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# Sequence-specific antibodies show that maturation of Moloney leukemia virus envelope polyprotein involves removal of a COOH-terminal peptide

(synthetic peptides/membrane protein processing)

NICOLA GREEN*, THOMAS M. SHINNICK*, OWEN WITTE†, ALFRED PONTICELLI†, J. GREGOR SUTCLIFFE*, AND RICHARD A. LERNER*

*Committee for the Study of Molecular Genetics, Research Institute of Scripps Clinic, La Jolla, California; and †Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California

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ABSTRACT **We followed maturation of the glycosylated envelope polyprotein $Pr80^{env}$ of a murine retrovirus by using antisera specific to subregions of the protein, including an antiserum directed against a synthetic peptide corresponding to the COOH-terminus of $Pr80^{env}$. Shortly after synthesis and glycosylation, $Pr80^{env}$ is cleaved into two species, gp70 and Pr15E, that are found associated, perhaps through disulfide bonds, in infected cells. Pr15E is further cleaved at the time of virus maturation to form virus protein p15E. $NH_2$-Terminal protein sequence analysis showed that Pr15E had an $NH_2$ terminus in common with p15E. Pr15E, but not p15E, is precipitated by antibody against the COOH-terminal peptide; hence, p15E is missing a peptide at the COOH-terminus. Our data indicate that Pr15E is the predominant species in cells and p15E is the major species in virus.**

The envelope (*env*) gene of murine retroviruses encodes a polymorphic family of proteins (1). The allele at this locus determines virus host range, and differences between highly leukemogenic viruses and their more benign relatives cluster within this gene (2–7). The primary protein product of the Moloney murine leukemia virus (Mo-MuLV) *env* gene is a glycosylated molecule that has an apparent $M_r$ 80,000 ($Pr80^{env}$) (8). The $Pr80^{env}$ polyprotein contains the information ($NH_2$ to COOH terminal) for gp70, the major envelope glycoprotein; p15E, a second viral membrane protein; and R, a COOH-terminal species (9, 10). This report describes the intermediates and end products of $Pr80^{env}$ processing and their times of appearance. We present evidence that the COOH-terminal peptide is removed during the formation of mature virions and may function in virus assembly.

From the data presented here and the work of others (9, 11–15), a scheme for *env* precursor processing emerges. After glycosylation of the primary *env* translation product, the resulting $Pr80^{env}$ molecule is cleaved into two products. One is derived from the $NH_2$-terminal region and contains gp70; the other contains the COOH-terminal 196 amino acids. All of the carbohydrate chains are attached to the gp70 portion. The 196-residue COOH-terminal product, which we designate Pr15E, contains p15E‡ and the R peptide. During virus maturation Pr15E is further processed by removal of the COOH-terminal R peptide to yield viral protein p15E. Such cleavage of COOH-terminal peptides may be representative of a general class of signals involved in membrane protein maturation.

## MATERIALS AND METHODS

**Metabolic Labeling of Cells and Viruses.** SCRF 60A cells, a murine thymoma line producing Mo-MuLV (16), were cultured in Eagle's minimal essential medium/10% fetal calf serum. Virus was harvested from culture supernatants by precipitation with 50% polyethylene glycol and isolated on 15–50% sucrose gradients. Cells were suspended at $2 \times 10^6$/ml in Hanks' balanced salt solution/5% minimal essential medium and [$^{35}$S]methionine; 1 Ci = $3.7 \times 10^{10}$ becquerels (970 Ci/mmol) or [$^3$H]leucine (120 Ci/mmol) at 100 $\mu$Ci/ml. Cultures were incubated at 37°C for 2 hr (steady-state label) or 15 min (pulse label). In pulse-chase experiments, labeled cells were washed in Hanks' balanced salt solution and resuspended in culture medium supplemented with 74.5 mg methionine per liter. SC-1 cells producing AKV-2 or AKR-247 virus and NIH 3T3 cells producing Rauscher murine leukemia virus (R-MuLV) were labeled with [$^{35}$S]methionine similarly. Immune precipitation and one-dimensional gel electrophoresis were performed as described (13). Antisera used for immune precipitation were as follows: (*i*) anti-gp70, raised against gp70 purified from R-MuLV (17); (*ii*) anti-p15E raised against p15E purified from R-MuLV (a gift from I. Fleissner); (*iii*) anti-p15E ascitic fluid from hybridoma 9E8, (a gift from R. Nowinski; ref. 18; and (*iv*) anti-R-pentadecamer (called anti-R hereafter), raised against a chemically synthesized peptide that corresponds to the 15 COOH-terminal amino acids of Mo-MuLV $Pr80^{env}$ as predicted by the *env* gene nucleotide sequence (13). Comparable results were obtained with the anti-p15E serum and the anti-p15E hybridoma ascitic fluid. Unless indicated, data shown were obtained with the anti-p15E serum.

**Two-Dimensional Gel Electrophoresis.** Immunoprecipitate complexes were washed twice with RIPA buffer (0.15 M NaCl/10 mM sodium phosphate, pH 7.5/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% $NaDodSO_4$/2% trasylol), suspended in 30 $\mu$l of $NaDodSO_4$ sample buffer [1% $NaDodSO_4$/0.05 M Tris, pH 6.8/10% (vol/vol) glycerol/2.5% 2-mercaptoethanol], heated for 5 min at 80°C, and centrifuged to remove the *Staphylococcus aureus*. Samples were prepared for isoelectric focusing by a variation of the procedure of Ames and Nikaido (19). To 10 $\mu$l of the supernatant was added 1 $\mu$l of bovine serum albumin in sample buffer (5 $\mu$g/ml) as an internal standard, 22 $\mu$l of sample dilution buffer (9.5 M urea/2% ampholines, pH 3.5–10/5% 2-mercaptoethanol/8% Nonidet P-40), and 8.25 mg of solid urea to bring the final concentration of urea to 9.5 M.

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Abbreviations: Mo-MuLV and R-MuLV, Moloney and Rauscher murine leukemia virus, respectively.

‡ Although the actual $M_r$ of p15E as determined from nucleic acid sequence analysis is ≈19,850, its apparent $M_r$ based on $NaDodSO_4$ gel migration is 15,000. We provisionally retain the p15E nomenclature here, based on the standard procedure of designating viral proteins according to their mobility characteristics. We intend the term Pr15E to indicate that this precursor is not the final form of the molecule. The actual $M_r$ of Pr15E is 21,740; its apparent $M_r$ on $NaDodSO_4$ gels is 17,000.

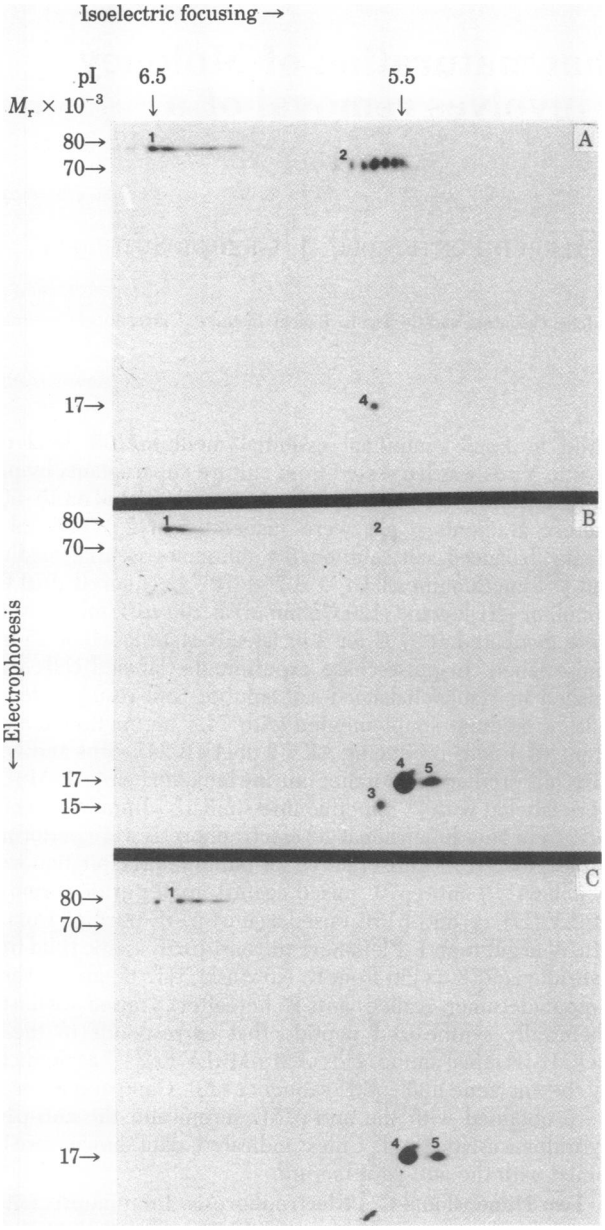


FIG. 1. Two-dimensional gel electrophoresis of Pr80$^{env}$ and its products. Extracts of [$^3$H]leucine-labeled SCRF 60A cells were allowed to react with anti-gp70 (*A*), anti-p15E (*B*), or anti-R serum (*C*).

The urea was dissolved at room temperature, and the sample was mixed in a Vortex and clarified in an Eppendorf centrifuge for 10 min at room temperature. Twenty microliters of sample was then directly loaded onto an isoelectric focusing tube gel, and two-dimensional electrophoresis was carried out as described (20), using an 11% polyacrylamide slab gel in the second dimension.

**Glycosylation Studies.** Cells were incubated for 45 min in culture medium containing tunicamycin at 1 μg/ml and then washed and incubated for 2 hr in labeling medium containing tunicamycin at 0.5 μg/ml and [$^{35}$S]methionine at 100 μCi/ml. Control cells were incubated for 2 hr in labeling medium containing [$^{35}$S]methionine.

[$^3$H]Glucosamine (40 Ci/mmol)- or [$^3$H]mannose (15 Ci/mmol)-labeled cell extracts were prepared as described above for metabolic labeling of SCRF 60A cells with [$^{35}$S]methionine.

**Sequence Analysis of Radiolabeled Protein.** Cell extracts of SCRF 60A cells labeled for 2 hours with [$^3$H]leucine or [$^{35}$S]methionine were allowed to react with anti-R serum, the precipitate was subjected to electrophoresis on a NaDodSO$_4$/polyacrylamide gel, and the $M_r$ 17,000 protein was cut out and eluted from the gel. The labeled $M_r$ 17,000 protein was mixed with sperm whale myoglobin, attached to a solid support, and subjected to stepwise Edman degradation in an automated protein sequenator. The product of each step was assayed for radioactivity, and selected steps were analyzed for unlabeled carrier-derived amino acid.

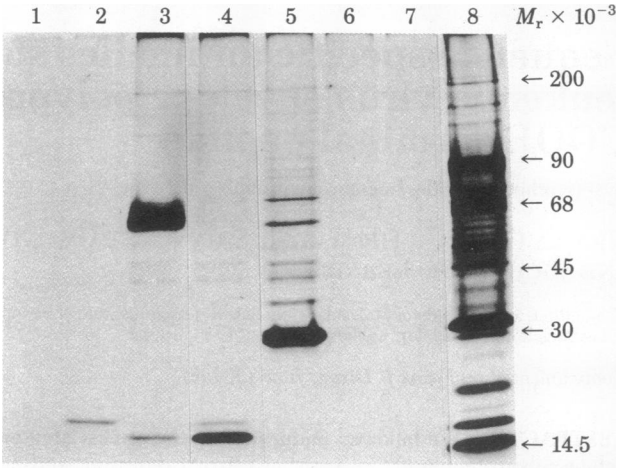


FIG. 2. Immunoprecipitation of [$^{35}$S]methionine-labeled virus. Lanes: 1, Normal rabbit serum; 2, rabbit anti-R; 3, goat anti-gp70; 4, αp15E hybridoma (9E8) ascites; 5, goat anti-R-MuLV p30; 6, normal goat serum; 7, control ascites; 8, nonimmunoprecipitated virus.

## RESULTS

**Virion p15E Does Not Contain the Pr80$^{env}$ COOH-terminal Peptide.** In infected cells labeled for 2 hr with [$^{35}$S]methionine, anti-gp70 serum detects target molecules that have NaDodSO$_4$/polyacrylamide gel mobilities corresponding to 80,000 and 70,000 daltons; anti-p15E serum detects molecules that have mobilities corresponding to 80,000, 17,000, and 15,000 daltons; and anti-R serum detects molecules that have mobilities corresponding to 80,000 and 17,000 daltons (see Fig. 4 and 5).

When immunoprecipitates of [$^3$H]leucine-labeled extracts of infected cells are examined in a two-dimensional gel electrophoresis system (Fig. 1), we find that anti-gp70 serum detects the 80,000-dalton species (pI 6.5), the 70,000-dalton species (pI 5.8), and a 17,000-dalton species (pI 5.5); anti-p15E serum detects the 80,000-dalton species; two 17,000-dalton species (pI 5.5 and 5.3), and a 15,000-dalton species (pI 5.7); and anti-R serum detects the 80,000-dalton species and the two 17,000-dalton species. Both anti-p15E and anti-R sera detect a trace spot that has a gel mobility of ≈18,000 daltons (pI 5.4).

The 80,000-dalton molecule precipitated by all three antisera has previously been shown to be Pr80$^{env}$ (13). The specificity of our antisera and the data presented below indicate that the 70,000-dalton molecule precipitated by anti-gp70 serum is gp70 and the 15,000-dalton molecule precipitated by anti-p15E serum is p15E. The 17,000-dalton molecules precipitated by anti-gp70, anti-p15E, and anti-R sera are the predominant anti-p15E reactive species in both [$^3$H]leucine- and [$^{35}$S]methionine-labeled infected cells.

To compare *env* products in virus-producing cells with molecules in mature virus, we isolated $^{35}$S-methionine-labeled virions and immunoprecipitated viral extracts with anti-gp70, anti-R, and anti-p15E sera (Fig. 2). Anti-gp70 serum precipitates a 70,000-dalton molecule (gp70) and a 15,000-dalton molecule (p15E); anti-R serum precipitates a 17,000-dalton target that is found in trace amounts in the lane displaying nonimmunoprecipitated virus; and anti-p15E serum precipitates a

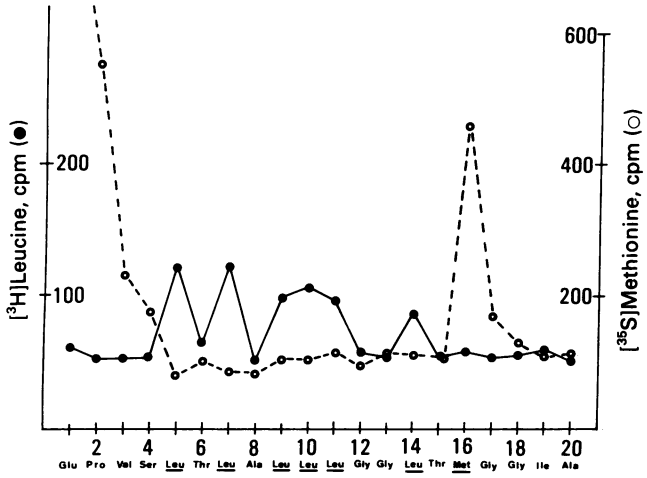


FIG. 3. Amino acid sequence analysis of Pr15E. Stepwise Edman degradation of [$^3$H]leucine- or [$^{35}$S]methionine-labeled Pr15E was accomplished in a Beckman 890 B protein sequencer. Each step was analyzed by scintillation counting for radioactivity and, in each run, the positions of all labeled and several unlabeled steps were confirmed by high-pressure liquid chromatography of the myoglobin carrier protein-derived amino acids. The $NH_2$-terminal sequence of Mo-MuLV p15E is shown at the bottom of the figure; the positions we have confirmed to be present in the $NH_2$ terminus of Pr15E are underlined. The methionine that appears in decreasing amounts in the first four steps is interpreted as washout, a frequently encountered artifact of radioactive protein sequence analysis and, hence, considered not significant.

major viral protein of 15,000 daltons and the trace 17,000 target. In contrast to cells, the 15,000-dalton molecule is the predominant anti-p15E-reactive molecule in virus. Anti-p15E serum precipitates the 17,000-dalton target apparently as efficiently as it precipitates the 15,000-dalton molecule; the ratio of band intensities of the 17,000- and 15,000-dalton molecules in the precipitated sample is the same as that in whole virus (Fig. 2). Hence, the 17,000-dalton molecule contains R determinants in addition to p15E determinants. Furthermore, as anti-R serum does not precipitate the 15,000-dalton molecule, p15E does not contain the determinants encoded by the COOH-terminal 15 amino acids of Pr80$^{env}$.

**Pr15E is 196 Amino Acids Long.** To further investigate the nature of the 17,000-dalton molecule, we determined the partial sequence of its $NH_2$ terminus (Fig. 3). This sequence fits exactly with the known sequence of the $NH_2$ terminus of p15E (T. Copeland and S. Oroszlan, cited in ref. 13). Therefore, because this 17,000-dalton molecule has the $NH_2$ terminus of p15E and is precipitated by an antiserum directed against the specific COOH-terminal amino acids of Pr80$^{env}$, it appears that the 17,000-dalton protein contains the 196 amino acids that span the region of the *env* gene from the $NH_2$ terminus of p15E, through p15E, ending at the COOH terminus (R) of the *env* polyprotein. As it contains all of the p15E information, we have designated this molecule Pr15E.

**Processing and Glycosylation of Pr80$^{env}$.** The processing of Pr80$^{env}$ and the possible precursor–product relationship of Pr15E and p15E were studied in pulse-chase experiments (Fig. 4). SCRF 60A cells were labeled with [$^{35}$S]methionine for 15 min, washed once to remove label, and then incubated in label-free medium with excess unlabeled methionine. Periodically, samples were taken. The cell lysates were allowed to react with antisera specific for gp70, p15E, or the R peptide. Immediately after the labeling period, all three precipitated only Pr80$^{env}$. After 15 min of chase, bands corresponding to gp70 and Pr15E begin to appear, and their intensities increased (accompanied by a decrease in intensity of the Pr80$^{env}$ band) until 1–2 hr of chase. The possibility that the p15E band is present at early times is obscured by a band precipitated by normal serum, but p15E does not seem to be present at < 1 hour. At 2 hr, the intensity of the gp70 band still appears to be increasing but that of the Pr15E band has dramatically decreased and there has been a corresponding increase in intensity of the 15,000-dalton region. This suggests that Pr80$^{env}$ is processed to produce gp70 and Pr15E and then Pr15E is processed to produce p15E. Interestingly, p15E is first detected at about the time one expects the labeled proteins to begin entering viruses.

The role of glycosylation in Pr80$^{env}$ processing was investigated in two ways. First, we determined whether radioactively labeled carbohydrates could be incorporated into any of the envelope polyprotein processing intermediates by labeling infected cells with [$^3$H]mannose and [$^3$H]glucosamine for 2 hr. The cell extracts were allowed to react with the three antisera, and immunoprecipitates were subjected to electrophoresis on $NaDodSO_4$/polyacrylamide gels (data not shown). As expected from the work of others (12, 21, 22), Pr80$^{env}$ and gp70 are labeled with both sugars but neither Pr15E nor p15E is labeled with either. Therefore, we conclude that Pr15E, and quite probably its cleavage products, does not contain mannose or glucosamine and most likely is not glycosylated. The absence of labeling p15E is in agreement with published reports that the p15E of R-MuLV is not glycosylated (14, 21, 23).

Second, to probe the role of glycosylation in Pr80$^{env}$ processing and, in particular, to determine whether the R peptide could be cleaved from the *env* polyprotein before the proteolytic clip that produces Pr15E, we treated infected cells with tunicamycin before and during labeling. Cell extracts were allowed to react with antisera to gp70, R, or p30 (one of the virus core proteins). The mobilities of the species precipitated by anti-p30 serum are unaffected by tunicamycin (Fig. 5), an observation consistent with the hypothesis that p30 and its precursor Pr65$^{gag}$ are not glycosylated (24). The mobility of the larger species precipitated by anti-R and anti-gp70 sera is clearly

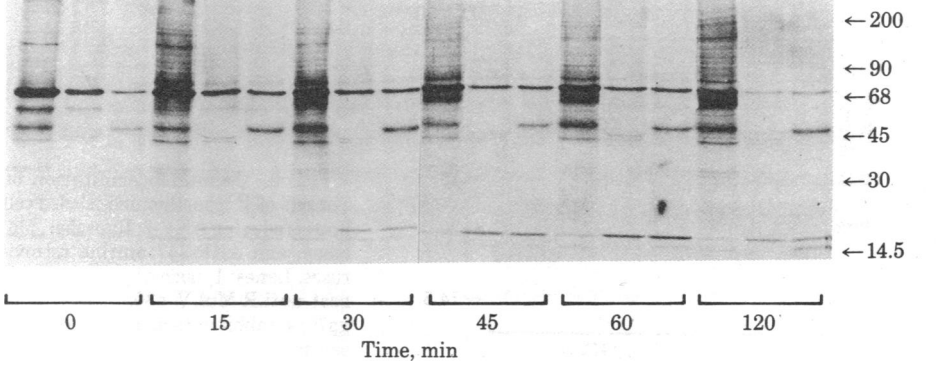


FIG. 4. Pulse-chase analysis of Pr80$^{env}$ maturation. SCRF 60A cells were labeled for 15 min with [$^{35}$S]methionine and then washed and incubated in medium containing five times the normal methionine concentration. At the indicated times of chase, an aliquot of cell suspension was removed, diluted with cold phosphate-buffered saline to prevent further metabolic reaction, and centrifuged, and the pellet was extracted with RIPA buffer and immunoprecipitated. Lanes: 1, 4, 7, 10, 13, and 16; goat anti-gp70; 2, 5, 8, 11, 14, and 17; rabbit anti-R; 3, 6, 9, 12, 15, and 18; anti-p15E hybridoma (9E8) ascites.

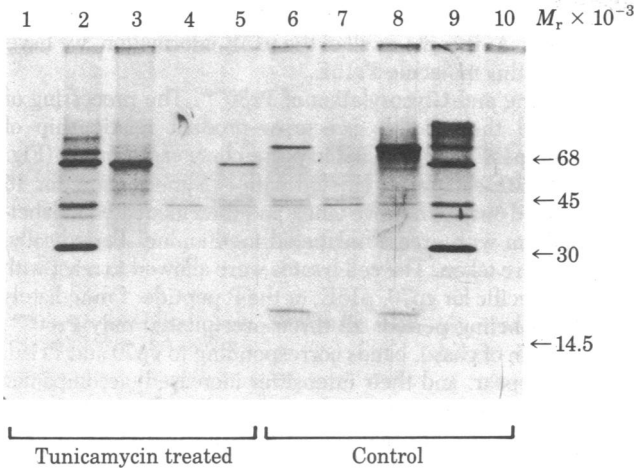


FIG. 5. Tunicamycin inhibits $Pr80^{env}$ processing. Lanes: 1 and 10, normal goat serum; 2 and 9, goat anti-R-MuLV p30; 3 and 8, goat anti-gp70; 4 and 7, normal rabbit serum; 5 and 6, rabbit anti-R.

greater when cells have been treated with tunicamycin. In addition, the band having 17,000-dalton mobility precipitated by anti-R in untreated cells is missing in tunicamycin-treated cells. We conclude, as have previous workers, that tunicamycin inhibits glycosylation of $Pr80^{env}$ and prevents cleavage of $Pr80^{env}$ into gp70 and p15E (11, 22). Because anti-R serum can apparently precipitate an equal amount of the envelope polyprotein precursor in treated cells or control cells (Fig. 5), we further conclude that treatment with tunicamycin prevents efficient removal of the R peptide from $Pr80^{env}$. This suggests that glycosylation is required for production of Pr15E and either glycosylation or production of Pr15E is required for removal of the COOH-terminal R peptide. Occasionally, as in the untreated sample in this experiment, we have observed that anti-gp70 precipitates the 17,000-dalton Pr15E molecule from cell extracts. We interpret this to mean that Pr15E is associated with gp70, perhaps through disulfide bonds, before the R peptide is removed from its COOH terminus and that the p15E–gp70 association persists in virions after the R peptide has been cleaved.

**The R peptide Is Different in Other Retroviruses.** Cells infected with AKV-2, Rauscher, and AKR-247 (an MCF recombinant) murine leukemia viruses were labeled with [$^{35}$S]methionine as described for SCRF 60A cells and allowed to react with anti-R, anti-gp70, and anti-p30 sera. Although all leukemia virus-infected cells produce appropriate envelope precursor, gp70, and anti-p30 reactive molecules (thereby indicating that the infection and labeling of cells worked), none produces a molecule of any size that reacts with anti-R serum (Fig. 6). Abelson leukemia and Moloney sarcoma virus-infected cells also fail to produce an anti-R serum target (data not shown). We conclude that the exact amino acid sequence of the R peptide is not duplicated in a protein expressed by any of these viruses. As envelope polyprotein maturation of other retroviruses has been observed to occur in an order consistent with the model for Moloney $Pr80^{env}$ (9) and these other viruses probably contain the equivalent of an R peptide, it appears that our anti-R serum is quite type specific.

## DISCUSSION

$Pr80^{env}$ reacts with anti-gp70, anti-p15E, and anti-R sera. If glycosylation of $Pr80^{env}$ is blocked by tunicamycin treatment, the determinants for anti-gp70, anti-15E, and anti-R sera remain in the nonglycosylated molecule—no smaller targets for these antisera are detected. If glycosylation is allowed to proceed normally in a pulse-chase experiment, label flows from $Pr80^{env}$ to smaller targets (gp70 and a 17,000-dalton target). Glucosamine and mannose residues are attached to $Pr80^{env}$ and gp70 but not to the smaller molecule. The 17,000-dalton target (Pr15E) then forfeits its label, apparently to a 15,000-dalton molecule. The $NH_2$ and COOH-termini of Pr15E, determined by partial $NH_2$-terminal amino acid sequence analysis and reactivity with anti-R serum, define it as containing the 196 COOH-terminal residues of $Pr80^{env}$. The 15,000-dalton molecule (p15E) has the same $NH_2$ terminus as Pr15E but lacks the Pr15E COOH-terminal antigenic determinants recognized by the anti-R-serum. Pr15E and p15E are both found in cells and virus but Pr15E is the predominant species in cells and p15E is the predominant species in virus. Both Pr15E and p15E are sometimes found associated with gp70. Anti-R serum finds no target in cells infected with a number of viruses related to Mo-MuLV.

These data provide the following insights into the steps involved in $Pr80^{env}$ processing. The order of *env* gene products ($NH_2$ to COOH) along $Pr80^{env}$ is gp70-p15E-R. After translation, the gp70 portion is glycosylated, containing both glucosamine- and mannose-derived carbohydrate. Cleavage then occurs, generating the 196-residue Pr15E molecule. This species is further cleaved to p15E at a time corresponding to that of virus budding. We interpret the small amount of Pr15E found in purified virus to be the result of either incomplete cleavage or, more probably, contaminating cellular membranes that copurify with virus. The p15E found associated with cells is probably due to virus in the process of budding, given the latency of p15E appearance in the pulse-chase experiment. We believe, therefore, that there may be a connection between the removal of the R peptide and virus budding.

We envision a model of virus maturation in which a species is formed that consists of gp70 disulfide bonded to Pr15E. [The occasional appearance of Pr15E in anti-gp70 serum immunoprecipitates suggests the association of gp70 and Pr15E similar to what has been reported for gp70 and p15E in virus (22, 25).] This complex would be anchored in the membrane by the hydrophobic domain of Pr15E, positions 135–164, which is fol-

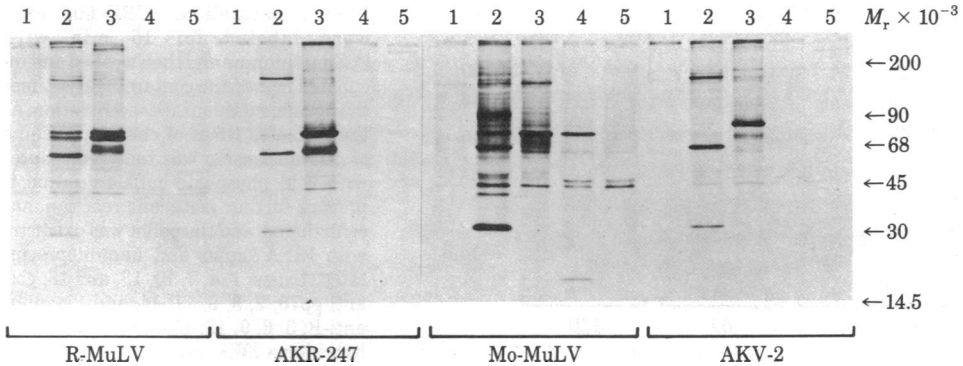


FIG. 6. Immunoprecipitation of extracts of [$^{35}$S]methionine-labeled cell lines producing AKV-2, Rauscher, Moloney, and AKR-247 murine retroviruses. Lanes: 1, normal goat serum; 2, goat anti-R-MuLV p30; 3, goat anti-gp70; 4, rabbit anti-R; 5, normal rabbit serum.

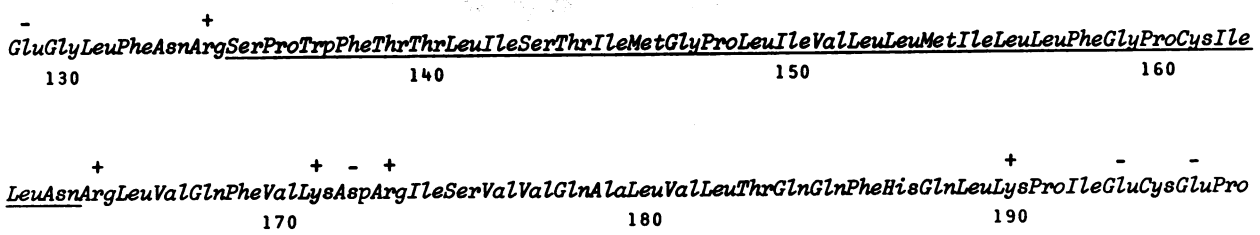


FIG. 7. COOH terminus of Pr15E. Residues 129–196 of Pr15E as deduced from the nucleotide sequence of the Mo-MuLV *env* gene (10). Residue 196 (proline) is the COOH terminus of $Pr80^{env}$. The hydrophobic region thought to span the membrane is underlined. Charged residues have the appropriate sign (+ or −) displayed above them.

lowed by several charged amino acids (Fig. 7). The COOH-terminal tail (R peptide) is cleaved off during the final stages of virus maturation. One can speculate about what role the existence and cleavage of the R peptide plays in virus morphogenesis. One possibility is that the portion of cell membrane that is in the process of actively forming virus is somehow stabilized in an extended position by this hydrophilic tail. Removal of the R peptide may trigger an association of the envelope-containing segment causing localized pinching of the membrane and virus budding.

We wish to relate our studies to the work done before the nucleotide sequence and the sequence-specific reagent were available. In studies of viral proteins, the nomenclature p15E has usually been used to describe our 15,000-dalton molecule (14, 23). However, in other studies using infected cells, molecules with apparent $M_r$ 15,000 and 12,000 (denoted p15E and p12E) were described (9). It now seems clear that these correspond to Pr15E and p15E, p15E being the major viral species. In this regard, the extreme hydrophobicity of the 196-residue Pr15E molecule renders its mobility on $NaDodSO_4$ gels misleading.

One is left to wonder about the generality of COOH-terminal protein sequences involved in protein compartmentalization. The existence of $NH_2$-terminal signal sequences has been well documented (26–28). The existence of non-$NH_2$-terminal sequences that govern protein destinies has also been suggested. The signal for ovalbumin is not $NH_2$ terminal, for this protein is secreted even though its RNA molecule prescribes no ephemeral $NH_2$-terminal sequence (29). Another case in point is the β-lactamase of gram-negative bacteria. Although the precursor of the enzyme contains an $NH_2$-terminal signal that is removed before the active enzyme arrives at its periplasmic destination, the COOH terminus of β-lactamase is also important for secretion (30, 31). Another virus structural protein, vesicular stomatitis virus G protein, interacts at its extreme COOH terminus with the membrane (32). It remains to be shown whether detachable COOH termini are representative of an important type of addressing mechanism for membrane proteins or are strictly a phenomenon of the requirements of the maturation of certain viruses.

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