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Tryptic Peptide Analysis of Avian Oncovirus *gag* and *pol* Gene Products

CARL W. RETTENMIER,* ROGER E. KARESS, STEVEN M. ANDERSON, AND HIDESABURO HANAFUSA

The Rockefeller University, New York, New York 10021

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Radiolabeled tryptic peptides of the *gag* and *pol* gene products of avian oncoviruses were examined. This analysis included Rous-associated virus 2 structural proteins and the $Pr76^{gag}$ and $P180^{gag\text{-}pol}$ proteins in Rous-associated virus 2-infected chicken embryo cells. The methionine- and cysteine-containing tryptic peptides of virion internal structural proteins were present in both $Pr76^{gag}$ and $P180^{gag\text{-}pol}$, suggesting that there was no loss of *gag* gene-coding sequences during the generation of $P180^{gag\text{-}pol}$. No overlap of *gag* and *pol* gene structural information was detected. Analysis of intermediates in the processing of $Pr76^{gag}$ and translation inhibition mapping with pactamycin yielded the following order of structural proteins within the Rous-associated virus 2 $Pr76^{gag}$ precursor: NH_2-p19-p12-p27-p15-COOH. The *gag* and *pol* sequences missing in the endogenous gs^+ P120 protein of uninfected gs^+ chicken cells were identified by comparison with those of Rous-associated virus 2 $P180^{gag\text{-}pol}$.

The internal structural proteins of avian leukosis-sarcoma viruses (ALSV) are cleaved from a 76,000-dalton polypeptide ($Pr76^{gag}$) (25) encoded by the *gag* gene (2) near the 5' end of the genome. Attempts to determine the order of individual *gag* proteins in the $Pr76^{gag}$ precursor have yielded the following result: NH_2-p19-(p12,p27)-p15-COOH, in which the relative order of p12 and p27 has not been firmly established (24, 25). Viral RNA-dependent DNA polymerase, the product of the *pol* gene (2), is apparently derived from a 180,000-dalton polyprotein ($P180^{gag\text{-}pol}$) containing the polymerase β subunit (molecular weight 92,000) and most or all of the *gag* proteins (9, 17). The α subunit of the polymerase is formed by proteolytic cleavage of the β subunit (5). Several lines of evidence indicate that $Pr76^{gag}$ and $P180^{gag\text{-}pol}$ are synthesized from an mRNA(s) similar or identical to the 35-39S genomic RNA of the virus (10, 15, 18, 20, 26, 28, 29). It has been hypothesized that $P180^{gag\text{-}pol}$ is produced either by suppression of the normal *gag* gene terminators at some low frequency during translation of $Pr76^{gag}$ (19) or as the result of "splicing" out those terminators to generate a second mRNA encoding $P180^{gag\text{-}pol}$ (28).

Certain replication-defective ALSV also produce polypeptides containing at least some *gag* gene-related information. The endogenous ALSV gs antigen found in uninfected gs^+ chicken embryo cells is a 120,000-dalton polyprotein, designated gs^+ P120, with partial sequences of both *gag* and *pol* gene products (3). In the present study we have examined the polypeptides specified by the nondefective ALSV *gag* and *pol* genes. Tryptic peptide mapping was used to study the individual virion proteins and their precursors. Analysis of intermediates in the processing of $Pr76^{gag}$ and translation inhibition mapping with pactamycin support the following order of structural proteins in $Pr76^{gag}$: NH_2-p19-p12-p27-p15-COOH. Comparison of the tryptic peptides in the exogenous viral $P180^{gag\text{-}pol}$ and the endogenous gs^+ P120 revealed that p15 and some polymerase sequences were missing in gs^+ P120.

MATERIALS AND METHODS

Cell culture and viruses. The preparation and maintenance of chicken embryo cell cultures were as previously described (7). C/E embryos used for the growth of virus were of the gs^-h^- phenotype (negative for both ALSV gs antigen and endogenous subgroup E chicken helper factor) (8). Subgroup B Rous-associated virus 2 (RAV-2) was used in all experiments. Chicken embryo cells were infected as secondary cultures and transferred two or three times before isotopic labeling. The gs^+ P120 protein was immunoprecipitated from SPAFAS gs^+h_H cells.

Isotopic labeling, virus purification, and preparation of cell extracts. For the preparation of radiolabeled virus, confluent cultures of RAV-2-infected cells were incubated in Eagle minimal essential medium containing Earle balanced salts and 2% bovine serum with the following modifications for the indicated isotopes. (i) A mixture of 15 uniformly ^{14}C-labeled L-amino acids (New England Nuclear NEC-445:

Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val) was used at a concentration of 20 μCi/ml in medium containing only 5% the normal quantities of unlabeled amino acids. (ii) L-[^{35}S]methionine (1,270 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) at 100 μCi/ml in methionine-free medium was used. (iii) L-[^{35}S]cystine (350 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was used at 100 μCi/ml in cystine-free medium which contained only 25% of the normal amount of methionine. Cultures were incubated for two successive 12-h intervals in radioactive medium, followed by a 12-h incubation in complete medium. Virus was purified from the supernatants at 4°C as follows. Culture fluids were clarified by centrifugation at 8,000 × *g* for 10 min. The supernatant was then centrifuged through a layer of 20% (wt/wt) sucrose in TEN buffer (10 mM Tris [pH 7.4] -1 mM EDTA-150 mM NaCl) onto a cushion of 60% (wt/wt) sucrose in TEN at 25,000 rpm for 2.5 h in a Beckman SW27 rotor. The material at the interface between the 20 and 60% sucrose was diluted threefold in TEN and layered on top of a 10-ml, linear 50 to 15% sucrose gradient in TEN in a Beckman SW27.1 centrifuge tube. The linear gradient was centrifuged for 18 h at 25,000 rpm, and the material banding at a density of 1.16 g/ml was diluted in TEN, centrifuged into a pellet at 100,000 × *g*, and stored at −70°C before electrophoresis.

For the preparation of cell extracts, RAV-2-infected gs^-h^- cultures or uninfected gs^+h_H cells in 100-mm tissue culture plates were incubated for 2.5 h in medium containing either [^{35}S]methionine or [^{35}S]cysteine at a concentration of 400 μCi/ml. The monolayers were then washed twice with ice-cold TG buffer (25 mM Tris [pH 7.4]-135 mM NaCl-5 mM KCl-0.4 mM Na_2HPO_4-5.5 mM glucose) and lysed in 1 ml of modified RIPA buffer (6) (50 mM Tris [pH 7.4]-150 mM NaCl-1 mM EDTA-1% Triton X-100-1% sodium deoxycholate-0.1% sodium dodecyl sulfate [SDS]) containing 1% Trasylol (a general protease inhibitor; FBA Pharmaceuticals, New York, N.Y.). Lysates were centrifuged at 100,000 × *g* for 20 min, and the supernatants were stored at −70°C before immunoprecipitation.

For pactamycin mapping experiments, RAV-2-infected cell monolayers were incubated in medium containing 5×10^{-7} M pactamycin for 15 s before addition of radioisotope. Medium containing pactamycin and 400 μCi of [^{35}S]methionine or [^{35}S]cysteine per ml was added for 15 min and then the cells were washed and lysed in modified RIPA buffer. Pactamycin was a gift from the Upjohn Co., Kalamazoo, Mich.

Immunoprecipitation. Monospecific rabbit antisera raised against avian myeloblastosis virus (AMV) p27 and p19 proteins were kindly provided by V. M. Vogt. Cell extracts were incubated with a mixture of these antisera (1:20 final dilution of each antiserum) for 10 min at 22°C followed by 30 min at 4°C. Four serum volumes of *Staphylococcus aureus* protein A-Sepharose CL 4B (a 50% [vol/vol] slurry in modified RIPA buffer; Sigma Chemical Co., St. Louis, Mo.) was added and mixed for 30 min at 4°C to adsorb immune complexes by the method of Kessler (13). After five washes in modified RIPA buffer, the immune complex-Sepharose pellet was suspended in 70 μl of gel sample buffer (62.5 mM Tris [pH 6.7]-10% glycerol-3% SDS-100 mM dithiothreitol-0.01% bromophenol blue) and heated to 95°C for 2 min. The supernatant was collected, and the pellet was washed with another 70 μl of sample buffer. Supernatants were combined before SDS-gel electrophoresis. The α and β subunits of viral RNA-dependent DNA polymerase were purified by immunoprecipitation from radiolabeled RAV-2 using a rat anti-AMV DNA polymerase serum generously supplied by R. C. Nowinski.

SDS-polyacrylamide slab gel electrophoresis. Polyacrylamide slab gels (30:0.8, acrylamide:*bis*-acrylamide) containing 0.1% SDS were prepared using the discontinuous buffer system described by Laemmli (14). Virus pellets were dissociated in 62.5 mM Tris (pH 6.8)-2% SDS-50 mM dithiothreitol-10% sucrose with 0.001% bromophenol blue as tracking dye. Samples were heated to 95°C for 2 min and then loaded into the gel slots. After electrophoresis at 12 mA, gels were stained with 0.05% Coomassie brilliant blue in 25% 2-propanol and 7% acetic acid and destained in 10% 2-propanol and 7% acetic acid. Gels were dried and exposed to Dupont Cronex 2DC safety film for autoradiography.

Tryptic peptide analysis of proteins. Radiolabeled proteins were separated by SDS-polyacrylamide gel electrophoresis and located by autoradiography. The appropriate gel slices were excised with a razor blade, washed twice in 10% methanol, and lyophilized. Performic acid (a 19:1 mixture of formic acid [88%]: hydrogen peroxide [30%] kept at 22°C for 1 h) was added to each gel slice for 1 h at 4°C to oxidize the proteins. The gel slices were lyophilized twice and then incubated for 16 h at 37°C in 0.6 ml of 50 mM ammonium bicarbonate buffer (pH 8.0) with 30 μg of L-(tosylamido 2-phenyl)ethyl chloromethyl ketone-treated bovine trypsin (252 U/mg; Worthington Biochemical Corp., Freehold, N.J.). Recovery of radioactivity from the gel slices was about 70%. Supernatants of the samples were removed and lyophilized twice. The tryptic peptides were analyzed in two dimensions on cellulose-coated, thin-layer plates (10 by 20 cm; EM Laboratories, Elmsford, N.Y.). Electrophoresis was done in the first dimension for about 20 min at 1,000 V in acetic acid-formic acid-water (15:5:80, pH 1.9) and was monitored using a mixture of 2% Orange G and 1% acid fuchsin as marker dyes as previously described (4). After the plates had dried, ascending chromatography in the second dimension was carried out in 1-butanol-pyridine-acetic acid-water (65:50:10:40, vol/vol). Dried plates were exposed to Dupont Cronex 2DC film for autoradiography. To quantitate the amount of radioactivity in the [^{35}S]methionine- or [^{35}S]cysteine-labeled spots, the corresponding areas of the cellulose layer of the plate were scraped into scintillation vials and radioactivity was measured using a toluene-based scintillant containing 10% NCS tissue solubilizer (Amersham Corp.).

RESULTS

Analysis of RAV-2 internal structural proteins. Figure 1 shows the electrophoretic

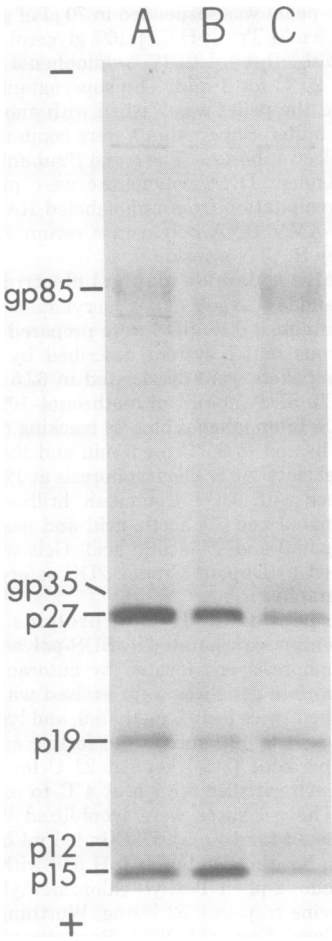

FIG. 1. *Autoradiogram showing the separation by SDS-polyacrylamide gel electrophoresis of RAV-2 virion proteins labeled with (A) mixed ^{14}C-amino acids, (B) [^{35}S]methionine, and (C) [^{35}S]cysteine. Approximately 50,000 cpm was loaded into each lane of a 12% polyacrylamide slab gel, and the autoradiogram was exposed for 3 days.*

separation in an SDS-polyacrylamide gel of RAV-2 virion proteins (1) labeled with the indicated amino acids. Densitometry scanning of the autoradiogram allows quantitation of the radioisotope incorporation in each protein. The amount of radioactivity in a particular protein was dependent on the isotope used for labeling. For example, relative to the incorporation of mixed ^{14}C-amino acids or [^{35}S]methionine, the glycoproteins gp85 and gp35 and the nonglycosylated structural proteins p19 and p12 were more efficiently labeled with [^{35}S]cysteine than were proteins p27 and p15. Estimates of the molar ratios and numbers of methionine and cysteine residues per molecule of the individual *gag* proteins were calculated from densitometry tracings (Table 1). The results suggest that p12 contains relatively little methionine, but, like p19, it is rich in cysteine. These findings are consistent with previously published data on the amino acid compositions of these proteins (11). The molar ratios of RAV-2 *gag* proteins were similar to those obtained for RAV-61 by Mosser et al. (16), except that we found less p12 in virions of RAV-2.

Tryptic peptide analysis of individual *gag* proteins labeled with ^{14}C-amino acids, [^{35}S]methionine, or [^{35}S]cysteine was performed, and the results are shown in Fig. 2 through 5. The ^{14}C-labeled tryptic peptides that were also labeled with [^{35}S]methionine or [^{35}S]cysteine are indicated in panels A and D of each figure. The methionine- and cysteine-containing peptides have been designated in a manner similar to that used by Vogt and co-workers (25), i.e., the number of the protein from which the peptide was derived, followed by a letter denoting the amino acid used for labeling (M for methionine, C for cysteine), and then an individual number—e.g., 27M1, 27M2, 27C1, etc. The unique identity of the methionine- and cysteine-containing tryptic peptides of each protein was established by experiments in which the samples were mixed before peptide analysis (data not shown). Since there was no overlap of the sets of [^{35}S]methionine- and [^{35}S]cysteine-labeled tryptic peptides for any protein, there was apparently no meta-

TABLE 1. *Incorporation of radioisotopes into RAV-2 internal structural proteins during metabolic labeling*[a]

Protein	Molar ratio in virions[b]	Relative no. of methionine residues/molecule[c]	Relative no. of cysteine residues/molecule[c]
p27	100	2.7	1.0
p19	31	2.0	3.0
p15	53	4.0	1.4
p12	21	1.0	3.0

[a] Determined by integrating the area under the peaks of individual proteins in a densitometer tracing of the autoradiogram in Fig. 1. Similar results were obtained by slicing and counting the gels.

[b] Estimated by dividing the radioactivity in ^{14}C-amino acids in the individual protein by its molecular weight as determined by gel filtration in 6 M guanidine hydrochloride (1). The value for p27 is arbitrarily set at 100.

[c] Estimated by dividing the radioactivity incorporated with [^{35}S]methionine or [^{35}S]cysteine by the apparent molar ratio of the protein. The values for p12 labeled with [^{35}S]methionine and p27 labeled with [^{35}S]cysteine are arbitrarily set at 1.0.

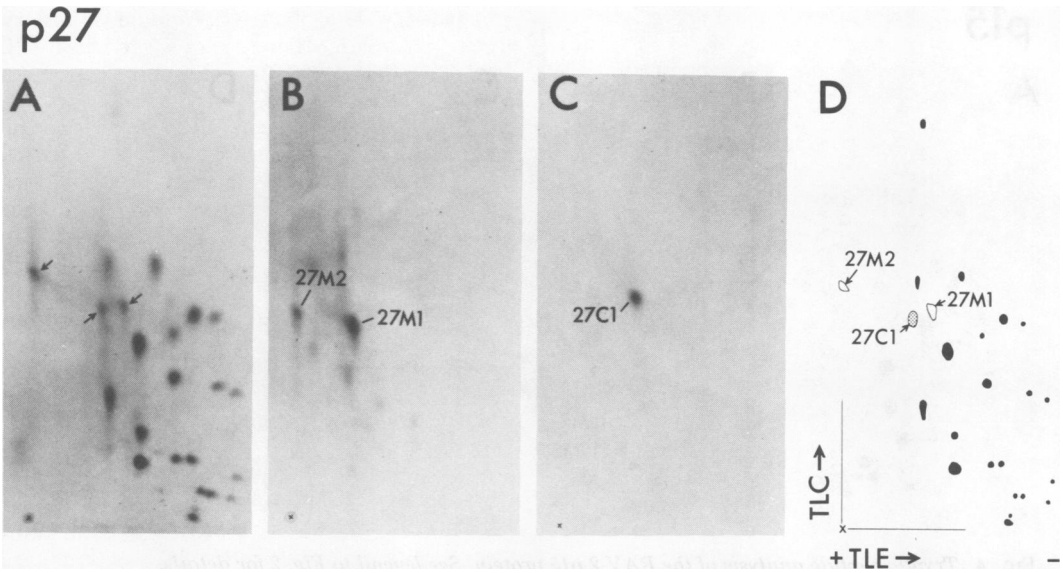

FIG. 2. *Two-dimensional analysis of tryptic peptides derived from RAV-2 p27 protein labeled with (A) mixed ^{14}C-amino acids, (B) [^{35}S]methionine, and (C) [^{35}S]cysteine. Samples were spotted in the lower left corner of each panel; the origin is marked with an "X". Electrophoresis (TLE) at pH 1.9 was done from left to right, followed by ascending chromatography (TLC) from bottom to top. The ^{14}C-peptides that were also labeled with [^{35}S]methionine or [^{35}S]cysteine are marked with arrows in (A). Panel D is a schematic diagram showing all ^{14}C-peptides (●), as well as the specific methionine- (○) and cysteine-containing (◎) peptides.*

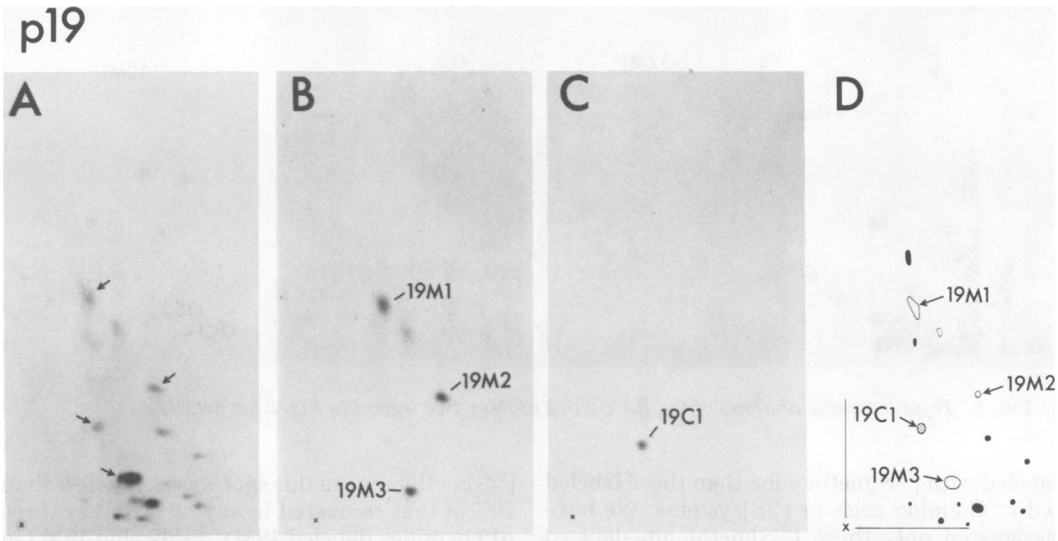

FIG. 3. *Tryptic peptide analysis of the RAV-2 p19 protein. See legend to Fig. 2 for details.*

bolic transfer of ^{35}S between these amino acids under our labeling conditions.

Tryptic digestion of radiolabeled proteins was done under conditions of excess enzyme. Peptides eluted from the gel slices were analyzed in two dimensions by thin-layer electrophoresis and chromatography on cellulose-coated glass plates. We could detect no qualitative differences in the tryptic maps when enzymatic digestion was continued for longer periods of time. Virtually all of the radioactivity was separated in the fingerprints, with little retained at the origin or spread along the axes. In general, the background was greater in the maps of proteins

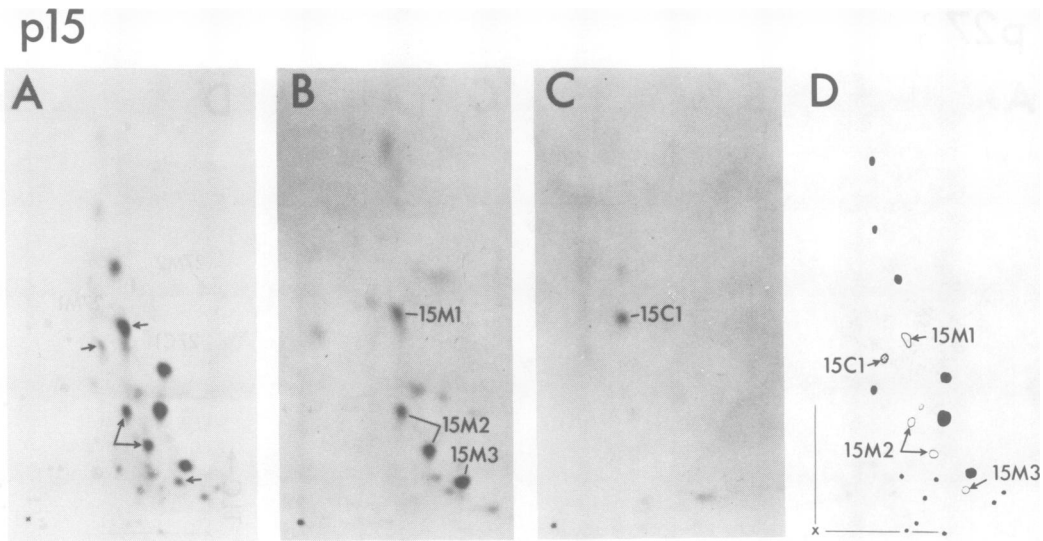

FIG. 4. *Tryptic peptide analysis of the RAV-2 p15 protein. See legend to Fig. 2 for details.*

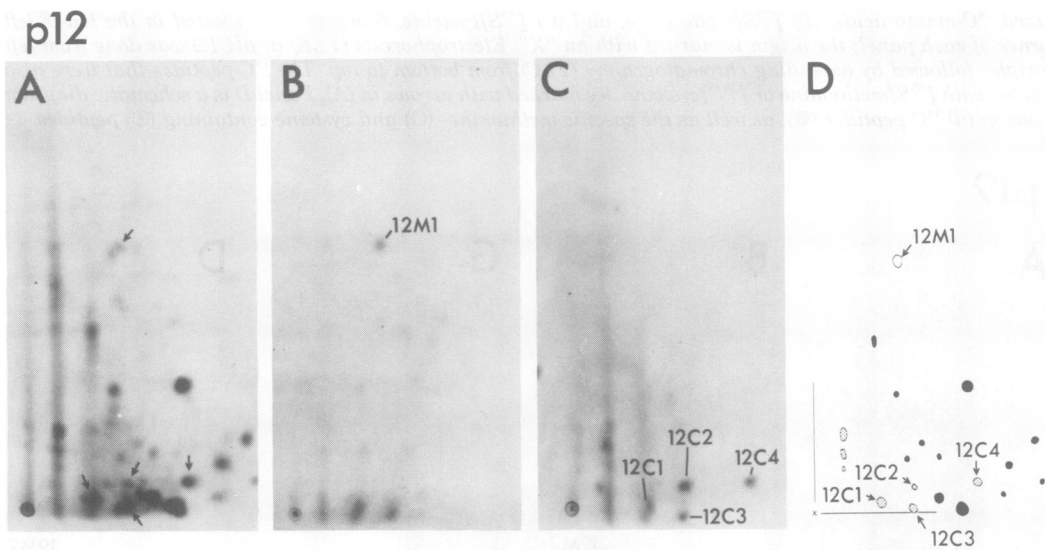

FIG. 5. *Tryptic peptide analysis of the RAV-2 p12 protein. See legend to Fig. 2 for details.*

labeled with [^{35}S]methionine than those labeled with ^{14}C-amino acids or [^{35}S]cysteine. We have designated only those [^{35}S]methionine-labeled spots that were also labeled with mixed ^{14}C-amino acids. The additional background spots may be partially digested intermediates of the authentic methionine-containing tryptic peptides.

We have not designated one p19 spot which was labeled both with mixed ^{14}C-amino acids and [^{35}S]methionine (Fig. 3A and B). This peptide was sometimes not seen in the tryptic digests of p19, and, when present, the amount of [^{35}S]methionine in this spot was always less than 20% of that recovered in any of the other three p19 peptides (labeled 19M1, 19M2, and 19M3 in Fig. 3B). Based on the amount of ^{35}S radioactivity recovered in these spots, the additional spot may be related to the 19M1 peptide. However, at the present time we cannot exclude the possibility that this peptide was a unique methionine-containing p19 peptide which was not efficiently recovered from the gel slice.

We have designated two methionine-containing p15 spots as 15M2 peptides (Fig. 4B). We believe that one of these spots was an incomplete

digestion product of the other for the following reasons. (i) The relative intensities of these two spots varied from one digest to the next, but the sum of the [^{35}S]methionine counts recovered from these spots in Pr76^{gag} was consistent with there being one methionine between them (see below). (ii) We have found a structural protein marker distinguishing the p15 of RAV-7 from that of other ALSV (21), and the only detectable difference in the tryptic peptide maps of RAV-7 and other viruses was in the spots labeled 15M2. On the other hand, chymotryptic digestion of p15's yielded three methionine-containing peptides of which only one differed between the RAV-7 p15 and other viral p15's (C. W. Rettenmier and H. Hanafusa, manuscript in preparation). Therefore, we believe that there are features of the p15 amino acid sequence near this methionine residue which render complete digestion with trypsin difficult even under conditions of 50- to 100-fold enzyme excess.

There were several ^{35}S-spots retained near the origin in the p12 cysteine maps (Fig. 5C). We have refrained from designating these peptides because the pattern and relative intensity of the spots was variable in our fingerprints of p12. At present we do not know whether these were unique p12 peptides or incomplete digestion products of this protein. Shaikh and co-workers (23) observed that p12 is not easily eluted from gel slices. These ^{35}S-spots appeared to be related to p12 because a similar pattern was also seen with [^{35}S]cysteine-labeled Pr76^{gag} (see Fig. 7).

Studies on Pr76^{gag}. Vogt et al. (25) have shown that the individual ALSV *gag* proteins are generated by proteolytic cleavage of a common precursor polypeptide Pr76^{gag}. We have examined the RAV-2 Pr76^{gag} in an effort to map the locations of the structural proteins. RAV-2-infected chicken embryo cells were labeled with either [^{35}S]methionine or [^{35}S]cysteine for 2.5 h and lysed in buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. The *gag*-related polypeptides were precipitated from the cell extract with a mixture of rabbit anti-p27 and anti-p19 sera using Sepharose-bound *S. aureus* protein A as an immunoadsorbent. The immunoprecipitates were analyzed by SDS-gel electrophoresis and autoradiograms of the [^{35}S]methionine-labeled proteins are shown in Fig. 6. Virus-specific proteins identified in the cell extracts are labeled in the margin of Fig. 6. These include the polyprotein P180$^{gag\text{-}pol}$, structural protein precursors Pr76^{gag}, Pr66^{gag}, Pr60^{gag}, and Pr32^{gag}, as well as the mature virion proteins p27 and p19. Pulse-chase experiments (data not shown) demonstrated that the *gag* precursors were, in fact, processed into mature RAV-2 structural proteins. Like previous investigators

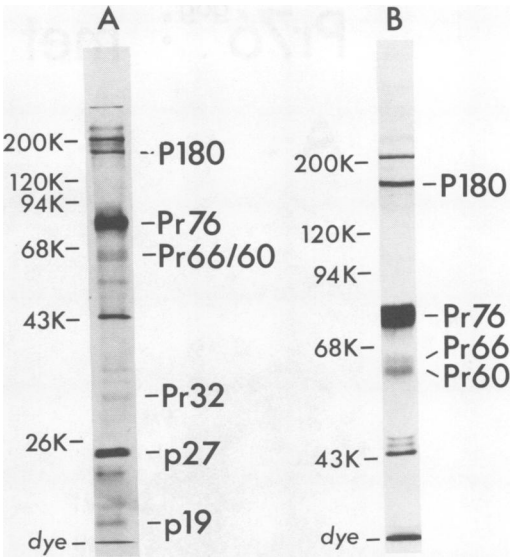

FIG. 6. *Electrophoretic separation in (A) 10% and (B) 7.5% polyacrylamide gels of [^{35}S]methionine-labeled, gag-related polypeptides immunoprecipitated from RAV-2-infected cells. The migration of molecular weight standards is marked in the left margin of each lane: rabbit myosin (200,000), Escherichia coli β-galactosidase (120,000), phosphorylase a (94,000), bovine serum albumin (68,000), ovalbumin (43,000), and chymotrypsinogen A (26,000). Virus-specific polypeptides are indicated in the right margin of each lane. The remainder of the proteins in the autoradiogram are apparently contaminating host cell proteins because they are also precipitated by nonimmune rabbit serum (not shown).*

(3, 9, 25), we found several cellular proteins contaminating the immunoprecipitates. The most prominent of these were two bands having molecular weights of about 200,000 and 45,000, which we believe were chicken myosin and actin, respectively. These cellular proteins were also seen in immunoprecipitates using normal rabbit serum and did not have tryptic peptides in common with ALSV *gag* proteins (data not shown).

Tryptic peptide analyses of [^{35}S]methionine- and [^{35}S]cysteine-labeled Pr76^{gag} are shown in Fig. 7. The peptides of individual *gag* proteins were identified by experiments in which Pr76^{gag} peptides were mixed with tryptic peptides of the structural proteins (data not shown) and are marked in Fig. 7. All of the major methionine- and cysteine-containing tryptic peptides in Pr76^{gag} were identified in one of the virion structural proteins. The autoradiogram of the [^{35}S]-methionine-labeled tryptic map in Fig. 7A is somewhat overexposed to show the background radioactivity; some of the minor spots were also seen in the maps of an individual *gag* protein, especially p27 (see Fig. 2B), and their intensity

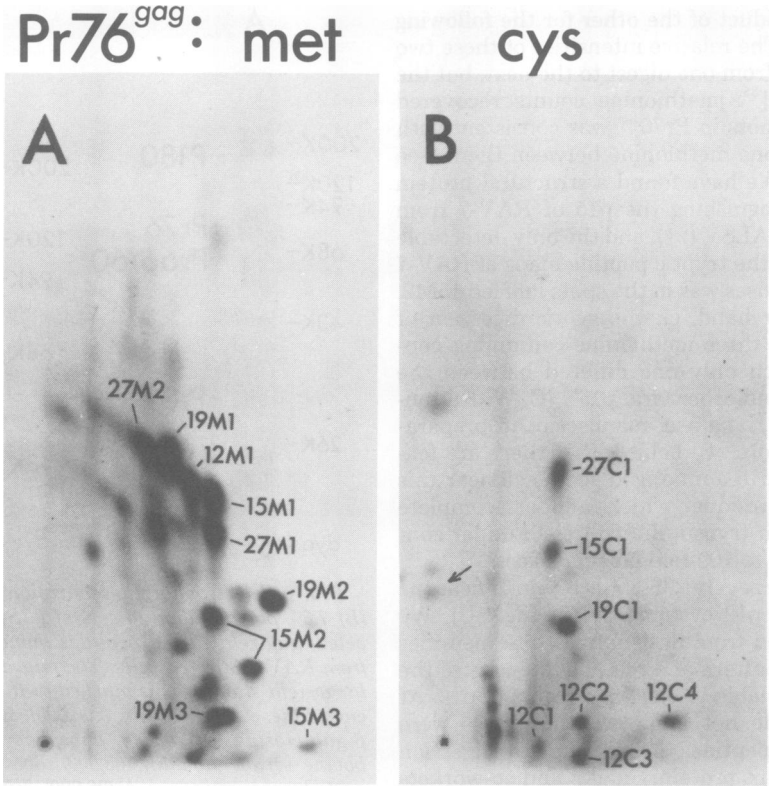

FIG. 7. *Autoradiograms of two-dimensional tryptic fingerprints of RAV-2 Pr76^{gag} labeled with (A) [^{35}S]methionine and (B) [^{35}S]cysteine. The tryptic peptides of individual gag proteins are identified in each panel. An arrow in (B) marks the spot that comigrates with $^{35}SO_4$.*

relative to the major spots was about the same in the fingerprint of the precursor. Quantitation of the radioactivity in the Pr76^{gag} tryptic peptides generally reflected the intensity of the spot in the map of the corresponding virion protein. An exception was the 15M3 peptide which was prominently labeled in the virion p15 fingerprint, but in which little radioactivity was detected in the Pr76^{gag} map. We do not know the reason for this. In general, the results are consistent with the interpretation that a single methionine was present in each methionine-containing tryptic peptide. Often those Pr76^{gag} peptides having low recoveries of radioactivity were derived from proteins with several background spots in their individual maps; we therefore believe that the background was incompletely digested material. Based on the recovery of radioactivity in [^{35}S]-cysteine-labeled spots, there appeared to be one cysteine residue in each of the tryptic peptides labeled in Fig. 7B except for the 19C1 peptide which apparently contained two. A group of p12-related ^{35}S-spots was seen near the origin of the cysteine map of Pr76^{gag}. Some radioactivity that comigrated with $^{35}SO_4$ was detected (Fig. 7B), suggesting that some free sulfate was released from cysteine residues during performic acid oxidation of the protein. However, the amount of ^{35}S lost as sulfate in the Pr76^{gag} maps did not seem to significantly interfere with quantitation of the spots (see Table 2).

Order of structural proteins in Pr76^{gag}. The antibiotic pactamycin preferentially inhibits the initiation of protein synthesis and can therefore be used in pulse-labeling experiments to map the order of peptides in a protein. At 15 s after the addition of 5×10^{-7} M pactamycin, we labeled RAV-2-infected cells with [^{35}S]methionine or [^{35}S]cysteine for 15 min. Pr76^{gag} was immunoprecipitated from the cell extracts and digested with trypsin, and the amount of radioactivity recovered in the individual tryptic peptides was determined (Table 2). The results with the [^{35}S]methionine-labeled extract indicated that p15 is located closest to the carboxyl terminus of the precursor because most of the radioactivity was detected in the 15M peptides. The 27M2 peptide also contained a significant

TABLE 2. *Relative recovery of radioactivity in tryptic peptides of RAV-2 Pr76^{gag} labeled with [^{35}S]methionine or [^{35}S]cysteine*[a]

Peptide spot	Ratio of radioactivity recovered relative to other spots	% of control radioactivity recovered in the pactamycin-treated sample[b]
19M1	1.0[c]	<0.5
19M2	0.8	<0.5
19M3	1.0[c]	<0.5
12M1	0.8	<0.5
27M1	0.6	<0.5
27M2	0.7	4.6
15M1	0.7	2.7
15M2	0.8	13
15M3	0.4	18
19C1	1.7	<3
12C1	0.8	<3
12C2	0.8	18
12C3	0.7	9
12C4	0.7	<3
12C other[d]	2.0	5
27C1	1.0[c]	33
15C1	1.0[c]	28
Free $^{35}SO_4$ in the cysteine maps	0.3	<3

[a] Recovery based on triplicate samples of spots scraped from cellulose-coated thin-layer plates as described in the text.

[b] Conditions for labeling cultures in the presence of 5×10^{-7} M pactamycin were as described in the text. Pr76^{gag} was immunoprecipitated from the cell extracts and isolated by SDS-polyacrylamide gel electrophoresis. Total incorporation of radioactivity into Pr76^{gag} was reduced in the pactamycin-treated culture by 90% with [^{35}S]methionine and 75% with [^{35}S]cysteine relative to the control.

[c] An arbitrary value of 1.0 represents 273 ± 5 cpm for a [^{35}S]methionine-labeled spot and 96 ± 3 cpm for a [^{35}S]cysteine-labeled spot.

[d] Includes all three or four spots that migrate close together near the origin in the two-dimensional peptide maps.

amount of radioactivity, suggesting that p27 is adjacent to p15. In the [^{35}S]cysteine-labeled sample, about 30% of the control counts was recovered in the 27C1 and 15C1 peptides, less in the 12C peptides, and none was detected in the 19C1 peptide. Therefore, translation inhibition mapping with pactamycin indicates that the order of structural proteins in the RAV-2 Pr76^{gag} is NH_2-p19-p12-p27-p15-COOH.

The order of structural proteins in the precursor was confirmed by peptide mapping of intermediates in the metabolic processing of Pr76^{gag} (Fig. 6). The results for the [^{35}S]methionine- and [^{35}S]cysteine-labeled intermediates are shown in Fig. 8 and indicate that p15 peptides were missing in both Pr66^{gag} and Pr60^{gag}. The methionine- and cysteine-containing p19 and p12 peptides were present in the RAV-2 Pr32^{gag} precursor. These results, together with the pactamycin mapping data, support the processing scheme for Pr76^{gag} shown in Fig. 9.

Tryptic peptide analysis of P180$^{gag\text{-}pol}$ and gs$^+$ P120. Viral *gag* and *pol* sequences are represented in a 180,000-dalton polypeptide detected in avian oncovirus-infected cells (9, 17) and by in vitro translation of viral RNA (15, 18, 20, 28). Although a definitive precursor-product relationship has not been demonstrated for P180$^{gag\text{-}pol}$ and the polymerase (17), to date, no other forms of the *pol* gene product have been found in infected cells. P180$^{gag\text{-}pol}$ also appears to be similar to a protein specified by endogenous viral information in certain chicken cells. Eisenman et al. (3) have shown that the gs antigen in uninfected gs$^+$ chicken cells is a 120,000-dalton polyprotein, gs$^+$ P120, which is immunoprecipitated both by anti-p27 serum and anti-viral DNA polymerase serum; this antigen is not detected in gs$^-$ chicken cells.

In the present study, methionine- and cysteine-containing tryptic peptides of RAV-2 P180$^{gag\text{-}pol}$ and gs$^+$ P120 were compared with those of the individual RAV-2 structural proteins and polymerase subunits. The results of this analysis are shown in Fig. 10, where polymerase peptides are marked with a *p* and the *gag*-related peptides are designated by the number of the structural protein in which they are present. The polymerase β subunit had two [^{35}S]-methionine-labeled tryptic peptides (designated *p'* in Fig. 10) that were not detected in the α subunit; the two subunits shared all cysteine-containing tryptic peptides (data not shown). Analysis of the RAV-2 P180$^{gag\text{-}pol}$ revealed that all of the Pr76^{gag} and polymerase peptides resolved in our fingerprints were present in this polyprotein. Three of the most prominent [^{35}S]methionine-labeled spots in the map of P180$^{gag\text{-}pol}$ resulted from comigration of polymerase tryptic peptides with the *gag* peptides 19M2, 19M3, and 12M1–15M1. However, none of the *gag* or *pol* cysteine-containing peptides were observed to migrate together in the fingerprints.

The gs$^+$ P120 protein contained many of the tryptic peptides found in the RAV-2 P180$^{gag\text{-}pol}$. However, p15-specific peptides and some polymerase peptides were missing in the maps of gs$^+$ P120. A similar result has been reported by Eisenman et al. (3). On the basis of these fingerprints, there appears to be extensive homology between the tryptic peptides of exogenous viral P180$^{gag\text{-}pol}$ and the gs$^+$ P120 specified by endogenous viral information in chicken cells. This is

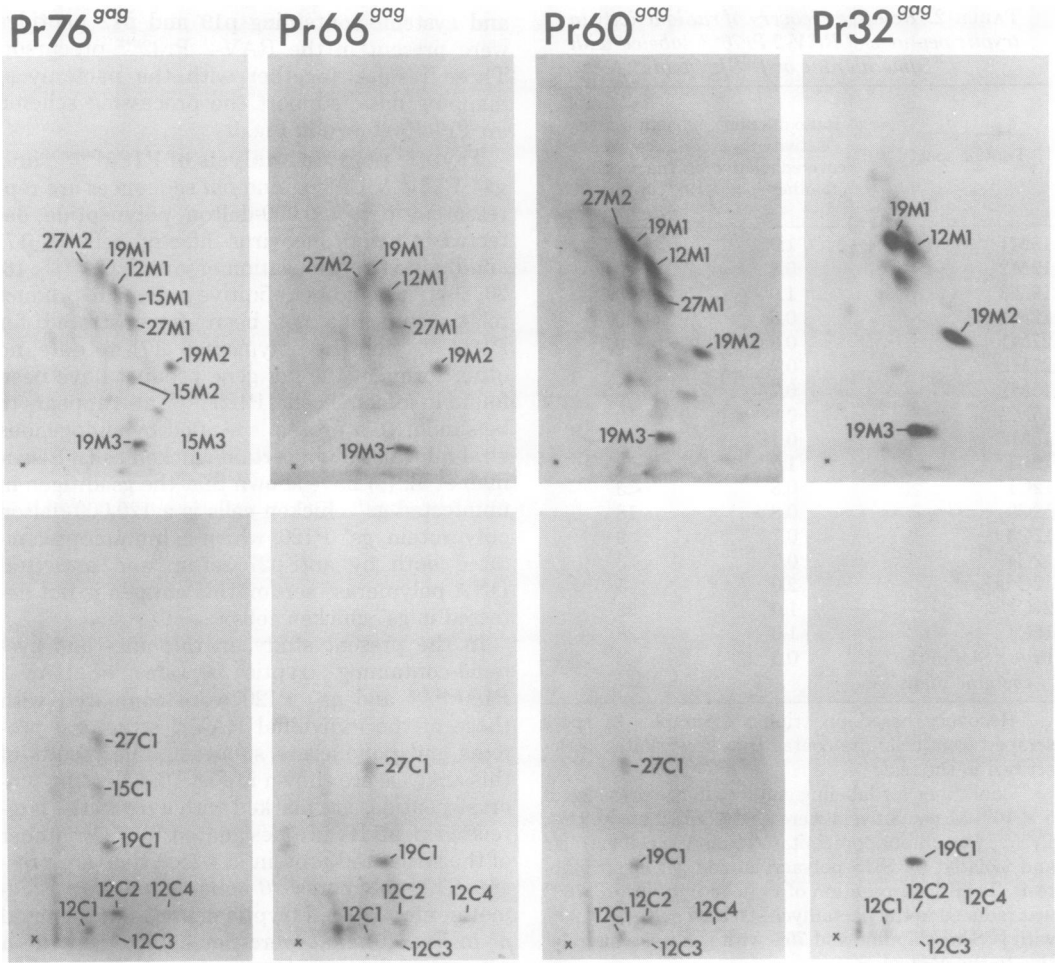

FIG. 8. *Autoradiograms of [^{35}S]methionine- (upper panels) and [^{35}S]cysteine-labeled (lower panels) tryptic peptides of intermediates in the processing of Pr76^{gag}. The individual peptides are marked in each panel. See the text regarding the methionine-containing spot which has not been designated in Pr32^{gag}.*

methionine- and cysteine-containing tryptic peptides of RAV-2 *gag* proteins comigrate with peptides of the corresponding proteins of endogenous virus RAV-0 (Rettenmier and Hanafusa, manuscript in preparation).

DISCUSSION

In the present study we prepared tryptic peptides of RAV-2 internal structural proteins with an interest toward studying precursor polypeptides and polyproteins that contain *gag* sequences. The numbers of methionine- and cysteine-containing tryptic peptides obtained for each protein were consistent with the amino acid compositions of these proteins (11). Vogt et al. (25) observed similar numbers of methionine-containing tryptic peptides in their analysis of AMV structural proteins. We used a slightly different numbering system to designate our tryptic peptides because we are not certain which of the peptides resolved by our two-dimensional mapping technique correspond to AMV peptides separated by cation-exchange chromatography. However, the methionine- and cysteine-containing peptides of *gag* proteins are generally conserved in the avian oncoviruses (Rettenmier and Hanafusa, manuscript in preparation). The *gag* proteins of RAV-2 are synthesized as part of a common precursor polypeptide, but different amounts of each protein are packaged into virions (Table 1). This selectivity in the assembly process probably reflects a specificity in the role of each structural protein in the virus particle.

We have used two approaches to establish the order of structural proteins in the RAV-2 Pr76^{gag}.

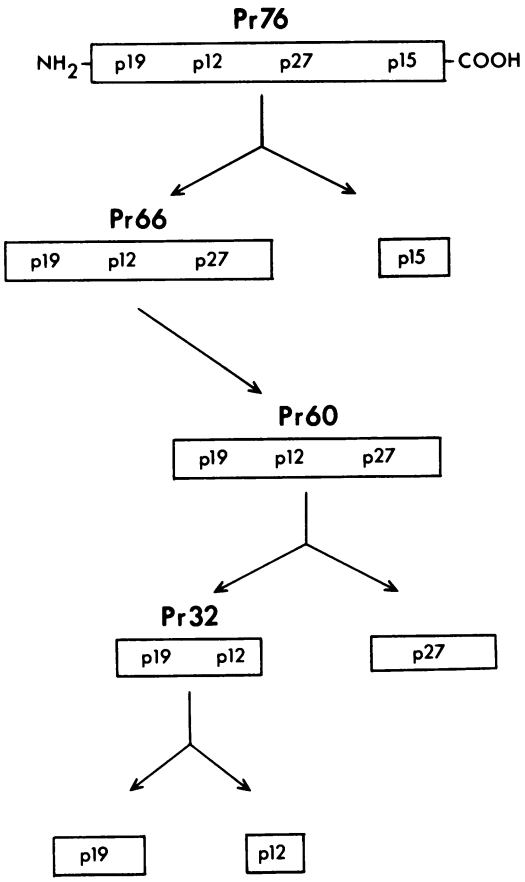

FIG. 9. *Proposed scheme for processing of the Pr76 gag gene product.*

First, infected cells were treated with pactamycin and pulse-labeled with [^{35}S]methionine or [^{35}S]cysteine, and the radioactivity recovered in each tryptic peptide of Pr76^{gag} was compared with the control. The results were consistent with an order of NH_2-p19-p12-p27-p15-COOH. When Vogt and co-workers (25) employed this approach with [^{35}S]methionine labeling, they arrived at the same order, but there was uncertainty regarding the relative position of p12 and p27. Because little [^{35}S]methionine or [^{3}H]leucine was incorporated into the virion p12, previous studies which compared the radiolabeling of mature viral structural proteins after pactamycin treatment did not resolve the relative order of p12 and p27 (24, 25).

The second approach was to carry out peptide analysis of the intermediates in Pr76^{gag} processing. The p19, p12, and p27 peptides were present in precursors Pr66^{gag} and Pr60^{gag}, but the p15 peptides were absent. This result is the same as that previously reported for the corresponding AMV precursors (25). However, in contrast to the AMV results, we found that the RAV-2 Pr32^{gag} contained p12 peptides in addition to p19 sequences. Vogt et al. (25) did not identify the [^{35}S]methionine-labeled p12 peptide in the AMV Pr32^{gag}. They did, however, detect an extra methionine-containing tryptic peptide in this precursor. Some evidence for this extra peptide is also seen in Fig. 8 of the present study. The origin of this spot is not known because it was not detected in the tryptic fingerprints of other *gag* precursors or mature virion proteins. Comparing the recovery of radioactivity in the peptides of Pr32^{gag}, the extra spot may represent a partial digestion product of the authentic 12M1 peptide. The order of NH_2-p19-p12-p27-p15-COOH for the RAV-2 Pr76^{gag} deduced from our experiments is the same as the order derived from antigenic analysis of avian viral *gag* gene products in nonproducer mammalian cells (22).

We were able to resolve *gag*- and *pol*-specific tryptic peptides in P180$^{gag\text{-}pol}$. Weiss and coworkers (28) recently postulated that P180$^{gag\text{-}pol}$ may be generated by translation of a "spliced" mRNA which is similar in size to the 35–39S genomic RNA, but which has the normal *gag* gene terminators removed. All of the mature virion *gag*-specific, methionine- and cysteine-containing tryptic peptides that were detected in our fingerprints were also found in P180$^{gag\text{-}pol}$. Therefore, if a splicing event does take place to form a separate mRNA for P180$^{gag\text{-}pol}$, it apparently does not delete any coding sequences for the *gag* proteins that can be detected by labeling with these two amino acids. However, it should be acknowledged that the resolution of the 12M1, 15M1, and the comigrating polymerase peptide is insufficient to make a definitive statement regarding this point. Although there was some overlap of *gag* and *pol* methionine-containing tryptic peptides in our fingerprints of P180$^{gag\text{-}pol}$, in general, our results do not support the suggestion of Hizi et al. (12) that structural protein p15 and at least part of p12 are also present in the polymerase. The order of *gag* proteins places p12 between p19 and p27, and, therefore, p27 is located between p12 and the polymerase sequences. Furthermore, although a polymerase peptide comigrated with the 15M1 and 12M1 peptides in our maps, methionine-containing polymerase peptides did not migrate with the 15M2 or 15M3 peptides, and there was no apparent overlap of cysteine-containing *gag* and *pol* tryptic peptides.

As has been previously reported by Eisenman et al. (3), the gs^{+} P120 protein was shown to contain many *gag* and *pol* sequences present in the nondefective viral P180$^{gag\text{-}pol}$. However, the

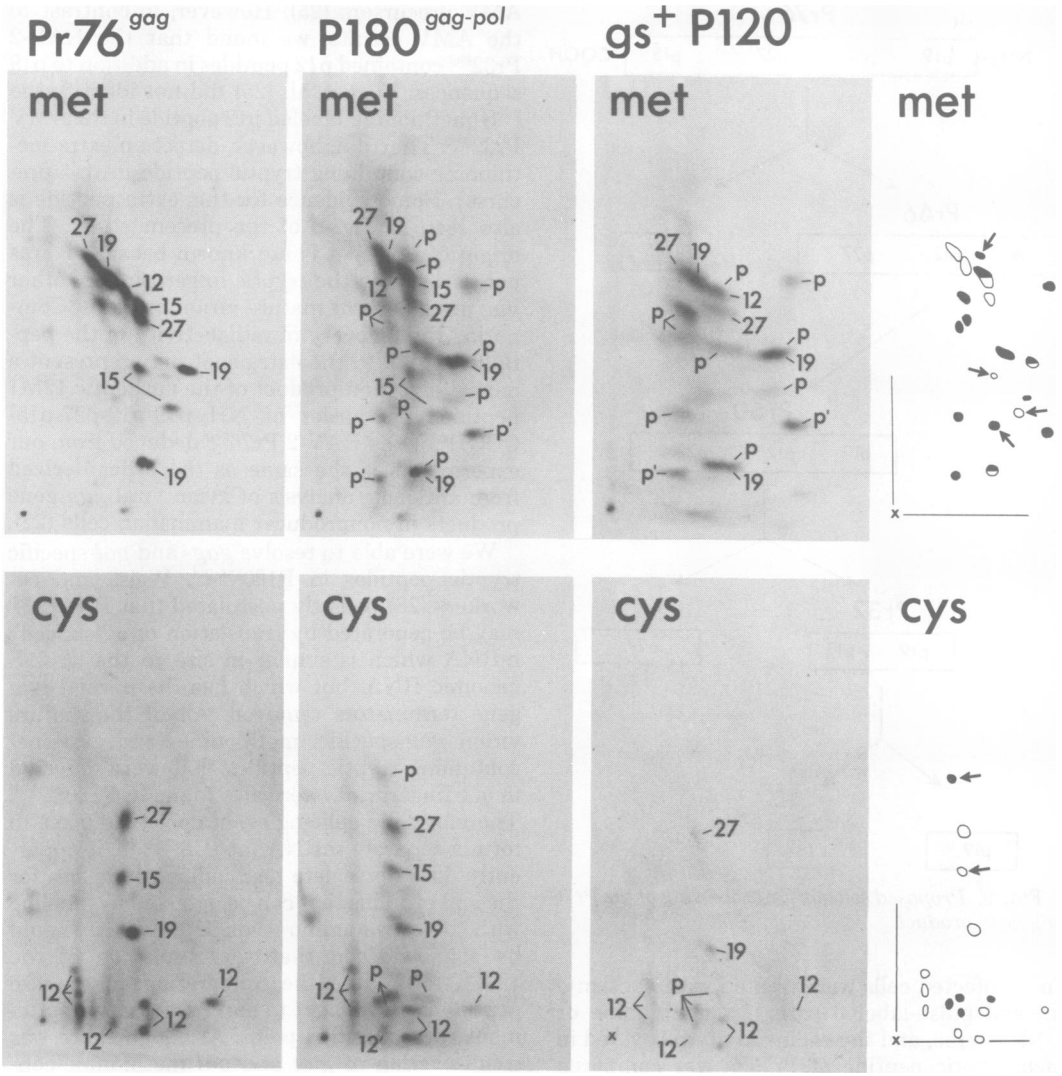

FIG. 10. *Two-dimensional analysis of [^{35}S]methionine- (upper panels) and [^{35}S]cysteine-labeled (lower panels) tryptic peptides of RAV-2 Pr76^{gag}, RAV-2 P180$^{gag\text{-}pol}$, and gs$^+$ P120. In each autoradiogram, the individual gag peptides are marked with the number of the structural protein in which they are found. Polymerase tryptic peptides common to both the α and β subunits of the enzyme are labeled with p; those peptides present in the β subunit, but absent in the α subunit, are indicated by p'. At the right is a schematic diagram of P180$^{gag\text{-}pol}$ showing the gag- (○) and pol-specific (●) spots, as well as those peptides (◓) that comigrate in our two-dimensional fingerprints. Peptides that are missing in gs$^+$ P120 are marked with arrows in the schematic diagram.*

gs$^+$ P120 lacked p15 and some polymerase peptides. The simplest interpretation of these data is that the RNA encoding gs$^+$ P120 contains a deletion encompassing the 3′ portion of the *gag* gene and extending into the 5′ portion of the *pol* gene. This hypothesis is consistent with the notion that gs$^+$ P120 in uninfected gs$^+$ chicken cells is translated from the endogenous viral 31S RNA, in which a similar deletion has been mapped (27). The proposed structure of gs$^+$ P120 assumes a single continuous deletion of *gag* and *pol* information rather than multiple smaller deletions in the two genes (3). There is some circumstantial evidence to support this model. Preliminary data based on pactamycin mapping (24) indicate that the α subunit of the polymerase is comprised of the amino terminal sequences of the β subunit. Therefore, a continu-

ous deletion extending from the 3′ end of *gag* into the 5′ end of *pol* would be expected to remove sequences found in both polymerase subunits, but to leave intact those sequences unique to the β subunit at the 3′ end of the *pol* gene. Examination of Fig. 10 reveals that this is the case. The methionine-containing polymerase peptides that were absent in gs^+ P120 were among those common to both the α and β subunits. Similarly, the two unique [^{35}S]methionine-labeled β peptides (designated p' in Fig. 10), which were missing in the α subunit, were present in gs^+ P120.

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LITERATURE CITED

1. **August, J. T., D. P. Bolognesi, E. Fleissner, R. V. Gilden, and R. C. Nowinski.** 1974. A proposed nomenclature for the virion proteins of oncogenic RNA viruses. Virology **60**:595–601.
2. **Baltimore, D.** 1974. Tumor viruses: 1974. Cold Spring Harbor Symp. Quant. Biol. **39**:1187–1200.
3. **Eisenman, R., R. Shaikh, and W. S. Mason.** 1978. Identification of an avian oncovirus polyprotein in uninfected chick cells. Cell **14**:89–104.
4. **Elder, J. H., R. A. Pickett, II, J. Hampton, and R. A. Lerner.** 1977. Radioiodination of proteins in single polyacrylamide gel slices. Tryptic peptide analysis of all the major members of complex multicomponent systems using microgram quantities of total protein. J. Biol. Chem. **252**:6510–6515.
5. **Gibson, W., and I. M. Verma.** 1974. Studies on the reverse transcriptase of RAV tumor viruses. Structural relatedness of two subunits of avian RNA tumor viruses. Proc. Natl. Acad. Sci. U. S. A. **71**:4991–4994.
6. **Gilead, Z., Y.-H. Jeng, W. S. M. Wold, K. Sugawara, H. M. Rho, M. L. Harter, and M. Green.** 1976. Immunological identification of two adenovirus 2-induced early proteins possibly involved in cell transformation. Nature (London) **264**:263–266.
7. **Hanafusa, H.** 1969. Rapid transformation of cells by RSV. Proc. Natl. Acad. Sci. U. S. A. **63**:318–325.
8. **Hanafusa, H., T. Hanafusa, S. Kawai, and R. E. Luginbuhl.** 1974. Genetic control of expression of endogenous virus genes in chicken cells. Virology **58**:439–448.
9. **Hayman, M. J.** 1978. Viral polyproteins in chick embryo fibroblasts infected with avian sarcoma leukosis viruses. Virology **85**:241–252.
10. **Hayward, W. S.** 1977. Size and genetic content of viral RNAs in avian oncovirus-infected cells. J. Virol. **24**:47–63.
11. **Herman, A. C., R. W. Green, D. P. Bolognesi, and T. C. Vanaman.** 1975. Comparative chemical properties of avian oncornavirus polypeptides. Virology **64**:339–348.
12. **Hizi, A., M. A. McCrae, and W. K. Joklik.** 1978. Studies on the amino acid sequence content of proteins specified by the *gag* and *pol* genes of avian sarcoma virus B77. Virology **89**:272–284.
13. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617–1624.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **McGinnis, J., A. Hizi, R. E. Smith, and J. P. Leis.** 1978. *In vitro* translation of a 180,000-dalton Rous sarcoma virus precursor polypeptide containing both the DNA polymerase and the group-specific antigens. Virology **84**:518–522.
16. **Mosser, A. G., R. C. Montelaro, and R. R. Reuckert.** 1977. Proteins of Rous-associated virus type 61: polypeptide stoichiometry and evidence that glycoprotein gp35 is not a cleavage product of gp85. J. Virol. **23**:10–19.
17. **Oppermann, H., J. M. Bishop, H. E. Varmus, and L. Levintow.** 1977. A joint product of the genes *gag* and *pol* of avian sarcoma virus: a possible precursor of reverse transcriptase. Cell **12**:993–1005.
18. **Paterson, B. M., D. J. Marciani, and T. S. Papas.** 1977. Cell-free synthesis of the precursor polypeptide for avian myeloblastosis virus DNA polymerase. Proc. Natl. Acad. Sci. U. S. A. **74**:4951–4954.
19. **Philipson, L., P. Anderson, U. Olshevsky, R. Weinberg, D. Baltimore, and R. Gesteland.** 1978. Translation of MuLV and MSV RNAs in nuclease-treated reticulocyte extracts: enhancement of the *gag-pol* polypeptide with yeast suppressor tRNA. Cell **13**:189–199.
20. **Purchio, A. F., E. Erikson, and R. L. Erikson.** 1977. Translation of 35S and of subgenomic regions of avian sarcoma virus RNA. Proc. Natl. Acad. Sci. U. S. A. **74**:4661–4665.
21. **Rettenmier, C. W., and H. Hanafusa.** 1977. Structural protein markers in the avian oncoviruses. J. Virol. **24**:850–864.
22. **Reynolds Jr., F. H., C. A. Hanson, S. A. Aaronson, and J. R. Stephenson.** 1977. Type C viral *gag* gene expression in chicken embryo fibroblasts and avian sarcoma virus-transformed mammalian cells. J. Virol. **23**:74–79.
23. **Shaikh, R., M. Linial, S. Brown, A. Sen, and R. Eisenman.** 1979. Recombinant avian oncoviruses. II. Alterations in the *gag* proteins and evidence for intragenic recombination. Virology **92**:463–481.
24. **Shealy, D. J., and R. R. Rueckert.** 1978. Proteins of Rous-associated virus 61, and avian retrovirus: common precursor for glycoproteins gp85 and gp35 and use of pactamycin to map translational order of proteins in the *gag*, *pol*, and *env* genes. J. Virol. **26**:380–388.
25. **Vogt, V. M., R. Eisenman, and H. Diggelmann.** 1975. Generation of avian myeloblastosis virus internal structural proteins by proteolytic cleavage of a precursor polypeptide. J. Mol. Biol. **96**:471–493.
26. **von der Helm, K., and P. H. Duesberg.** 1975. Translation of Rous sarcoma virus RNA in a cell-free system from ascites Krebs II cells. Proc. Natl. Acad. Sci. U. S. A. **72**:614–618.
27. **Wang, S. Y., W. S. Hayward, and H. Hanafusa.** 1977. Genetic variation in the RNA transcripts of endogenous virus genes in uninfected chicken cells. J. Virol. **24**:64–73.
28. **Weiss, S. R., P. B. Hackett, H. Oppermann, A. Ullrich, L. Levintow, and J. M. Bishop.** 1978. Cell-free translation of avian sarcoma virus RNA: suppression of the *gag* termination codon does not augment synthesis of the joint *gag/pol* product. Cell **15**:607–614.
29. **Weiss, S. R., H. E. Varmus, and J. M. Bishop.** 1977. The size and genetic composition of virus-specific RNAs in the cytoplasm of cells producing avian sarcoma-leukosis viruses. Cell **12**:983–992.