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Murine Mammary Tumor Virus Structural Protein Interactions: Formation of Oligomeric Complexes with Cleavable Cross-Linking Agents

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Murine mammary tumor virus protein interactions in the intact virion structure were studied with the use of the cleavable cross-linking reagents dithiobis(succinimidyl propionate) and methyl 4-mercaptobutyrimidate hydrochloride. Cross-linked oligomeric complexes of murine mammary tumor virus proteins were analyzed by two-dimensional gel electrophoresis. Among the complexes most consistently formed were a heterodimer of the two glycoproteins gp36 and gp52, the homodimer of gp36, and the homotrimer of gp52. A very prominent oligomer formed at higher concentrations of dithiobis(succinimidyl propionate) was a complex of about 230,000 molecular weight, made up of three molecules each of gp36 and gp52. A number of lines of evidence, including electron microscopic analysis, suggest that the 230,000-molecular-weight complex actually represents the murine mammary tumor virus spike structure. Of the murine mammary tumor virus core proteins, p14 forms homooligomers most readily. Upon cross-linking with methyl 4-mercaptobutyrimidate hydrochloride a small amount of what seems to be a heterodimer made up of the N-terminal *gag* protein p10 and the hydrophobic membrane glycoprotein gp36 can be observed.

The mature murine mammary tumor virus (MuMTV; type B particle) is an enveloped virus whose prominent structural features are an eccentrically located electron-dense core surrounded by less dense material and a unit membrane covered with projections arranged in a regular pattern. The structural proteins of MuMTV can be classified into two groups: the *gag* gene-derived internal core proteins (p10, p14, p23, and p28), and the *env* gene-derived membrane-associated glycoproteins (gp36 and gp52). What is presently known about the structural location or function of some of these proteins within the MuMTV structure can be summarized as follows: gp52 is located on the outside of the virion envelope (24, 30), gp36 is mostly embedded within the bilayer membrane (15, 24), p14 is the nucleic acid-binding protein (1), and p10 has an affinity for the membrane fraction (5, 16). However, the structural role of all these proteins is not fully understood, and it has not yet been possible to define biochemically the structures that are observed in electron micrographs of MuMTV particles.

One of the most successful approaches to the study of viral protein interactions, especially those of enveloped viruses, has been the use of cross-linking reagents (7-9, 18, 20, 22, 25, 26, 29). The principal conclusions that have emerged from cross-linking studies of enveloped viruses are that the spike structures observed on the membrane of these viruses are oligomeric complexes of the viral membrane glycoproteins (8, 9, 20, 25, 26, 29) and that there is often a demonstrable interaction between a membrane protein and an internal core protein (8, 18, 26).

The cross-linking reagents that have proven to be most valuable in these types of studies are the bifunctional molecules, such as dithiobis(succinimidyl propionate) (DTBSP) (14) and methyl 4-mercaptobutyrimidate hydrochloride (MMB) (11), that contain a cleavable internal disulfide bond. The reactive ends of the molecules readily form covalent bonds at room temperature and neutral pH with primary amine groups such as the ϵ amino group of lysine residues. The presence of the cleavable bonds in these reagents permits the regeneration and identification of the monomeric components from the cross-linked oligomeric complexes.

To explore MuMTV protein interactions in greater detail, we used cleavable bifunctional cross-linking agents in conjunction with two-dimensional gel electrophoresis. The most readily formed cross-linked complexes that we observed were oligomers of the two glycoproteins, with a very prominent one of about 230,000 molecular weight, made up of three molecules each of gp36 and gp52. On the basis of a number of lines of evidence presented in this report, we propose that the 230,000-molecular-weight complex represents the MuMTV spike structure.

MATERIALS AND METHODS

Reagents. The reagents DTBSP and MMB were purchased from Pierce Chemical Co. *N*-ethylmaleimide was obtained from Aldrich Chemical Co., and dithiothreitol was purchased from Calbiochem. *U*-^{14}C-labeled amino acids and [^{35}S]methionine were obtained from New England Nuclear Corp. All of the reagents used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, including 2-mercaptoethanol, were from Bio-Rad Laboratories. Agarose was obtained from Seakem, and the nonionic detergent Nonidet P-40 (NP-40) was obtained from Shell Chemical Co.

Viruses. Unlabeled MuMTV produced by the tissue culture cell line Mm5mt/c_l was obtained from J. Gruber through the Virus Cancer Program of the National Cancer Institute. MuMTV from RIII mouse milk was purified as previously described (24). Labeled virus was obtained from cell line Mm5mt/c_l by labeling the cells with ^{14}C-amino acids as previously described (24).

Cross-linking of MuMTV proteins. (i) DTBSP. Fresh stock solutions of DTBSP in dimethyl sulfoxide were prepared before each experiment, at a concentration of 40 mg/ml. The stock solution was then diluted with phosphate-buffered saline to a concentration which would yield the final desired concentration when added to the virus sample in a ratio of 1:5. Viral samples were suspended in phosphate-buffered saline, and the cross-linking reaction was carried out at 23°C for 20 min. The reaction was terminated by adding SDS-polyacrylamide gel electrophoresis loading buffer and heating the samples for 3 min at 100°C.

(ii) MMB. Cross-linking with MMB was carried out with a modification of the method of Kenny et al. (11). A 200 mM stock solution of MMB in 500 mM triethanolamine hydrochloride, pH 8.0, was added to the viral sample to give a final concentration of 20 mM MMB. After the reaction was allowed to proceed for 20 min at 23°C, hydrogen peroxide was added to a concentration of 40 mM to promote cross-linking between adjacent sulfhydryl groups by disulfide bond formation. The oxidation was allowed to proceed for 15 min at 23°C, after which the reaction was terminated as described above.

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis in the first dimension was carried out in 0.75-mm-thick, 5 to 20% gradient gels, using the discontinuous buffer system of Laemmli (13) as previously described (21). For analysis of samples cross-linked by the cleavable reagent DTBSP or MMB, reducing agents (2-mercaptoethanol or dithiothreitol) were omitted from the loading buffer. At the end of the electrophoretic run, lanes containing samples to be analyzed in the second dimension were excised, sealed in Saran Wrap, and stored at −20°C. For analysis in the second dimension, the gel strips were incubated in Laemmli stacking gel buffer containing 5% 2-mercaptoethanol for 30 min at 37°C or in buffer containing 100 mM dithiothreitol for 10 min at 60°C. Both techniques yielded identical results. After the reducing step, the gel strip was washed for 5 min in loading buffer to get rid of excess reducing agent and to absorb some bromophenol blue tracking dye. The gel strip was then placed on top of a 1.5-mm-thick, 5 to 20% gradient gel containing a 1.5-cm-wide stacking gel. The gel strip was immobilized by pouring on stacking gel buffer containing 1% agarose. Slots for application of marker virus or other samples were formed at either end of the gel strip in the agarose solution. Gels were fixed and treated by the method of Bonner and Laskey (4) for analysis by fluorography.

^{14}C-labeled molecular weight markers (with molecular weights in parentheses) used for calibration purposes were the following: ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B (94,000), β-galactosidase (116,000), and myosin (200,000). Fibronectin, for use as a marker, was obtained by immunoprecipitating tissue culture medium supernatant of ^{14}C-amino acid-labeled Mm5mt/c_l cells after virus harvest with anti-fibronectin serum (21). Electrophoresis of the immunoprecipitates in the presence of reducing agent yields fibronectin monomer (molecular weight, 230,000), and electrophoresis in the absence of reducing agent displays the predominant disulfide-bonded dimer form (molecular weight, 460,000) (12).

Electron microscopy. Virus suspension was placed on a microscopic grid covered with carbon-coated Formvar film. After 20 s, the excess fluid was withdrawn by touching the grid with a piece of filter paper. The grid was then washed three times by flotation on 3 drops of double-distilled water and stained for 30 s with 2% sodium phosphotungstic acid, pH 7.0 (23). Grids were examined in a Philips 300 electron microscope.

RESULTS

Cross-linking of MuMTV. These studies were initially performed with unlabeled MuMTV isolates derived from tissue culture cells (line Mm5mt/c_l) as well as from mouse milk (strain RIII mice), and the results were analyzed on Coomassie brilliant blue-stained polyacrylamide gels (Fig. 1). Note that we have adopted the more widely used nomenclature for MuMTV structural proteins (p10, p14, p23, p28, gp36, and gp52 [2, 27]) in lieu of our previous designations (p12, p16, p23, p27, gp34, and gp47 [21]). A comparison between the two control viral samples revealed that considerable differences existed between the SDS-polyacrylamide gel electrophoresis profiles of the tissue culture-derived (Fig. 1c) and milk-derived (Fig. 1d) virus isolates. Primarily, the two glycoproteins (gp36 and gp52) of the RIII milk virus (Fig. 1d) migrated noticeably faster than their counterparts of the C3H tissue culture virus (Fig. 1c). The apparent faster migration of the milk virus glycoproteins might have been due to a decreased polysaccharide content of these proteins resulting from the action of glycosidases present in milk. In addition, the RIII milk virus contains a number of proteins not seen in the C3H virus, with a prominent one of about 68,000 molecular weight and another migrating between p10 and p14. These additional proteins are probably milk proteins which either adsorb onto the virus or

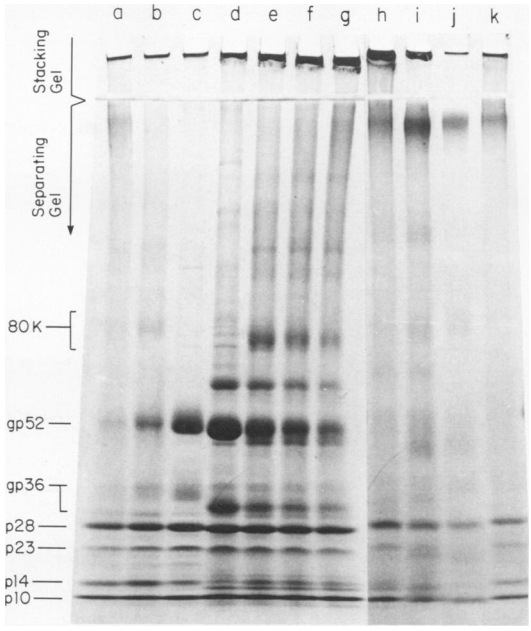

FIG. 1. *Coomassie brilliant blue-stained gel of C3H tissue culture and RIII milk MuMTV preparations cross-linked with DTBSP. (a) C3H virus plus DTBSP (100 μg/ml); (b) C3H virus plus DTBSP (30 μg/ml); (c) untreated C3H virus; (d) untreated RIII virus; (e) RIII virus plus DTBSP (30 μg/ml); (f) RIII virus plus DTBSP (60 μg/ml); (g) RIII virus plus DTBSP (100 μg/ml); (h) RIII virus plus DTBSP (400 μg/ml); (i) RIII virus plus 0.1% NP-40 plus DTBSP (800 μg/ml); (j) C3H virus plus 0.1% NP-40 plus DTBSP (800 μg/ml); (k) C3H virus plus DTBSP (400 μg/ml). 80K, 80,000 molecular weight.*

are part of milk vesicles that copurify with virus (17). We have observed that these differences in protein profiles are not due to viral strain differences, but are typical of milk-derived viral isolates versus tissue culture-derived viral isolates, regardless of strain.

Treatment of intact MuMTV with the cross-linking agent DTBSP followed by analysis on SDS-polyacrylamide gel electrophoresis under nonreducing conditions resulted in the appearance of new protein bands and the disappearance or decreased intensity of some MuMTV protein bands. Treating the virus preparations with relatively low concentrations of DTBSP (30 μg/ml) resulted in a marked decrease in the relative intensities of the glycoprotein (gp36 and gp52) bands (Fig. 1b and e), whereas the core protein bands remained relatively unchanged. A new band of about 80,000 apparent molecular weight appeared in the cross-linked samples (Fig. 1b and e) which was not present in the control samples (Fig. 1c and d). This new band in the tissue culture-derived virus (Fig. 1b) migrated with a slightly higher apparent molecular weight than the new band seen in the milk-derived virus (Fig. 1e). At higher DTBSP concentrations (100 μg/ml), we noticed an increase of Coomassie brilliant blue staining material at very high apparent molecular weight ranges (near the top of the separating gel [Fig. 1a and g]). At a DTBSP concentration of 400 μg/ml, all of the glycoproteins had reacted, and a cross-linked complex of greater than 200,000 molecular weight had become very prominent (Fig. 1h and k). Note that in all of the cross-linked samples, and even in the nonreduced controls, there was a considerable amount of Coomassie brilliant blue-stained material that was present at the bottom of the loading slots that had not entered the stacking gel. Furthermore, in the cross-linked samples, there was a diffusion of most of the minor discrete nonviral protein bands seen in control samples, especially in the higher-molecular-weight ranges.

Cross-linking of NP-40-lysed virus. One possible explanation for the apparent lack of reactivity of the core proteins in the presence of DTBSP might have been that the MuMTV envelope was impermeable to the cross-linking agent. To test this possibility, we began studying NP-40-lysed viral preparations for cross-linking with DTBSP. Treating MuMTV preparations with NP-40 causes disruption of the viral membrane, freeing of the cores, and the formation of rosettes which consist chiefly of a viral membrane containing the two glycoproteins gp36 and gp52 and exhibiting the surface spikes (24). We observed, however, that under these conditions, higher concentrations of DTBSP were required to form detectable oligomeric protein complexes. The most striking cross-linked complex observed in the NP-40-lysed viral preparations was the species of very high molecular weight, which only migrated about 1 to 2 cm into the separating gel (Fig. 1i and j).

Use of ^{14}C-labeled MuMTV. After the initial studies with unlabeled MuMTV isolates, all further cross-linking experiments were conducted with ^{14}C-amino acid-labeled MuMTV preparations because autoradiography of isotopically labeled viral preparations is a more sensitive technique of analysis than is staining of unlabeled viral protein. The greatly reduced concentration of virus required in reactions with ^{14}C-amino acid-labeled MuMTV also reduces the potential for artifacts that may occur when cross-linking virus at the relatively high concentrations needed for Coomassie brilliant blue staining.

Cross-linking of a type C virus. In structural studies of type C viruses (20, 25), it has been reported that oligomeric complexes of

greater than 200,000 molecular weight are also formed as a result of cross-linking. Because type B and type C viruses have considerable structural similarity, we thought it would be of interest to compare the cross-linked products formed with both types of viruses. To that end, ^{14}C-amino acid-labeled Rauscher leukemia virus and ^{14}C-amino acid-labeled C3H MuMTV were reacted with DTBSP under identical conditions and analyzed. To get a better estimate of the molecular weights of the cross-linked complexes, the analysis was performed on a 30-cm-long, 3 to 20% hyperbolic gradient gel formed in such a way as to expand the high-molecular-weight range. In addition to our standard molecular weight markers, fibronectin in its reduced monomer (molecular weight, 230,000) and nonreduced dimer (molecular weight, 460,000) forms (12), obtained as described above, was run on the gel as well. As reported by Pinter and Fleissner (20), oxidation of the type C virus with *N*-ethylmaleimide resulted in the formation of a 90,000-molecular-weight complex (Fig. 2i) which was composed of the two membrane proteins gp70 and p15E linked by disulfide bonds. Oxidation of MuMTV with *N*-ethylmaleimide (Fig. 2c) caused the disappearance of p14 and of the small band migrating just above p28. Formation of new complexes after *N*-ethylmaleimide oxidation of MuMTV was not observed, however. Further analysis of *N*-ethylmaleimide-oxidized MuMTV preparations (data not shown) revealed that p14 was complexed into aggregates too large to enter the gel. Furthermore, it was found that, even in untreated virions, a portion of the p14 molecules existed in an aggregated state that could be dissociated by reduction with 2-mercaptoethanol or dithiothreitol. Treatment of Rauscher leukemia virus with a low concentration (10 μg/ml) of DTBSP caused the appearance of a prominent band of 60,000 molecular weight (Fig. 2h) which was shown to be a dimer of p30 (20). A similar cross-linking of MuMTV (Fig. 2d) resulted in the formation of the 80,000-molecular-weight complex, which was also observed at a higher (150 μg/ml) DTBSP concentration (Fig. 2e). If a dimeric form of p28 was formed, it might have been masked by comigration with gp52. However, as will be discussed later, two-dimensional gel analysis did not reveal any such complexes (Fig. 3). Cross-linking of NP-40-disrupted Rauscher leukemia virus with DTBSP resulted in the formation of a complex of greater than 200,000 molecular weight (Fig. 2g) which migrated just slightly above the complex of a similarly treated MuMTV preparation (Fig. 2f). Note that in the NP-40-lysed MuMTV sample (Fig. 2f) cross-linked at a high DTBSP concentration (400 μg/ml) the gp52 band was very broad and extended toward a lower-molecular-weight range. This phenomenon was probably caused by internal cross-links formed within gp52 molecules, thereby decreasing their relative size and permitting migration with an apparent lower molecular weight in the SDS-polyacrylamide gel. The core protein p28 showed a similar, although less pronounced, altered migration as well (Fig. 2f). A streaking to a lower apparent molecular weight range was also exhibited by both the 80,000-molecular-weight complex (Fig. 2f) and the complex with a molecular weight greater than 200,000 and was again probably due to internal cross-links in the molecules making up the complexes. Although it is not discernible in Fig. 2, an examination of the original X-ray film clearly shows that most of the material in the

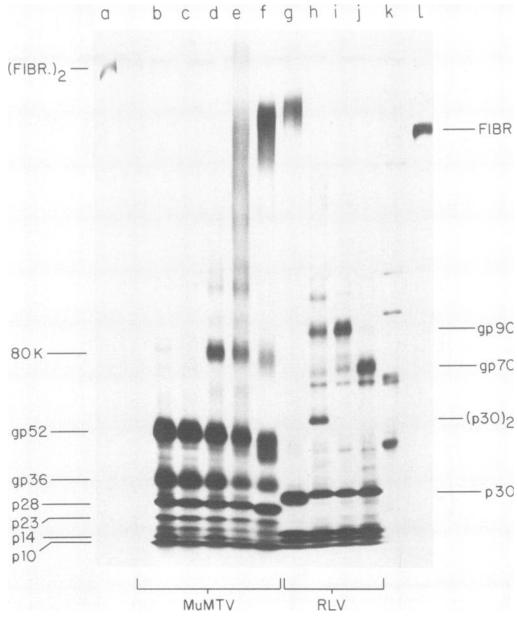

FIG. 2. *Cross-linking of ^{14}C-amino acid-labeled MuMTV and ^{14}C-amino acid-labeled Rauscher leukemia virus with DTBSP. (a) Nonreduced immunoprecipitated fibronectin (molecular weight, 460,000); (b) untreated MuMTV; (c) MuMTV oxidized with N-ethylmaleimide; (d) MuMTV plus DTBSP (20 μg/ml); (e) MuMTV plus DTBSP (150 μg/ml); (f) MuMTV plus 0.1% NP-40 plus DTBSP (400 μg/ml); (g) Rauscher leukemia virus plus 0.1% NP-40 plus DTBSP (400 μg/ml); (h) Rauscher leukemia virus plus DTBSP (10 μg/ml); (i) Rauscher leukemia virus oxidized with N-ethylmaleimide; (j) untreated Rauscher leukemia virus; (k) ^{14}C-labeled molecular weight markers (followed by molecular weights in parentheses): myosin (200,000), β-galactosidase (116,000), phosyphorylase B (94,000), bovine serum albumin (68,000), and ovalbumin (43,000); (l) reduced, immunoprecipitated fibronectin (molecular weight, 230,000). 80K, 80,000 molecular weight.*

MuMTV complex with a molecular weight greater than 200,000 (Fig. 2f) is located at the top of the smeared band, in a region no larger than the analogous complex of Rauscher leukemia virus (Fig. 2g).

Molecular weight estimation of the large cross-linked complexes. By plotting the observed mobilities of the molecular weight markers in Fig. 2 on a semilogarithmic plot, we obtained a linear relationship for mobility versus molecular weight from ovalbumin (molecular weight, 43,000) through fibronectin monomer (molecular weight, 230,000) (data not shown). The migration of the fibronectin dimer (molecular weight, 460,000), however, departed from linearity relative to the other marker proteins. As a means of obtaining a rough estimate of the molecular weights of the large cross-linked MuMTV and Rauscher leukemia virus complexes, we plotted their migration on a line joining the fibronectin monomer position to the fibronectin dimer position. Such an estimate gave approximate apparent molecular weights of 270,000 for the MuMTV complex and 300,000 for the Rauscher leukemia virus (RLV) complex. It has been observed previously (18) that large oligomeric complexes of cross-linked polypeptides migrate in SDS-Laemmli discontinuous gels with an apparent molecular weight approximately 20% greater than that calculated from their polypeptide composition. If we apply such a correction factor to the large MuMTV and RLV cross-linked complexes, then we obtain molecular weights of approximately 250,000 for the RLV complex and 230,000 for the MuMTV complex.

Two-dimensional gel analysis of cross-linked MuMTV polypeptides. Cross-linked MuMTV samples were analyzed by cutting out the appropriate strip from the first-dimension gel, incubating it in the presence of reducing agents to reverse cross-linking, and then running the entire strip in a second dimension. Figure 3 shows a typical two-dimensional gel of MuMTV proteins cross-linked with DTBSP at a concentration of 30 μg/ml. The origin of electrophoresis in the first dimension is in the upper left-hand corner, with the direction of migration to the right. Electrophoresis in the second dimension is from top to bottom. The gel strip shown on top is a duplicate of the cross-linked sample that was reduced and run in the second dimension. The MuMTV pattern seen on the right side is an MuMTV standard that was run adjacent to the first-dimension gel strip in the second-dimension gel.

After reduction and electrophoresis in the second dimension, the polypeptides that were not affected by the cross-linking reagent are located on a diagonal line extending from upper left to lower right. Spots running below this diagonal identify polypeptides that were components of higher-molecular-weight cross-linked complexes in the first dimension. Polypeptide spots located above the diagonal indicate that these polypeptides migrated with an apparent lower molecular weight under nonreducing conditions, probably because of internal cross-linking. The most prominent spots below the diagonal in Fig. 3 are gp52 and gp36, which are vertically aligned beneath the 80,000-molecular-weight band seen in cross-linked samples. This alignment indicates that the 80,000-molecular-weight cross-linked complex is a heterodimer of gp52 and gp36. Only a gp36 spot can be observed beneath the 65,000-molecular-weight band, identifying it as a homodimer of gp36. Note that all of the major MuMTV protein bands show streaks beneath the diagonal. These streaks probably represent cross-links in the first dimension with the contaminating cellular proteins, which are present in MuMTV preparations, as in all enveloped viruses. Two-dimensional gels of MuMTV preparations cross-linked at higher DTBSP concentrations (data not shown) showed increased streaking, with more material at higher-molecular-weight ranges, but still displayed the prominent spots derived from the homodimer gp36-gp36 and the heterodimer gp36-gp52 as seen in Fig. 3. At the higher DTBSP concentrations, oligomeric complexes of about 140,000 molecular weight, made up of gp52, also became more noticeable.

Two-dimensional gel analysis of an MuMTV preparation lysed with NP-40 and cross-linked at a high DTBSP concentration (800 μg/ml) is shown in Fig. 4. As was discussed above, under these conditions a large complex of about 230,000 molecular weight was formed. As shown in Fig. 4, this large cross-linked complex was composed of gp52 and gp36. The faster-migrating portion of the gp52 spot probably represented internally cross-linked gp52 molecules that were not fully reduced. A similar phenomenon can be observed in the square-shaped gp52 "spot" running on the diagonal. These observations suggest that the internal cross-links are the most resistant to cleavage by reduction.

When the spots derived from the 230,000-molecular-weight complex were cut out of the gel and the radioactivity was eluted and counted, it was found that the ratio of gp52 to gp36 in the 230,000-molecular-weight complex was the same as that found in the whole virus. A small amount of the heterodimer gp36-gp52 can be seen in Fig. 4 as well.

Cross-linking with MMB. The cross-linking agent MMB was also used in these studies since

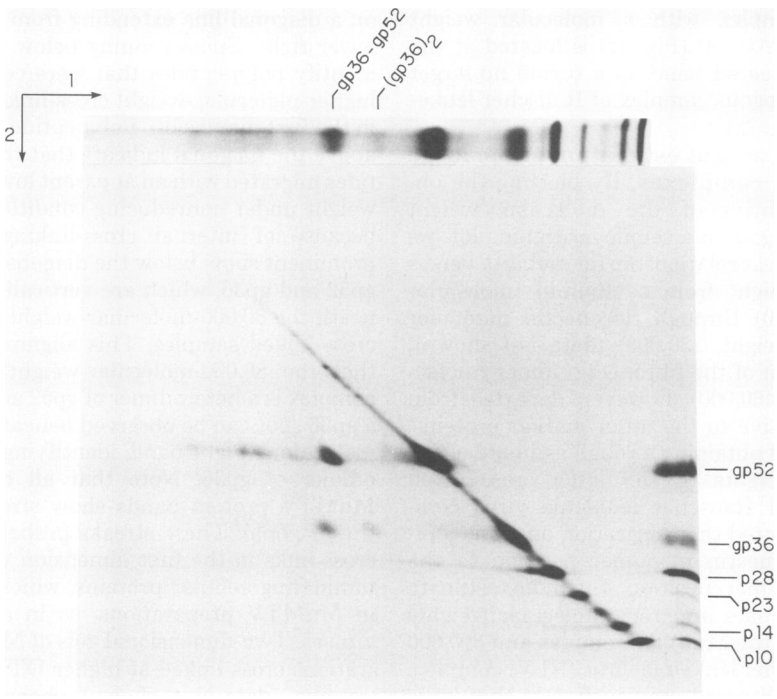

FIG. 3. *Two-dimensional gel of a MuMTV preparation cross-linked with DTBSP (30 μg/ml). The band on top is an exact duplicate of the strip that was electrophoresed in the second dimension. The lane on the right side is an MuMTV marker which was run simultaneously with the second-dimension electrophoresis.*

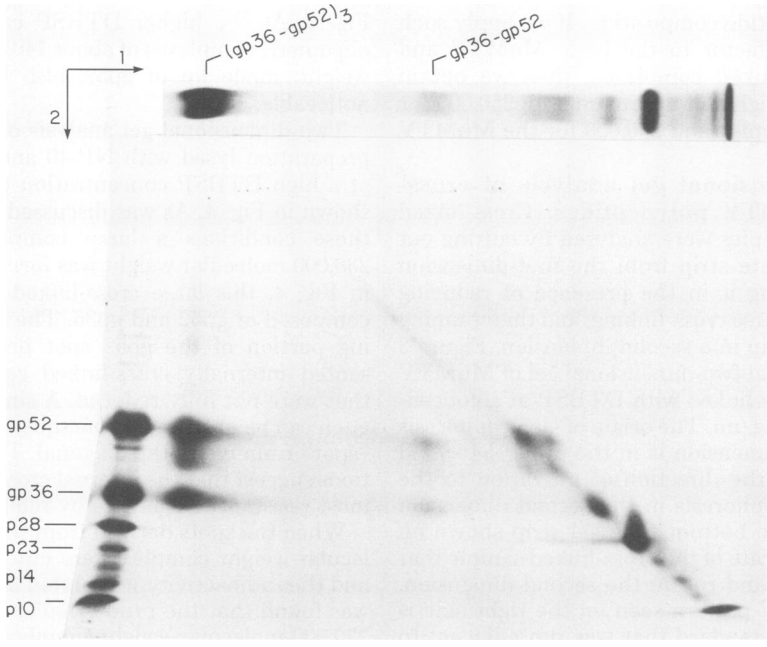

FIG. 4. *Two-dimensional gel of an NP-40-lysed MuMTV preparation, cross-linked with DTBSP (400 μg/ml). The strip on top is an exact duplicate of the gel lane that was run in the second dimension. The lane on the left side is an MuMTV marker.*

it penetrates the viral membrane more readily than does DTBSP (8) and might therefore react more extensively with internal core proteins. The major discrete spots visible in the two-dimensional gel of MMB cross-linked MuMTV (Fig. 5) again corresponded to the complexes gp36-gp52 and gp36-gp36. A major spot of gp52 was visible underneath a molecular weight range of about 140,000, which might have represented a cross-linked homotrimer of gp52. As we have seen, gp52 tended to form internal cross-links, which might have accounted for the elongated form of the spot and its relatively fast migration. On the left side of Fig. 5 is a lane of marker MuMTV, and on the right side is a lane that represents material which was cross-linked in such large complexes that it did not enter the first dimension gel, as was shown in Fig. 1. These highly cross-linked polypeptide complexes were recovered by cutting out the top portion of the stacking gel, cleaving the disulfide bonds with reducing agent, and running the products alongside the two-dimensional gel. Most MuMTV proteins were present in these large complexes, including most of the p14; however, what was striking was that no gp52 was detectable. Similar analyses were done after cross-linking MuMTV at high DTBSP concentrations (data not shown), in the presence and absence of NP-40, and the same observation was made; namely, gp52 did not participate in the formation of cross-linked complexes too large to enter the gel under nonreducing conditions.

Underneath a molecular weight range of about 55,000, there was an elongated spot of p28, which might have been derived from dimeric forms of p28. On the bottom of Fig. 5 we can observe three p10 spots under the diagonal; the first two nearest the diagonal (no. 1 and 2) probably arose from dimer and trimer forms of p10. The third rather faint spot of p10 (no. 3) coincided vertically with a distinct gp36 spot, underneath a molecular weight range of about 45,000, raising the possibility of their derivation from a gp36-p10 heterodimer. The longer distance between spots 3 and 2, relative to the distance between spots 1 and 2, also suggests that the third p10 spot did not arise from a tetramer of p10, but probably derived from a heterodimer of p10 and gp36.

Morphology of cross-linked MuMTV. Tissue culture-derived MuMTV preparations treated with DTBSP (400 μg/ml) under conditions which cross-link gp36 and gp52 to generate

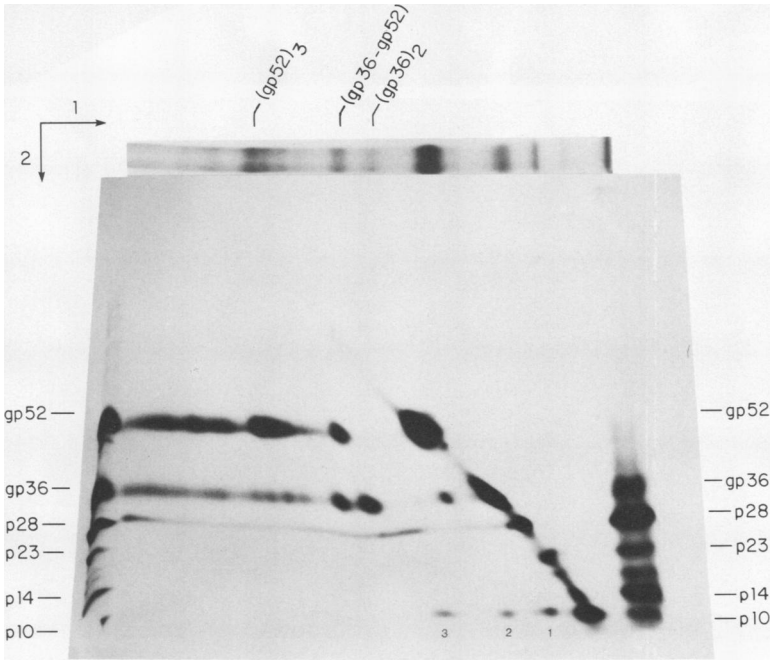

FIG. 5. *Two-dimensional gel of a MuMTV preparation cross-linked with MMB (20 mM). The strip on top is a duplicate of the one analyzed in the second dimension. The lane on the left side is marker virus. MuMTV proteins that were cross-linked into oligomeric complexes too large to enter the separating gel in the first-dimension gel were removed, reduced, and run in the lane shown on the right side of the two-dimensional gel.*

the 230,000-molecular-weight component were examined under the electron microscope. Most of the cross-linked virions appeared spherical (Fig. 6A and B), and about 40 to 50% of the viral particles were penetrated by phosphotungstic acid (Fig. 6A). Whereas the virions penetrated by stain usually formed large aggregates (Fig. 6A), the unpenetrated particles were uniformly distributed (Fig. 6B). Unlike the untreated particles (Fig. 6D), the cross-linked virions (Fig. 6A, B, and C) did not show head and tail configurations but, instead, were found to exhibit small blebs. The morphology of the projections of the cross-linked virions did not appear to have undergone any drastic alteration, particularly in those particles that were not penetrated by stain.

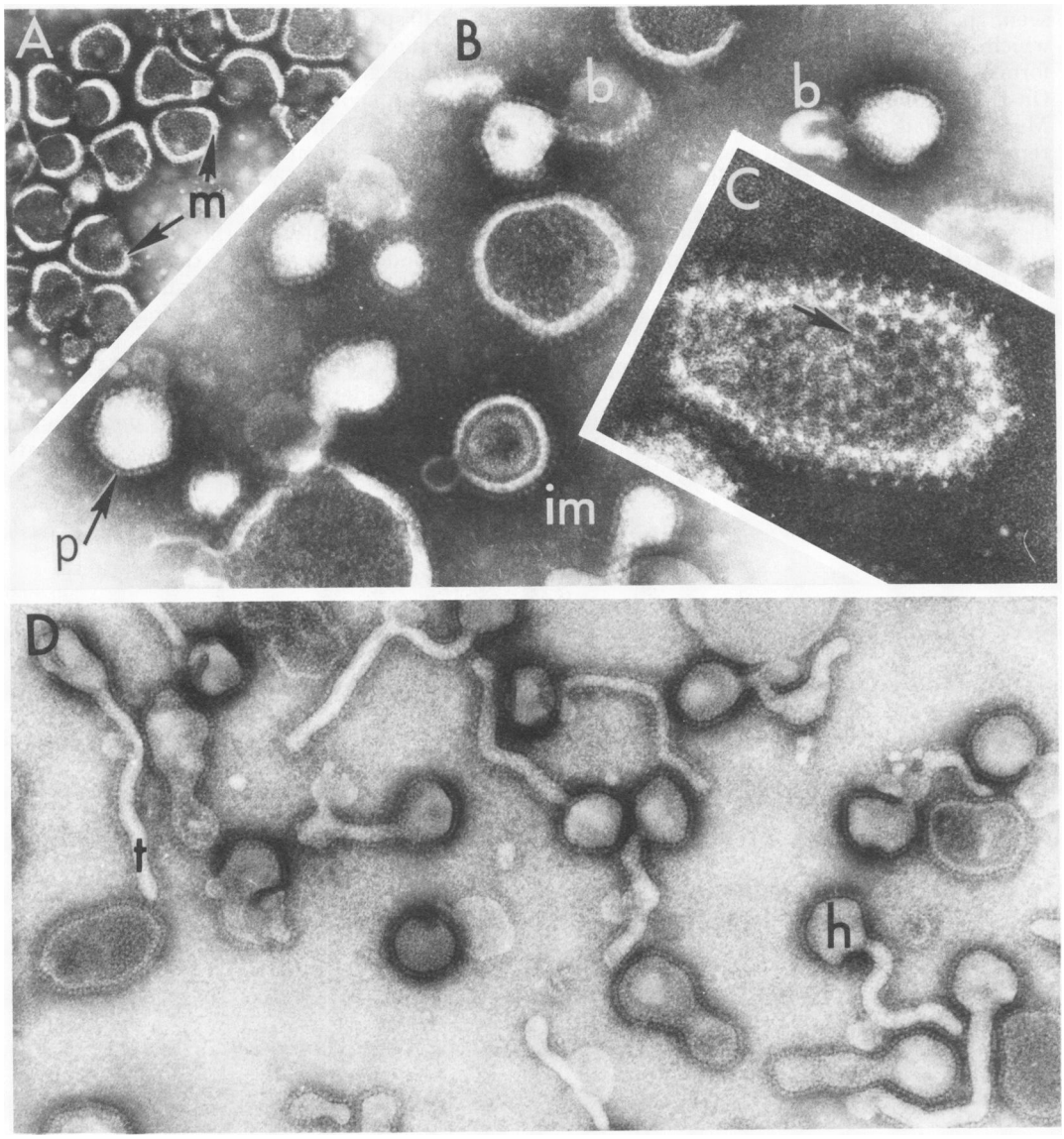

FIG. 6. *Appearance of tissue culture-grown MuMTV after treatment with the cross-linking reagent DTBSP (400 μg/ml) (A to C). The membrane (m) of the particles in (A) is clearly visible. An immature particle (im) is shown in (B). Most of the particles in (B) are not penetrated by stain; they show a small bleb (b). P denotes projection. The particle in (C) shows a reticular structure (arrow). (D) shows the morphology of untreated MuMTV (control). Most of the untreated particles show head (h) and tail (t) configurations. (A) ×40,000; (B) ×100,000; (C) ×280,000; (D) ×80,000.*

The knob portion of the projections of many stained particles appeared slightly smaller in size, whereas the stalks that connect the knobs to the viral membrane were much more visible (Fig. 6C) than those in untreated virions. These observations suggest that the knobs and stalks formed stable aggregates after treatment with cross-linking reagents. Furthermore, the previously described (23) surface reticular structure of sixfold symmetry was much more frequently exhibited by the cross-linked virions (Fig. 6C) than by the control viral preparations.

Subunit structure of the knobs of MuMTV projections. In an earlier study on the surface structure of milk-derived MuMTV (23), it was observed that the viral surface projection knobs seemed to be composed of at least three subunits. In this study, we examined in detail the knob structure of tissue culture particles and established that the knobs of MuMTV are indeed made of three subunits. Figure 7A shows the morphology of an MuMTV particle, the surface of which is covered with projections. In some areas of the viral surface (squared area in Fig. 7A), the projection knobs are seen to have a sixfold symmetry; i.e., one projection knob is surrounded by six immediate neighbors (Fig. 7B). The knobs have the appearance of triangles, and two or three subunits are readily seen in some of these knobs; the presence of a hole (arrow, Fig. 7B) is also evident. To establish the number of subunits making up the projection knobs, we employed the technique of image rotation as described earlier (23). Figures 7C, D, E and F represent the appearance of the projection knob shown by the arrow in Fig. 7B after three-, four-, five- and sixfold rotations, respectively. It appears from these rotation images that this particular knob is composed of three units, as only threefold rotation resolved the subunits clearly. It should be stressed that the structures that are analyzed here are close to the resolution (practical) of the microscope, which contributes to some distortions and imperfections of the images. That the knobs are arranged in a sixfold symmetry on the viral surface is also evident from the sixfold rotation image (Fig. 7F); note the resolution of the six knobs in Fig. 7F. The resolution of the subunits of the knobs is also dependent on how tightly they are grouped together. When the subunits are very tightly packed, they appear as a triangular structure and are not resolved by rotation (Fig. 7G). In knobs in which the subunits are spaced apart due to mechanical stress, the subunits could be resolved, and their number could be estimated from threefold rotation pictures as shown in Fig 7H through K. Rotations other than threefold did not resolve the subunits of the knobs analyzed in these figures. These observations strongly suggest that the knobs of MuMTV projections are composed of three subunits and support the biochemical results reported above.

DISCUSSION

Cross-linking reagents have become major tools for exploring subunit structures and molecular associations in various biological systems. The underlying assumption of this technique, that the cross-linked complexes provide chemical evidence of in situ interactions, has been generally accepted and borne out. The significant cross-linked products (those that reflect noncovalent interactions in the unperturbed state) are the ones that are rate favored, i.e., those that are formed at the lowest cross-linker concentrations and over a wide range of conditions. Among the cross-linked complexes of MuMTV proteins that we have observed that satisfy these criteria are the heterodimer gp36-gp52 and the homodimer gp36-gp36. The formation of these complexes indicates that in the native state, in the MuMTV envelope, the two glycoproteins gp36 and gp52 are in intimate contact and that gp36 molecules are in close association with each other. This close association of the two glycoproteins was suggested by the earlier observation (28) that gp36 and gp52 copurify together through lectin-agarose and molecular sieve chromatography. Another consistently observed cross-linked complex is what appears to be a homotrimer of gp52. This trimer of gp52 exhibits a rather fast migration in the SDS-polyacrylamide gels, which is probably caused by extensive internal cross-links within the gp52 molecule. The most interesting cross-linked complex that we have observed, however, is the 230,000-molecular-weight complex composed of gp36 and gp52. Although the large complex is only detectable at higher DTBSP concentrations, it is formed over a wide range of cross-linker concentrations and most notably is also formed in the presence of the detergent NP-40. The detergent treatment of MuMTV results in the disruption of the virus and formation of rosettes, which are viral membrane fragments in the form of vesicles carrying the typical MuMTV spike structures on their surface (24). On the basis of its size, composition, and the ratio of the two glycoproteins, we conclude that the large cross-linked complex is made up of three molecules each of gp52 and gp36. Since the rate-favored cross-linking reaction is the formation of gp36-gp52 heterodimers, it might be more accurate to describe the 230,000-molecu-

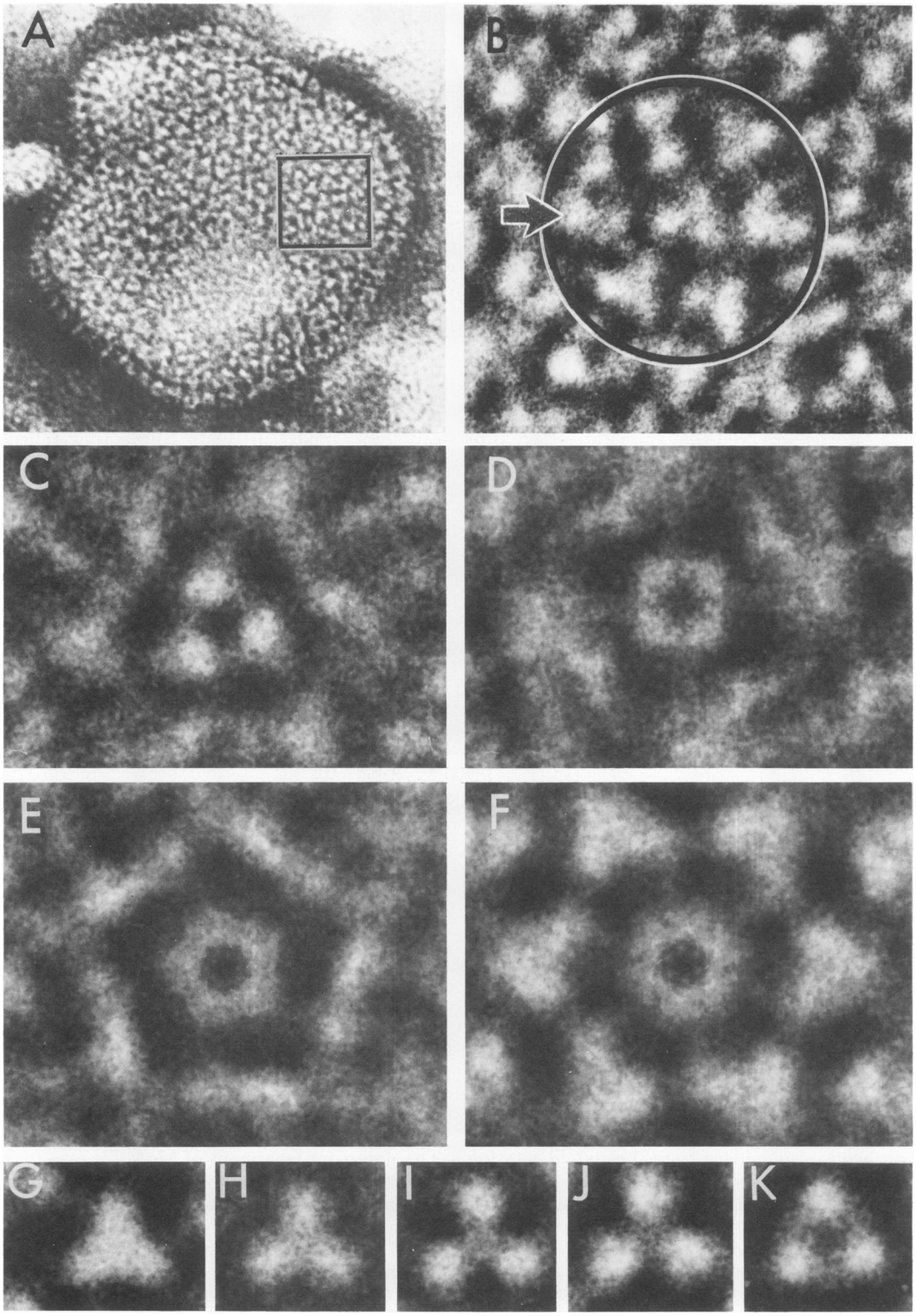

FIG. 7. *Surface morphology of tissue culture-grown MuMTV. The central part of the enclosed area in (A) shows projections in a sixfold symmetry. A highly magnified view of the projections is shown in (B). Three subunits (C) of the projection knob marked by an arrow in (B) are resolved by a threefold (n = 3) rotation about the central part of the structure; n = 4 in (D); n = 5 in (E); n = 6 in (F). (G) to (K) show threefold rotation images of five selected knobs in which the subunits were either held together very tightly or loosely. (A) ×350,000; (B) ×1,800,000; (C to K) ×2,900,000.*

lar-weight complex as made up of three gp36-gp52 heterodimers. The presence of cross-linked gp52 homotrimers indicates that the gp52 molecules are all in contact with one another in the larger complex. The absence of gp52 molecules from very large cross-linked complexes (those too large to enter the gel) suggests that, in the viral or rosette structures, gp52 molecules exist in isolated groupings of limited size, probably clusters of three. All of these observations lead to the conclusion that the 230,000-molecular-weight complex is actually the MuMTV spike that one observes in the electron microscope. Similarly, the 250,000-molecular-weight complex formed upon cross-linking Rauscher leukemia virus preparations must represent a trimer of gp70-p15E heterodimers and is probably the spike structure. Formation of homotrimers of gp85 (gp70-p15E complexes) after cross-linking of type C virus has been previously reported (25). Furthermore, Takemoto et al. (25) reported that no complexes larger than the 240,000-molecular-weight homotrimer of gp85 could be detected after reaction with various concentrations of cross-linking agents. A major difference between type C and type B virus envelope protein interactions emerges from these studies; namely, in type C viruses, the envelope proteins are held together by disulfide bonds (20), whereas in MuMTV, the two glycoproteins seem to interact by hydrophobic forces (15, 28).

The above conclusions about the nature of the 230,000-molecular-weight MuMTV complex, obtained by biochemical means, complement the electron microscopic observation that MuMTV surface projections seem to be composed of three subunits. This observation was originally made in a study on the surface structure of RIII milk virus (23). The present studies on tissue culture-derived MuMTV show even more clearly the subunit structure of the MuMTV surface projections. The three-part substructure of the knobs is clearly evident upon analysis by the image rotation technique. Electron micrographs of DTBSP cross-linked MuMTV preparations show that the membrane is permeable to stain and that the morphology of the virion generally resembles that of glutaraldehyde-fixed virions.

Of the MuMTV core proteins, p14 will most readily form homooligomers; it does so even in the absence of cross-linking agents. In nonreduced virion preparations, a large fraction of p14 molecules is complexed in disulfide-bonded oligomers too large to penetrate into the stacking gel. Upon exposing the virus preparation to oxidizing agents, all of the p14 molecules complex into these large oligomers. Oxidation of virus also causes the disappearance of the protein band of 30,000 molecular weight, just above p28, that is present in all MuMTV isolates. Tryptic peptide map studies of MuMTV proteins (6, 10) have demonstrated that all of the p14 peptide spots are contained in the p30 tryptic peptide map. Although the role of p30 in MuMTV assembly is not known, its behavior upon oxidation probably reflects the fact that the p14 sequence is contained within it. The major core protein p28 shows a very slight tendency to form homooligomers in the presence of cross-linkers, much less so than the major core proteins of type C viruses.

While this work was in progress, a report appeared describing DTBSP cross-linking studies with RIII milk MuMTV (7). Our results agree with and confirm a number of their observations, including that p14 forms large homooligomers and that DTBSP cross-linking generates homodimers of gp36 and heterodimers of gp36-gp52. However, Dion et al. (7) report that heterodimers of the major core protein p28 and membrane glycoprotein gp36 are present both in control nonreduced viral preparations and in DTBSP cross-linked virions. We have never observed such a p28-gp36 complex under any conditions and have also not observed the gp52 homodimers which Dion et al. (7) also report. Furthermore, these workers did not report the formation of the 230,000-molecular-weight complex, even though they studied isolated rosette preparations at high (400 μg/ml) DTBSP concentrations. The reason for these discrepancies is not known, although it is possible that the use of high concentrations of virus needed for staining and a lower-resolution gel system (7) might account for the differing interpretations.

As for the interaction of any core proteins with any of the envelope proteins of MuMTV, the only evidence that we have is a possible formation of a p10-gp36 heterodimer in the presence of the cross-linker MMB. Although the amount of this complex that we observe is quite small, it is the reaction that one would expect in view of the studies (5, 15, 16) which have shown that p10 has an affinity for the MuMTV envelope. Recently, it has been shown that the N-terminal *gag* region protein of avian and murine type C viruses can be linked to viral membrane lipids with dimethyl suberimidate (19). Since p10 seems to be the equivalent protein in the MuMTV system (6, 16), by analogy one would expect that it too is membrane associated and hence possibly in contact with gp36. Failure to detect more p10-gp36 heterodimers is not unexpected, however, since a number of studies with type C viruses (3) and a recent report on MuMTV (16) suggest that the recognition be-

tween the N-terminal *gag* protein and the hydrophobic membrane protein might occur at the *gag* polyprotein precursor stage, before processing.

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