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gag-Related Polypeptides Encoded by Replication-Defective Avian Oncoviruses

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The content of viral structural (*gag*) protein sequences in polypeptides encoded by replication-defective avian erythroblastosis virus (AEV) and myelocytomatosis virus MC29 was assessed by immunological and peptide analyses. Direct comparison with *gag* proteins of the associated helper viruses revealed that MC29 110K polypeptide contained p19, p12, and p27, whereas the AEV 75K polypeptide had sequences related only to p19 and p12. Both of these polypeptides contained some information that was unrelated to *gag*, *pol*, or *env* gene products. In addition, no homology was detected between these unique peptides of MC29 110K and AEV 75K. The AEV 75K polypeptide shared strain-specific tryptic peptides with the p19 encoded by its naturally occurring helper virus; this observation suggests that *gag*-related sequences in 75K were originally derived from the helper viral *gag* gene. Digestion of oxidized MC29 110K and AEV 75K proteins with the *Staphylococcus aureus* V8 protease generated a fragment which comigrated with *N*-acetylmethionylsulfoneglutamic acid, a blocked dipeptide which is the putative amino-terminal sequence of structural protein p19 and *gag* precursor $Pr76^{gag}$. This last finding is evidence that the *gag* sequences are located at the N-terminal end of the MC29 110K and AEV 75K polypeptides.

Oncoviruses encode replicative functions required for the production of infectious progeny and oncogenic functions responsible for their ability to cause neoplastic disease. Identification of the specific gene products involved in these processes is central to an understanding of the biology of these viruses. The known replicative functions include the *gag*, *pol*, and *env* genes which direct the synthesis of viral internal structural proteins, RNA-dependent DNA polymerase, and envelope glycoproteins, respectively (2). The role of these proteins in the viral life cycle has been elucidated by genetic and biochemical techniques (for reviews, see 3, 11, 57, 58).

The oncogenic functions can be used to divide avian oncoviruses into three groups based on their pathogenic properties (18): sarcoma viruses, lymphatic leukemia viruses, and acute leukemia viruses. Sarcoma viruses, many strains of which possess all replicative functions, carry the *src* gene responsible for induction of fibrosarcomas in vivo and transformation of fibroblasts in vitro; the product of the *src* gene is apparently a 60,000-dalton polypeptide which has a protein kinase activity (8, 38). In general, lymphatic leukemia viruses cause a lymphoid leukosis after long latent periods in infected birds and contain all replicative functions, but they do not effect morphological transformation of fibroblasts or hematopoietic cells in tissue culture. Acute leukemia viruses, all of which lack one or more replicative functions, induce myeloid or erythroid leukemias, carcinomas, and in some instances fibrosarcomas; these replication-defective viruses can transform hematopoietic cells, and in some cases fibroblasts, in vitro (16) and may encode distinct oncogenic functions analogous to the *src* gene of sarcoma viruses (9, 15, 30). The oncogenic functions of the acute leukemia viruses are apparently unrelated to *src*, because *src*-specific nucleotide sequences are neither detected in the genomes of these viruses nor induced in infected cells (51) and because there is not uniform expression of transformation parameters in fibroblasts infected by sarcoma or acute leukemia viruses (44).

Internal structural proteins (*gag* proteins) of avian oncoviruses are derived by proteolytic cleavage of a common 76,000-dalton precursor polypeptide ($Pr76^{gag}$) (59). The order of viral structural proteins in this precursor has been determined as NH_2-p19-p12-p27-p15-COOH (42, 59). Some *gag* genetic information can also be found in polyproteins encoded by replication-defective viruses. Two acute leukemia viruses, myelocytomatosis virus MC29 and avian eryth-

roblastosis virus (AEV), synthesize polypeptides containing some *gag* sequences (4, 23, 24, 30). In the present study we have carried out antigenic and peptide analysis of the *gag*-related proteins of MC29 and AEV. We have sought to determine the amount of *gag*-specific information present in these polyproteins and whether there was detectable homology among the remaining sequences. The amino terminus of structural protein p19 was identified as *N*-acetylmethionylglutamic acid, and this blocked dipeptide was shown to be present in both $Pr76^{gag}$ and the *gag*-related polypeptides of acute leukemia viruses. These results indicate that the amino terminus of $Pr76^{gag}$ is also the amino terminus of p19 and suggest that the *gag* sequences comprise the N-terminal residues of replication-defective viral polyproteins.

MATERIALS AND METHODS

Cells and viruses. Primary cell cultures of SPAFAS chicken and Japanese quail embryos were prepared as previously described (17). Chicken embryo cells used in the present experiments were of the gs^-h^- phenotype (20). Subgroup B Rous-associated virus 2 (RAV-2) and other leukosis viruses were propagated on gs^-h^- chicken cells. Nonproducer (NP) quail cell clones transformed by myelocytomatosis virus MC29 (MC29-Q5 and MC29-Q8) were generously supplied by P. K. Vogt (4) and were grown in F10 medium supplemented with 10% tryptone phosphate broth, 5% bovine serum, 2% chicken serum, and 0.5% dimethyl sulfoxide. A stock of MC29A and the ES4 strain of AEV were provided by R. Ishizaki. AEV-transformed NP chicken cell clones were obtained by infecting gs^-h^- chicken embryo cells with a diluted stock of AEV, isolating colonies of transformed cells in soft agar, and assaying the clones for virus particle production by RNA-dependent DNA polymerase. AEV-NP clones were grown in F10 medium supplemented with 10% tryptone phosphate broth, 5% bovine serum, and 0.5% dimethyl sulfoxide. Myelocytomatosis-associated helper virus (MCAV) of subgroup A and erythroblastosis-associated virus (EAV) of subgroup B were isolated by endpoint dilution from stocks of MC29A and AEV, respectively.

Radiolabeling cells and immunoprecipitation. Extracts of cells labeled for 4 h at a concentration of 400 μCi/ml with either L-[^{35}S]methionine or L-[^{35}S]-cystine were prepared, and immunoprecipitation using Sepharose-bound *Staphylococcus aureus* protein A as an immunoadsorbent was done as previously described (42). The following specific antisera were used: rabbit antisera raised against avian myeloblastosis virus structural proteins p27, p19, and p12 (provided by V. M. Vogt), a rat antiserum against avian myeloblastosis virus RNA-dependent DNA polymerase (from R. C. Nowinski), and a rabbit antiserum against the subgroup E glycoprotein of RAV-60 (prepared by J. H. Chen). The latter antiserum is able to immunoprecipitate the cell-associated viral glycoproteins of subgroups A through F (7; Rettenmier, unpublished observation).

SDS-polyacrylamide gel electrophoresis and peptide analysis of proteins. Radiolabeled polypeptides were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (41). For peptide analysis, the bands of interest in the gel were located by autoradiography, excised, and oxidized with performic acid (42). The samples were then incubated for 18 h at 37°C in 0.6 ml of 0.05 M ammonium bicarbonate buffer (pH 8.0) containing either (i) 30 μg of L-(tosylamido 2-phenyl)ethyl chloromethyl ketone-treated bovine trypsin (252 U/mg; Worthington Biochemicals Corp., Freehold, N.J.) or (ii) 30 μg of *S. aureus* V8 protease (520 U/mg; Miles Laboratories, Elkhart, Ind.). Under these conditions the V8 protease specifically cleaves peptide bonds on the carboxy-terminal side of glutamic acids (25). The supernatants were lyophilized twice, and peptides were separated in two dimensions on cellulose-coated glass plates (10 by 20 cm; EM Laboratories, Elmsford, N.Y.). In the first dimension, samples were spotted 1.5 cm from the lower edge of the plate, either at the center for electrophoresis at pH 6.5 in pyridine-acetic acid-water (100:3:879) or 1.0 cm from the lateral edge of the plate for separation at pH 1.9 in acetic acid-formic acid-water (15:5:80). Electrophoresis was monitored using a mixture of 2% Orange G and 1% acid fuchsin dissolved in electrophoresis buffer as marker dyes. Ascending thin-layer chromatography (TLC) in 1-butanol-pyridine-acetic acid-water (65:50:10:40) was then done in the second dimension. Dried plates were exposed to Dupont Cronex 2DC safety film for autoradiography.

Preparation of Ac-Met-Glu. L-Methionyl-L-glutamic acid (Met-Glu) was purchased from Sigma Chemical Co. (St. Louis, Mo.). For N-terminal acetylation (56), 110 mg of the dipeptide was mixed with 2.0 ml of water and 2.0 ml of acetic anhydride with rapid stirring for 15 h at 22°C. The product, which was found to be a single spot on TLC, was freed of solvent by rotary evaporation under reduced pressure and lyophilized twice. The structure of the product was determined to be *N*-acetyl-L-methionyl-L-glutamic acid (Ac-Met-Glu) on the basis of the following criteria. (i) By TLC on silica gel-coated plates in chloroform-methanol-acetic acid (85:10:5) solvent, the Met-Glu starting material (R_f 0.0) was readily distinguished from the product (R_f 0.3). Mobility in this solvent suggested that the product had a blocked N-terminus. (ii) TLC on cellulose-coated plates in 1-butanol-pyridine-acetic acid-water (65:50:10:40) also resolved the starting material (R_f 0.4) from the product (R_f 0.8). (iii) Nuclear magnetic resonance of the product in trifluoroacetic acid revealed two findings in support of the structure Ac-Met-Glu. First, when compared with the spectrum of the starting material, the product had an additional signal, consistent with the presence of 3 mol of protons of the acetyl group per mol of starting material. Second, the peaks of the α protons were superposed in the spectrum of the product, whereas the signal of these two protons was split in the starting material due to the free amino terminus of Met-Glu. (iv) The product was not stained by ninhydrin at 22°C; the unblocked Met-Glu starting material was ninhydrin positive after about 2 h at this temperature. The location of the product could be visualized after nin-

hydrin treatment by heating the TLC plates at 160°C for 3 min under vacuum, which were conditions that presumably broke peptide bonds and generated reactive amines.

Performic acid treatment, such as that used for radiolabeled proteins during peptide analysis, oxidizes methionine residues to the methioninesulfone [Met(O_2)] derivative (32). This was confirmed by hydrolysis of peptides in 6 N HCl at 110°C for 24 h and separation of the amino acids in an automated Beckman 121 analyzer. After hydrolysis, the Met-Glu starting material revealed peaks of Met and Glu in a 1:1 ratio; on performic acid oxidation and subsequent hydrolysis, the Met was replaced by a peak comigrating with Met(O_2) which eluted near aspartic acid in the chromatogram (50). On TLC in 1-butanol–pyridine–acetic acid–water (65:50:10:40) solvent, Met(O_2)-Glu had an R_f of 0.2 and Ac-Met(O_2)-Glu had an R_f of 0.5 (see Fig. 6).

Carboxypeptidase Y digestion of [^{35}S]methionine-labeled 19* peptide. The 19* peptide was obtained by digestion of [^{35}S]methionine-labeled RAV-2 p19 with *S. aureus* V8 protease, separated from other peptides by two-dimensional fingerprinting, eluted from the cellulose layer of the TLC plates, and lyophilized. The material was dissolved in 2.0 ml of 0.1 M pyridine acetate (pH 5.5) and divided into two aliquots. One aliquot served as an untreated control, and to the other was added 10 μg of carboxypeptidase Y (71 U/mg; Worthington). The samples were incubated for 4 h at 37°C, lyophilized twice, and then analyzed by two-dimensional fingerprinting on cellulose-coated TLC plates (10 by 10 cm). Marker standards included Ac-Met(O_2)-Glu, which comigrated with the 19* peptide (see Fig. 6), and Ac-Met(O_2), which was prepared by performic acid oxidation of *N*-acetylmethionine obtained from Sigma. Fluorography was done by dipping the dried TLC plates into molten naphthalene containing 0.4% 2,5-diphenyloxazole (6) and exposing the plates to film at −70°C.

RESULTS

Antigenic analysis of viral polyproteins in avian cells. Figure 1 shows the electrophoretic separation of viral polypeptides immunoprecipitated from RAV-2-infected chicken cells by specific antisera. The indicated viral proteins have been described previously and included translation products of all the known replicative genes (7, 13, 22, 31, 33, 59). These same proteins were also found in detergent lysates of cells infected with MCAV and EAV, which were nontransforming helper viruses isolated by endpoint dilution from stocks of MC29 and AEV, respectively. The detection of mature viral glycoproteins (gp85 and gp35) in Fig. 1D was a consequence of the 4-h labeling interval in this experiment and probably reflected the presence of progeny virions at the cell surface (29). There was also evidence for processing of the Pr76^{gag} precursor to mature structural protein p27 in Fig. 1B.

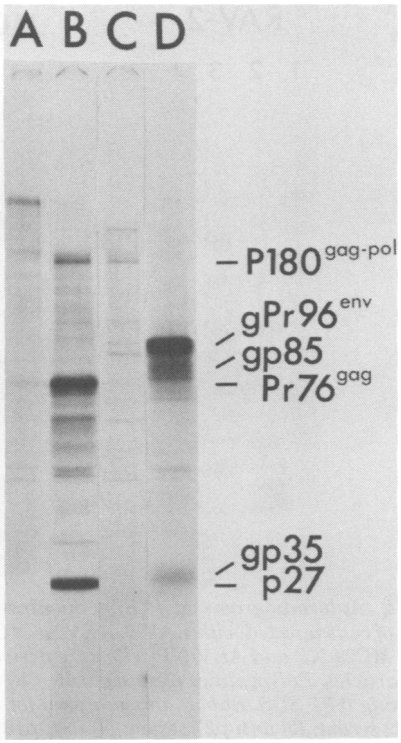

FIG. 1. *Autoradiogram of [^{35}S]methionine-labeled viral polypeptides immunoprecipitated from detergent extracts of RAV-2-infected chicken cells by the indicated sera. The precipitated proteins were separated by SDS-gel electrophoresis in a 5 to 15% gradient polyacrylamide slab. (A) Nonimmune rabbit serum; (B) rabbit anti-p27 serum; (C) rat anti-viral DNA polymerase serum; (D) rabbit anti-viral glycoprotein serum. Virus-specific polypeptides are indicated in the right margin: Pr76^{gag} and p27 are present in (B); P180$^{gag-pol}$ is in both (B) and (C); gPr96^{env}, gp85, and gp35 are in (D).*

Previous investigators have documented the presence of *gag*-related polypeptides in NP cells infected by avian acute leukemia viruses (4, 23, 24, 27). The MC29 110K and AEV 75K polypeptides, both of which contain some *gag* sequences, are not cleaved to form mature viral structural proteins in the transformed NP cells (4, 24). We have examined the *gag* information present in these polypeptides using rabbit antisera raised against individual virion proteins p19, p27, and p12. The results are shown in Fig. 2. Specificities of these antisera were assessed from the control precipitations of RAV-2-infected cell lysates: the anti-p27 serum (lane 3 in each panel) appeared to be monospecific because it did not precipitate other mature structural proteins. The anti-p19 serum (lane 2) precipitated p19, but it also had some weak reactivity with p27. The p12 antise-

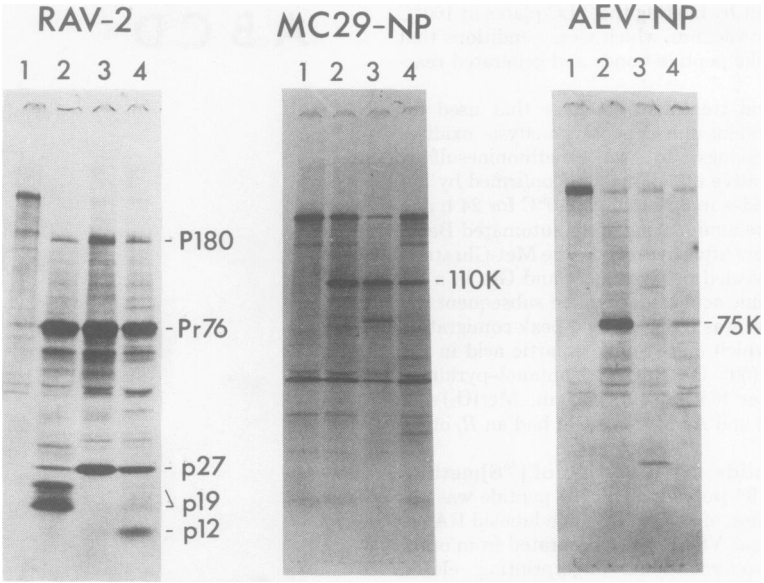

FIG. 2. *Autoradiograms of [^{35}S]methionine-labeled, gag-related polypeptides immunoprecipitated from lysates of cells infected with RAV-2, AEV, or MC29. The RAV-2-infected cells were producing progeny viruses, but the MC29-NP and AEV-NP cells were derived from transformed clones that were not releasing detectable virus particles. Polypeptides were separated by electrophoresis in 5 to 15% gradient polyacrylamide slab gels containing 0.1% SDS. Rabbit antisera used for the respective lanes of each lysate: (1) nonimmune serum; (2) anti-p19 serum; (3) anti-p27 serum; (4) anti-p12 serum. Virus-specific, gag-related polypeptides are indicated at the right margin of each panel.*

rum (lane 4) had some anti-p27 and, to a lesser extent, anti-p19 activity.

Equivalent amounts of RAV-2 Pr76^{gag} and P180$^{gag\text{-}pol}$ were immunoprecipitated by the p19, p27, and p12 antisera. All of these anti-*gag* sera also reacted with the MC29 110K protein. However, the anti-p19 serum precipitated AEV 75K protein much more efficiently than did the other two structural protein antisera. Some 75K protein was brought down by the anti-p12 serum; however, the p27 antiserum did not detectably precipitate a polypeptide in the 75K region above the background that was occasionally seen with nonimmune serum (see lane 1 in the panels in Fig. 2). The MC29 110K and AEV 75K proteins were not precipitated by antisera raised against the viral RNA-dependent DNA polymerase or envelope glycoproteins (data not shown).

Tryptic peptide analysis of MC29 110K and AEV 75K proteins. We next attempted to determine the amount of *gag* information in MC29 110K and AEV 75K by tryptic peptide fingerprinting. First, the structural proteins (1) of nondefective MCAV and EAV helper viruses were compared. For this analysis, MCAV and EAV were purified from culture fluids of infected cells grown in the presence of [^{35}S]methionine or [^{35}S]cysteine. The structural proteins of these viruses were separated by SDS-polyacrylamide gel electrophoresis, and tryptic peptides of the individual *gag* proteins were prepared. The peptides were analyzed in two dimensions on cellulose-coated thin-layer plates by electrophoresis at pH 1.9 and chromatography in 1-butanol–pyridine–acetic acid–water (65:50:10:40). Details of this procedure have been described (42). In general, there was extensive homology among the methionine- and cysteine-containing tryptic peptides of the helper viral *gag* proteins. However, as shown in Fig. 3, we did detect strain-specific variation in [^{35}S]methionine-labeled tryptic peptides of the p19 proteins. The methionine-containing peptides of MCAV p19 were identical to those of p19's from many other avian oncoviruses (42; C. W. Rettenmier, unpublished observation). On the other hand, the EAV p19 had a higher electrophoretic mobility in SDS gels than did the p19 of any other virus in our laboratory collection, and the four novel tryptic peptides shown in Fig. 3 were also unique to the EAV p19.

Next, NP cells transformed by MC29 or AEV were radiolabeled with [^{35}S]methionine and [^{35}S]cysteine, and detergent lysates were prepared. The MC29 110K and AEV 75K proteins were purified from the extracts by immunoprecipitation and SDS-gel electrophoresis, and the

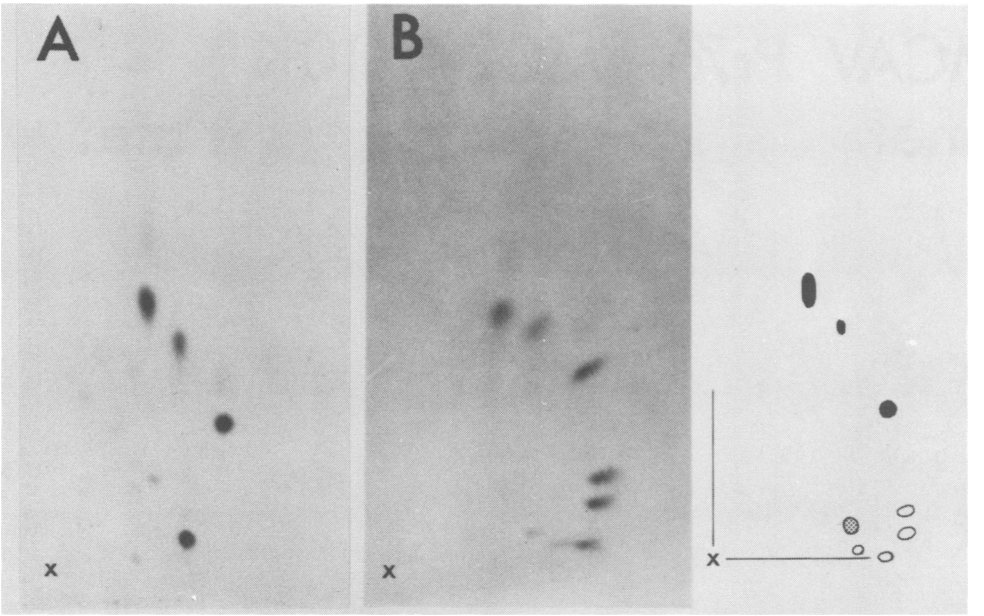

FIG. 3. *Comparison of [^{35}S]methionine-labeled tryptic peptides of MCAV p19 (A) and EAV p19 (B) by two-dimensional fingerprinting. Peptides were spotted on cellulose-coated thin-layer plates in the lower left corner (marked with an ×) of each panel. Electrophoresis at pH 1.9 was carried out from left to right in the horizontal direction with the anode at the left, and then ascending chromatography was done from bottom to top. At the right is a schematic diagram showing the methionine-containing tryptic peptides common to both p19 proteins (●), those unique to MCAV p19 (◎), and those unique to the EAV p19 (○). The relative positions of the spots were confirmed by experiments in which the samples were mixed before fingerprinting (data not shown).*

tryptic peptides were analyzed as described above. We also examined the peptides of individual structural proteins and the Pr76^{gag} precursors of MCAV and EAV helper viruses. The results are presented in Fig. 4 and 5, where the tryptic peptides obtained from Pr76^{gag} and the *gag*-related polyproteins are compared. Each *gag*-related peptide spot was identified by analysis of the individual structural proteins and is designated in Fig. 4 and 5 by the number of the corresponding *gag* protein. For example, peptides marked with 19 comigrated with tryptic peptides of the mature viral p19, etc. The identity of individual *gag* peptides was confirmed by experiments in which samples were mixed before peptide analysis (data not shown).

Figure 4 demonstrates that methionine- and cysteine-containing tryptic peptides of p19, p12, and p27 were detected in the MC29 110K protein. However, none of the p15 peptides identified in the MCAV Pr76^{gag} were found in the 110K polypeptide. In addition, several peptides that were not present in the Pr76^{gag} of MCAV helper virus were observed in the 110K protein. The fingerprints of 110K derived from both MC29-Q5 and MC29-Q8 NP cells were identical (data not shown). Only a small number of *gag*-related tryptic peptides were detected in the AEV 75K protein (Fig. 5); these included several p19 methionine-containing peptides, the p19 cysteine-containing peptide, and some p12 cysteine peptides. Two of the strain-specific, [^{35}S]methionine-labeled peptides of EAV p19 were present in AEV 75K (see arrows in the schematic diagram in Fig. 5). We did not detect any peptides related to the EAV p27 or p15 proteins in AEV 75K; the methionine-containing p12 peptide and two of the methionine peptides of EAV p19 were also absent. Fingerprints of 75K protein from several AEV-NP clones were identical to each other (data not shown). There were many additional peptides in AEV 75K that were not shared with *gag* proteins, and these unique sequences of 75K had no detectable homology with those of MC29 110K. Similarly, the unique peptides of these acute leukemia viral proteins were not related to viral polymerase peptides (data not shown).

Identification of the N-terminal dipeptide of p19. Palmiter and co-workers (35) have determined the N-terminal amino acid sequence of Prague strain Pr76^{gag} to be Met-Glu-Ala-Val-Ile-Lys- . . . , and they have shown that the amino terminus of the protein is blocked, probably as

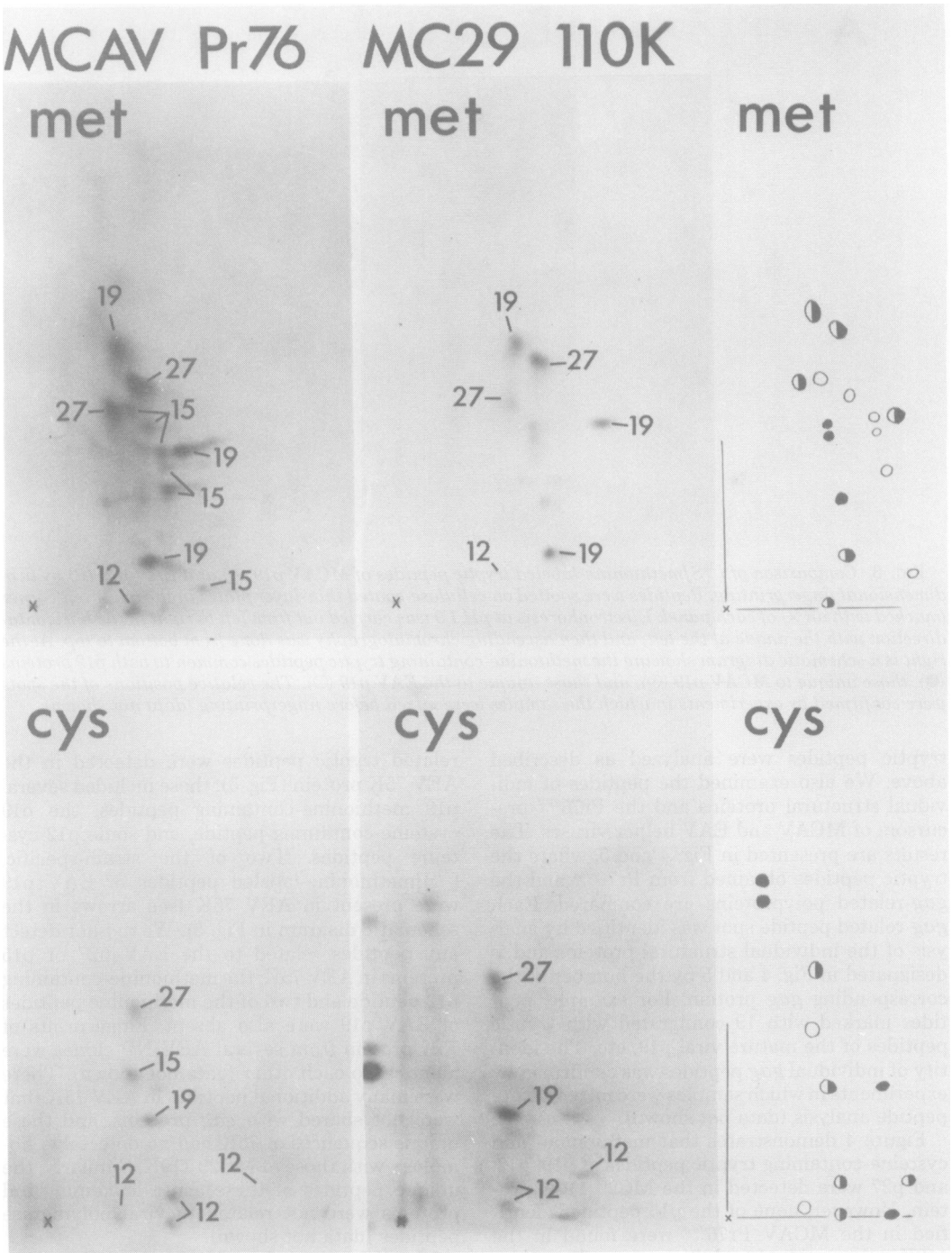

FIG. 4. *Two-dimensional tryptic peptide analysis of MCAV Pr76^{gag} (left) and MC29 110K (center) proteins labeled with [^{35}S]methionine (upper) and [^{35}S]cysteine (lower). Details of the analysis were as described in the legend to Fig. 3. The gag-peptides in the autoradiograms are designated by the number of the individual structural protein in which they were detected. At the right are schematic diagrams showing tryptic peptides found in MCAV Pr76^{gag} (○), MC29 110K (●), and those gag-related peptides present in both proteins (◑).*

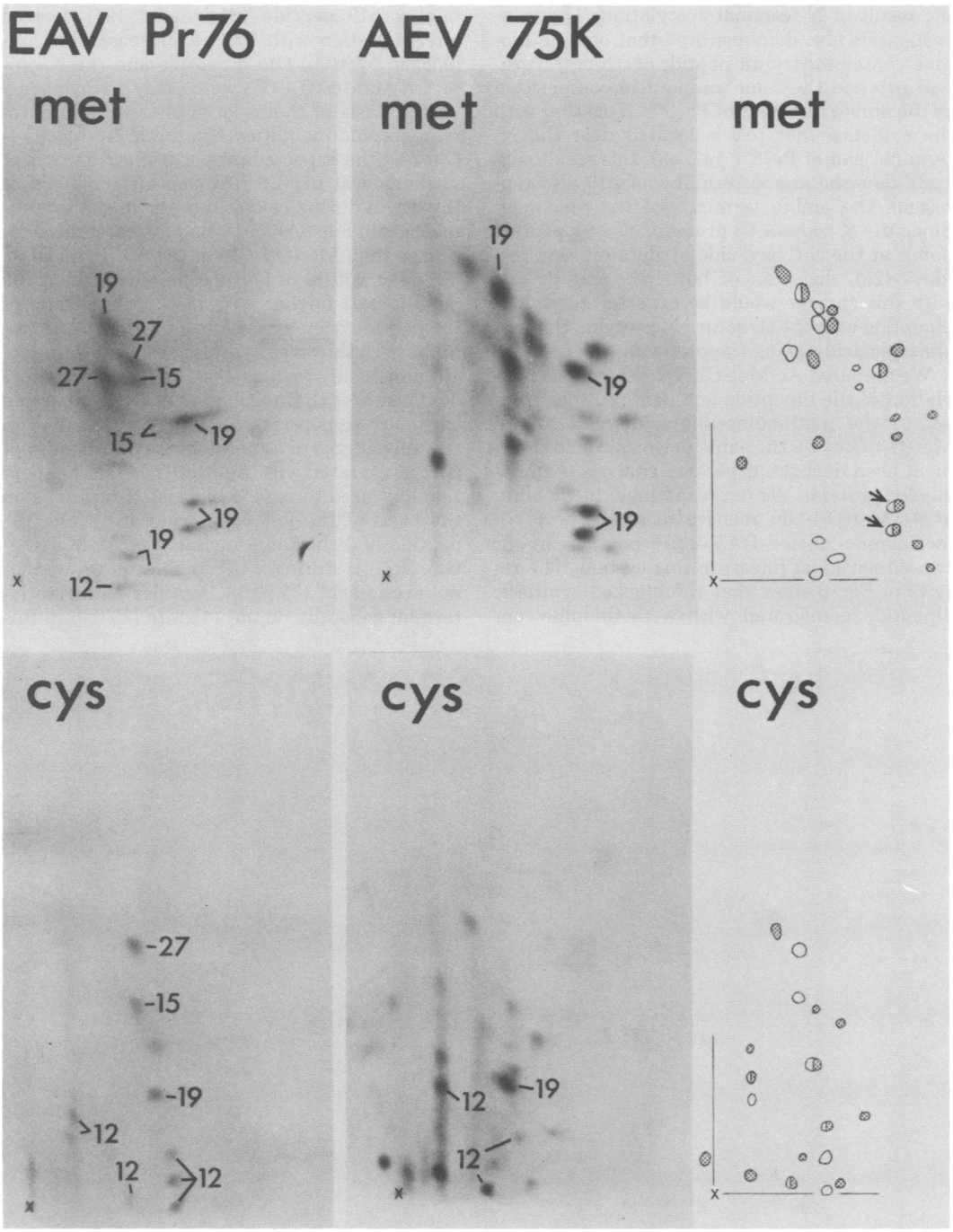

FIG. 5. *Two-dimensional tryptic peptide analysis of EAV Pr76^{gag} (left) and AEV 75K (center) proteins labeled with [^{35}S]methionine (upper) and [^{35}S]cysteine (lower). Details of the analysis were as described in the legend to Fig. 3. At the right are schematic diagrams showing tryptic peptides found in EAV Pr76^{gag} (○), AEV 75K (⊙), and those gag-related peptides present in both (○). Two type-specific tryptic peptides unique to the EAV p19 are marked with arrows (→) in the schematic diagram of the [^{35}S]methionine-labeled fingerprints.*

the result of N-terminal acetylation. These investigators also demonstrated that one methionine-containing tryptic peptide of structural protein p19 had the same amino acid composition as the amino terminus of $Pr76^{gag}$. Together with the evidence that p19 is located near the N-terminal end of $Pr76^{gag}$ (42, 59), this result suggests that the amino terminus of p19 also represents the amino terminus of the precursor. Since the *S. aureus* V8 protease cleaves peptide bonds at the carboxyl side of glutamic acid residues (25), digestion of both p19 and $Pr76^{gag}$ with this enzyme would be expected to yield a dipeptide with the structure *N*-acetylmethionylglutamic acid among the products.

We prepared Ac-Met-Glu by N-terminal acetylation of the dipeptide and oxidized this product to the methioninesulfone derivative [Ac-Met(O_2)-Glu] by the same performic acid treatment used during our peptide analysis of radiolabeled proteins. We next examined the mobility of Ac-Met(O_2)-Glu relative to authentic [^{35}S]-methionine-labeled RAV-2 p19 peptides in our two-dimensional fingerprinting system. The results in Fig. 6 show that the blocked synthetic dipeptide comigrated with a methionine-containing p19 peptide (designated 19*) derived after digestion with the V8 protease. The unblocked Met(O_2)-Glu dipeptide and the N-protected Ac-Met(O_2)-Glu were easily distinguished on the basis of their electrophoretic and chromatographic mobilities. Synthetic Ac-Met(O_2)-Glu had the expected properties because it was uncharged at pH 1.9 (the peptide remained at the origin during electrophoresis in the first dimension in Fig. 6A) and it had a greater negative charge than Met(O_2)-Glu at pH 6.5 (Fig. 6B).

The structure of [^{35}S]methionine-labeled 19* peptide was further examined after elution of the peptide from TLC plates. Carboxypeptidase Y has been shown to hydrolyze carboxy-terminal glutamic acid residues at pH 5.5 (21). Figure 7 demonstrates that incubation of the 19* peptide with carboxypeptidase Y at pH 5.5 resulted in the appearance of a methionine-containing spot that comigrated with Ac-Met(O_2). The 19* peptide appeared to have a blocked N-terminus on the basis of the following criteria. (i) The 19* peptide had the same mobility as Ac-Met(O_2)-Glu (R_f 0.1) during TLC in chloroform-methanol-acetic acid (85:10:5). Peptides with free N-termini generally do not migrate (R_f 0.0) in this

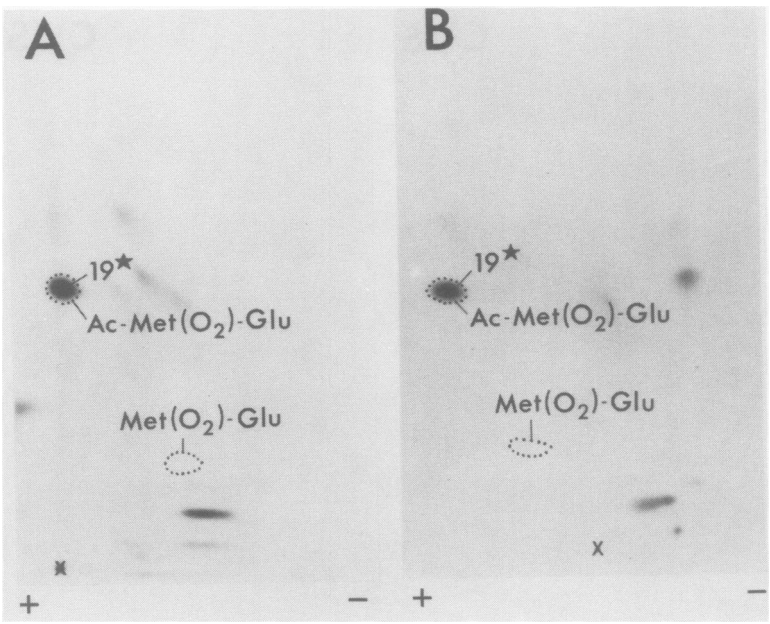

FIG. 6. *Autoradiograms showing two-dimensional analysis of S. aureus V8 protease-derived fragments of [^{35}S]methionine-labeled RAV-2 p19. Electrophoresis in the first dimension was done in the horizontal direction with the anode at the left: (A) pH 1.9 with the origin (marked with an ×) in the lower left corner; (B) pH 6.5 with the origin (×) in the lower center. Ascending chromatography in 1-butanol–pyridine–acetic acid–water was done in the vertical direction from bottom to top. The mobilities of two marker peptides, Met (O_2)-Glu and Ac-Met(O_2)-Glu, detected by the ninhydrin reaction are indicated by dotted circles. Note that the [^{35}S]methionine-labeled V8 peptide designated 19* comigrated with Ac-Met(O_2)-Glu in both panels.*

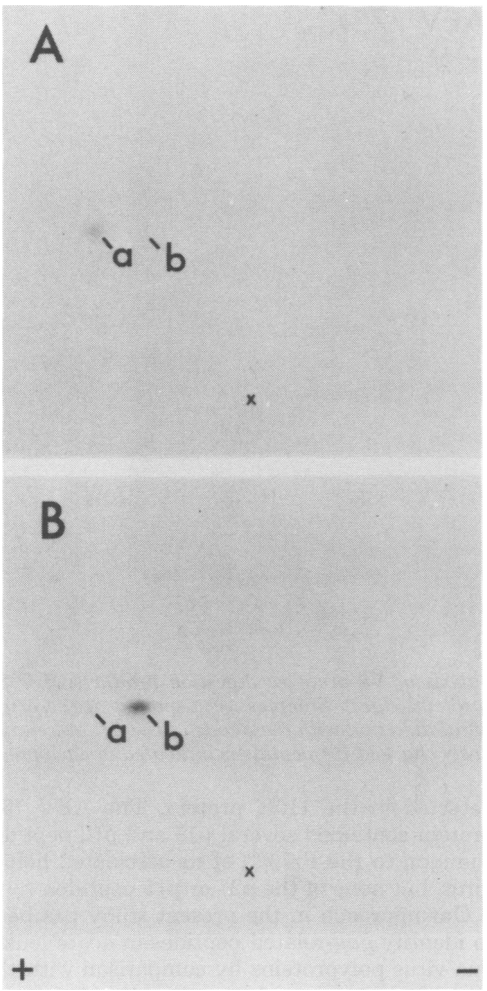

FIG. 7. *Autoradiograms showing two-dimensional separation of proteolytic digestion products derived from [^{35}S]methionine-labeled 19* peptide: (A) untreated control; (B) treated by carboxypeptidase Y. Details of the digestions were as described in the text. Samples were spotted at the origin (×), and then electrophoresis at pH 6.5 was done in the horizontal direction with the anode at the left, followed by ascending chromatography from bottom to top. Letters a and b in each panel indicate the mobilities of marker peptides Ac-Met(O_2)-Glu and Ac-Met(O_2), respectively.*

solvent system. (ii) The 19* peptide was resistant to digestion by leucine aminopeptidase (10 U/ml; 155 U/mg) in 75 mM Tris (pH 8.8)–2.5 mM $MnCl_2$ for 4 h at 37°C. These studies support the conclusion that 19* is the oxidized form of the amino-terminal dipeptide of RAV-2 p19 and has the structure Ac-Met(O_2)-Glu. Digestion of the EAV p19 with the V8 protease revealed that the 19* peptide was also present in this protein (data not shown).

The MC29 110K, AEV 75K, and Pr76^{gag} proteins of RAV-2, MCAV, and EAV were labeled with [^{35}S]methionine, purified from detergent lysates of cells by immunoprecipitation and SDS-gel electrophoresis, oxidized with performic acid, and digested with the *S. aureus* V8 protease. The resulting peptides were separated by two-dimensional fingerprinting, and the 19* peptide was present in all cases. As illustrated for RAV-2 Pr76^{gag} and AEV 75K proteins in Fig. 8, the 19* peptide was the only methionine-containing V8 peptide having a net negative charge at pH 6.5. These results indicate that the sequence of the 19* peptide is highly conserved among the avian oncoviruses, as might be expected if it represents the amino-terminal dipeptide of p19. The finding of 19* in the *gag*-related polyproteins of replication-defective viruses suggests that the *gag* sequences are present at the N-termini of MC29 110K and AEV 75K proteins. The 19* peptide was also recovered after V8 protease digestion of [^{35}S]methionine-labeled Pr76^{gag} obtained by in vitro translation of RAV-2 35S virion RNA in the nuclease-treated, cell-free rabbit reticulocyte system (36) (data not shown). This last result indicates that the reticulocyte lysate contained an activity which blocked the N-terminus of Pr76^{gag} by a mechanism similar to that present in infected chicken embryo cells.

DISCUSSION

All isolates of avian acute leukemia viruses thus far studied have been shown to be defective and dependent upon helper lymphatic leukosis viruses for their replication (16, 18, 27). The genetic defects of these acute leukemia viruses appear to be very extensive (5, 16, 27); they do not encode any functions capable of complementing lesions in the *gag*, *pol*, or *env* genes of superinfecting helper viruses. Instead of the normal products of retroviral replicative genes, polypeptides unique to each strain of acute leukemia virus have been detected in transformed NP cells. For MC29 and AEV studied here, 110K and 75K proteins, respectively, have been identified by virtue of the fact that they contained some *gag* antigenic determinants and were precipitated by anti-*gag* sera (4, 23, 24). Antisera against reverse transcriptase or envelope glycoproteins had no cross-reactivity with these polyproteins. From these observations, it appears that the polyproteins are unique products of the acute leukemia viruses and consist of a portion of the *gag* proteins linked to sequences that are structurally unrelated to *pol* or *env* proteins.

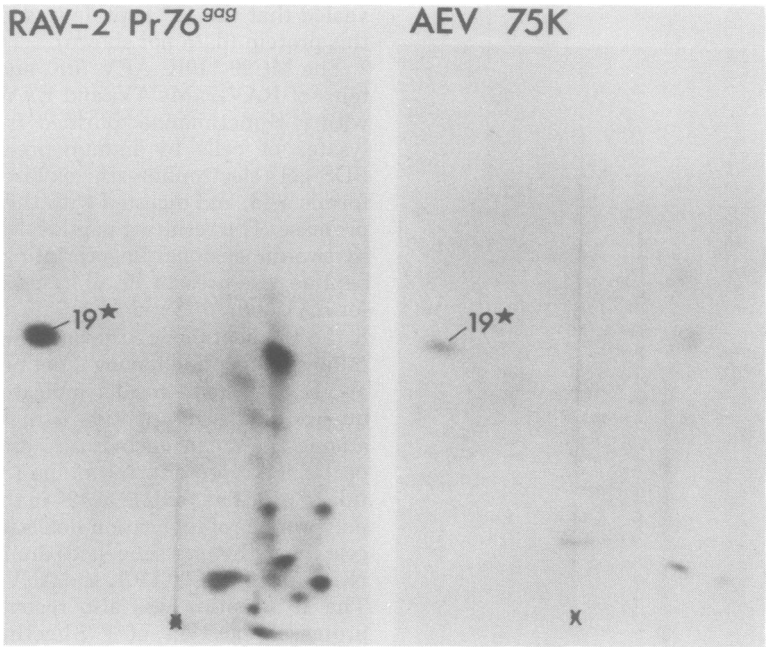

FIG. 8. *Autoradiograms showing two-dimensional analysis of V8 protease digestion products of [^{35}S]-methionine-labeled RAV-2 Pr76^{gag} (left) and AEV 75K protein (right). Samples were spotted at the origin (×), and electrophoresis at pH 6.5 was done in the horizontal direction with the anode at the left. Ascending chromatography was done from bottom to top. The negatively charged 19* peptide is indicated in each panel.*

Radioimmunoassay techniques have been used to characterize the *gag* information present in MC29 110K and AEV 75K proteins. Bister et al. (4) showed that the MC29 110K protein contained p19 and some p27 antigenic determinants, but no detectable p15. Hayman et al. (24) demonstrated the existence of only p19 determinants in the AEV 75K protein. Using peptide fingerprinting, these latter investigators also detected two [^{35}S]methionine-labeled tryptic peptides of B77 virus p19 in the 75K polypeptide. The presence of p12 peptides in these acute leukemia viral polyproteins was not established in these studies. Our immunoprecipitation data are consistent with the previously published results. The MC29 110K and AEV 75K proteins were also precipitated by an anti-p12 serum; however, these results may be due to anti-p27 and weak anti-p19 reactivities in the p12 antiserum (see Fig. 2). Analysis of [^{35}S]methionine- and [^{35}S]cysteine-labeled MC29 and AEV polyproteins by tryptic peptide fingerprinting provided more convincing evidence regarding the presence of *gag*-related sequences. Tryptic maps of [^{35}S]cysteine-labeled proteins were particularly useful in studying p12 peptides because of the relative abundance of this amino acid in p12 (42). Our results confirmed the presence of p19 and p27 peptides and the absence of p15 in the MC29 110K. In addition, p12 peptides were also detected in the 110K protein. The AEV 75K protein contained several p19 and p12 peptides common to the Pr76^{gag} of its associated helper virus, but none of the p27 or p15 peptides.

Our approach in the present study has been to identify *gag*-related peptides in acute leukemia virus polyproteins by comparison with the structural proteins of the respective helper viruses. We assume that the *gag* sequences in the helper viral proteins are closely related to those in MC29 110K and AEV 75K. However, this analysis is limited by the extent of homology between the two sets of *gag* sequences. In general, the methionine- and cysteine-containing tryptic peptides of individual avian oncoviral structural proteins are highly conserved (Rettenmier and Hanafusa, manuscript in preparation). On the other hand, if corresponding *gag* proteins have different tryptic fingerprints (41, 47), then such strain-specific variation would result in a low estimate of the amount of *gag* sequences present in MC29 110K and AEV 75K. For example, not all of the p19 or p12 peptides of EAV Pr76^{gag} were present in the AEV 75K protein; this result suggests that some sequences of p19 and p12 were missing in the polyprotein. However, we cannot exclude the possibility that 75K contained complete sequences of p19 and p12 proteins different from those encoded by the EAV helper. The AEV 75K protein had two

type-specific methionine-containing tryptic peptides of EAV p19, which indicates at least part of the *gag* sequences in 75K are closely related to those of the helper virus. However, Tsichlis and Coffin (55) have recently shown that avian acute leukemia viruses are able to recombine with lymphatic leukosis viruses. Therefore, AEV may have acquired some of the strain-specific *gag* peptides of EAV through recombination during passage in the presence of the helper. The inability to detect all of the EAV p19 and p12 peptides in AEV 75K does not eliminate the possibility that the entire sequences of some other p19 and p12 are present in the replication-defective viral polyprotein.

Both the MC29 110K and AEV 75K proteins contained additional peptides that were unrelated to *gag* or *pol* proteins. At least some of these sequences were presumably derived from the portions of these polyproteins encoded by the MC29- and AEV-specific regions of the genomes (26, 30, 48, 51). The nature of the unique sequences in 110K and 75K is not known. One possibility is that these proteins result from fusion of the *gag* and *env* genes similar to the apparent *gag-pol* fusion in the endogenous gs^+ P120 protein (10, 42). However, Hayman and coworkers (24) found no homology among methionine-containing tryptic peptides of AEV 75K protein and the $gPr95^{env}$ precursor of B77 virus. The MC29 110K and AEV 75K proteins are not glycosylated (S. M. Anderson, unpublished observation), and in the present study they were not immunoprecipitated by a broadly reactive antiserum which detects group-specific determinants in viral glycoproteins of subgroups A through F. It is tempting to speculate that the novel sequences in these *gag*-related polyproteins represent the oncogenic functions of these viruses. No homology was detected among the non-*gag* peptides of MC29 110K and AEV 75K proteins. If these proteins do have an oncogenic function, then the lack of homology is consistent with the observations that these viruses induce different types of leukemias and cause differential expression of transformation parameters in infected fibroblasts (16, 44).

A number of studies with avian and murine systems have strongly suggested that sarcoma viruses and certain leukemia viruses were generated by recombination between replication-competent lymphatic leukemia viruses and cellular oncogenes (12, 14, 19, 39, 40, 46, 49, 53, 54, 60). It is possible that avian acute leukemia viruses were also formed by insertion of certain cellular sequences into the lymphatic leukemia virus genome. If the MC29 110K and AEV 75K proteins are involved in oncogenesis, then the relevant sequences have been joined to the 5′ portion of the viral *gag* gene. It is conceivable that the only essential *gag* sequences in the replication-defective transforming viruses are those involved in the initiation of transcription and translation near the 5′ end of the gene. This would result in synthesis of the *gag*-linked proteins subject to regulation of the viral genome. Variable amounts of *gag* information are expressed in cells transformed by defective mammalian transforming viruses (28, 34, 37, 43, 45, 61). Tronick et al. (52) have postulated an obligatory role for the 5′-terminal nucleotide sequences which these viruses share with their associated helpers.

We have demonstrated that at least the amino terminus of p19 is retained in the MC29 110K and AEV 75K proteins. The V8 protease-derived 19* peptide, which comigrated in our fingerprints with the putative N-terminal sequence of Ac-Met-Glu, may serve as a general marker for the amino terminus of *gag*-related polyproteins of avian acute leukemia viruses. A proposed structure of the MC29 110K and AEV 75K proteins is shown in Fig. 9. As discussed above, we do not know whether all of the p19 and p12

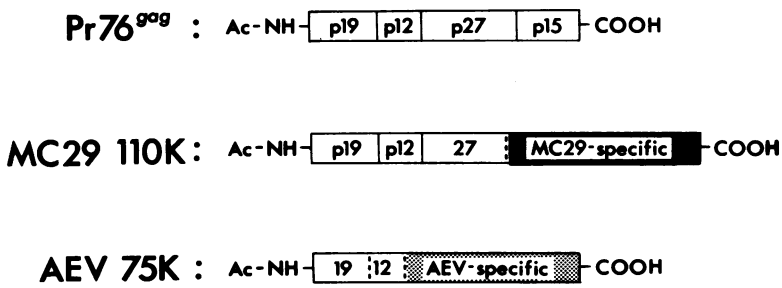

FIG. 9. *Proposed structure of $Pr76^{gag}$, MC29 110K, and AEV 75K proteins. Complete sequences of a gag-protein are indicated by the designation p19, p12, p27, or p15; partial sequences are designated 19, 12, or 27, respectively. The MC29 110K protein is shown to contain only part of p27 from the antigenic analysis of Bister et al. (4). The order of gag proteins in $Pr76^{gag}$ is based on our previous findings (42). An acetylated (Ac-) N-terminus is shown for each protein, with the gag sequences located at the amino-terminal end of MC29 110K and AEV 75K proteins.*

sequences are present in AEV 75K; if only partial sequences of both *gag* proteins are present, then a segment encompassing the carboxy terminus of p19 and the amino terminus of p12 may have been deleted. Detection of the 19* peptide in the MC29 and AEV polyproteins provides direct evidence that the *gag*-related sequences are located at the amino terminus of the molecules. Other investigators have recently identified the amino-terminal tryptic peptide of p19 in these polyproteins (R. N. Eisenman, M. Linial, M. Groudine, R. Shaikh, S. Brown, and P. E. Neiman, Cold Spring Harbor Symp. Quant. Biol., in press).

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