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Sequence Relationship of Glycosylated and Unglycosylated *gag* Polyproteins of Moloney Murine Leukemia Virus

STEVEN A. EDWARDS† AND HUNG FAN*

Tumor Virology Laboratory, The Salk Institute, San Diego, California 92138

Both glycosylated and unglycosylated polyproteins coded by the *gag* gene are produced in cells infected with Moloney murine leukemia virus. GpP80^{gag} is a glycosylated precursor of a larger *gag* glycoprotein exported to the cell surface, whereas Pr65^{gag} is an unglycosylated precursor of the virion internal structural proteins. GpP80^{gag} contains not only carbohydrate, but also additional polypeptide sequences not found in Pr65^{gag}. In the experiment reported here, we localized the differences between GpP80^{gag} and Pr65^{gag} with respect to the domains of the individual *gag* proteins. This was done by comparison of partial proteolytic cleavage fragments from Pr65^{gag}, from GpP80^{gag}, and from the unglycosylated form of GpP80^{gag} (P75^{gag}) which had been immunoprecipitated by antisera specific for *gag* proteins p30, p15, and p10. We conclude that the additional polypeptide sequences in GpP80^{gag} are located at or very near the amino terminus of the polyprotein. The carbohydrate in GpP80^{gag} is attached to polypeptide sequences held in common between GpP80^{gag} and Pr65^{gag}

The *gag* gene of murine leukemia virus (MuLV) has at least three pathways of expression in infected cells: (i) as Pr65^{gag}, which is subsequently cleaved to give the major internal structural proteins of MuLV (10, 20, 24, 31); (ii) as GpP80^{gag}, which is further glycosylated to give a 85,000- to 95,000-dalton cell surface-associated glycoprotein (6, 7, 16, 26); and (iii) as part of a fusion product with the *pol* gene of 180,000 daltons, Pr180$^{gag\text{-}pol}$, which is the precursor of reverse transcriptase (10). Kinetic and stoichiometric considerations rule out the possibility that Pr180$^{gag\text{-}pol}$ is also the major precursor of the other *gag* products (10).

In a recent report, we proposed that GpP80^{gag} and Pr65^{gag} were synthesized independently in Moloney MuLV (M-MuLV)-infected cells, perhaps from separate initiation sites for protein synthesis in the *gag* gene (6). We found that GpP80^{gag} was a mannose-containing glycoprotein with a polypeptide core of 75,000 daltons. The unglycosylated 75,000-dalton core of GpP80^{gag} (P75^{gag}) could be identified either by labeling cells in the presence of tunicamycin or by digesting Gp80^{gag} with endoglycosidase H. Cell-free translation of M-MuLV genomic RNA resulted in two major *gag* gene products which comigrated on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) with in vivo-labeled Pr65^{gag} and P75^{gag}. Both of the cell-free translation products could be labeled with [^{35}S]formylmethionine, which specifically labels the amino terminus, indicating that the 65,000-dalton polypeptide was not derived from the 75,000-dalton polypeptide by cleavage of the amino-terminal portion in vitro (6). In addition, Schultz and Oroszlan compared in vivo-labeled polyproteins from Rauscher MuLV-infected cells and reported that GpP80^{gag} differed from Pr65^{gag} only at the amino terminus (25). It appeared that the most plausible explanation for these results was that GpP80^{gag} and Pr65^{gag} are translated from different initiation sites in the *gag* gene.

Other models for the relationships of Pr65^{gag}, GpP80^{gag}, and Pr180$^{gag\text{-}pol}$ have been proposed, based on different experimental findings. Kopchick et al. (12) found that GpP80^{gag} has tryptic peptides in common with reverse transcriptase, and they have therefore proposed that GpP80^{gag} has an extension from the carboxy terminus of Pr65^{gag} (12). Pr180$^{gag\text{-}pol}$ would be processed such that the extra sequences in GpP80^{gag} would be present as part of the reverse transcriptase. Schultz et al. (26) have proposed that whereas GpP80^{gag} differs at the amino terminus, the unglycosylated P75^{gag} is the primary translation product of the *gag* gene, serving as a precursor to both of these molecules.

In the investigations reported here, we have tried to localize the differences between Pr65^{gag}, P75^{gag}, and GpP80^{gag} with respect to the domains of the individual *gag* proteins. This was done by a variation of the partial proteolysis mapping technique of Cleveland et al. (4). Individual *gag* polyproteins were subjected to partial

† Present address: Peptide Biology Laboratory, The Salk Institute, San Diego, CA 92138.

proteolysis followed by immunoprecipitation with antisera specific for *gag* protein p30, p15, or p10 and then analyzed by SDS-PAGE. In this manner, it was possible to order the cleavage fragments obtained from each polyprotein and to identify the regions in which the polyproteins differ.

MATERIALS AND METHODS

Cell line and virus used. M-MuLV clone 1 cells, a line of NIH-3T3 cells productively infected with M-MuLV, were grown as previously described (8, 12). M-MuLV virus particles were harvested from these cells at 3-h intervals, using a Smith-Kozoman autoharvester (Bellco), and purified as before (8).

Preparation of viral RNA. Viral genomic RNA was isolated by phenol-chloroform extraction of virus, followed by sedimentation on sucrose gradients as previously described (6). Virion 70S RNA was denatured by incubation at 100°C in water for 45 s before use in cell-free translation.

Cell-free translation of *gag* polyproteins. Cell-free translation of virion RNA was performed as before (6) in the messenger-dependent reticulocyte lysate as described by Pelham and Jackson (20). Viral RNA concentration was 25 μg/ml, and reaction volumes were 0.1 to 0.5 ml. Immunoprecipitation of the synthesized products was performed as described previously (6).

Labeling and immunoprecipitation of cell lysates. Subconfluent 9-cm tissue culture dishes were labeled with 250 μCi of either [^{3}H]leucine (40 to 60 Ci/mmol) or [^{35}S]methionine (800 to 1,000 Ci/mmol) per ml for 1 h at 37°C in Dulbecco modified Eagle medium lacking the appropriate amino acid. [^{3}H]leucine was purchased from New England Nuclear Corp.; [^{35}S]methionine was purchased from Amersham/Searle.

Preparation of cell lysates and immunoprecipitation. Procedures were as described before (6).

Antiserum. Rabbit anti-p30 serum was prepared in this laboratory from gel-purified p30 protein and has been described previously (18). Goat anti-Rauscher MuLV p10 and goat anti-Rauscher MuLV p15 sera were obtained from the Office of Program Resources and Logistics, Biological Carcinogenesis Branch, National Cancer Institute. Goat antiserum to SCRF 60A MuLV (8) gp70 was a gift from Stephen Kennel, Oak Ridge National Laboratory.

PAGE. Preparatory gels were 12.5% acrylamide–0.1% methyl bisacrylamide. Analytical gels were either the same (Fig. 1) or 15% acrylamide–0.09% bisacrylamide. The buffer system and other conditions were those described by Laemmli (14).

Elution of radioactive proteins from gels. Immunoprecipitates were separated according to size by electrophoresis in preparatory acrylamide gels. After electrophoresis, the gels were washed for approximately 15 min in water and then quickly dried under vacuum in a rubber apparatus suspended over a boiling water bath. Radioactively labeled bands were visualized by autoradiography on Kodak NS 54T film. [^{3}H]leucine bands could not be visualized in this manner; therefore, [^{35}S]methionine markers in adjacent tracks were co-electrophoresed in order to locate ^{3}H-labeled proteins. After autoradiography, the portion of the gel containing the radioactive protein was cut out, and the filter paper backing was scraped off. The gel slice was then placed in a dialysis bag with 1 to 2 ml of electroelution buffer (0.1 M ammonium bicarbonate, pH 8.8, 0.1% SDS, and 5% mercaptoethanol), and the dialysis bag was then placed in a tank containing approximately 100 ml of 0.1 M ammonium bicarbonate–0.1% SDS and bordered on each side with a platinum electrode connected to a power supply. The polyproteins were eluted by electrophoresis at 200-mA constant current for 2 h. Under these conditions, greater than 90% of the labeled polyproteins were eluted. (Larger proteins or more concentrated gels required longer elution times.)

After electroelution, the eluant was removed from the dialysis bag and lyophilized. Samples were then suspended in the same volume of water and lyophilized again, as it was important to remove the mercaptoethanol since this compound inhibited the protease used.

Proteolysis. Samples were suspended in 0.1 M ammonium bicarbonate to a final concentration of 0.5% SDS (the SDS from the electroelution buffer also remained in the samples after lyophilization). Proteolysis was performed at a concentration of 100 μg of *Staphylococcus aureus* V8 protease (Miles Laboratories) per ml for 30 min at 37°C. These conditions gave relatively large fragments suitable for our purposes. Proteolysis was terminated by the addition of 5% mercaptoethanol and incubation at 100°C for 2 min. The samples were then lyophilized, resuspended, and lyophilized two more times.

Immunoprecipitation of peptides. The samples were suspended in phosphate-buffered saline containing 1% Nonidet P-40 to a final SDS concentration of 0.5%. Insoluble material was removed from the samples by centrifugation in a Brinkman microfuge for 10 min at 4°C. Immunoprecipitation was performed in a volume of 0.5 ml with 2.5 to 5 μl of antiserum, and incubation was for 16 h at 0°C. Immune complexes were collected with fixed *S. aureus* and washed as previously described (6). Precipitated fragments were analyzed by SDS-PAGE followed by fluorography as described by Laskey and Mills (15).

RESULTS

Comparison of the V8 protease cleavage patterns of Pr65^{gag}, P75^{gag}, and GpP80^{gag}: identity of cellular and cell-free translation products. In previous investigations (6), we identified *gag*-related cell-free translation products of M-MuLV virion RNA as similar to in vivo-labeled Pr65^{gag} and P75^{gag} on the basis of the fact that they comigrated on SDS-PAGE. To substantiate this identification, we compared proteolytic fragments of the in vivo- and in vitro-labeled *gag* polyproteins (Fig. 1). Our procedure differed from the standard peptide mapping procedure of Cleveland et al. (4) in that partial proteolysis was performed in solution, which allowed us to immunoprecipitate the proteolytic fragments in subsequent experiments (see below). In the experiment shown in Fig. 1, M-

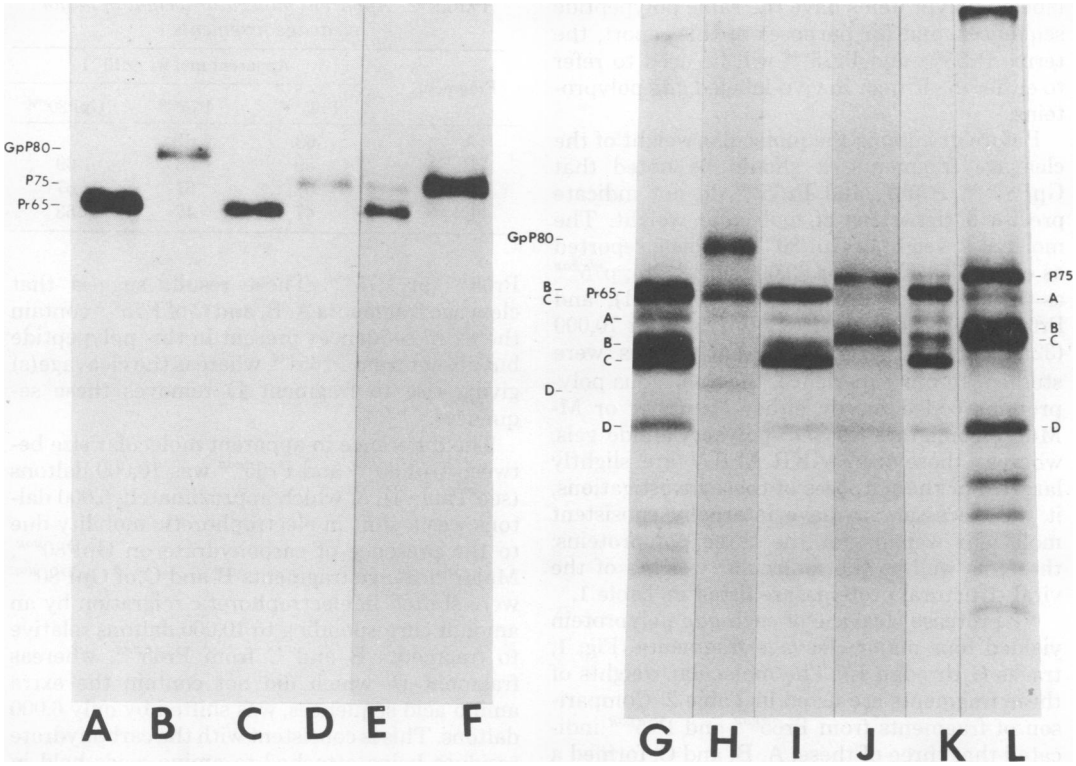

FIG. 1. *V8 protease cleavage profiles of gag polyproteins. gag polyproteins were isolated by immunoprecipitation of either cell extracts or cell-free translation products followed by SDS-PAGE and autoradiography; appropriate gel slices were cut out, and the protein was eluted as described in the text. Tracks A through F represent the uncleaved polyproteins which were re-electrophoresed and visualized by fluorography. Tracks A and B contain Pr65^{gag} and GpP80^{gag}, respectively, isolated from cytoplasmic extracts of [^{35}S]methionine-labeled M-MuLV clone 1 cells. Tracks C and D contain Pr65^{gag} and P75^{gag}, respectively, obtained from parallel cultures of tunicamycin-treated cells. Tracks E and F represent the 65,000- and 75,000-dalton gag-related [^{35}S]methionine-labeled translation products of clone 1 M-MuLV virion RNA in the messenger-dependent reticulocyte lysate translation system. Tracks G through L represent V8 cleavage profiles of the gag profiles presented in tracks A through F. Tracks G and H are the profiles of Pr65^{gag} and GpP80^{gag} from M-MuLV clone 1 cells, I and J are profiles of the cleavage profiles of Pr65^{gag} and P75^{gag} from tunicamycin-treated M-MuLV clone 1 cells, and K and L show the cleavage profiles of the 65,000- and 75,000-dalton gag-related translation products of clone 1 M-MuLV virion RNA.*

MuLV clone 1 cells were labeled for 1 h with [^{35}S]methionine. A cytoplasmic lysate was prepared, and *gag*-related polyproteins were immunoprecipitated with antiserum to p30, one of the internal structural proteins coded by the *gag* gene. The same procedure was performed for a parallel culture which was treated with tunicamycin, an inhibitor of glycosylation (13), which results in the synthesis of P75^{gag} rather than GpP80^{gag} (6, 26). The 65,000- and 75,000-dalton *gag*-related polypeptides were also immunoprecipitated from cell-free translation products of MuLV virion RNA. The gag-related proteins from the immunoprecipitates were purified by electrophoresis on SDS-gels. Individual polyproteins were located by autoradiography, excised, and electroeluted. The *gag* polyproteins were eluted from gel slices with minimal degradation (tracks A through F, Fig. 1). There was some cross-contamination between the 65,000- and 75,000-dalton cell-free translation products in this preparation (which was also reflected in the cleavage fragments to some degree).

The eluted polypeptides were subjected to partial proteolysis with *S. aureus* V8 protease, and the resultant cleavage fragments were analyzed by SDS-PAGE. The V8 cleavage profiles of Pr65^{gag} and P75^{gag} from tunicamycin-treated cells were substantially the same as those of the comigrating polyproteins from cell-free translation products of MuLV virion RNA (Fig. 1). Thus we conclude that the in vivo- and in vitro-

labeled polyproteins have the same polypeptide sequences, and for purposes of this report, the terms $Pr65^{gag}$ and $Pr75^{gag}$ will be used to refer to either in vitro- or in vivo-labeled *gag* polyproteins.

Before discussing the molecular weight of the cleavage fragments, it should be noted that $GpP80^{gag}$, $P75^{gag}$, and $Pr65^{gag}$ do not indicate precise designations of molecular weight. The molecular weight of $GpP80^{gag}$ has been reported as either 75,000 (16) or 80,000 (6, 12, 26); $p75^{gag}$ as 72,000 (9), 75,000 (6), or 78,000 (21); and $Pr65^{gag}$ as either 65,000 (6, 10, 12, 16) or 70,000 (32), although several different viruses were studied. In our experience, the analogous polyproteins coded for by either Rauscher or M-MuLV comigrate on SDS-polyacrylamide gels, whereas those from AKR MuLV are slightly larger. For the purposes of these investigations, it was necessary to have internally consistent molecular weights for the three polyproteins; these, as well as the molecular weights of the viral structural proteins, are listed in Table 1.

V8 protease cleavage of each *gag* polyprotein yielded four major cleavage fragments (Fig. 1, tracks G through L). The molecular weights of these fragments are listed in Table 2. Comparison of fragments from $Pr65^{gag}$ and $P75^{gag}$ indicated that three of these, A, B, and C, formed a "ladder" staggered in molecular size such that the fragments from $P75^{gag}$ were 4,000 daltons larger than the corresponding fragments from $Pr65^{gag}$; 4,000 daltons was the total difference in molecular size between the two polyproteins (see Table 1). Fragment D, on the other hand, had the same molecular size, whether derived from $Pr65^{gag}$ or $P75^{gag}$. These results suggest that cleavage fragments A, B, and C of $P75^{gag}$ contain the extra sequences present in this polypeptide but absent from $Pr65^{gag}$, whereas the cleavage(s) giving rise to fragment D removes these sequences.

TABLE 1. *Estimated molecular weights by SDS-PAGE of gag polyproteins and virion proteins*[a]

Species	Apparent mol wt
$GpP80^{gag}$	78,000
$P75^{gag}$	72,000
$Pr65^{gag}$	68,000
gp70	70,000
p30	32,000
p15E	14,000
p15	13,000
p12	12,000
p10	8,500

[a] Molecular weights of the *gag* polyproteins are based on comparison with M-MuLV marker proteins. [^{3}H]leucine-labeled M-MuLV proteins were included on every gel in these investigations in order to have a consistent molecular weight standard. The molecular weights of the virion proteins shown above are based on comparison with the published molecular weights of the following marker proteins: β-galactosidase, bovine serum albumin, ovalbumin, myoglobin, soybean trypsin inhibitor, and cytochrome *c*.

TABLE 2. *Apparent molecular weight of major cleavage fragments*

Fragment	Apparent mol wt ($\times 10^{-3}$)		
	$Pr65^{gag-}$	$P75^{gag}$	$GpP80^{gag}$
A	63	67	
B	59	63	69
C	57	61	67
D	47	47	53

The difference in apparent molecular size between $GpP80^{gag}$ and $Pr65^{gag}$ was 10,000 daltons (see Table 1), of which approximately 6,000 daltons was a shift in electrophoretic mobility due to the presence of carbohydrate on $GpP80^{gag}$. Major cleavage fragments B and C of $GpP80^{gag}$ were shifted in electrophoretic migration by an amount corresponding to 10,000 daltons relative to fragments B and C from $Pr65^{gag}$, whereas fragment D, which did not contain the extra amino acid sequences, was shifted by only 6,000 daltons. This is consistent with the carbohydrate residues being attached to amino acids held in common between $GpP80^{gag}$ and $Pr65^{gag}$ in a region of major cleavage fragment D that overlaps fragments B and C.

Specificity of antisera. To place the polyprotein cleavage fragments with respect to different regions of the *gag* gene, the fragments were immunoprecipitated with antisera specific for different individual *gag* proteins. Three antisera were used for this purpose: anti-p30, anti-p15, and anti-p10 sera. Anti-p30 serum was prepared in this laboratory and has been used in previous investigations (6, 18). Anti-p15 and anti-p10 sera were obtained from the National Cancer Institute. Figure 2 shows the specificity of these antisera with respect to [^{3}H]leucine-labeled proteins of M-MuLV. All three antisera precipitated the protein to which they are directed, and none precipitated other *gag* proteins. Anti-p30 serum recognized some larger minor virion proteins which have not been characterized, but they may represent intermediate cleavage products of *gag* precursors.

Ordering the V8 cleavage fragments: immunoprecipitation with either anti-p15 or anti-p10 serum. The M-MuLV internal structural protein domains of $Pr65^{gag}$ have been ordered as follows: NH_2-p15-p12-p30-p10-COOH (26). Therefore, immunoprecipitation of V8 cleavage fragments with anti-p15 serum would

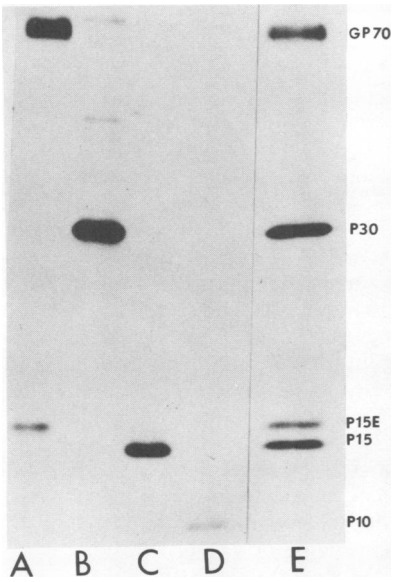

FIG. 2. *Specificity of antisera. [^{3}H]leucine-labeled M-MuLV was lysed in 0.5% Nonidet P-40–0.5% SDS in phosphate-buffered saline, and insoluble material was removed by brief centrifugation. The supernatant was immunoprecipitated with various monospecific antisera and S. aureus, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. (Track A) Anti-gp70 serum; (B) anti-p30 serum; (C) anti-p15 serum; (D) anti-p10 serum; (E) total [^{3}H]-leucine-labeled viral proteins in the starting material. Anti-gp70 serum also precipitates p15E, probably because these proteins are linked by disulfide bonds.*

be expected to yield fragments containing polypeptide sequences close to the amino terminus, whereas immunoprecipitation with anti-p10 serum would be expected to yield fragments containing polypeptide sequences close to the carboxy terminus of the polyprotein. Pr65^{gag} and P75^{gag} were isolated from [^{35}S]methionine-labeled cell-free translation product, and fragments were immunoprecipitated with either anti-p15 or anti-p10 serum and analyzed by SDS-PAGE. In the immunoprecipitations with anti-p10 serum (Fig. 3, tracks C and D), one major cleavage fragment was precipitated from either polyprotein, with a molecular size of 10,000 daltons, and the 10,000-dalton fragments from Pr65^{gag} and P75^{gag} were indistinguishable in size. This suggests that the carboxyl termini of Pr65^{gag} and P75^{gag} are the same and that the additional sequences present in P75^{gag} lie elsewhere. It should be noted that the most dominant cleavage fragments from the total cleavage profiles of Fig. 1 were the C fragments, indicating that they are generated by a very active cleavage site in the polyproteins. The 10,000-dalton p10-related cleavage fragments were the appropriate size to represent the remaining portion of Pr65^{gag} or P75^{gag} resulting from a cleavage 10,000 daltons from the carboxyl termini which also gives rise to the C fragments. The C fragments themselves were not immunoprecipitated with anti-p10 serum, but they were precipitated with anti-p15 serum (see below). Major fragments larger than 10,000 daltons were not precipitated with anti-p10 serum, presumably due to the fact that the cleavage site giving rise to the 10,000-dalton fragment is such a strong one.

Immunoprecipitation with anti-p15 serum yielded a set of nonidentical fragments from Pr65^{gag} and P75^{gag}, each of which reflected the difference in molecular size between the two *gag* polyproteins. These were as follows: major cleavage fragments B and C, and corresponding fragments of 55,000 and 59,000 daltons: 36,000 and 40,000 daltons; and 32,000 and 36,000 daltons, from Pr65^{gag} and P75^{gag}, respectively (see Fig. 3 and Table 3). The 36,000-dalton fragments from Pr65^{gag} and P75^{gag} migrated very close together; however, a consideration of the relative intensities of the various bands and a comparison with p15-containing fragments from GpP80^{gag} (see Fig. 6 and Table 3) make it likely that the 36,000-dalton band from Pr65^{gag} was not identical to the 36,000-dalton band from P75^{gag}, but corresponded to the 40,000-dalton fragment from p75^{gag}.

Fragments from the two polyproteins of identical molecular size were also immunoprecipitated by anti-p15 serum, although these were less prominent in the profile. These identical fragments migrated at 51,000, 47,000 (major cleavage fragment D), 20,000, and approximately 10,000 daltons. There was a minor fragment at 15,000 daltons from P75^{gag} for which there was no apparent corresponding fragment from Pr65^{gag}.

It seems likely that the large nonidentical major cleavage fragments, B and C, as well as the corresponding fragments of 55,000 and 59,000 daltons, were generated from Pr65^{gag} and P75^{gag} by one cleavage. Since these fragments were immunoprecipitated by anti-p15 serum but not anti-p10 serum, it is likely that they contained the amino termini but lacked the carboxy termini. The location of these fragments within the *gag* polyprotein as well as the position of the V8 cleavage sites giving rise to them are shown in the proposed cleavage map in Fig. 4. The region of nonidentity between the two polyproteins is shown as being at the extreme amino termini. The assignment of the nonidentical sequences to the amino terminus is done to accom-

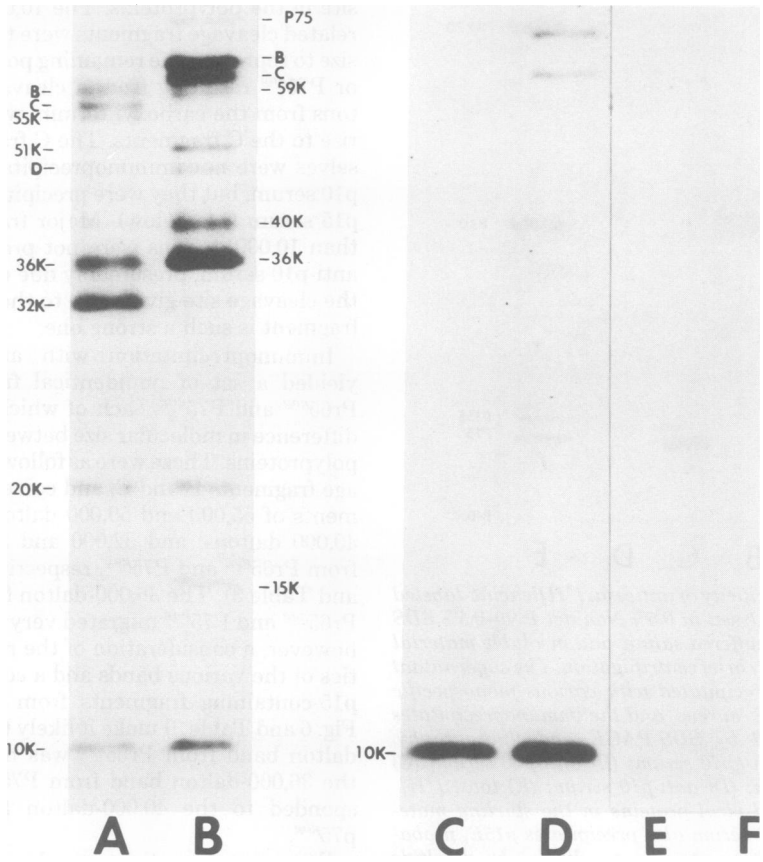

FIG. 3. *Immunoprecipitation of V8 cleavage fragments with anti-p15 and anti-p10 serum. [^{35}S]methionine-labeled Pr65^{gag} and P75^{gag} were isolated from cell-free translation products and treated with V8 protease as before. V8 cleavage fragments were immunoprecipitated with either anti-p15 or anti-p10 serum as described in Materials and Methods, and analyzed by SDS-PAGE. (A) Pr65^{gag} fragments immunoprecipitated with anti-p15 serum; (B) P75^{gag} fragments immunoprecipitated with anti-p15 serum; (C) Pr65^{gag} fragments immunoprecipitated with anti-p10 serum; (D) P75^{gag} fragments immunoprecipitated with anti-p10 serum; (E) immunoprecipitation of Pr65^{gag} fragments with normal serum; (F) immunoprecipitation of P75^{gag} fragments with normal serum.*

TABLE 3. *Apparent molecular weights (×10^{-3}) of homologous V8 cleavage fragments containing p15 antigenic determinants*[a]

Translation products			In vivo-labeled products		
Pr65^{gag}	P75^{gag}	Mol wt difference	Pr65^{gag}	GpP80^{gag}	Mol wt difference
59	63	4	59	69	10
57	61	4	57	67	10
55	59	4	55	65	10
(51)	(51)	0	(51)	57?	6
(47)	(47)	0	47	53	6
36	40	4	36	46	10
32	36	4	32	42	10

[a] Estimates of molecular weights are based on data presented in Fig. 3 and 6. This list does not include all fragments that were immunoprecipitated. Figures in parentheses indicate minor cleavage products in the anti-p15 immunoprecipitation profiles.

modate the largest identical fragment (51,000 daltons) obtained from the two polyproteins (see the next section). Proposed cleavages giving rise to the p15-related fragments of 32,000 and 36,000 daltons and 36,000 and 40,000 daltons from Pr65^{gag} and P75^{gag}, respectively, are also shown in Fig. 4.

Immunoprecipitation of V8 cleavage fragments with anti-p30 serum. Kopchick et al. (12) reported that GpP80^{gag} from Rauscher MuLV-infected cells is lacking two tryptic peptides held in common between Pr65^{gag} and p30. One possible explanation for this finding is that the migration of these peptides was altered in the mapping procedure due to the presence of carbohydrate in the p30 domain of GpP80^{gag}. Alternatively, the polypeptide core of GpP80^{gag} could differ due to the presence of extra amino acid sequences within the p30 region. The latter

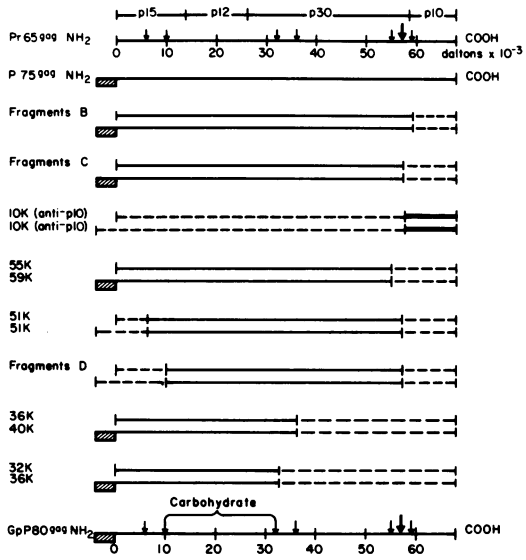

FIG. 4. *Proposed sequence relationship of gag polyproteins and assignment of V8 cleavage sites. Numbers refer to the position in kilodaltons from the amino terminus of Pr65^{gag}. V8 cleavage sites are indicated by arrows, with the large arrow indicating the most active site, which generates fragments C and the p10-containing 10,000-dalton fragments. (Note: The boundaries of the internal structural proteins are only approximate.) In the diagrams of the V8 cleavage fragments, the upper fragment in each pair is derived from Pr65^{gag}, whereas the lower one is derived from P75^{gag} and the unique sequences in P75^{gag} are indicated by the cross-hatched lines. The assignment of V9 cleavage sites limits the region in which the unique sequences in P75 are contained to within 6,000 daltons of the amino terminus of the corresponding region in Pr65^{gag}. With the exception of the 10,000-dalton p10-containing fragments from the carboxy terminus, all of the fragments diagramed above had corresponding fragments from GpP80^{gag} displaced in electrophoretic migration, reflecting the apparent molecular size addition due to carbohydrate. Therefore, the carbohydrate must be attached to the polypeptide in a region held in common between the fragments, as indicated above.*

possibility would not support the placement of V8 cleavage fragments as shown in Fig. 4, and therefore we investigated the possibility of additional polypeptide sequences in the p30 region of GpP80^{gag}. To avoid confusion due to the presence of carbohydrate, the unglycosylated form of GpP80^{gag}, P75^{gag}, was again studied.

Pr65^{gag} and P75^{gag} were isolated from cell-free translation products of virion RNA and cleaved with V8 protease as above. Cleavage fragments were immunoprecipitated (Fig. 5). The prominent fragments visible in tracks A and B have been previously identified as having p15 determinants. The nonidentical major cleavage fragments, A, B, and C, were immunoprecipitated, as well as the identical-sized fragments of 51,000 daltons and major fragment D. These results are consistent with the placement of p15-containing fragments as shown in Fig. 4, but the fragments were too large to allow a detailed analysis of the p30 region. To generate smaller fragments, Pr65^{gag} and P75^{gag} were treated for twice the length of time with V8 protease (60 instead of 30 min) and then immunoprecipitated with anti-p30 serum, as shown in tracks C and D. Major cleavage fragments D and the 51,000-dalton pair were again immunoprecipitated, but the larger fragments were only minor components in this preparation. As expected, the longer incubation with protease resulted in the generation of many smaller fragments immunoprecipitable with anti-p30 serum. In this situation, all of the p30 immunoprecipitable fragments smaller than 51,000 daltons from P75^{gag} had corresponding fragments of identical size from Pr65^{gag}. These results indicate the polypeptide cores of the two polyproteins are the same in the region of p30, and that the extra sequences in P75^{gag} come from elsewhere in the polyprotein, supporting the arrangement of fragments in Fig. 4.

Identical fragments from Pr65^{gag} and P75^{gag} of 51,000 and 47,000 daltons were immunoprecipitated with anti-p30 serum and somewhat less efficiently with anti-p15 serum, but not with anti-p10 serum. These fragments must be generated by two cleavages, one which removes the region of nonidentity between the two polyproteins and another which removes p10 determinants, since they were not immunoprecipitated with anti-p10 serum nor are they long enough to contain both p15 antigenic determinants and the carboxy terminus of the *gag* gene. We have tentatively placed the carboxy terminus of these fragments at the most active cleavage site (which generates fragment C) and their amino termini within the p15 domain (Fig. 4). The extra polypeptide sequences in P75^{gag} must lie outside the region of identity defined by the 51,000-dalton fragment and therefore close to the amino terminus of the polyprotein.

Cleavage fragments from in vivo-labeled polyproteins: assignment of carbohydrate-containing fragments. The migration of GpP80^{gag} on SDS-PAGE was displaced from that of Pr65^{gag} by an amount corresponding to 10,000 daltons (78,000 daltons for GpP80^{gag} versus 68,000 for Pr65^{gag}), of which 4,000 daltons was attributable to extra amino acid sequences, and the remaining 6,000 daltons to carbohydrate. GpP80^{gag} V8 cleavage fragments would also be displaced; therefore, immunoprecipitation of GpP80^{gag} cleavage fragments allows an identification of carbohydrate-containing frag-

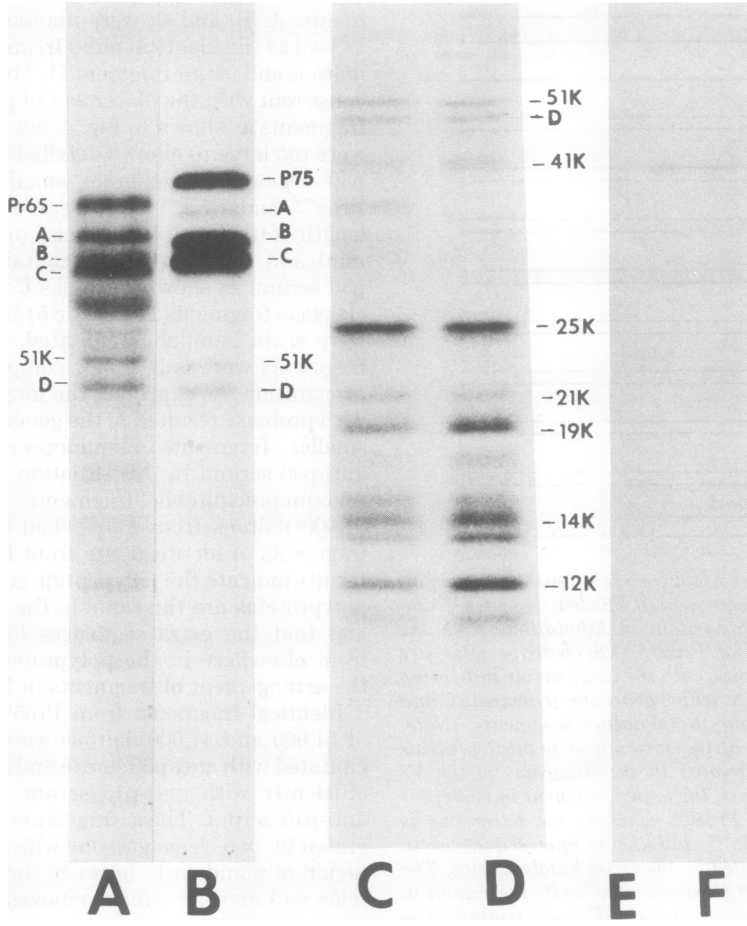

FIG. 5. *gag polyprotein cleavage fragments immunoprecipitated with anti-p30 serum. In tracks A, B, E, and F, [^{35}S]methionine-labeled Pr65^{gag} and P75^{gag} were treated with V8 protease, immunoprecipitated with anti-p30 serum, and analyzed as before. (A) Pr65^{gag} fragments immunoprecipitated with anti-p30 serum; (B) P75^{gag} cleavage fragments immunoprecipitated with anti-p30 serum; (E) Pr65^{gag} fragments immunoprecipitated with normal serum; (F) P75^{gag} fragments immunoprecipitated with normal serum. In tracks C and D, Pr65^{gag} and P75^{gag} were isolated from [^{3}H]leucine-labeled cell-free translation products, and these polyproteins were incubated with V8 protease for twice the normal length of time (60 min instead of 30 min) in order to generate smaller fragments. The immunoprecipitated fragments were electrophoresed on a 20% rather than 15% polyacrylamide gel. Track C shows Pr65^{gag} cleavage fragments immunoprecipitated with anti-p30 serum, and track D shows P75^{gag} fragments immunoprecipitated with anti-p30 serum.*

ments and the partial localization of the glycosylated portions of the molecule.

Pr65^{gag} and GpP80^{gag} were isolated from cytoplasmic lysates of M-MuLV clone 1 cells labeled for 1 h with [^{3}H]leucine. The V8 cleavage pattern of the two polyproteins (Fig. 6, tracks A and B) was basically the same as that shown for [^{35}S]methionine-labeled fragments in Fig. 1. Immunoprecipitation of Pr65^{gag} cleavage fragments with anti-p15 serum (track C) yielded results compatible with those for the cell-free translation product, although this preparation gave more of the fragments of higher molecular weight. The fragments from in vivo-labeled Pr65^{gag} that were immunoprecipitated with anti-p15 serum were major cleavage fragments, A, B, C, and D, and fragments of 55,000, 36,000, and 32,000 daltons. No prominent bands migrating further were seen in this preparation.

The total difference in apparent molecular size between Pr65^{gag} and GpP80^{gag} (10,000 daltons) was conserved in many of the p15-containing fragments from GpP80^{gag}, including major cleavage fragments B and C and fragments of 65,000, 46,000, and 42,000 daltons (see Fig. 6, track D, and Table 3). The latter two are likely

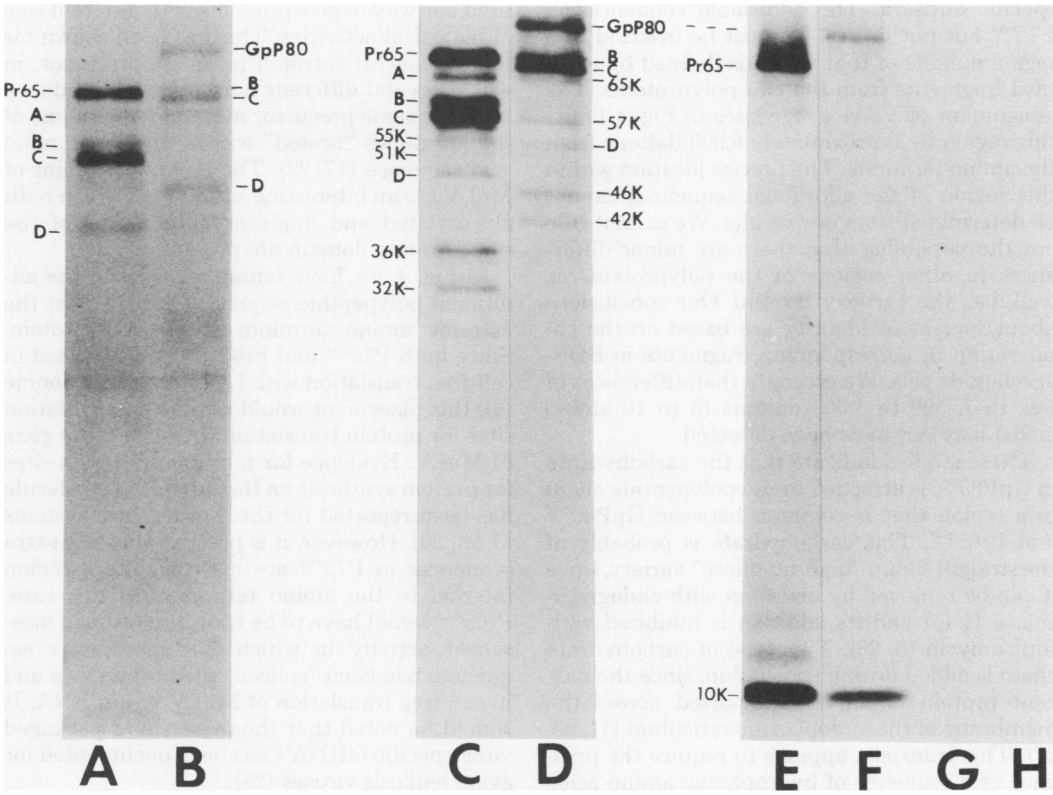

FIG. 6. *Immunoprecipitation of in vivo-labeled Pr65^{gag} and GpP80^{gag} V8 cleavage fragments with either anti-p15 or anti-p10 serum. Pr65^{gag} and GpP80^{gag} were isolated from cytoplasmic extracts of [^{3}H]leucine-labeled M-MuLV clone 1 cells and treated with V8 protease as before. (A and B) Cleavage profiles of Pr65^{gag} and GpP80^{gag}, respectively; (C) Pr65^{gag} cleavage fragments immunoprecipitated with anti-p15 serum; (D) GpP80^{gag} fragments immunoprecipitated with anti-p15 serum; (E) Pr65^{gag} fragments immunoprecipitated with anti-p10 serum; (F) GpP80^{gag} fragments immunoprecipitated with anti-p10 serum; (G) Pr65^{gag} fragments immunoprecipitated with normal serum; (H) GpP80^{gag} fragments immunoprecipitated with normal serum.*

glycosylated versions of the 40,000- and 36,000-dalton fragments from P75^{gag} (see Fig. 5 and Table 3). Major cleavage fragment D was also immunoprecipitated from GpP80^{gag} with anti-p15 serum. Fragment D from either Pr65^{gag} or P75^{gag} was identical in size, but the corresponding fragment from GpP80^{gag} was 6,000 daltons larger, reflecting the difference due to glycosylation between Pr75^{gag} and GpP80^{gag} as discussed previously. Finally, there was a fairly prominent fragment of 57,000 daltons for which a corresponding p15-containing fragment from Pr65^{gag} was difficult to assign. However, a minor fragment in the Pr65^{gag} profile at 51,000 daltons (also seen in the cell-free translation products, Fig. 3) may have been the unglycosylated form of the 57,000-dalton fragment.

Since cleavage of GpP80^{gag} gave p15-containing fragments of 42,000 and 46,000 daltons instead of the 36,000- and 40,000-dalton fragments obtained from P75^{gag}, the carbohydrate of GpP80^{gag} must lie within the 36,000-dalton fragment which comes from the amino end of P75^{gag}. On the other hand, major cleavage fragment D from GpP80^{gag} also contained a similar amount of carbohydrate. We conclude that the region of overlap between major cleavage fragment D and the p15-containing 42,000-dalton fragment (corresponding to the 32,000-dalton fragment from Pr65^{gag}) contains the glycosylation sites in GpP80^{gag} (Fig. 4).

DISCUSSION

In the results reported here, the additional polypeptide sequences present in P75^{gag} and GpP80^{gag} but not present in Pr65^{gag} are located at or near the amino terminus. These results were obtained by partial proteolysis of GpP80^{gag}, P75^{gag}, and Pr65^{gag}, followed by immunoprecipitation of cleavage fragment with different *gag*-

specific antisera. The additional sequences in $P75^{gag}$ but not in $Pr65^{gag}$ must be inserted in a region outside of that which is defined by identical fragments from the two polyproteins. The assignment of V8 cleavage sites in Fig. 4 limits this region to approximately 6,000 daltons from the amino terminus. The precise location within this region of the additional sequences cannot be determined from our results. We cannot rule out the possibility that there are minor differences in other regions of the polyproteins as well, i.e., the carboxy termini. Our conclusions about regions of identity are based on the comigration of corresponding fragments in SDS-acrylamide gels. We estimate that differences of less than 500 to 1,000 daltons (5 to 10 amino acids) may not have been detected.

These studies indicate that the carbohydrate in $GpP80^{gag}$ is attached to the polypeptide chain in a region that is common between $GpP80^{gag}$ and $Pr65^{gag}$. This carbohydrate is probably of the straight-chain "high-mannose" variety, since it can be removed by digestion with endoglycosidase H (6) and its addition is inhibited with tunicamycin (6, 26). This type of carbohydrate chain is added during translation, since the nascent protein chain is transported across the membrane of the endoplasmic reticulum (11, 23, 27). This transport appears to require the presence of a sequence of hydrophobic amino acids at the amino terminus of the polypeptide (the "signal peptide") (2), and the extra amino terminal sequences present in $GpP80^{gag}$ might provide this function. The absence of a signal peptide in $Pr65^{gag}$ would explain why this polyprotein is not glycosylated even though a glycosylation site(s) is available.

Kopchick et al. (12) have reported that Rauscher MuLV $GpP80^{gag}$ lacks two tryptic peptides which are shared by $Pr65^{gag}$ and mature p30. However, the result shown in Fig. 5, in which the unglycosylated form of $GpP80^{gag}$, $P75^{gag}$, was compared with $Pr65^{gag}$, revealed no differences in the cleavage fragments from the p30 regions of these polyproteins. A possible explanation for the results of Kopchick et al. (12) is that glycosylation of $GpP80^{gag}$ occurs within the p30 region, with a resulting alteration in mobility of the glycosylated tryptic peptides. The location of the carbohydrate in $GpP80^{gag}$ deduced from these experiments would be compatible with such an explanation (see Fig. 4).

Alternate forms of expression of a protein-coding sequence have been observed in several cases. In papovaviruses, alternate processing of viral RNA transcripts leads to different mRNA's which are translated to yield distinct proteins with some common sequences (1, 5). At another level, translation products are processed in more than one way to give products with different sets of biological activities. This has been shown for the adrenocorticotropin hormone precursor, in which several different hormones are produced from the same precursor molecule, with some of the hormones "nested" within the same amino acid sequence (17, 22). The *gag* polyproteins of MuLV are an interesting situation in which both glycosylated and unglycosylated forms of the same protein domain are produced.

In Fig. 4, we have tentatively placed the additional polypeptide sequences in $P75^{gag}$ at the extreme amino terminus of the polyprotein. Since both $P75^{gag}$ and $Pr65^{gag}$ can be labeled in cell-free translation with [^{35}S]formylmethionine (6), this placement would require two initiation sites for protein translation within the *gag* gene of MuLV. Evidence for multiple initiation sites for protein synthesis on the same RNA molecule has been reported for three other viral systems (3, 24, 30). However, it is possible that the extra sequences in $P75^{gag}$ are inserted into a region internal to the amino terminus. In this case, $Pr65^{gag}$ would have to be translated from a messenger activity in which the appropriate sequences had been "spliced out" both in vivo and in cell-free translation of MuLV virion RNA. It should be noted that the presence of packaged virus-specific mRNA's has been documented for avian leukosis viruses (28).

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