JOURNAL OF VIROLOGY, Dec. 1980, p. 872-877
0022-538X/80/12-0872/06$02.00/0

Vol. 36, No. 3

NOTES

Structure of and Alterations to Defective Murine Sarcoma Virus Particles Lacking Envelope Proteins and Core Polyprotein Cleavage

ANTHONY DEMSEY,[1]† FULMER COLLINS,[1] AND DOUGLAS KAWKA[2]

Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205,[1] and Merck Institute for Therapeutic Research, Rahway, New Jersey 07065[2]

HTG2 hamster cells produce a defective murine sarcoma virus lacking gp70 and, consequently, viral surface projections (knobs), but the lack of knobs appears to have no effect on intramembrane particle distribution. In addition, it has been noted that the core of the virus remains in the "immature" form as a result of the failure of the polyprotein precursor (p65) to undergo cleavage. However, incubation of HTG2 virus with avian myoblastosis virus was found to yield specific cleavage products of p65.

Recently, reports from this and other laboratories have described the protein composition and structure of a defective murine sarcoma virus produced in HTG2 hamster cells (5, 16). By a number of procedures, the cells and their viruses have been shown to lack detectable gp70, the major viral envelope glycoprotein, as well as p15E, p10 antigenicity, and reverse transcriptase; the viruses are consequently noninfectious. Additionally, the core of this C-type virus retains its spherical, "immature" form and fails to reorganize into the amorphous "collapsed" or "mature" form.

Such a retention of the immature core has been correlated with a lack of proteolytic cleavage of the core precursor polyprotein molecule, $Pr65^{gag}$ (20-22). Thus, appropriately, gels of purified and disrupted HTG2 virus reveal only a single 65,000-molecular-weight band and none of the usual, lower-molecular-weight bands corresponding to p30, p15, p12, and p10. Furthermore, radioimmunoprecipitations of radiolabeled virus with anti-p30, anti-p15, or anti-p12 serum precipitated only the 65,000-molecular-weight protein (p65) (5, 16); however, anti-p10 serum was not immunoreactive (16).

We report here some further investigations into the morphological abnormalities in HTG2 virus particles relating to these proteins. Such studies may have broader implications since similar abnormalities, i.e., virus assembly in the absence of envelope proteins (4) and the retention of a core precursor lacking p10 antigenicity (14), have been reported for other retroviruses.

HTG2 cells were provided by A. Gazdar (Veterans Administration Hospital, Washington, D.C.). Cells were grown in McCoy 5A modified medium with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml in an atmosphere of 5% CO_2. $JLSV_9$-RLV, Rauscher murine leukemia virus (R-MuLV)-infected mouse fibroblasts, were obtained from The John L. Smith Memorial for Cancer Research (Pfizer and Co., Maywood, N.J.) and were cultivated similarly, but in modified essential medium with Earle salts and glutamine. Virus was harvested from cells grown in roller bottles and purified as previously described (7).

Cells were prepared for thin sectioning by fixation for 1 h at room temperature in 0.08 M cacodylate-buffered 2% glutaraldehyde and gentle scraping of monolayers into centrifuge tubes followed by a buffer rinse and fixation in buffered 1% osmium tetroxide (OsO_4). Purified virus pellets were fixed in buffered 1% OsO_4 alone, whereas negatively stained virus was unfixed. Specimens were stained, dehydrated, and embedded as described previously (7). Cells were freeze-fractured using a Balzers 350M unit (Balzers A. G., Liechtenstein) after fixation in cacodylate-buffered 2% glutaraldehyde, rinsing in buffer, infiltration with 20% glycerol, and freezing in a supercooled liquid nitrogen slurry. After growth for 2 to 3 days on glass cover slips, cells were prepared for freeze-drying by fixation in situ with buffered 1% OsO_4 followed by rinsing in distilled water. Carbon-platinum surface replicas of freeze-dried (−82°C for 25 min) cells

† Present address: Laboratory of Experimental Pathology, National Institutes of Health, Bethesda, MD 20205.

were prepared as previously described (7, 8). Preparations were viewed with either a JEM 100B or a Philips 300 electron microscope, and all micrographs of replicas were printed with white shadows directed upward.

For radioimmunoprecipitations, virus was labeled with either ^{14}C-amino acids or [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.). Cells were grown in petri dishes (150-mm diameter) to two-thirds confluency and incubated for 15 min with Dulbecco modified essential medium with 1% normal amino acid content or without methionine. Cells were then incubated for 2 h in 9 ml of this same medium with the addition of the appropriate isotope (10 μCi of ^{14}C-amino acids or 50 μCi of [^{35}S]methionine per ml) and subsequently incubated overnight with the addition of 1 ml of complete medium. Radioimmunoprecipitations of purified virus were performed by the procedures of Racevskis and Sarkar (18), preparations were electrophoresed on 5 to 20% exponential gradient sodium dodecyl sulfate-polyacrylamide gels, and gels were autoradiographed with Kodak XR2 X-Omat film. For experiments involving in vitro cleavage of the p65, HTG2 virus (approximately 100,000 counts) was mixed with 100 to 500 μg of R-MuLV, Moloney MuLV, or avian myoblastosis virus in the presence of 0.5% Nonidet P-40 to disrupt the virus and incubated at 37°C for 1 to 24 h before radioimmunoprecipitation. Goat anti-R-MuLV gp70 (6S-0508), p30 (6S-0492), p15 (5S-0716), and p12 (4S-0455) sera were obtained from Huntington Research Center (provided by R. Wilsnack), and rabbit anti-Friend MuLV gp71 and p31 sera were obtained from W. Schäfer, Tübingen, West Germany; 10 to 50 μl of each antiserum was used per precipitation, depending on the titer of the particular antiserum.

Sodium dodecyl sulfate-polyacrylamide gels of labeled HTG2 virus and of virus immunoprecipitated with antisera to MuLV components confirmed that HTG2 virus was composed of only one structural protein, p65, and that this molecule possessed core protein antigenicities (Fig. 1). In addition, negative staining and thin sectioning of purified HTG2 virus demonstrated corresponding retention of the immature structure of the viral core after budding (Fig. 2).

We previously demonstrated that viral gp70 on budding virus surfaces is organized into 10-nm globular projections (knobs) which are also present on the rest of the cell surface in addition to normal, uninfected cell surface particulation (7, 8). In contrast, the lack of gp70 on HTG2 cells was reflected in a sparse cell surface particulation, and virus buds themselves appeared to be devoid of knobs (Fig. 3). Interestingly, the

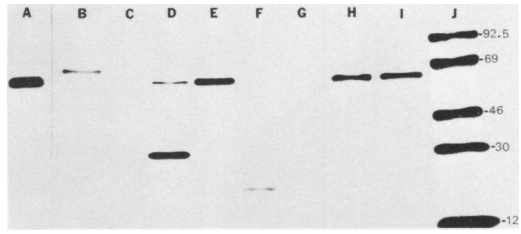
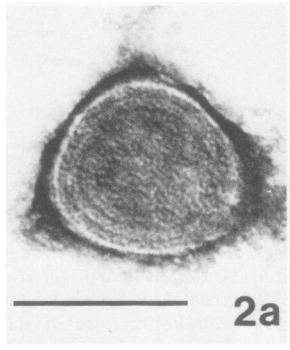


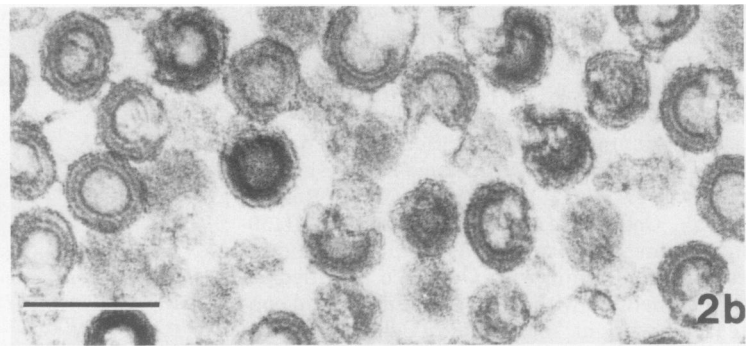
FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of ^{14}C-amino acid-labeled viruses: (A) Purified HTG2 virus showing single protein band; (B) R-MuLV immunoprecipitated with anti-gp70; (C) HTG2 virus immunoprecipitated with anti-gp70; (D) R-MuLV immunoprecipitated with anti-p30; (E) HTG2 virus immunoprecipitated with anti-p30; (F) R-MuLV immunoprecipitated with anti-p15E; (G) HTG2 virus immunoprecipitated with anti-p15E; (H) HTG2 virus immunoprecipitated with anti-p15; (I) HTG2 virus immunoprecipitated with anti-p12; and (J) molecular weight standards: phosphorylase B, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; and cytochrome, 12,300.*

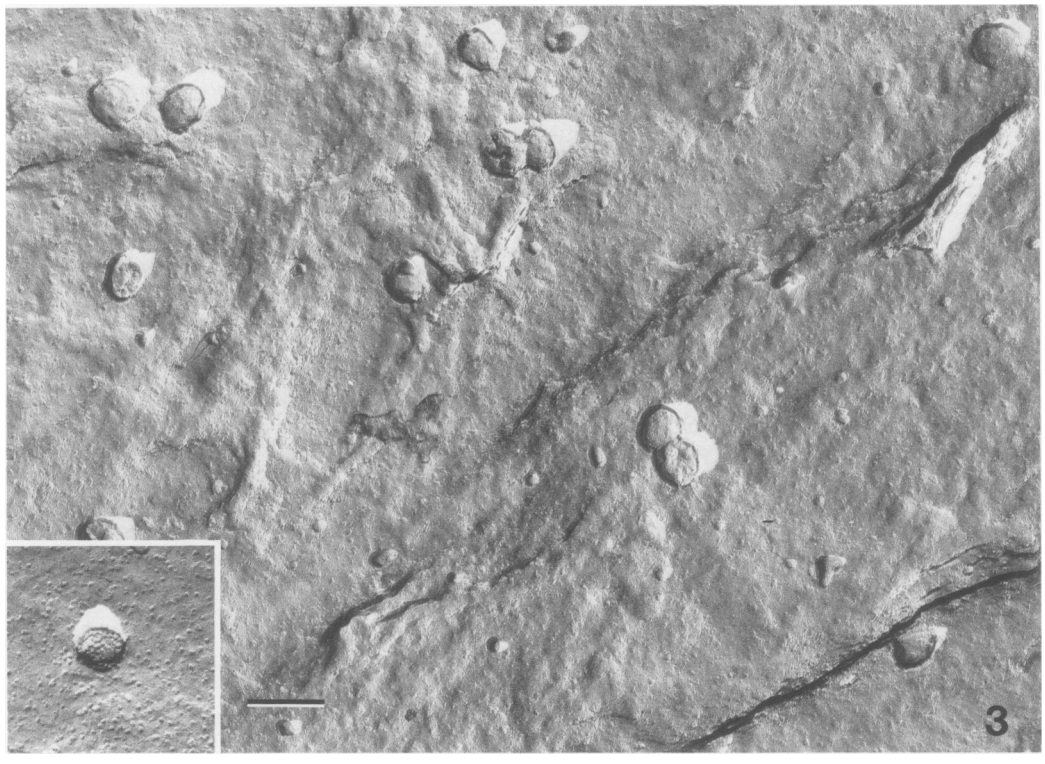
viral envelope was often seen, both in replicas of freeze-dried cells (Fig. 3) and in thin sections of budding virus (Fig. 4), to fit loosely around the virus core. Furthermore, the lack of virus gp70 and knobs did not seem to alter the distribution of intramembranous particles. An apparently random arrangement of numerous intramembranous particles within the membrane was seen in freeze-fracture preparations (Fig. 5), except for the region of the putative virus bud, which was lacking intramembranous particles in the same manner as for wild-type virus-infected cell lines (7, 19). Thus, the lack of knobs appears to have little effect on the disposition of other intrinsic membrane proteins, even within the virus envelope membrane itself.

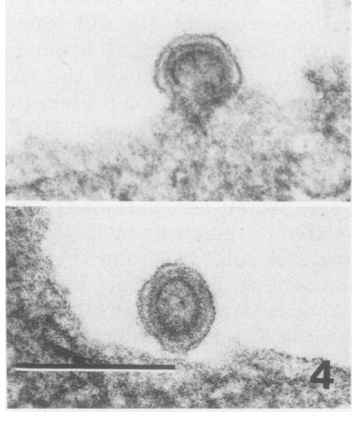
It has been proposed that virus-specific envelope glycoprotein inserted into the cell membrane can associate transmembranously with underlying viral core components and convert parts of the cell membrane into budding patches (1, 2). Consequently, the assembly of the virus might be regulated and directed via protein-protein interactions (3, 11). Although recent studies (15, 17) employing protein cross-linking reagents on purified MuLV have failed to demonstrate such envelope-core protein associations, this failure might be related to the spanning distance of the cross-linking molecules used and the availability of amino groups. In addition, as Pinter and Fleissner (17) have suggested, envelope-core associations existing during budding are probably not retained after the virus is budded from the membrane.

Our recent studies have suggested that self-


2a


2b


3


4


5

assembly or "nucleation" phenomena may indeed be involved in MuLV morphogenesis (6; A. Demsey, D. Kawka, S. Galuska, I. Margulies, and U. Heine, Arch. Virol., in press). We have demonstrated that transmembrane associations of envelope and core components appear to exist during growth of cells infected with a temperature-sensitive mutant of R-MuLV (ts25) at the restrictive temperature before bud formation (Demsey et al., in press). Subsequently, ts25 bud formation can be induced simply by incubation at temperatures as low as 4°C or at the restrictive temperature by incubation with antiserum to viral envelope gp70 (6). In this latter instance, a "mimicking" of the budding process is presumably achieved when patching of virus surface glycoproteins imposes concomitant core component associations in the subjacent cytoplasm, associations which may otherwise be sterically hindered. We have noted in this same system, however, that buds may form at the permissive temperature without the presence of gp70-containing knobs (Demsey et al., in press). Although one cannot exclude the possibility in this case that gp70 is actually present but not formed into knobs, the total lack of gp70 in HTG2 cells and virus does not allow a similar explanation. This is in contrast to the suggestion of Bolognesi et al. (2) that virus particle synthesis is unlikely in the absence of virus glycoprotein. Despite the absence of gp70, however, minimum requirements for bud formation are presumably still maintained and might simply consist of the partitioning of slightly hydrophobic p30 or moderately hydrophobic p15 moieties (12, 15) of p65 into the membrane. The interactions between p30 sequences of p65 could then initiate and drive the budding process (16). In addition, the apparent loose fit of the virus envelope around the core of HTG2 virus might reflect the suggestion of Blough and Tiffany (1) that the shape and rigidity of the viral envelope is maintained by the transmembrane association of a specific number of envelope components with a specific number of core components.

The interaction, then, of the p30 moities of p65 with one another in this scheme for virus development is clearly not dependent on further core polyprotein processing, and this independence may be a general principle in C-type virus budding (2, 6). Moreover, the persistent retention of p65 in HTG2 virus provides a useful model for determining the conditions under which the core polyprotein undergoes cleavage, and subsequent experiments were undertaken for this purpose. In these studies, labeled HTG2 virus was incubated with purified virions of R-MuLV or Moloney MuLV for up to 24 h without detectable cleavage of p65. However, when labeled HTG2 virus was incubated for 1 h with concentrates of avian myoblastosis virus, extensive cleavage was seen. Autoradiograms of radioimmunoprecipitations employing antisera to p30, p15, and p12 revealed several cleavage products (Fig. 6). After 1 h at 37°C, the only cleavage product precipitated by anti-p30 serum was found at molecular weights of 40,000 to 42,000. A similar cleavage product was obtained by Yoshinaka and Luftig (20–22) during treatment of R-MuLV with low levels of Nonidet P-40, but we have not found a similar response of HTG2 virus to Nonidet P-40 alone. However, unlike the 40,000- to 42,000-molecular-weight product in the Yoshinaka and Luftig studies, p12 may be present in this HTG2 virus cleavage product as demonstrated by coprecipitation with anti-p12 serum. Since viral p10 is not present in the p65 of HTG2 virus but is evidently substituted with another 10,000-molecular-weight polypeptide (16), part of this broad 40,000- to 42,000-molecular-weight band might be p30 plus the substituted 10,000-molecular-weight segment. Although the predominant reactivity of our anti-p12 serum was found at molecular weight 42,000, a minor reactivity was seen with a 25,000- to 27,000-molecular-weight polypeptide doublet. Anti-p15 serum strongly reacted with this doublet, and with the 15,000-molecular-weight segment as well, but to a lesser degree. The additional reactivity of anti-p15 serum seen at molecular weight 42,000 was distinctly less than that seen with either anti-p30 or anti-p12 serum and might not be significant, since a similar faint band is usually precipitated with normal goat serum and presumably represents actin.

The proteolytic activity of avian RNA tumor viruses is commonly associated with structural protein p15, which is a C-terminal protein (10, 13). It is interesting, therefore, that in HTG2 virus the normal C-terminal virus protein, p10, is not present. This lack of p10 itself is probably not responsible for the core cleavage defect of

FIG. 2. *(a) Purified HTG2 virus showing ring-like immature core structure after negative staining with 1% uranyl acetate, pH 4.5. Bar, 100 nm. (b) Thin section of HTG2 virus pellet revealing immature core structure. Bar, 200 nm.*

FIG. 3. *Surface replica of freeze-dried HTG2 cell revealing sparse cell surface particulation and virus buds devoid of gp70-containing knobs. Inset: in contrast, the surface of a JLSV$_9$-RLV fibroblast possesses many knob-like particles on cell and virus surfaces. Bar, 200 nm.*

FIG. 4. *Thin sections of HTG2 cells showing virus buds with loose-fitting envelopes. Bar, 200 nm.*

FIG. 5. *Freeze-fracture of an HTG2 cell showing numerous intramembranous particles, except in the region of an apparent virus bud (arrow). Bar, 200 nm.*

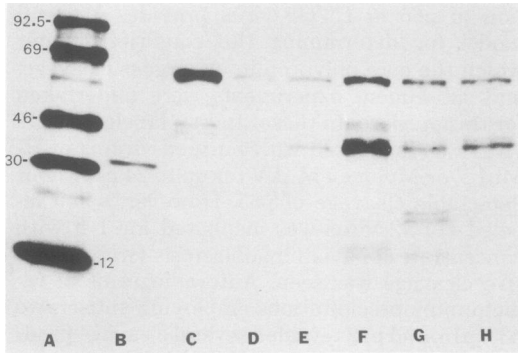


FIG. 6. *Autoradiogram of immunoprecipitates of ^{14}C-amino acid-labeled HTG2 virus and cleavage products: (A) molecular weight markers; (B) R-MuLV precipitated with anti-p30; (C) HTG2 virus incubated for 1 h at 37°C and precipitated with anti-p30; (D) HTG2 virus incubated for 1 h at 37°C and precipitated with normal goat serum; (E) HTG2 virus incubated for 1 h at 37°C with unlabeled avian myoblastosis virus and precipitated with normal goat serum; (F) HTG2 virus incubated for 1 h at 37°C with unlabeled avian myoblastosis virus and precipitated with anti-p30; (G) HTG2 virus incubated 1 h at 37°C with unlabeled avian myoblastosis virus and precipitated with anti-p15; (H) HTG2 virus incubated for 1 h at 37°C with unlabeled avian myoblastosis virus and precipitated with anti-p12.*

HTG2 virus, however, since previous studies could not correlate murine proteolytic activity with any of the viral structural polypeptides (23). The effectiveness of the avian myoblastosis virus protease in a murine system is particularly noteworthy because the avian virus protease appears to be papain-like (9), whereas the murine protease is reportedly similar to trypsin in its activity (21). In this regard, our inability to obtain cleavage of p65 with purified R-MuLV or Moloney MuLV will be pursued further, particularly in light of recent studies (13) suggesting that critical parameters may exist for the proteolytic reaction. In addition, further studies on the morphology of the core itself, both in its immature form and after in vitro cleavage, are presently under way.

Finally, cleavage of the HTG2 virus core polyprotein using a crude virus preparation cannot define the precise mechanisms of the cleavage process. Subsequent studies will require the use of purified viral components, alone or in combination, to determine these mechanisms and possibly their temporal order.

We thank Douglas Jones for photographic expertise and Nurul Sarkar for helpful discussions, advice, and encouragement.

LITERATURE CITED

1. **Blough, H. A., and J. M. Tiffany.** 1975. Theoretical aspects of structure and assembly of viral envelopes. Curr. Top. Microbiol. Immunol. **70**:1-30.
2. **Bolognesi, D. P., R. C. Montelaro, H. Frank, and W. Schafer.** 1978. Assembly of type C oncornaviruses: a model. Science **199**:183-186.
3. **Caspar, D. L. D.** 1965. Design principles in virus particle construction, p. 51-93. *In* F. L. Horsfall, Jr., and I. Tamm (ed), Viral and rickettsial infections of man. Lippincott, Philadelphia.
4. **DeGiuli, C., S. Kawai, S. Dales, and H. Hanafusa.** 1975. Absence of surface projections on some noninfectious forms of RSV. Virology **66**:253-260.
5. **Demsey, A.** 1979. Structure and composition of a noninfectious C-type retrovirus produced by a cell line established from a MSV-induced transplantable hamster tumor, p. 42-43. *In* G. W. Bailey (ed), Electron Microscopy Society of America Proceedings. 1979. Calitor's Publishing Division, Baton Rouge, La.
6. **Demsey, A., D. Kawka, S. Galuska, and C. W. Stackpole.** 1979. Assembly of a temperature-sensitive mutant of Rauscher murine leukemia virus at the cell surface induced by low temperature and by ligands. Virology **95**:235-240.
7. **Demsey, A., D. Kawka, and C. W. Stackpole.** 1977. Application of freeze-drying intact cells to studies of murine oncornavirus morphogenesis. J. Virol. **21**:358-365.
8. **Demsey, A., D. Kawka, and C. W. Stackpole.** 1978. Cell surface membrane organization revealed by freeze-drying. J. Ultrastruct. Res. **62**:13-25.
9. **Dittmar, K. J., and K. Moelling.** 1978. Biochemical properties of p15-associated protease in an avian RNA tumor virus. J. Virol. **28**:106-118.
10. **Kahn, A. S., and J. R. Stephenson.** 1979. Feline sarcoma virus-coded polyprotein: enzymatic cleavage by a type C virus-coded structural protein. J. Virol. **29**:649-656.
11. **Klenk, H.-D.** 1974. Viral envelopes and their relationship to cellular membranes. Curr. Top. Microbiol. Immunol. **68**:29-58.
12. **Marcus, S. L., S. W. Smith, J. Racevskis, and N. H. Sarkar.** 1978. The relative hydrophobicity of oncornaviral structural proteins. Virology **86**:398-412.
13. **Moelling, K., A. Scott, K. E. J. Dittmar, and M. Owada.** 1980. Effect of p15-associated protease from an avian RNA tumor virus on avian virus-specific polyprotein precursors. J. Virol. **33**:680-688.
14. **Oskarsson, M. K., J. H. Elder, J. W. Gautsch, R. A. Lerner, and G. F. VandeWoude.** 1978. Chemical determination of the ml Moloney sarcoma virus pP60^{gag} gene order: evidence for unique peptides in the carboxy terminus of the polyprotein. Proc. Natl. Acad. Sci. U.S.A. **75**:4694-4698.
15. **Pepinsky, R. B., and V. M. Vogt.** 1979. Identification of retrovirus matrix proteins by lipid-protein cross-linking. J. Mol. Biol. **131**:819-837.
16. **Pinter, A., and E. deHarven.** 1979. Protein composition of a defective murine sarcoma virus particle possessing the enveloped type-A morphology. Virology **99**:103-110.
17. **Pinter, A., and E. Fleissner.** 1979. Structural studies of retroviruses: characterization of oligomeric complexes of murine and feline leukemia virus envelope and core components formed upon cross-linking. J. Virol. **30**: 157-165.
18. **Racevskis, J., and N. H. Sarkar.** 1978. Synthesis and processing of precursor polypeptides to murine mammary tumor virus structural proteins. J. Virol. **25**:374-383.
19. **Sheffield, J. B.** 1974. Membrane alterations which accompany MuMTV maturation. I. Studies by freeze-cleave techniques. Virology **57**:287-290.
20. **Yoshinaka, Y., and R. B. Luftig.** 1977. Murine leukemia virus morphogenesis: cleavage of p70 *in vitro* can be accompanied by a shift from a concentrically coiled internal strand ("immature") to a collapsed ("mature")

form of the virus core. Proc. Natl. Acad. Sci. U.S.A. **74:** 3446–3450.

21. **Yoshinaka, Y., and R. B. Luftig.** 1977. Partial characterization of a p70 proteolytic factor that is present in purified virions of Rauscher leukemia virus (RLV). Biochem. Biophys. Res. Commun. **76:**54–63.
22. **Yoshinaka, Y., and R. B. Luftig.** 1977. Characterization of Rauscher leukemia virus (RLV) p. 40–42, an intermediate cleavage product of the group specific antigen (*gag*) precursor polypeptide, p. 65–70. Biochem. Biophys. Res. Commun. **79:**319–325.
23. **Yoshinaka, Y., and R. B. Luftig.** 1977. Properties of a p70 proteolytic factor of murine leukemia virus. Cell **12:**709–719.