1 M NaCl). By using previously described procedures (6, 7), the resuspended virus solution was serially centrifuged in discontinuous sucrose gradients, followed by isopycnic centrifugation in linear sucrose gradients. Virus protein was estimated by the technique of Lowry et al. (12). All virus preparations were tested by immunodiffusion for the presence of MTV and other antigens.

Internal labeling of virus ([^{3}H]MTV). MTV was labeled in tissue culture by adding either ^{3}H-reconstituted protein hydrolysate-algal profile (Schwarz/Mann) (15 μCi/ml) or D-[^{3}H]glucosamine (New England Nuclear Corp.) (20 μCi/ml) in Earle balanced salt solution containing 2% dialyzed FCS, insulin, cortisol, and antibiotics. Virus was purified as described above from the culture fluid after 24 h of continuous labeling. Radioactivity was determined, after the addition of 10 ml of Aquasol (New England

Nuclear Corp.) to 0.025-ml samples of each gradient fraction, by counting in a liquid scintillation counter (Nuclear-Chicago).

External labeling of virus ([^{125}I]MTV). Gradient-purified MTV was externally labeled with ^{125}I (New England Nuclear Corp.) by using the lactoperoxidase technique (22, 25). Intact virions were labeled directly. Virion polypeptides were also labeled after disruption for 30 min at 4 C with 2% NP-40 (Shell Chemical Co.). Radioactivity was determined by counting in a gamma spectrometer (Packard).

Immunodiffusion assay. Immunodiffusion tests were done by using 0.6% Noble agar in 0.85% NaCl containing 1:10,000 merthiolate and buffered to pH 7.2 (2). The plates were incubated at 37 C in a humidified chamber, and precipitin lines usually developed within 24 h.

Antiserum against MTV purified from mouse milk was kindly provided by Phyllis Blair, University of California, Berkeley. Antisera against FCS, cell-free extracts of BALB/c lactating mammary glands, and purified mouse casein (10, 16) were prepared in rabbits according to previously described procedures (2). Goat antiserum against AKR murine leukemia virus was provided by Huntingdon Laboratories under National Cancer Institute contract NIH-NCI-69-54.

BALB/c milk was kindly provided by Phyllis Blair. AKR virus, lot 270-32-13, was provided by Electro-Nucleonics Laboratories under National Cancer Institute contract NIH-NCI-72-3249. Type-C virus, spontaneously arising from cell cultures of a BALB/c splenic tumor, was kindly provided by C. M. McGrath, Michigan Cancer Foundation, Detroit, Mich., under National Cancer Institute contract NIH-NCI-E-71-2421.

Polyacrylamide gel electrophoresis. Virion polypeptides were prepared for polyacrylamide gel electrophoresis by pelleting purified MTV at 250,000 × g for 20 min, resuspending the virus in 50 to 100 μliters of 4% sodium dodecyl sulfate (SDS), 8 M urea, and 0.2 M dithiothreitol (DTT) in 0.01 M PO_4 buffer, pH 6.8, and heating for 4 min at 100 C. Polypeptides from disrupted virions were precipitated in 10% cold trichloroacetic acid, centrifuged at 5,000 × g for 15 min and resuspended and heated as described above.

SDS-polyacrylamide gel electrophoresis was performed according to Maizel (13) by using 10% acrylamide (recrystallized from acetone) with 0.33% bis-acrylamide, 0.1% SDS, and 4 mM DTT in 0.1 M PO_4 buffer at pH 6.8. Gels (10 cm) were polymerized with 0.2% ammonium persulfate in glass columns (12.5 by 0.5 cm) and were pre-electrophoresed for 1 h at 5 mA/gel. Approximately 100 to 150 μg, or 30,000 counts/min ([^{3}H]MTV) to 100,000 counts/min ([^{125}I]MTV), were applied to each gel with bromophenol blue as a marker dye. The samples were electrophoresed at ambient temperature (22 to 25 C) at 12 mA/gel for 3 h. Standard proteins of known molecular weights were electrophoresed in parallel gels with each run.

Gel staining and analysis of radioactivity. Gels containing unlabeled virus proteins were extruded from the glass tubes, washed, and fixed overnight in a solution of 20% sulfosalicylic acid. The gels were stained with 0.25% Coomassie blue in 50% methanol and 7% acetic acid for 2 to 3 h. Gels were destained for 3 h in 50% methanol and 7% acetic acid, then overnight in 7% acetic acid.

A modified PAS technique similar to that described by Bolognesi and Bauer (4) was used to stain polysaccharides (personal communication, F. Troy, University of California, Davis). Gels were rinsed in 50% methanol in 7.5% acetic acid for 5 to 18 h at room temperature. After five 15-min washings in distilled water, the gels were immersed in 1% periodic acid in 3% acetic acid for 1 h, washed overnight in running tap water, and stained in 0.4% basic fuchsin for 5 min in the dark. Finally, the gels were washed five times in fresh 0.5% metabisulfite at 10-min intervals, washed overnight in tap water, and stored in 7.5% acetic acid.

Stained gels were scanned on an Ortec Integrating Microdensitometer connected to a Gilford linear transport system (Gilford Instruments). Coomassie blue-stained gels were scanned at 570 nm, and PAS-stained gels were scanned at 530 nm.

Gels containing radioactive preparations were extruded from the glass tubes, frozen at −70 C, and then sliced into 1-mm fractions. Iodinated gel slices were placed in scintillation vials and counted in a gamma spectrometer (Packard). Gel slices containing tritiated polypeptides were put into scintillation vials and incubated overnight at 60 C with 0.2 ml of 30% H_2O_2, and 10 ml of Aquasol (New England Nuclear Corp.) was then added to each vial. Each vial was counted in a liquid scintillation counter (Nuclear-Chicago).

RESULTS

When cells from spontaneous mammary tumors of BALB/cfC3H mice were grown in densely crowded primary cultures under the appropriate hormonal stimulation, the monolayers formed morphological units referred to as domes (13). The production of MTV in primary culture appears to be related to, or dependent upon, dome formation (14, 15). Domes developed in culture 3 to 4 days after plating. Once domes had formed, 10 to 15 μg of MTV protein was harvested from the medium from each 75-cm^2 flask per 24-h period.

The final step of purification, isopycnic centrifugation, resulted in a light-scattering band with a density of 1.17 g/ml in sucrose gradients. Analysis of light-scattering bands revealed particles containing 60 to 70*s* and 4*s* RNAs and RNA-dependent DNA polymerase activity. The particles were identified as type-B particles by their characteristic electron microscopic morphology and their reactions in immunodiffusion (Fig. 1) and radioimmune assays (R. D. Cardiff, J. Immunol., in press). The final MTV preparations used for polypeptide analysis did not contain detectable amounts of host cell, FCS, or murine leukemia antigens as shown by the immunodiffusion plates in Fig. 1 and 2.

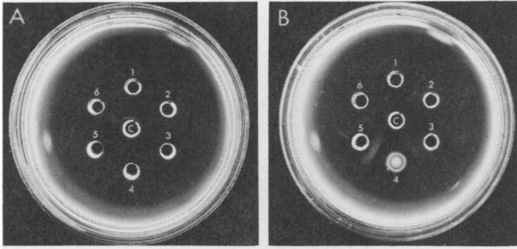

FIG. 1. *Immunodiffusion tests showing: A, the characteristic reaction of the mouse mammary tumor virus (MTV) B particle (3) and the absence of detectable quantities of contaminants in the virus preparation; and B, the immunologic reactivity of the antisera used in A. A, Center well, purified MTV; 1, anti-MTV; 2, anti-fetal calf serum; 3, anti-BALB/c lactating mammary gland extract; 4, anti-mouse casein; 5, anti-AKR virus; 6, blank. B, Center well, anti-MTV; 1, anti-fetal calf serum; 2, fetal calf serum; 3, anti-BALB/c lactating mammary gland extract; 4, BALB/c milk; 5, anti-mouse casein; 6, blank.*

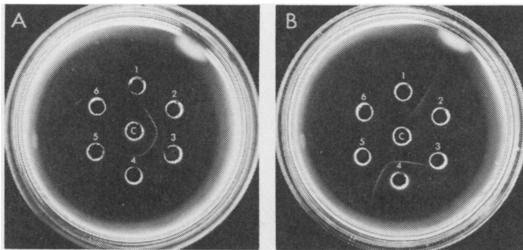

FIG. 2. *Immunodiffusion tests showing the absence of detectable quantities of murine leukemia virus antigens in the mouse mammary tumor virus (MTV) preparation. A, Center well, anti-AKR virus; 1, 100 µg of purified MTV disrupted with 2% NP-40; 2–6, NP-40-disrupted AKR virus in ranges from 0.32 to 5 µg of total virus protein; 2, 5 µg; 3, 2.5 µg; 4, 1.25 µg; 5, 0.63 µg; 6, 0.32 µg. B, Center well, NP-40 disrupted C-type virus (1.1 µg) from spontaneous BALB/c splenic tumor; 1, anti-MTV; 2–3, NP-40-disrupted MTV; 4, anti-AKR virus; 5–6, NP-40-disrupted AKR virus (2 µg).*

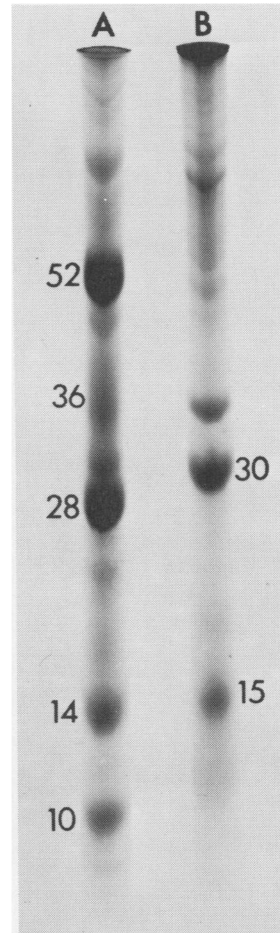

FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels comparing purified mouse mammary tumor virus (MTV) and murine leukemia virus. Migration is from top to bottom. A, MTV; B, AKR virus. Numbers refer to estimated molecular weights in thousands of daltons.*

Polyacrylamide gel electrophoresis. Electrophoresis in 10% polyacrylamide-SDS gels of dissociated MTV from tissue culture resulted in the separation of at least 12 polypeptides. The polypeptide pattern for MTV was distinctly different from that observed with murine leukemia virus (Fig. 3), confirming previous observations (11). A polypeptide was considered major when it comprised more than 10% and minor when it comprised less than 10% of the total counts or stain. Percentages were determined by integration of the area under the peaks of radioactivity or stain. The same polypeptides were consistently major or minor in each technique, but the precise proportion varied among runs and cultures. The data presented below represent typical examples in which all procedures were applied to virus from the same group of cultures.

^{3}H-amino acid labeled virus. Electropherograms of isopycnically purified MTV, internally labeled with ^{3}H-amino acids-MTV, clearly showed 10 polypeptides (Fig. 4A). Four major polypeptides contained 67.5% of the total label. Their estimated molecular weights were 52,000, 36,000, 28,000, and 10,000. The 52,000- and 28,000-dalton polypeptides had the highest proportions of total label with 19.9 and 17.8%, respectively. The 36,000-dalton polypeptide

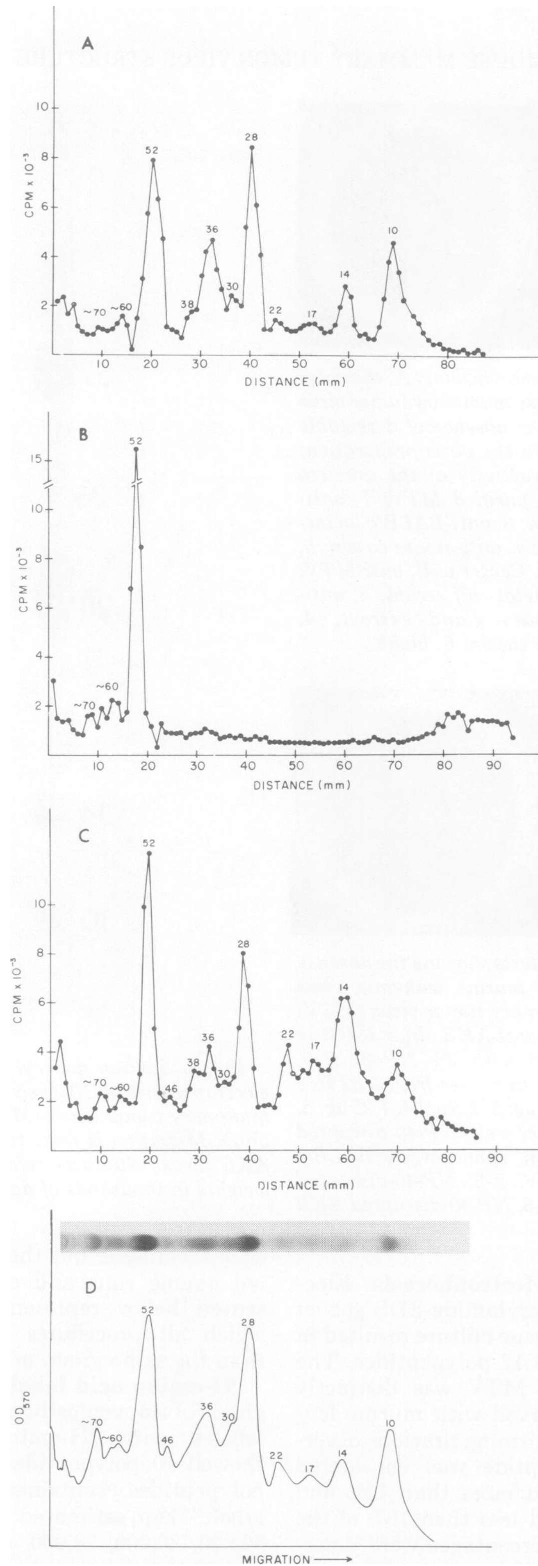

Fig. 4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified mouse mammary tumor virus (MTV) on 10% gels. Migration is from left to right. Numbers above peaks refer to molecular weights in thousands of daltons. A, ^{3}H amino acid-labeled MTV; B, ^{125}I-MTV labeled without NP-40 treatment; C, ^{125}I-MTV labeled after treatment with 2% NP-40; D, Coomassie blue-stained MTV polypeptides with photograph and densitometer tracing of gel.*

contained less radioactivity in the peak fraction, but was heterodispersed, occupying more fractions. Three polypeptides each contained less than 4% of the label. These three polypeptides had estimated molecular weights of 60,000, 38,000, and 22,000. A 46,000-dalton polypeptide was not clearly resolved, but was possibly represented by a shoulder in front of the 52,000-dalton polypeptide. Three polypeptides each contained between 4 and 10% of the total counts and had molecular weights of 30,000, 17,000, and 14,000.

Varying amounts of radioactivity with low electrophoretic mobility were observed. These peaks had estimated molecular weights above 70,000. Because of the variability in the number of peaks and amount of radioactive label present in this region, it was not clear whether these peaks represented polymeric aggregates or distinct polypeptides.

^{125}I-labeled MTV. Purified MTV was enzymatically iodinated either before or after disruption with NP-40. When intact virus was iodinated and isopycnically centrifuged, the ^{125}I counts were found in the 1.17 g/ml region of the sucrose gradient. When intact iodinated virus was disrupted and electrophoresed, approximately 10% of the counts were found to be associated with the 70,000- and 60,000-dalton polypeptides, whereas 90% of the counts migrated in a single peak corresponding to the 52,000-dalton polypeptide (Fig. 4B).

If purified virions were first disrupted with NP-40, then iodinated, and subsequently electrophoresed, several polypeptides were resolved (Fig. 4C). Three of these polypeptides, with molecular weights of 52,000, 28,000, and 14,000, were considered major and contained 52.2% of the total counts. Although the 52,000- and 28,000-dalton polypeptides had more counts in their peak fractions, the 14,000-dalton polypeptide region occupied more fractions and, thus, the greatest proportion of the total counts with 19.1%. The 36,000- and 10,000-dalton polypeptides, major by internal label, each contained less than 10% of the total counts. The 17,000-, 22,000-, and 38,000-dalton polypeptides had slightly higher proportions of the total counts than in the ^{3}H-amino acid labeled preparations. Small amounts of radioactivity (less than 3% each) were found in the 70,000-, 60,000-, 46,000-, and 30,000-dalton regions. Again, irregularly labeled regions were frequently found at the "top" of the gel.

Coomassie blue staining. When MTV from BALB/cfC3H tissue cultures was disrupted and electrophoresed in SDS-polyacrylamide gels, 12 polypeptides were resolved (Fig. 4D) by Coomassie blue staining. Three of these polypeptides, 52,000, 36,000, and 28,000, were major and contained 44.8% of the stain. The 10,000- and 14,000-dalton polypeptides, which were major by the labeling procedures, were proportionately denser than any of the other "minor" polypeptides, but still fell below the 10% limit. Bands at 70,000, 60,000, 22,000, and 17,000 daltons were all resolved as discrete polypeptides.

Stained bands of 46,000 and 30,000 were clearly resolved with Coomassie blue, even though they were somewhat more difficult to evaluate in the labeling procedures.

The major departure in the run illustrated in Fig. 4D was the absence of a stainable peak in the 38,000-dalton region. Peaks of radioactivity were clearly resolved in the isotope labeling procedures. A stainable band was, however, intermittently observed between the 46,000- and 36,000-dalton peaks in other runs with virus from this and other cultures.

Another variation from the labeled preparations was the frequent appearance of a second polypeptide in the 60 to 67,000-dalton range. The resolution of a discrete stainable band was clearly observed, as in Fig. 4D. As with the other techniques, variable amounts of stain were found above the 70,000-dalton region.

Glycoproteins. When MTV-containing gels were stained for the presence of polysaccharides by using the PAS technique, four bands were detected (Fig. 5A). The two major regions of stain corresponded in mobility to the 52,000- and 36,000-dalton polypeptides with 36.5 and 34.9% of the total stain, respectively. Although the heaviest concentration of stain was in the 52,000-dalton region, the staining in the 36,000-dalton region was heterodispersed; therefore, the percentages of stain in these two regions were nearly equal. Fainter staining bands were better revealed by the densitometer scan and corresponded in mobility to the 70,000- (9.9%) and the 10,000- (19.6%) dalton polypeptides.

The electrophoresis of MTV, which had incorporated radioactive glucosamine in tissue culture ([^{3}H]glucosamine MTV), resulted in an electrophoretic pattern which was somewhat different from that obtained by PAS staining (Fig. 5B). The 52,000- and 36,000-dalton regions were again major, containing 31.2 and 36.9% of the total radioactivity, respectively, whereas the 70,000-dalton region contained 9.1%. The 10,000-dalton region did not contain significant [^{3}H]glucosamine counts, whereas 8% of the glucosamine radioactivity was found in the 60,000-dalton region.

Table 1 is a summary of the relative percent-

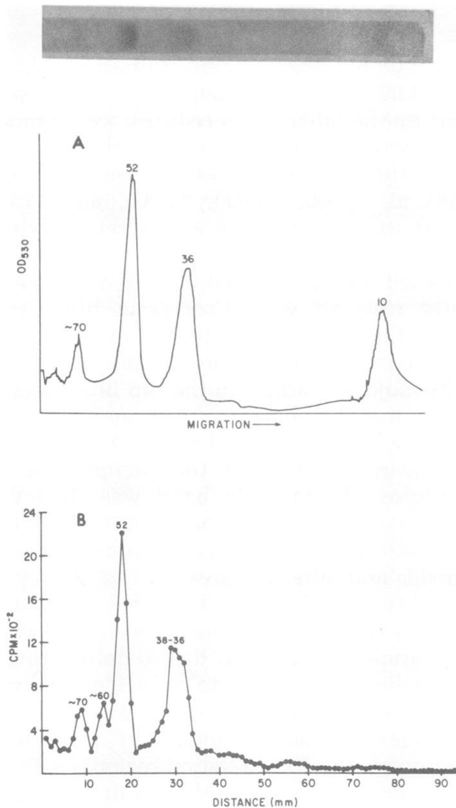

FIG. 5. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified mouse mammary tumor virus (MTV) on 10% gels. Migration is from left to right. Numbers above peaks refer to molecular weights in thousands of daltons. A, Densitometer scan of periodic acid Schiff-stained MTV polypeptides; B, radioactive electrophoretic pattern of [^{3}H]glucosamine-labeled MTV.*

ages of radioactivity or stain found in each of the polypeptides, as detected by the various methods used in this study. Although a few of the polypeptides were not clearly resolved by any single technique, their percentages were estimated by the correlation of similar regions found by another technique. These estimated percentages are included for comparative purposes.

DISCUSSION

The dissociation and electrophoresis of MTV purified from culture fluids of primary cultures of BALB/cfC3H mammary tumor cells resulted in the separation of five major and seven minor polypeptides. These polypeptides were detected by external and internal labeling and Coomassie blue staining. A comparison of [^{3}H]glucosamine labeling and PAS staining showed five of these polypeptides to also contain carbohydrate moieties.

The use of a tissue culture system and multiple detection methods permitted an interpretation of the results that would not be available by using any single technique. If a polypeptide could not be readily detected by one technique, its presence could be confirmed by using another technique. The 46,000-dalton polypeptide, for example, appears as a shoulder, which is not clearly separated from the 52,000-dalton polypeptide in either the iodinated or tritiated preparations (Fig. 4A and 4C). It is, however, seen as a clearly resolved band with the Coomassie blue stain (Fig. 4D). Correlation of the data obtained by several techniques further allowed an interpretation of any single polypeptide as being a contaminant or not. An iodinated or stained polypeptide, which corresponded to a peak in electropherograms of internally labeled preparations, is not likely to be an exogenous contaminant, e.g., FCS. Nearly identical electrophoretic patterns were repeatedly obtained by using internal and external labeling and protein specific staining, even in the resoluton of minor polypeptides

TABLE 1. *Polypeptide and glycoprotein composition of MTV*[a]

Mol wt of polypeptide[b]	Total counts or stain/gel (%)				
	^{3}H-AA-MTV	^{125}I-MTV	COOM-MTV	PAS-MTV	[^{3}H] Glu-MTV
>70,000	5.9	5.8	7.1		4.9
~70,000	1.9	2.2	6.2	9.9	9.1
~60,000	2.4	2.4	6.3		8.0
52,000	19.9	18.4	16.3	36.5	31.2
46,000	1.5	2.0	3.5		
38,000	2.6	4.2			
36,000	13.5	5.1	14.1	34.9	36.9
30,000	4.1	2.5	3.8		
28,000	17.8	14.7	14.5		
22,000	2.8	6.6	5.9		
17,000	4.0	8.5	5.3		
14,000	7.3	19.1	7.8		
10,000	16.3	8.5	8.9	18.6	

[a] MTV, Mouse mammary tumor virus; ^{3}H-AA-MTV, ^{3}H-labeled amino acid-MTV; COOM-MTV, Coomassie blue staining-MTV; PAS-MTV, periodic acid Schiff staining-MTV; [^{3}H]Glu-MTV, [^{3}H]glucosamine-MTV.

[b] Estimated molecular weights as compared with the following protein standards: bovine serum albumin (65,000), ovalbumin (45,000), carbonic anhydrase (30,000), myoglobin (17,000), and cytochrome C (12,400).

(Fig. 4). Thus, the consistency of these data suggests that the 12 polypeptides are integral components of the MTV virion.

Similar correlations between stained and labeled preparations could be made with the glycoprotein preparations. The 70,000-, 52,000-, 36,000-dalton glycoproteins appeared in all preparations. Tritiated glucosamine appeared in a 60,000-dalton peak which was not stained with PAS. On the other hand, a 10,000-dalton glycoprotein was stained with PAS and labeled with ^{3}H amino acids, but did not incorporate glucosamine. These two apparent inconsistencies probably reflect the relative total carbohydrate and glucosamine contents in the respective polypeptides, but will require more detailed study.

Labeling and staining techniques both revealed the presence of polypeptides above 70,000 daltons. These polypeptides represented 7.1% or less of the total protein and were variable in number. It was not clear whether these polypeptides represented intermolecular aggregates, large polypeptides, or both. These questions will have to be resolved with gel and electrophoretic systems better suited for study of molecules in this size range.

The differences between the data presented here and that of other investigations could be differences in contaminants, source of virus, or interpretation. Of the possible contaminants considered, C-type particles and host cell protein could not be excluded, but were reduced to minor possibilities by our immunological tests. Previous studies have been based upon MTV from milk (11, 19, 23, 25) and not tumor cell monolayer cultures as in the present study and, thus, represent another possible explanation for the differences in protein composition. After careful examination of the published data, however, we feel that the major difference is based on the differing interpretations of the stained gel patterns. The use of several different techniques reinforces our interpretation of the polypeptide composition of MTV.

The current results allow only limited speculation about the virion structure; however, several observations might be correlated. Most viral glycoproteins thus far described have been envelope proteins (5, 8, 9, 20, 24). Five glycoproteins have been identified in this study. Three of these (52,000, 70,000, and 60,000 daltons) were enzymatically iodinated in the intact virion. Experiments with erythrocytes, lymphocytes, and other viruses suggest that only surface polypeptides are labeled with the lactoperoxidase method (18, 20-22, 24, 25). This suggests that at least the 52,000-, 70,000-, and 60,000-dalton glycoproteins are on the virion surface. The 52,000-dalton polypeptide was a major polypeptide in all procedures used and corresponds in molecular weight to the MTV-Sl protein of Nowinski et al. (19). It has been suggested that this protein is an internal polypeptide (19, 23), but the experiments reported herein and those of Witte et al. (25) are more suggestive of an external position for the 52,000-dalton protein.

Within this context, it should be noted that the 36,000- and 10,000-dalton polypeptides were also glycoproteins, but were not readily identified by iodination of the intact virion. They were, however, iodinated when the virus was disrupted, indicating that these glycoproteins do contain tyrosine or histidine residues, or both. These amino acids were apparently not readily accessible to lactoperoxidase iodination in the intact virion. This is possibly due to the influence of other glycoproteins, lipids, carbohydrates, or other envelope components and their structural arrangement in the virion (22). Although the 36,000- and 10,000-dalton polypeptides may be envelope glycoproteins, their precise positions on the virion await additional study.

The MTV virion composition is much more complex than previously appreciated (1, 23). The studies described in this report extend previous studies through the use of multiple techniques. Additional work, particularly concerning the high-molecular-weight range and the carbohydrate composition will, no doubt, modify the current interpretations. Future studies hopefully will also lead to a better understanding of the structure-function relationships of the MTV proteins.

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