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Intracellular Synthesis of Mouse Mammary Tumor Virus Polypeptides: Indication of a Precursor Glycoprotein

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Mouse mammary tumor virus polypeptides were detected in the cytoplasm of mouse mammary tumor cell cultures using immunological precipitation techniques. The anti-mouse mammary tumor virus serum precipitated the major virion glycoproteins gp49 and gp37.5/33.5 and a viral-related nonvirion glycoprotein of 76,000 daltons. Subcellular fractionation studies revealed that the cell-associated virion glycoproteins were present in the membrane fraction. Pulse-chase experiments indicated that a viral-related nonvirion glycoprotein of 76,000 daltons may be a precursor to one or more of the virion glycoproteins.

Mouse mammary tumor virus (MTV), a member of the oncornavirus group, contains an aggregated 70*S* single-stranded RNA genome which can be dissociated into 30-35*S* subunits (7). The virion contains 9 to 12 polypeptides including two or three major glycopeptides which are present in the outer membrane of the virion (6, 9, 21, 22, 28, 31). Viral replication proceeds in a manner which would appear to preclude the use of drugs to selectively inhibit host protein synthesis (29). Furthermore, viral protein synthesis constitutes only a small percentage of the total cellular protein synthesis (25, 30). Immunological precipitation procedures in conjunction with polyacrylamide gel electrophoresis have allowed the selection and identification of viral-related cytoplasmic proteins (8, 10, 19, 25, 30). Using these techniques on whole cell cytoplasmic extracts from mouse mammary tumor cells, the major MTV glycoproteins gp49 and gp37.5/33.5 and a large viral related nonvirion glycopeptide of 76,000 daltons (gp76) have been identified. Cell fractionation procedures show that these glycopeptides are present in the membrane fraction of the cytoplasm. The major internal polypeptide p24 was found in only small amounts in the cytoplasmic extracts, and the other virion polypeptides were not detected. These findings and their possible significance in the replication of MTV are discussed.

MATERIALS AND METHODS

Cell cultures. Tumor cell cultures were prepared from spontaneous mammary gland adenocarcinomas of the BALB/cfC3HCrg1 mouse strain by a previously described method (5, 17). Cells were seeded at a density of 5×10^5 cells per cm^2 in 10-cm petri dishes (Falcon) in Dulbecco modified Eagle medium (DME) containing 15% fetal calf serum, 10 μg of bovine insulin per ml (Sigma), 5 μg of hydrocortisone per ml, 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml.

C3H3T7 cells chronically infected with gross passage A virus were a generous gift of A. DeCléve, Stanford University Medical School, Calif. The cells were propagated in Dulbecco modified Eagle medium containing 10% fetal calf serum, high glucose (4.5 g/liter) and antibiotics.

Labeling of cultures. The cell cultures were routinely labeled with 4 ml of medium composed of 9 volumes of Hanks balanced salt solution and 1 volume of Dulbecco modified Eagle medium, with usual concentrations of vitamins, hormones, and antibiotics. The medium contained, at 10 μCi/ml, ^{3}H- or ^{14}C-labeled amino acid mixtures, D-[^{3}H]glucosamine (224 mCi/mmol), or [^{35}S]methionine (171 Ci/mmol) (New England Nuclear). For pulse-chase experiments, cell cultures were pulsed for 15 min with 2 ml of labeling medium containing 125 μCi of ^{3}H-labeled amino acid mixture per ml and then chased for various periods with culture medium.

Isolation of virus. Radioactively labeled MTV was isolated from the tissue culture fluid of tumor cultures as described (6).

Preparation of cell extracts. (i) Whole cell: labeled cell cultures were washed three times with 5 ml of cold phosphate-buffered saline, scraped from the dishes, and washed twice with a further 20 ml of phosphate-buffered saline by centrifugation at 400 $\times$ *g* for 5 min. The cell pellet derived from two to eight 10-cm petri dishes of tumor cultures was resuspended in 1 to 2 ml of buffer (0.01 M Tris-hydrochloride, pH 7.5, 0.025 M KCl, and 0.005 M $MgCl_2$) and detergent was added to a final concentration of 0.5% (vol/vol) Triton X-100 and 0.5% (wt/vol) deoxycholate. The cell pellet was resuspended and homogenized in a glass homogenizer with a tight-fitting teflon pestle until at least 90% of the cells were disrupted as determined by phase-contrast microscopy. The ho-

mogenate was centrifuged at 100 × g for 5 min, followed by centrifugation of the supernatant at 150,000 × g for 35 min. The resulting supernatant was used directly in the immunoprecipitation procedure. (ii) Subcellular fractionation: the labeled cells were washed as described for the total cell extracts and homogenized in buffer without the addition of detergents. The homogenate was centrifuged at 100 × g for 5 min, the nuclear pellet was re-extracted with a small volume of homogenization buffer, and the combined supernatants were centrifuged at 2,300 × g for 5 min. The supernatant was layered over a discontinuous sucrose density gradient composed of 1.5 ml of 10% (wt/vol) sucrose in buffer and 1.5 ml of 50% (wt/vol) sucrose in buffer, and then centrifuged at 220,000 × g for 90 min. The portion of the gradient above the 10% sucrose layer was taken as the soluble fraction (cytosol) and the interface between the 10% sucrose and 50% sucrose as the membrane fraction. Each of these fractions was made 0.5% with respect to both Triton X-100 and deoxycholate and used in the immunoprecipitation procedure.

Protein determinations. The method of Lowry et al. (16) was used with bovine serum albumin as a standard.

Antiserum. Rabbit anti-MTV serum was prepared by using virus isolated from mouse milk of the BALB/cfC3HCrg1 strain, disrupted with Tween 80 and ether as described previously (20). The first inoculation was composed of an equal volume of dissociated viral protein and complete Freund adjuvant. Subsequent inoculations contained an equal volume of incomplete Freund adjuvant and viral proteins and were administered at 3- to 4-week intervals, at multiple subcutaneous sites. Antisera obtained after the fifth, sixth, and seventh immunizations were used in the experiments. The antiserum was absorbed with an equal volume of skimmed milk from virus-free mice of BALB/cCrg1 strain, by incubation at 37 C for 30 min, storage at 4 C overnight, and finally clarification by centrifugation at 150,000 × g for 30 min.

Immunoprecipitation procedures. To obtain a quantitative immunoprecipitation of antigens, the antiserum was titrated against detergent-disrupted purified virus and against whole cell cytoplasmic extract. Increasing amounts of antiserum were added to a constant known amount of antigen, either disrupted MTV, or cytoplasmic extract. The final volume of each reaction was adjusted to 250 μl by the addition of buffer. The reactions were incubated at 37 C for 30 min and then at 4 C overnight. The resulting precipitates were collected by centrifugation at 13,000 × g for 30 min and washed three times with 200 μl of phosphate-buffered saline. The washed precipitates were dissolved in 200 μl of 0.1% sodium dodecyl sulfate and counted in a Triton X-100-toluene-based scintillant (Triton X-100-toluene, 1:2 containing 4 g of Omnifluor per liter [New England Nuclear]).

Immune precipitation of the cytoplasmic extracts for analysis on polyacrylamide gels was performed as described using an optimal ratio of antiserum to extract as determined from the titration curves. The washed precipitates were subjected to polyacrylamide gel electrophoresis as described below.

Polyacrylamide gel electrophoresis. The dissociation and electrophoresis of samples on columns was based on the method of Maizel et al. (18). The samples were disrupted with urea, sodium dodecyl sulfate, and mercaptoethanol at a final concentration of 5 M and 0.1 and 2%, respectively. The mixture was heated to 100 C for 2 min and applied directly to the gels after cooling. The electrophoresis was carried out on 12.5 or 10% polyacrylamide gels at 2.5 V/cm for 22 h (6). After the electrophoresis was complete, the gels were cut into 1-mm slices, treated with 0.25 ml of Protosol (New England Nuclear) at 70 C for 2 h, and counted in a toluene-Triton X-100 scintillant as described. (An additional 6 ml of acetic acid per liter of scintillant was added to prevent chemiluminescence.)

RESULTS

Quantitation of the immunoprecipitation procedures. To determine the optimal volume of anti-MTV serum to immunoprecipitate viral-related proteins from cytoplasmic extracts, a constant amount of whole cell cytoplasmic extract was reacted against increasing amounts of immune and control serum. Figure 1a demonstrates that the most efficient precipitation of viral polypeptides occurred at a ratio of approximately 100 μl of anti-MTV serum per mg of extract protein. Greater amounts of antisera did not increase nor decrease the amount of precipitation. The specific precipitable material was approximately 0.5% of the input, whereas the nonspecific radioactivity accounted for up to 1% of the input. However, when precipitates were analyzed on polyacrylamide gels, the contribution by nonspecific radioactivity did not interfere with the resolution of the viral polypeptides.

To examine the multivalency of the anti-MTV serum, purified virions were detergent disrupted and immune precipitated under optimal conditions, as predetermined by an immunoprecipitation titration (Fig. 1b). The immune precipitates were analyzed on polyacrylamide gels which showed that the major MTV polypeptides gp49, gp37.5/33.5, p29, and p24 were effectively precipitated (Fig. 2). The polypeptides gp58, p17, p13.5, and p8 were precipitated less efficiently.

Cytoplasmically synthesized MTV-related polypeptides. Mouse mammary tumor cells were cultured for several days to obtain a good level of virus replication before labeling with either ^{3}H-labeled amino acids or [^{35}S]methionine. After 18 to 20 h of labeling, the cultures were washed, and a whole cell cytoplasmic extract was prepared. The labeled extracts which contained 50 to 70% of the total trichloroacetic acid-precipitable radioactivity were immune precipitated, and the precipitates were

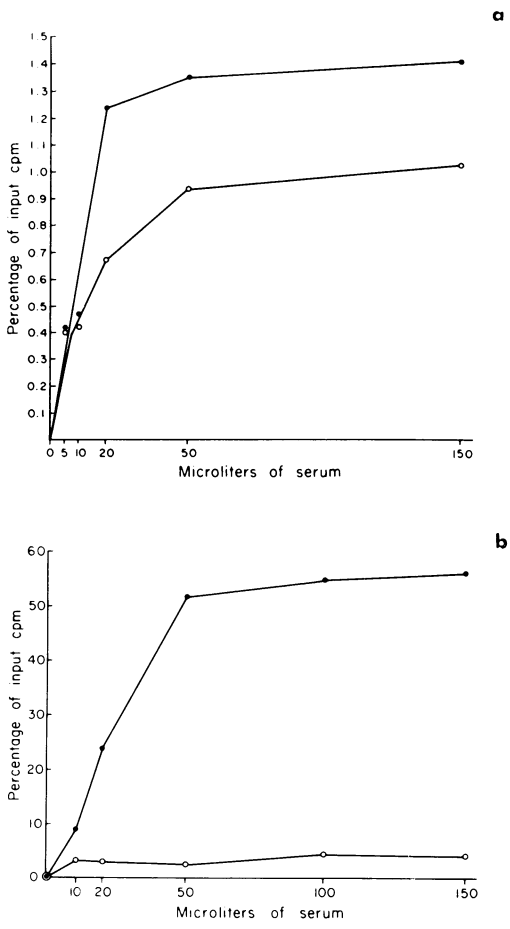

FIG. 1. (a) *Immunoprecitation curve of a whole cell cytoplasmic extract. 190 μg of extract (2 × 10⁵ counts/min) was immunoprecipitated with increasing amounts of either anti-MTV serum (●) or normal rabbit serum (○). (b) Immunoprecipitation curve using detergent disrupted purified MTV (2.3 μg or 1,670 counts/min).*

electrophoresed as described. The polyacrylamide gel profiles show the presence of the virion glycopeptides gp49 and gp37.5/33.5 and a new polypeptide of molecular weight 76,000 (Fig. 3a and b). The virion polypeptide p24 was present in very small amounts and the polypeptides gp58, p29, p17, p13.5, and p8 were not detected. The polypeptide profiles of cell extracts labeled with either ^{3}H-labeled amino acid mixtures or [^{35}S]methionine were similar. However, the amount of gp49 relative to the gp37.5/33.5 double peak was always less in [^{35}S]methionine-labeled extracts, a feature also reflected in the profiles obtained with virions from similarly labeled cultures (Fig. 2).

Cytoplasmic extracts prepared from cell cultures labeled with D-[^{3}H]glucosamine showed incorporation of the isotope into the glycoproteins gp49 and gp37.5/33.5 and the polypeptide of molecular weight 76,000, indicating that this polypeptide is also a glycoprotein (Fig. 3c).

The specificity of the immunoprecipitation procedure was established by demonstrating that cytoplasmic extracts, derived from cell cultures of normal mammary gland epithelium of BALB/c mice (MTV free), did not show viral-related polypeptides after immunoprecipitation and analysis on polyacrylamide gels (data not shown). Furthermore, similar procedures performed with cultures of C3H3T7 cells chronically infected with gross passage A virus gave similar results (Fig. 3d).

Pulse-chase experiments. To investigate the possible precursor nature of gp76, tumor cell cultures were pulsed with a ^{3}H-labeled amino acid mixture for 15 min and then chased with unlabeled culture medium for periods of 1 or 4 h. The resulting polyacrylamide gel profiles show that only gp76 was detected immediately after the pulse and subsequently diminished during the chase, whereas gp49 and gp37.5/33.5 were present only in the profiles from the chase (Fig. 4). Since gp76 is a glycoprotein and appears before the virion glycoproteins, a precursor role for this polypeptide is indicated.

Subcellular localization of viral polypeptides. The results of a cell fractionation experiment are shown in Fig. 5. After homogenization, the ^{3}H-labeled amino acid cell extract was centrifuged to yield a supernatant containing approximately 44% of the total trichloroacetic acid-precipitable radioactivity. Fractionation on a step sucrose density gradient resulted in 64% of the supernatant remaining in the soluble fraction, 12% collecting on the 10 to 50% sucrose interface as the membrane fraction, and 5% recovered as a pellet. Addition of detergents to the membrane fraction resulted in solubilization of 76% of the radioactivity. Hence, an overall yield of 28% as the cytosol fraction, 4% as soluble membrane fraction and 1% as insoluble membrane fraction, was obtained. The electrophoretic analysis of the immunoprecipitates from the cytosol and solubilized membrane fraction demonstrated that MTV proteins were present in the solubilized membrane fraction, whereas none were detected in the cytosol (Fig. 5).

DISCUSSION

Immunological precipitation techniques have enabled us to demonstrate intracellular synthesis of viral polypeptides. The immunoprecipita-

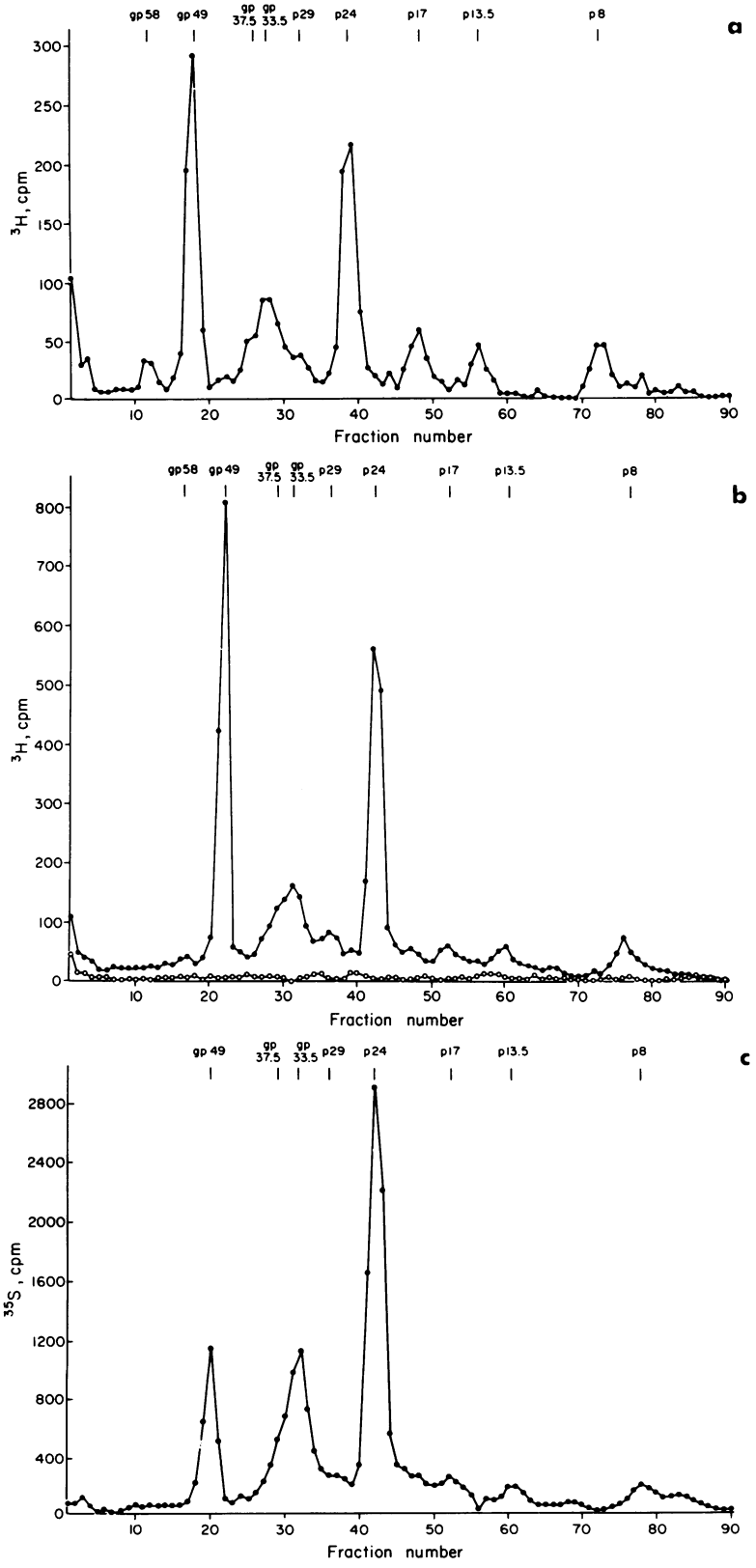

FIG. 2. *(a) Electrophoretic gel profile of MTV polypeptides separated by electrophoresing purified virus labeled with ^{3}H-labeled amino acids. (b) Gel profile obtained by electrophoresing the immunoprecipitate obtained with disrupted MTV and anti-MTV serum (●) or normal rabbit serum (○). (c) Electrophoretic gel profile of [^{35}S]methionine-labeled MTV.*

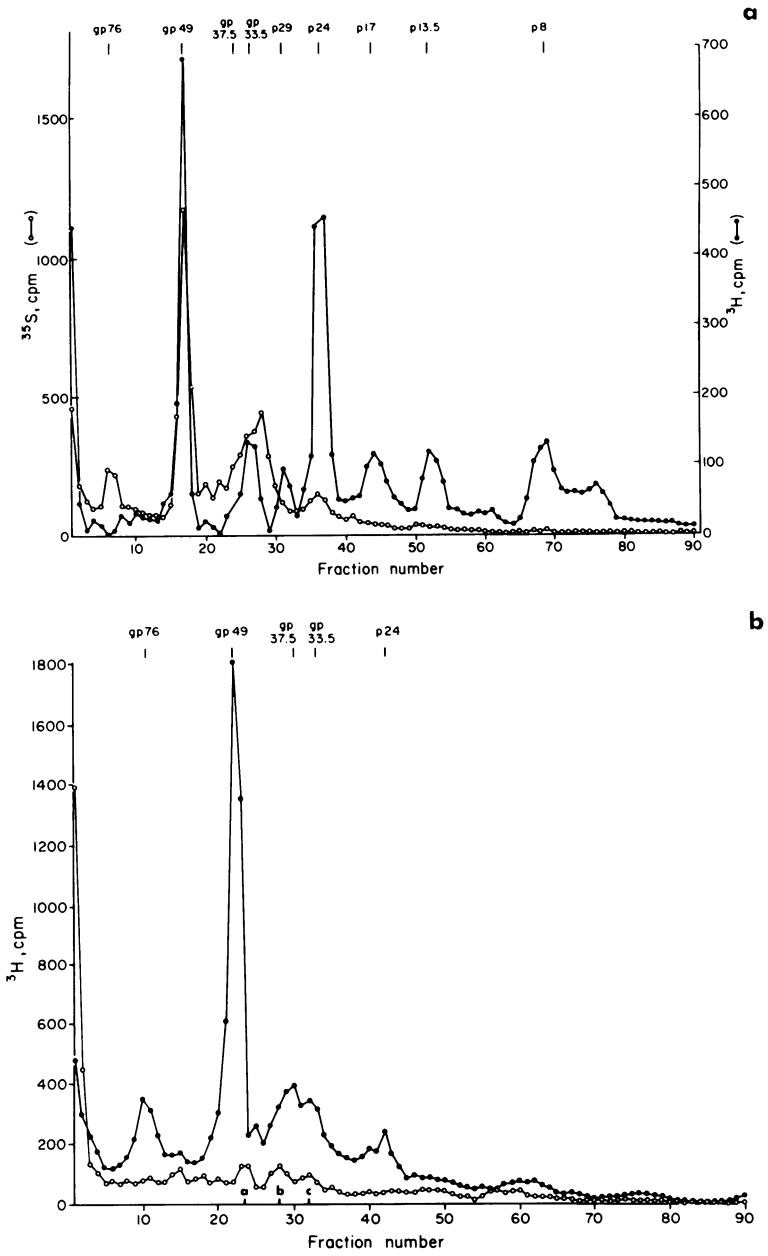

FIG. 3. (a) *Electrophoretic gel profile of viral polypeptides obtained by co-electrophoresis of ^{3}H-labeled amino acid mixture-labeled MTV* (●) *and immunoprecipitated [^{35}S]methionine-labeled cytoplasmic extract* (○). *The ^{3}H channel count was corrected for ^{35}S spillover. (b) Gel profile of ^{3}H-labeled amino acid mixture-labeled cytoplasmic extract immunoprecipitated with either anti-MTV serum* (●) *or normal rabbit serum* (○). *The normal rabbit serum profile shows several minor peaks, a, b, and c, with estimated molecular weights of 55,000, 45,000, and 40,000, respectively. (c) Gel profile of an immunoprecipitated cytoplasmic extract labeled with D-[^{3}H]glucosamine. (d) Gel profile of a [^{35}S]methionine-labeled cytoplasmic extract prepared from C3H3T7 cells chronically infected with gross passage A virus and immunoprecipitated with anti-MTV serum.*

tion titrations show that up to 0.5% of the cytoplasmic extracts are viral related, but there is also a substantial amount of nonspecific radioactivity precipitated (Fig. 1a). However, an examination of the precipitates obtained with immune and control sera reveal no poly-

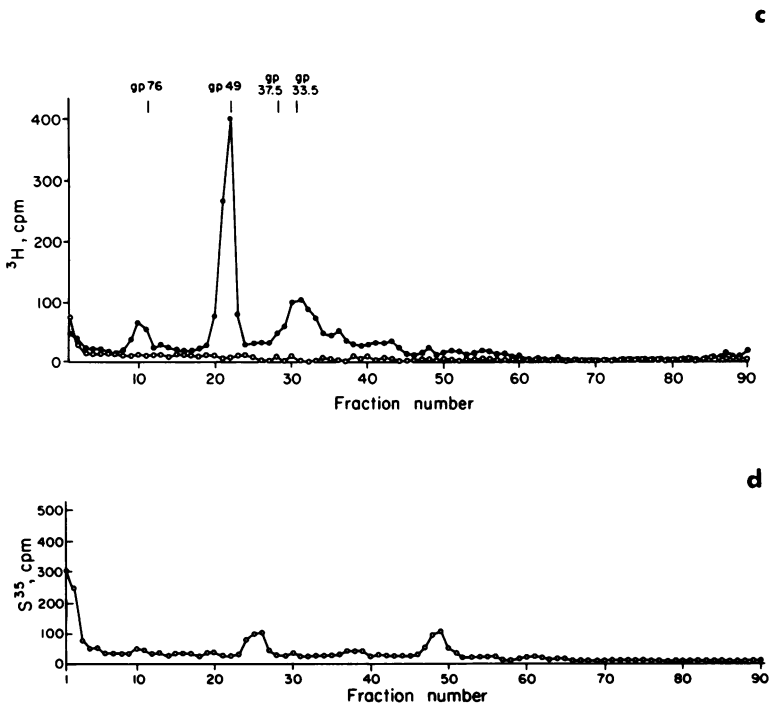

FIG. 3. *c and d.*

peptides which interfere with the resolution of viral proteins on polyacrylamide gels. From the immunoprecipitation data and the estimated recovery of the MTV proteins on gels, 0.1 to 0.5% of the total cell protein was extractable as viral related. The detectable polypeptides constitute 47% of the total virion proteins (6), and the immunoprecipitation curve for detergent-disrupted purified MTV shows a recovery of about 55% of the input (Fig. 1b). Consequently, the intracellular viral protein was estimated to constitute between 0.2 to 1.5% of the total tumor cell protein.

The polypeptides found in the tumor cell cytoplasm are primarily restricted to the major glycoproteins of the virion and a glycoprotein of 76,000 daltons (Fig. 3). The cytoplasmic glycoproteins of 37.5/33.5 × 1,000 daltons often vary slightly in molecular weight compared to the equivalent virion glycoproteins. The reason for this is unclear but may indicate incomplete glycosylation of the nascent polypeptides. Previous studies have established the polypeptides gp49, gp37.5/33.5, and p24 as the major virion proteins (6), although some authors have reported the molecular weights of these proteins to be slightly higher, i.e., gp52, gp36, p28, or p27, respectively (9, 21, 22, 28, 31).

The pulse-chase experiments show that the nonvirion glycopeptide gp76 is rapidly labeled during a short pulse and then loses its label significantly by 1 h of chase and almost completely by 4 h of chase with unlabeled culture medium. The polypeptides gp49 and gp37.5/33.5 are not labeled during the pulse but become progressively labeled in the chase. This suggests a precursor nature for gp76, although a more quantitative kinetic analysis, and the demonstration of amino acid sequence homology between proposed precursor and its product, are required to establish its precursor role.

The intracellular localization of viral polypeptides was examined by subcellular fractionation procedures, which demonstrated that the detectable glycopeptides, with the possible exception of gp76, were present in the membrane fraction, whereas no viral polypeptides were detected in the cytosol fraction. However, this may reflect the sensitivity of the techniques, as polypeptides could be synthesized in the cytosol and rapidly transferred to the membrane. Alternatively, a nonglycosylated form of the polypeptide from the cytoplasm may be present but not detected by the immune serum. In the case of the gp49, the use of trypsin on whole cells before homogenization resulted in a 70% reduction in the amount of this polypeptide in gel profiles (unpublished data), indicating that the plasma membrane could be the prime site of localization of this viral glycopeptide.

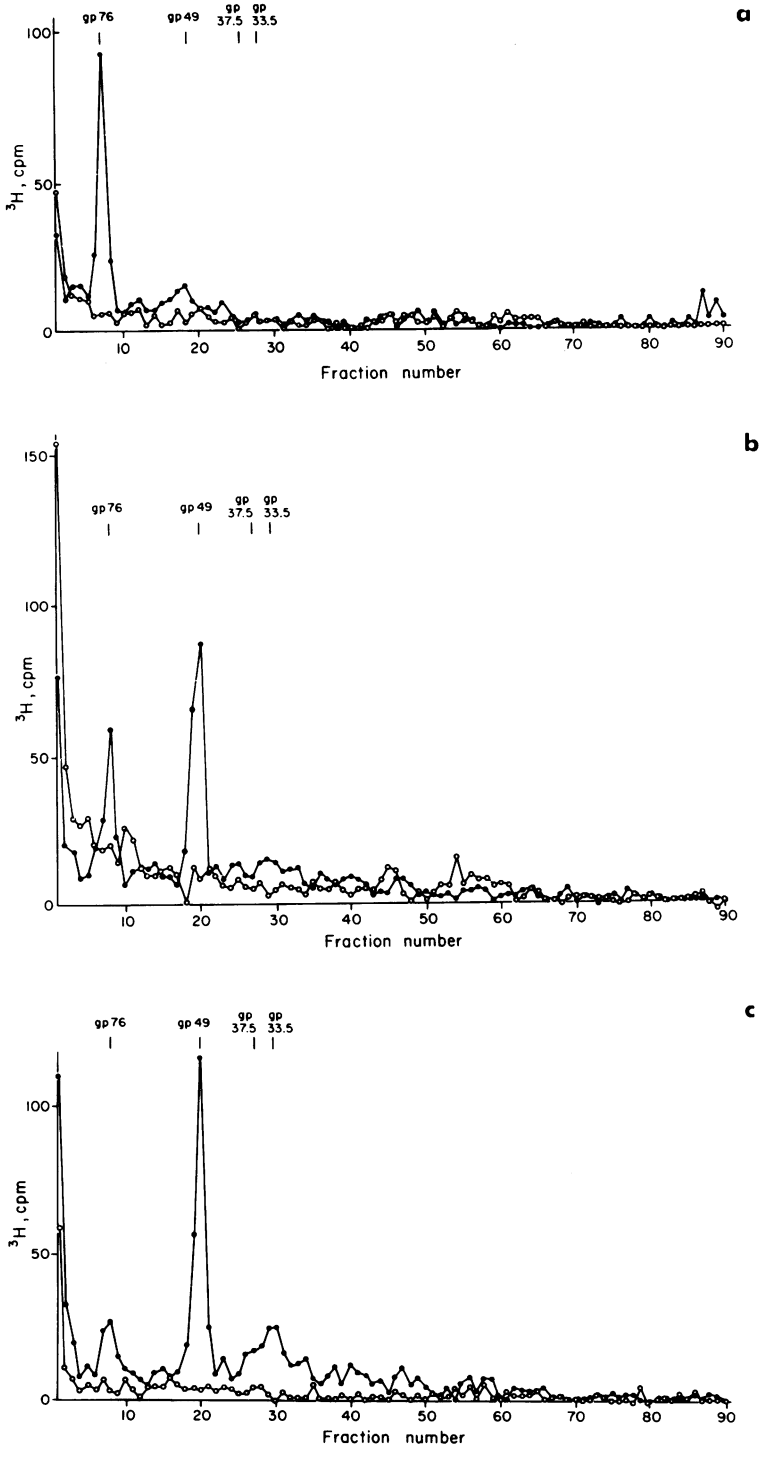

FIG. 4. *Electrophoretic gel profile of cytoplasmic extracts labeled with ^{3}H-labeled amino acid mixture at 125 μCi/ml for (a) 15 min, (b) 15 min followed by a 1-h chase, (c) followed by a 4-h chase. Each extract was immunoprecipitated with anti-MTV serum (●) and normal rabbit serum (○).*

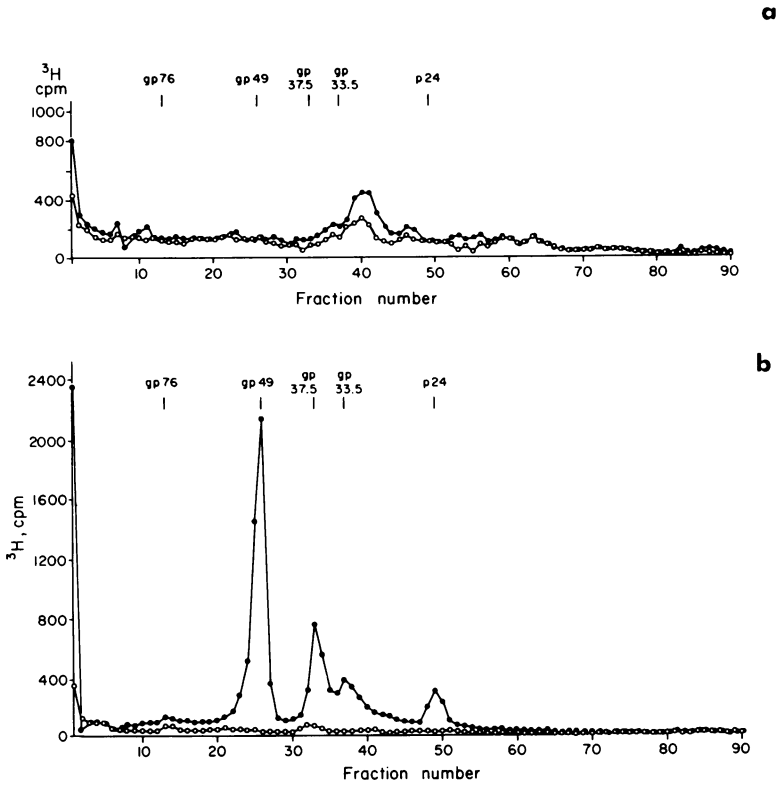

FIG. 5. *Electrophoretic gel profiles of immunoprecipitates obtained from different cell fractions isolated as described. (a) Cytosol fraction; (b) solubilized membrane fraction, immunoprecipitated with anti-MTV serum (●) and normal rabbit serum (○).*

In tumor cell extracts, the major internal virion polypeptide p24 (4) was poorly recovered, and the other virion polypeptides of low molecular weight (p17, p13.5 and p8) were not detected at all (Fig. 3). However, virus isolated from the culture fluid of tumor cell cultures, labeled under standard conditions, showed good incorporation into these polypeptides (Fig. 2). Therefore, minimal labeling due to a large cytoplasmic pool of polypeptides is an unlikely explanation. An isolation artifact was ruled out by finding that p24 could be easily recovered from cytoplasmic extracts if radioactively labeled virions were added to unlabeled cells before the extraction procedure (unpublished data). A possible explanation for these results is that p24 and possibly the other low-molecular-weight virion polypeptides also exist in a precursor form, which does not react with the antiserum. In fact, the maturation of viral proteins by means of proteolytic cleavage mechanisms is now an established route in viral processing, with reported examples in several groups, including the picornaviruses (2, 3, 11, 13–15, 27), toga-viruses (23, 24), adenoviruses (1, 12), myxoviruses (26), and more recently the oncornaviruses (19, 30).

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