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Structural Protein Markers in the Avian Oncoviruses

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The proteins of purified avian oncoviruses were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and isoelectric focusing. Certain members of the avian leukosis-sarcoma viruses (ALSV) had group-specific antigens with altered electrophoretic properties. (i) The p27 protein of Rous-associated virus 0 (RAV-0) had a lower electrophoretic mobility in SDS gels and a lower isoelectric point than the p27 of other ALSV. (ii) The p19 proteins of RAV-1, RAV-2, and the Bryan high-titer strain of Rous sarcoma virus had higher mobilities in SDS gels than did the corresponding protein of other viruses. This altered electrophoretic mobility was correlated with specific differences in the tryptic peptides of radioiodinated p19s. (iii) The p15 protein of RAV-7 had a lower mobility in SDS gels than did the p15 of other ALSV. These markers were used in a study of the structural proteins of subgroup E RAV-60 produced after infection of chicken embryo cells by exogenous ALSV. Although exogenous group-specific protein markers could often be identified in the subgroup E isolates, one RAV-60 had a p27 that comigrated with the p27 of RAV-0. The p19s of two other RAV-60 isolates had electrophoretic properties that were different than those of p19s from either RAV-0 or the exogenous viruses. These results support the hypothesis that RAV-60 is generated by recombination between endogenous and exogenous oncoviruses and indicate that at least the p27 encoded by RAV-0 is closely related to a protein specified by endogenous viral information in chicken cells.

Avian leukosis-sarcoma viruses (ALSV) consist of an RNA genome and RNA-dependent DNA polymerase encapsidated by four structural proteins within a lipid-containing envelope (6, 44). Glycoprotein complexes on the virion surface (10, 30, 36, 40) exhibit type-specific antigenic determinants (4) and specify the virus subgroup (14, 47). The nonglycosylated internal proteins have molecular weights of 27,000, 19,000, 15,000, and 12,000 and are designated p27, p19, p15, and p12, respectively (1); they have strong group-specific (gs) antigenic characteristics (5, 12) and are generated by cleavage of a common precursor polypeptide (49) encoded by the viral *gag* gene (2). There have also been reports of chemical polymorphism (13, 25) and type-specific antigenic determinants (5, 21, 43) in some of these internal structural proteins.

Chicken embryos contain information related to ALSV in the form of proviral DNA (3, 39, 45), and expression of this information is under genetic controls (9, 17, 34, 38, 52). Certain lines of chicken cells spontaneously release a virus, known as Rous-associated virus 0 (RAV-0), which has subgroup E envelope glycoprotein specificity (9, 46). In the absence of virus production, other embryos synthesize varying amounts of viral RNA (8, 22), gs antigens (7, 8, 42), and subgroup E envelope protein (16). Infection of these virus-free chicken cells with ALSV results in the production of a subgroup E virus known as RAV-60 (19, 20), which replicates in permissive cells more efficiently than does RAV-0 (18, 31, 37). There is evidence from nucleic acid hybridization studies that RAV-60 is formed by recombination between exogenous ALSV and the endogenous information in chicken cells (23, 41).

In the present study we used electrophoresis in high-resolution polyacrylamide gels to analyze the virion proteins of ALSV. We have shown that variant forms of p27, p19, and p15 are found in certain strains of these avian oncoviruses. One of the electrophoretic markers in viral p19s was correlated with specific differences in the ^{125}I-labeled tryptic peptides of these proteins. Virion polypeptides of several RAV-60 isolates were compared with structural proteins of the exogenous viruses and RAV-0. One RAV-60 had a p19 apparently derived from the exogenous virus used in its isolation and a p27 that comigrated with the corresponding protein of RAV-0. This result indicates that RAV-60-type viruses are generated by recombination between endoge-

nous and exogenous viral genomes and that RAV-0 is related to endogenous viral information in normal, uninfected chicken cells.

MATERIALS AND METHODS

Cell culture and viruses. The preparation of cell cultures from SPAFAS chicken and Japanese quail embryos has been described previously (15). Chicken embryo cells were analyzed for the presence of viral gs antigen and endogenous subgroup E helper activity (17), and three types of embryos were used in the present experiments: gs^-h^- (negative for both gs antigen and helper factor), gs^+h_H (high gs antigen titer and high helper activity), and gs_Lh_E (low gs antigen titer and extremely high helper activity). RAV-0-producing line 100 and line 7 × 15 chicken cells were kindly supplied by L. B. Crittenden. The following viruses were used: nondefective Schmidt-Ruppin strain Rous sarcoma virus of subgroup A (SR-RSV-A), nondefective Prague strain RSV of subgroup C specificity (PR-RSV-C), glycoprotein-defective Bryan high-titer strain RSV [BH-RSV(−)], RAV-2 of subgroup B, RAV-7 of subgroup C, RAV-0 of subgroup E, and several isolates of subgroup E RAV-60. With the exception of RAV-60 isolates that were grown in quail cells, all virus stocks were passaged in gs^-h^- chicken embryo cells.

As previously described (19), RAV-60s were obtained after infection of SPAFAS C/E chicken embryo cells with an exogenous ALSV. Supernatants were harvested, and the virus progeny were passaged twice in quail cells, which are permissive for subgroup E viruses. Individual RAV-60 isolates were then obtained by two successive cycles of end-point dilution on quail cells. The subgroup specificity of these isolates was confirmed by their ability to interfere with focus formation by BH-RSV(chf) in quail cells.

Proposal for numbering RAV-60 isolates. The designation RAV-60 has been used previously for any nontransforming subgroup E virus recovered after infection of chicken cells by exogenous ALSV of a different subgroup (18–20). As shown in the present study, these isolates are not identical, even for a single exogenous virus, and there is evidence that RAV-60-type viruses are generated by recombination between endogenous and exogenous oncovirus genes (23, 41). Therefore, it is necessary to provide each isolate with a unique designation. We propose a numbering system in which the general term of RAV-60 will be retained for these viruses, and appended in brackets will be a specification of the exogenous virus used for the generation of that RAV-60, the laboratory of origin, and an individual isolate number. The use of brackets rather than parentheses is suggested to avoid confusion with the notation for describing oncovirus pseudotypes, which have envelope glycoprotein properties defined by a helper virus, e.g., BH-RSV(RAV-2). For the designation of RAV-60 isolates, the laboratories may be abbreviated as recommended in the nomenclature for ALSV mutants (48). In this way two of our RAV-60s used in the present study will be numbered RAV-60[RAV-2/NY1] and RAV-60[BH-RSV/NY1] to denote that the respective exogenous viruses were RAV-2 and BH-RSV. RAV-60[RAV-1/NY1] and RAV-60[RAV-2/NY1] were kindly supplied by T. Hanafusa. RAV-60[RAV-1/NY2] and RAV-60[RAV-2/NY2] were isolated by S. Kawai.

Isotopic labeling of viruses. Virus-infected cells were incubated for two successive labeling intervals in medium containing radioisotopes at the following concentrations: L-[^{35}S]methionine (404 Ci/mmol; New England Nuclear, Boston, Mass.), 25 μCi/ml; L-[^{14}C]leucine (312 mCi/mmol; Schwarz/Mann, Orangeburg, N.Y.), 5 μCi/ml; or D-[1-^{14}C]glucosamine hydrochloride (58 mCi/mmol; Amersham/Searle, Arlington Heights, Ill.), 4 μCi/ml. Labeling intervals were 12 h for amino acids and 24 h for glucosamine. Supernatants were collected from these cultures, and viruses were purified by sucrose density gradient centrifugation as previously described (40).

SDS-polyacrylamide slab gel electrophoresis. Polyacrylamide slab gels (10.5%) with 4% stacking gels, both containing 0.1% (wt/wt) sodium dodecyl sulfate (SDS), were prepared by using the discontinuous buffer system described by Laemmli (28). Purified virus pellets were dissociated in 0.0625 M Tris (pH 6.8)–2% SDS–0.05 M dithiothreitol–10% sucrose with 0.001% bromophenol blue as the tracking dye. Samples were heated for 2 min in boiling water prior to electrophoresis. The total volume, usually 20 μl, loaded into the gel slots was the same for all samples. Electrophoresis was carried out at 10 mA/gel in buffer consisting of 0.025 M Tris (pH 8.5), 0.2 M glycine, and 0.1% SDS. The gels were fixed for 8 h in 10% (wt/wt) trichloroacetic acid–25% (vol/vol) 2-propanol, and staining was carried out with 0.05% Coomassie brilliant blue in 25% 2-propanol and 7% (vol/vol) acetic acid. The destaining solution was 10% 2-propanol and 7% acetic acid.

Slab gels were dried onto sheets of Whatman chromatography paper in an apparatus consisting of a piece (18.7 by 18.7 cm) of porous polyethylene placed on top of the circular plastic base plate (32.5-cm diameter; Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) of a vacuum bell jar. The system was attached to a vacuum line and sealed with cellophane. This inexpensive apparatus was able to dry our gels (1.25 mm thick) in 12 to 16 h, and we encountered no problems with cracking of the gels at polyacrylamide concentrations as high as 15%. Dried gels were exposed against Dupont Cronex 2DC safety film for autoradiography.

Tryptic peptide mapping of radioiodinated proteins. Tryptic peptide analysis of ^{125}I-labeled ALSV proteins was performed as described by Elder et al. (10a). Viral proteins were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue in 25% 2-propanol–10% acetic acid. After destaining, the regions of the gel containing individual proteins were cut out with a razor blade, washed extensively in 10% methanol, and lyophilized. Proteins were iodinated in the gel slices by the addition of 1 mCi of ^{125}I (17 mCi/mmol; Amersham/Searle) and 5 μg of chloramine T in 30 μl of 0.5 M sodium phosphate buffer (pH 7.5). The reaction was terminated after 1 h by the addition of 1 ml of 0.1% sodium bisulfite. The gel slices were incubated in two changes of 10% acetic acid, washed extensively in 10% methanol to remove free ^{125}I, and lyophilized. Dried gel slices

were placed in siliconized glass tubes and incubated for 12 h at 37°C in 0.5 ml of 0.05 M ammonium bicarbonate buffer (pH 8.0) with 25 μg of L-(tosylamido 2-phenyl)ethyl chloromethyl ketone-treated trypsin (271 U/mg; Worthington Biochemicals Corp., Freehold, N.J.). Supernatants of the samples were removed and lyophilized in siliconized glass tubes. The tryptic peptides were analyzed in two dimensions on cellulose-coated thin-layer chromatography plates (10 by 10 cm; EM Laboratories, Elmsford, N.Y.) by electrophoresis for 30 min at 1,000 V in acetic acid-formic acid-water (15:5:80) followed by ascending chromatography in 1-butanol–pyridine–acetic acid–water (32.5:25:5:20). Dried plates were exposed against Kodak XRP-1 X-ray film for autoradiography.

Two-dimensional electrophoresis of ALSV proteins. Two-dimensional separation of proteins, using isoelectric focusing and SDS-gel electrophoresis, was carried out by the procedure of O'Farrell (32). Isoelectric focusing in the first dimension was done at constant power to a final value of about 6,000 V · h in 4% polyacrylamide tube gels containing 9 M urea, 2% Nonidet P-40, and 2% ampholines (pH 3.5 to 10; LKB Instruments Inc., Rockville, Md.). Optimal resolution of the basic p27 proteins found in exogenous ALSV was obtained when samples were loaded at the anode of the isoelectric focusing gel. After separation in the first dimension, gels were equilibrated for 30 min at room temperature in 0.0625 M Tris (pH 6.8) containing 2.3% SDS, 5% 2-mercaptoethanol, and 10% glycerol. The second dimension was a 10 to 14% gradient polyacrylamide slab gel with a 3-cm 4% polyacrylamide stacking gel, both containing 0.1% SDS. This gradient slab gel provided good separation of viral gs proteins (molecular weights of 10,000 to 30,000), but we routinely observed some streaking of the p27 and p15 proteins in the second dimension (see Fig. 5). The streaking of these proteins was largely independent of equilibration conditions (32), but it was usually eliminated when a steeper polyacrylamide gradient was used in the slab gel.

RESULTS

SDS-polyacrylamide gel electrophoresis of ALSV proteins. Figure 1 shows the separation of ^{14}C-labeled proteins of purified RAV-2 after electrophoresis in an SDS-polyacrylamide slab gel. The pattern is similar to previously published data for ALSV (5, 12, 40, 49), and the major virion proteins have been designated according to the proposal for a uniform nomenclature (1). In our gel system the major viral glycoprotein, gp85, migrated as a diffuse band having an apparent molecular weight of 80,000 to 95,000. Several minor bands were also seen in the region of the gp85 in Fig. 1A. Our preliminary work indicates that the proteins with molecular weights of 62,000 and 94,000 were the α and β subunits of the viral RNA-dependent DNA polymerase, and the closely spaced bands with molecular weight of about 70,000 were contaminating host proteins that copurified with virions (data not shown). The material designated gp35

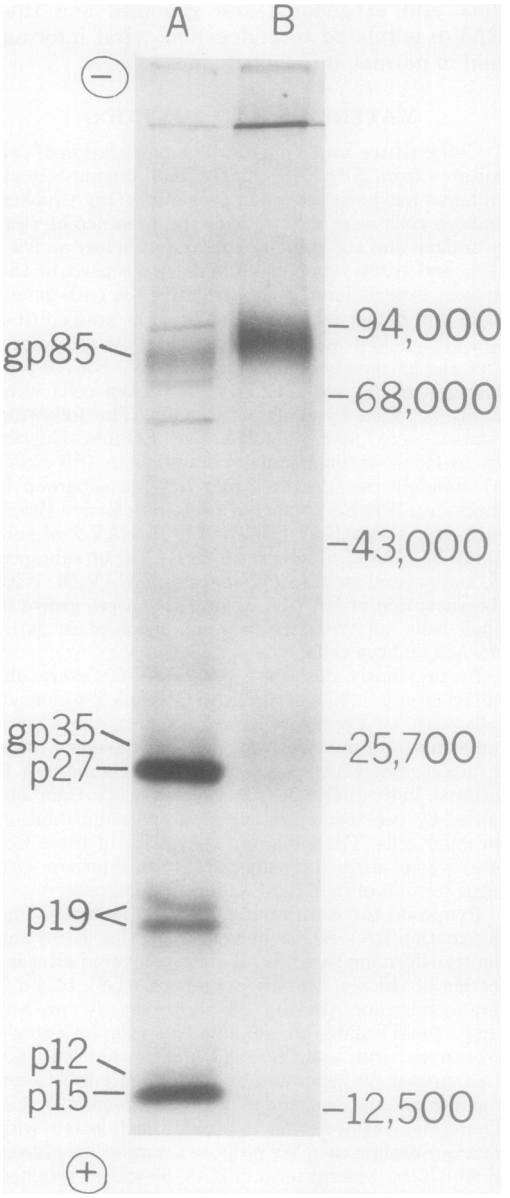


FIG. 1. *Autoradiogram of radiolabeled proteins of purified RAV-2 after electrophoresis in a 10.5% polyacrylamide slab gel containing 0.1% SDS. Migration in the gel was from top to bottom. Infected chicken embryo cells were labeled with (A) [^{14}C]leucine or (B) [^{14}C]glucosamine, and the virus was purified by sucrose density gradient centrifugation. Approximately 20,000 cpm was loaded into slot (A), and 4,000 cpm was loaded into slot (B). The autoradiogram was exposed for 10 days. The major virion proteins are noted in the left margin. The mobilities of several marker proteins are indicated in the right margin: phosphorylase a, 94,000 daltons; bovine serum albumin, 68,000 daltons; ovalbumin, 43,000 daltons; chymotrypsinogen A, 25,700 daltons; and cytochrome c from Candida krusei, 12,500 daltons.*

in Fig. 1, which was labeled with both leucine and glucosamine, had about the same electrophoretic mobility as the viral p27 protein. Apparently, this was due to fortuitous comigration of the two proteins. When RAV-2 was dissociated in 2% SDS with 0.05 M iodoacetamide to prevent reduction of disulfide bonds (data not shown), the mobility of p27 was unaffected, whereas the gp85 and gp35 bands were not seen. Instead, all of the glucosamine-labeled material migrated as a diffuse band with a molecular weight of 100,000 to 120,000, which corresponded to the glycoprotein complex described by Leamnson and Halpern (30).

As has been previously reported for these viruses (12, 49), the mobilities of the gs proteins in SDS gels did not precisely reflect their molecular weights as determined by gel filtration in 6 M guanidine hydrochloride. In addition, at least two bands were always seen in the region of the viral p19 protein. On the basis of tryptic peptide analysis, Vogt and co-workers (49) concluded that the two bands of avian myeloblastosis virus p19 were derived from a single polypeptide, and Lai (29) has reported that the ALSV p19 is a phosphoprotein. Erikson et al. (11) have recently demonstrated that the slower electrophoretic form of this protein is phosphorylated, and the higher mobility form is not. In the present study we will refer to these two bands as the p19 doublet.

Electrophoretic variants of gs proteins. The electrophoretic separation in SDS gels of [^{35}S]methionine-labeled proteins from several ALSV is shown in Fig. 2. Under the conditions for labeling and autoradiography used in this and subsequent experiments, the p12 proteins were not seen because little [^{35}S]methionine was incorporated into them. The electrophoretic mobilities of the respective p27 and p15 proteins were virtually identical for the viruses shown in Fig. 2. However, the p19 doublets of RAV-2 and BH-RSV(−) had higher mobilities than the corresponding proteins of PR-RSV-C and SR-RSV-A. Assuming that these differences in migration are functions only of molecular weight, the altered mobility of RAV-2 and BH-RSV p19 proteins reflected a molecular weight difference of about 500 from the p19s of the other two viruses. We examined the virion proteins of other ALSV and found that most had p19 doublets that comigrated with those of PR-RSV-C and SR-RSV-A (data not shown). Only the RAV-1 p19 showed an increased electrophoretic mobility similar to that of RAV-2 and BH-RSV.

As shown in Fig. 3, we also found examples of an electrophoretic variant for the p27 and p15 proteins of ALSV. The p27 of RAV-0 (Fig. 3B) and the p15 of RAV-7 (Fig. 3C) had reduced mobilities in SDS gels relative to the corresponding proteins of RAV-2. Based on comparisons with molecular weight markers, the migration of the RAV-0 p27 represented an apparent difference in molecular weight of 700 to 1,200 from the p27 of RAV-2. The altered mobility of the RAV-7 p15 corresponded to an apparent molecular weight difference of 150 to 300.

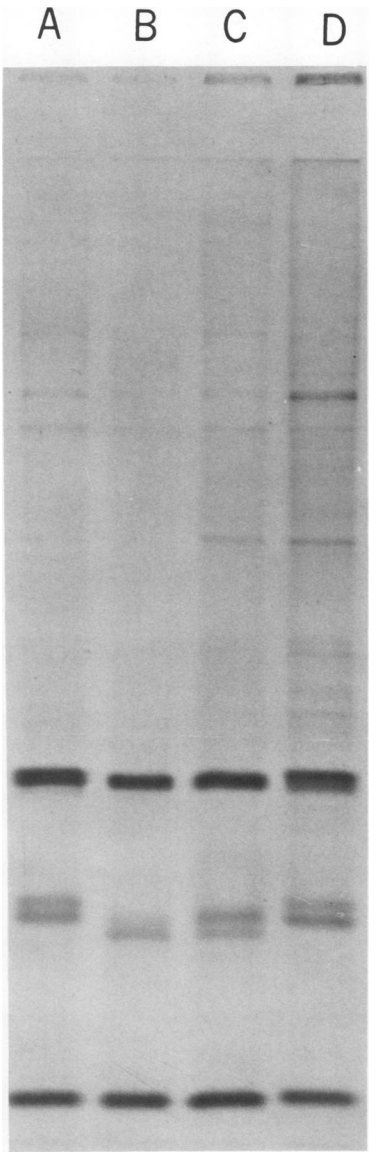


FIG. 2. *Autoradiogram of [^{35}S]methionine-labeled proteins of purified ALSV after separation by SDS-polyacrylamide gel electrophoresis. (A) PR-RSV-C, (B) RAV-2 (subgroup B), (C) BH-RSV(−), and (D) SR-RSV-A.*

Tryptic peptide analysis of radioiodinated ALSV proteins. Elder and co-workers (10a) have recently developed a technique for

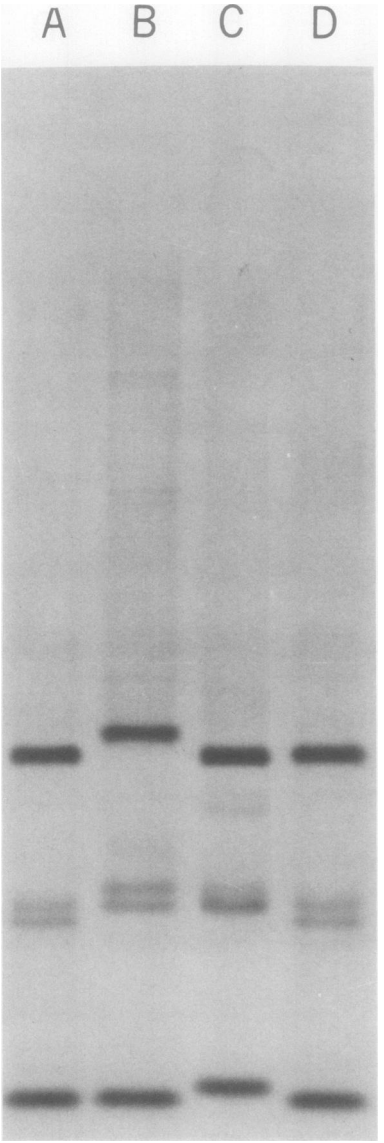
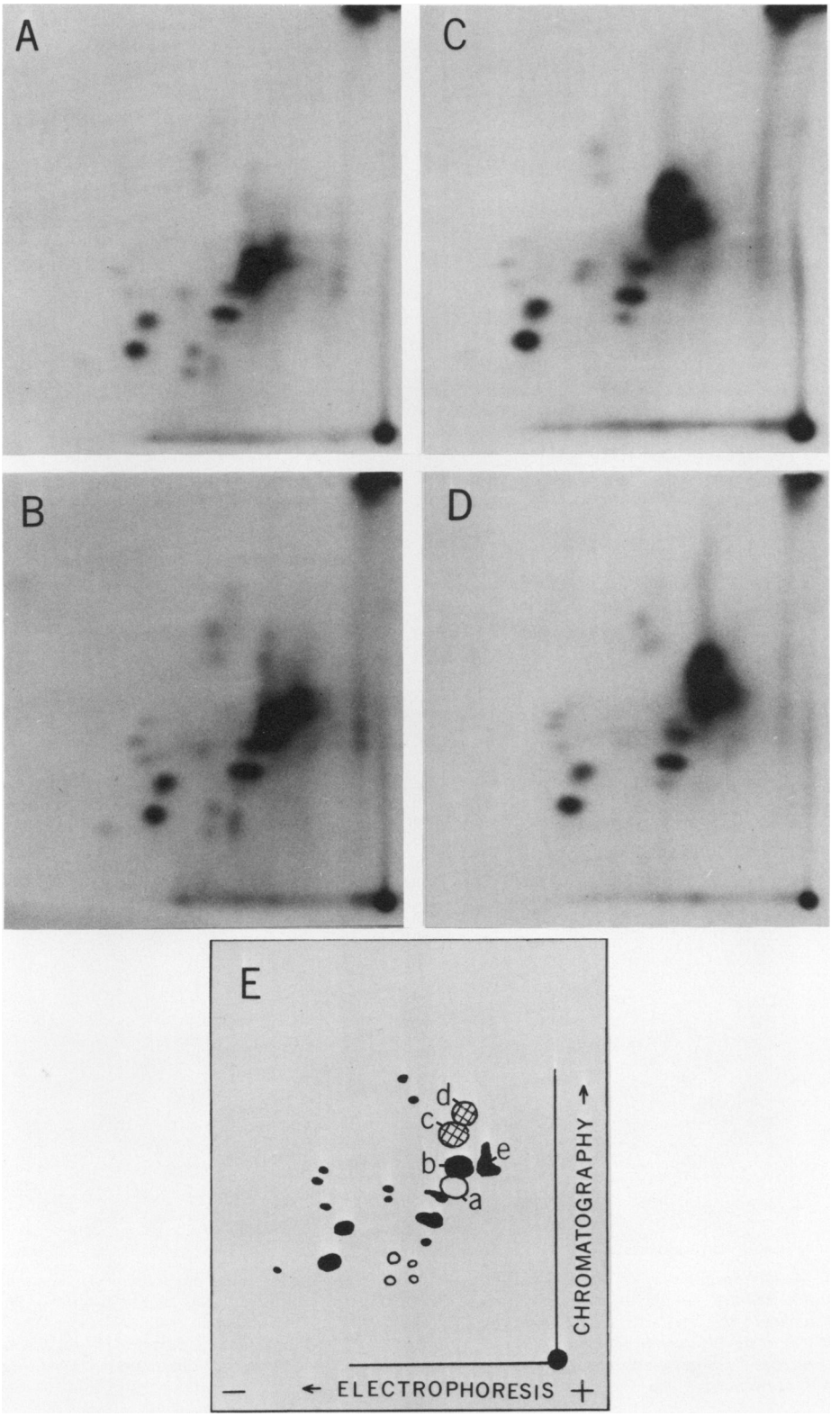


FIG. 3. *Autoradiogram of [^{35}S]methionine-labeled ALSV proteins demonstrating electrophoretic variants of the p27 and p15 proteins in SDS-slab gels. (A) RAV-2, (B) RAV-0, (C) RAV-7, and (D) RAV-2.*

comparing the primary structures of proteins in polyacrylamide gel slices by radioiodination and peptide mapping. We have used this procedure to analyze the tryptic peptides of ALSV internal proteins. Virion proteins were separated in SDS slab gels and stained with Coomassie brilliant blue. The regions of the gels containing individual proteins were excised and radioiodinated by the chloramine T procedure as described above. Tryptic peptides of these proteins were prepared and analyzed on cellulose-coated thin-layer plates by electrophoresis in one dimension and chromatography in the second. As shown in Fig. 4, the ^{125}I-labeled peptides of phosphorylated and unphosphorylated forms of the p19 protein of each virus were found to be identical. Occasionally, we saw a third form of ALSV p19 that migrated with an apparent molecular weight of 23,000 in SDS gels; traces of such a protein are seen for RAV-7 in Fig. 3C. We found that the ^{125}I-labeled peptides of this protein were identical to those of the other forms of p19 (data not shown).

Although there was considerable homology among the p19 proteins from all ALSV that we examined, strain-specific differences in the iodinated p19 peptides were detected. As shown in Fig. 4E, five major ^{125}I-labeled tryptic peptides of ALSV p19's formed a closely spaced group (labeled a to e) in the peptide maps. The b-peptide and e-peptide were common to p19 proteins from all viruses that we examined. The a-peptide was specific for the p19's of RAV-7, RAV-0, and all other viruses having the p19 doublet with lower electrophoretic mobility in SDS gels. This a-peptide was not seen among the ^{125}I-labeled peptides of RAV-2 or BH-RSV p19's; instead, two additional peptides (labeled c and d in Fig. 4E) were seen for these latter viruses. Therefore, the differences in the major ^{125}I-labeled p19 peptides diagramed in Fig. 4E correlated with the altered electrophoretic properties of these proteins shown in Fig. 2 and 3. We could detect no differences in the ^{125}I-labeled peptides of the RAV-0 p27 or RAV-7 p15 when compared with the corresponding proteins of other viruses (data not shown). A detailed study

FIG. 4. *Comparison of the ^{125}I-labeled tryptic peptides of RAV-7 and RAV-2 p19 proteins. The proteins were radioiodinated in polyacrylamide gel slices as described in the text. Tryptic peptides were prepared and analyzed on cellulose-coated glass plates. Samples were spotted in the lower right corner of each panel. Electrophoresis was in the horizontal direction from right to left, toward the cathode. Ascending chromatography was carried out in the vertical direction. (A) to (D) are autoradiograms of ^{125}I-labeled peptides of the indicated proteins: (A) RAV-7 p19, phosphorylated form; (B) RAV-7 p19, unphosphorylated form; (C) RAV-2 p19, phosphorylated form; and (D) RAV-2 p19, unphosphorylated form. (E) is a drawing that shows the differences between the ^{125}I-labeled peptides of RAV-7 and RAV-2 p19's: ●, spots common to both p19 proteins; ○, spots unique to the p19 of RAV-7; ⊗, spots unique to the p19 of RAV-2. The five major ^{125}I-labeled peptides of ALSV p19's are labeled a to e in (E) and form a cluster in the peptide maps. The b- and e-peptides are common to all p19's. The a-peptide is specific to ALSV, like RAV-7, having the slower electrophoretic form of the p19 doublet, and is not seen in the peptide maps of RAV-2 or BH-RSV p19. The c- and d-peptides are unique to RAV-2 and other viruses having the higher mobility form of this protein.*


A
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of ALSV proteins by peptide mapping will be presented in a subsequent communication.

Two-dimensional electrophoresis of ALSV proteins. A two-dimensional procedure for the separation of proteins by isoelectric focusing and SDS-gel electrophoresis has been described by O'Farrell (32). In this system, proteins are first focused by isoelectric point in a polyacrylamide tube gel containing 2% ampholines, 9 M urea, and 2% Nonidet P-40. The cylindrical gel is then aligned on top of a polyacrylamide slab gel containing 0.1% SDS, and electrophoresis is carried out in the second dimension. The resolution of [^{35}S]methionine-labeled proteins of RAV-2 by this technique is shown in Fig. 5. The major spot of the RAV-2 p27 protein had a pI of about 8.3, and the p15 had a pI of 7.4. The two forms of the RAV-2 p19 doublet had pI's of 6.9 and 7.6, respectively. Erikson et al. (11) have found that the more

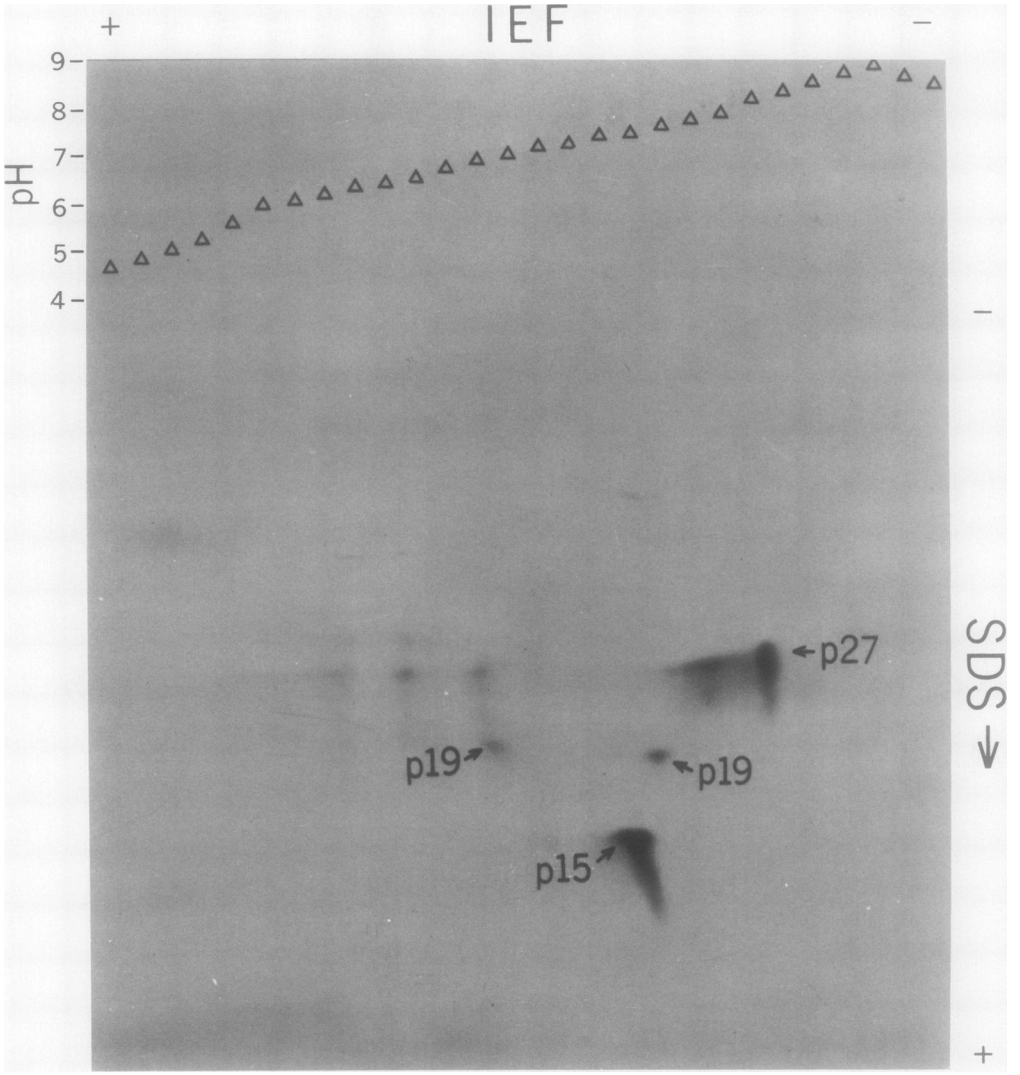

FIG. 5. *Autoradiogram of [^{35}S]methionine-labeled proteins of RAV-2 after electrophoretic separation by the two-dimensional gel technique of O'Farrell (32). The sample was applied at the anode of the first-dimension isoelectric focusing (IEF) tube gel, and migration of proteins was from left to right. The pH gradient (△) of an isoelectric focusing gel run in parallel with this sample is superimposed on the stacking gel used for the SDS-gel electrophoresis. Migration of proteins in the second-dimension SDS-slab gel was from top to bottom.*

acidic spot of the PR-RSV-C p19 is the phosphorylated form of the protein.

We next used the two-dimensional gel system to compare the [^{35}S]methionine-labeled proteins of RAV-2 and RAV-0. Figure 6 shows only the regions of the gels containing the viral gs proteins. The major spot of the RAV-0 p27 had a pI of 7.2, and the p15 had a pI of 7.4. The two forms of RAV-0 p19 protein had pI values of 6.6 and 6.9. When RAV-0 and RAV-2 were mixed and subjected to coelectrophoresis (Fig. 6C), the p15's of these viruses migrated together, but the p27 proteins were easily resolved. The lower-mobility form of the RAV-2 p19 had about the same electrophoretic properties in the two-dimensional system as the higher-mobility form of RAV-0 p19. However, the slower electrophoretic form of RAV-0 p19 (pI 6.6) could be distinguished from the faster electrophoretic form of RAV-2 p19 (pI 7.6). RAV-0 used in the experiments shown in Fig. 3 and 6 was virus spontaneously released from line 7 × 15 chicken cells. We observed identical results for the virion proteins of RAV-0 produced by line 100 cells (data not shown). We used the two-dimensional gel system to separate the proteins of several other ALSV, and the results are shown in Table 1. With the exception of the p19 of RAV-0, the p19's of all other viruses examined had the same isoelectric points, despite the previously discussed differences of mobility in SDS gels. Therefore, both the p27 and p19 proteins of RAV-0 were unique among the ALSV that we tested.

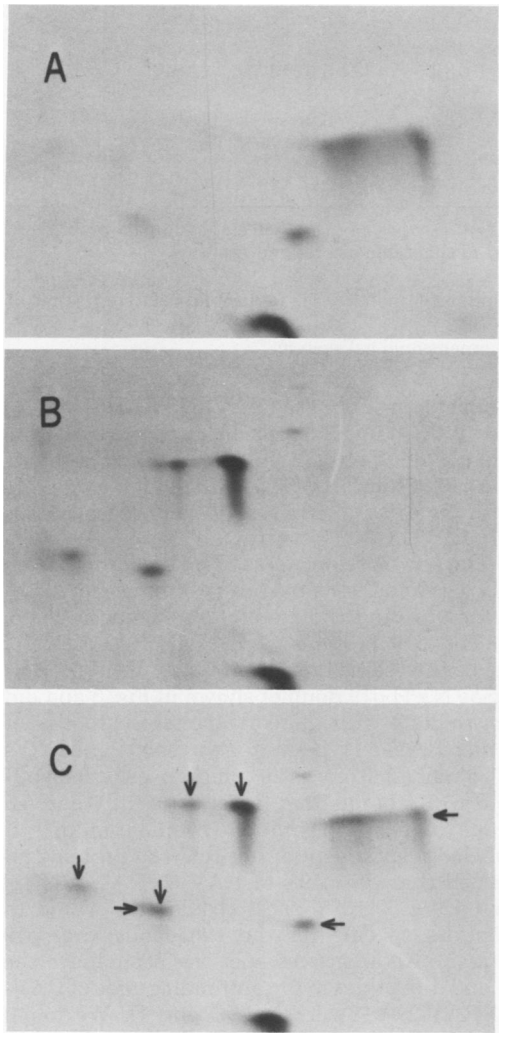

FIG. 6. *Autoradiograms showing the [^{35}S]methionine-labeled gs proteins of RAV-2 and RAV-0 after separation by two-dimensional electrophoresis. The orientation of the gels is the same as shown in Fig. 5, and only the regions of the gels containing the gs proteins are shown. (A) RAV-2, (B) RAV-0, and (C) coelectrophoresis of RAV-2 and RAV-0. In (C), the p27 and p19 proteins of RAV-2 are indicated by horizontal arrows (→ and ←), and those of RAV-0 are shown by vertical arrows (↓).*

Several technical aspects of the two-dimensional gel system deserve further comment. In previous studies on isoelectric focusing of ALSV proteins in urea, Hung et al. (27) found that the p15 and p27 proteins of BH-RSV(RAV-1) had isoelectric points of pH 7.4 and 8.9, respectively. We obtained a lower pI value for the p27 proteins of these viruses, but it often appeared that the p27 of exogenous ALSV did not focus sharply in the first dimension. Using the ampholine mixture described by O'Farrell (32) that gave a pH gradient of 5.2 to 8.0, Erikson and co-workers (11) observed that the p27 of PR-RSV-C did not enter the isoelectric focusing gel and concluded that this protein had a pI higher than 8.0. With the ampholine mixture used in the present experiments, the p27 of exogenous viruses did enter the isoelectric focusing gel, but the resolution of this protein was sometimes poor. These effects may be related to the fact that the pH gradient flattened at the basic end of the gel in the region close to the pI of the exogenous viral p27. Although it is not clear how accurately the pI's of these proteins could be measured in our two-dimensional gel system, the p27 proteins of exogenous viruses could be readily separated from the p27 of RAV-0.

Analysis of the virion proteins of RAV-60s. The existence of structural protein markers in ALSV encouraged us to examine the proteins of putative recombinant viruses. Figure 7 shows autoradiograms of [^{35}S]methionine-labeled pro-

TABLE 1. *Electrophoretic properties of the gs antigens of several strains of avian oncoviruses*

Virus	Protein[a]					
	p27		p19[b]		p15	
	Mol wt[c] ($\times 10^{-3}$)	pI[d]	Mol wt ($\times 10^{-3}$)	pI	Mol wt ($\times 10^{-3}$)	pI
PR-RSV-C	24.0	8.3	18.6	6.9	12.8	7.4
			18.1	7.6		
RAV-7	24.0	8.3	18.6	6.9	13.0	7.4
			18.1	7.6		
BH-RSV	24.0	8.3	18.1	6.9	12.8	7.4
			17.6	7.6		
RAV-1	24.0	8.3	18.1	6.9	12.8	7.4
			17.6	7.6		
RAV-2	24.0	8.3	18.1	6.9	12.8	7.4
			17.6	7.6		
RAV-0	25.2	7.2	18.6	6.6	12.8	7.4
			18.1	6.9		

[a] Extensive homology was observed among ^{125}I-labeled tryptic peptides of the corresponding gs proteins of all viruses. Therefore, the electrophoretic variants described in this table are related proteins.

[b] Includes both forms of the p19 doublet.

[c] Apparent molecular weight, estimated from the electrophoretic mobility of the protein relative to that of several marker proteins in an SDS-polyacrylamide gel.

[d] Determined by the migration of the protein in the two-dimensional gel system.

teins from several RAV-60 isolates after separation in SDS-slab gels. The virion proteins of RAV-0 and the exogenous virus used for each isolate of RAV-60 were included in the adjacent lanes. Several RAV-60s had structural protein markers from the exogenous virus: the p15 of RAV-60[RAV-7/NY1] shown in Fig. 7E and the p19 proteins of RAV-60[RAV-2/NY1] and RAV-60[RAV-2/NY2] shown in Fig. 7H and I, respectively. However, certain of the RAV-60s obtained from gs^+h_H cells had one gs protein that did not comigrate with the corresponding polypeptide of the exogenous ALSV. Instead, these latter proteins migrated with RAV-0 marker proteins; examples were the p27 of RAV-60[RAV-2/NY1] shown in Fig. 7H and the p19 doublet of RAV-60[BH-RSV/NY1] shown in Fig. 7B.

Using the proteins of RAV-0 and the respective exogenous virus as a reference, we next examined the virion polypeptides of RAV-60[RAV-2/NY1] and RAV-60[BH-RSV/NY1] in the two-dimensional gel system. The results shown in Fig. 8 confirm that the p19 doublet of the RAV-2-derived virus (Fig. 8B) and the p27 of the BH-RSV-derived virus (Fig. 8E) comigrated with the corresponding proteins of the exogenous viruses. On the other hand, the p27 of RAV-60[RAV-2/NY1] migrated with the p27 of RAV-0 (Fig. 8C). Although the p19's of RAV-60[BH-RSV/NY1] had lower mobilities in SDS gels than the corresponding proteins of BH-RSV, these two sets of proteins had the same isoelectric points and are seen as closely spaced doublets in Fig. 8E. RAV-60[BH-RSV/NY1] p19 proteins could also be distinguished from the p19 doublet of RAV-0 (Fig. 8F). Therefore, the p19 of this RAV-60 isolate did not comigrate with the p19's of either RAV-0 or the exogenous BH-RSV.

The structural proteins of several other RAV-60s were analyzed by the two-dimensional gel technique, and the results are shown in Table 2. The p19 proteins of RAV-60[RAV-1/NY1] gave the same result as the RAV-60[BH-RSV/NY1] p19 doublet shown in Fig. 8 and did not migrate with the corresponding proteins of either RAV-1 or RAV-0. Two isolates of RAV-60 obtained from gs_Lh_E chicken cells had p27 and p19 proteins that were apparently derived from the exogenous virus. Examination of ^{125}I-labeled tryptic peptides of RAV-60 proteins revealed that the p19's of RAV-60[RAV-2/NY1] and RAV-60[RAV-2/NY2] had the c- and d-peptides specific for the exogenous viral p19 (Fig. 9). An additional spot was seen above the c- and d-peptides in the autoradiograms of RAV-60[RAV-2] p19's in Fig. 9C and D. We found the appearance of this spot to be quite variable, and in some experiments it was absent from the peptide maps of these proteins. This spot seemed to be an artifact related to radioiodination and did not appear to represent novel genetic information in these proteins. The p19 peptide maps of RAV-60[BH-RSV/NY1] and RAV-60[RAV-1/NY1], unlike those of the exogenous viruses, were missing the c- and d-peptides, but did have the a-peptide unique to RAV-

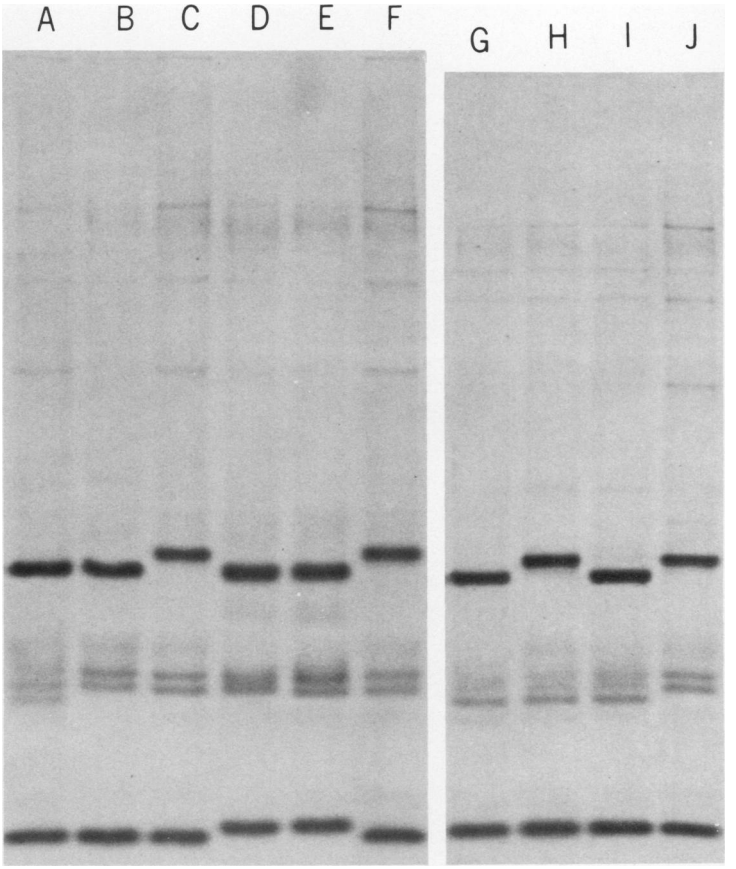

FIG. 7. *Autoradiogram showing the analysis of [^{35}S]methionine-labeled proteins of RAV-60-type viruses by SDS-slab gel electrophoresis. (A) BH-RSV (–), (B) RAV-60[BH-RSV/NY1], (C) RAV-0, (D) RAV-7, (E) RAV-60[RAV-7/NY1], and (F) RAV-0. On a separate gel: (G) RAV-2, (H) RAV-60[RAV-2/NY1], (I) RAV-60[RAV-2/NY2], and (J) RAV-0.*

0 and other viral p19's having lower electrophoretic mobility in SDS gels (data not shown).

DISCUSSION

We have demonstrated that certain members of the ALSV have internal structural proteins with altered electrophoretic properties. Wang et al. (50) have previously reported differences in the electrophoretic mobilities of p27 proteins of RAV-6 and *ts*LA337PR-C, and similar observations have been made for the murine leukemia viruses (26, 35). In our studies one of the electrophoretic variants in the viral p19's was correlated with specific differences in ^{125}I-labeled tryptic peptides of these proteins. This last result is preliminary evidence that, at least in this one case, the altered electrophoretic properties are due to small differences in the primary structures of homologous proteins. We are continuing our characterization of these strain-specific electrophoretic markers in ALSV gs proteins.

Like previous investigators (11, 49), we found at least two bands in the region of the viral p19 protein. Erikson and co-workers (11) demonstrated that the [^{35}S]methionine-labeled tryptic peptides of these two bands were identical, the slower electrophoretic species was phosphorylated, and the phosphorylated band had a lower isoelectric point than the unphosphorylated protein. We have confirmed their results to the extent that, in the viruses we examined, the slower electrophoretic form was a more acidic protein. In addition, although strain-specific differences were seen in the ^{125}I-labeled tryptic peptides of p19 proteins, the iodopeptides of the two p19 bands from each isolate of ALSV were identical. The simplest interpretation of these data is that the two bands are the same polypeptide and that the slower electrophoretic species is a phosphorylated form.

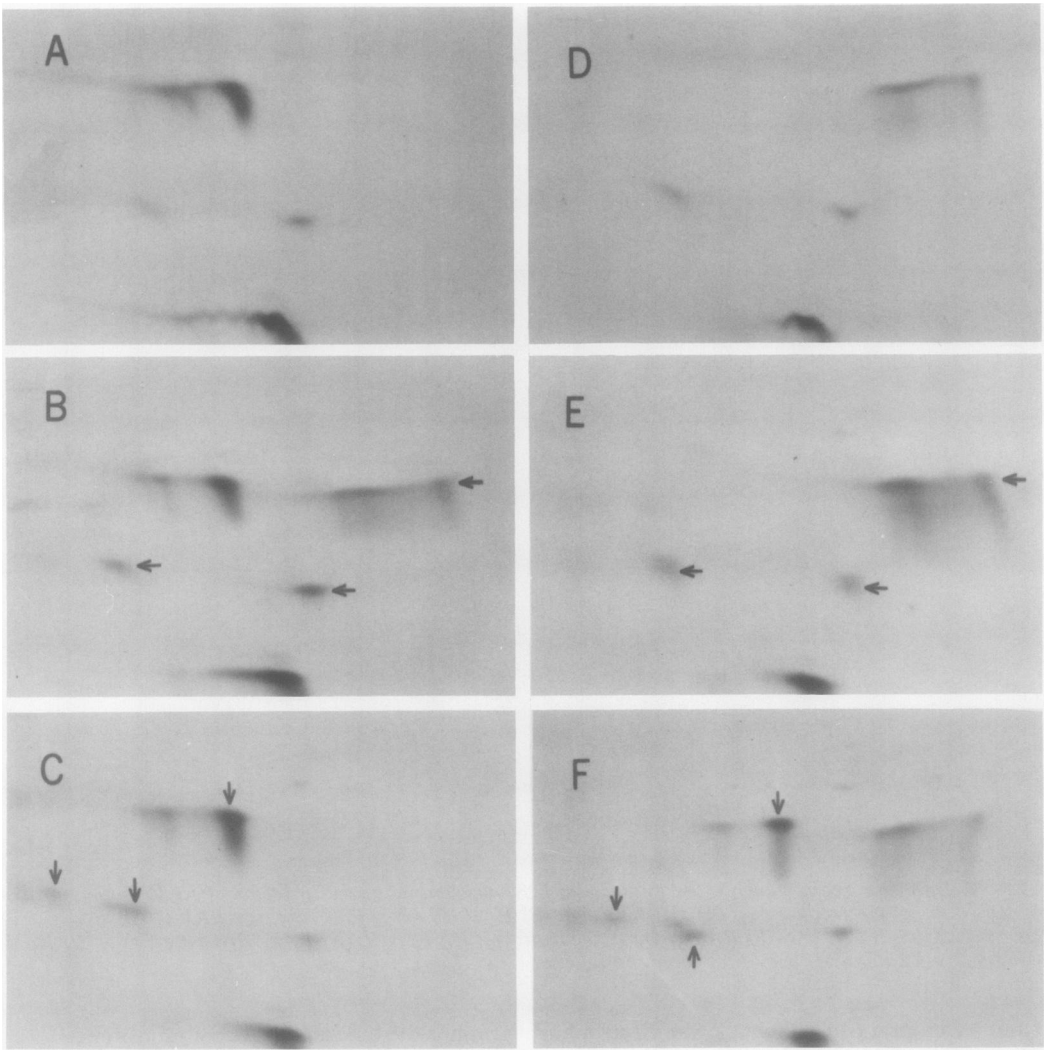

FIG. 8. *Autoradiograms showing two-dimensional gel analysis of [^{35}S]methionine-labeled gs proteins of two RAV-60 isolates. The orientation of the gels is the same as shown in Fig. 5 and 6. (A) RAV-60[RAV-2/NY1], (B) coelectrophoresis of RAV-60[RAV-2/NY1] and RAV-2, (C) coelectrophoresis of RAV-60[RAV-2/NY1] and RAV-0, (D) RAV-60[BH-RSV/NY1], (E) coelectrophoresis of RAV-60[BH-RSV/NY1] and BH-RSV, and (F) coelectrophoresis of RAV-60[BH-RSV/NY1] and RAV-0. Horizontal arrows (←) in (B) and (E) indicate the p27 and p19 proteins of the exogenous viruses, and vertical arrows (↓ and ↑) in (C) and (F) mark the p27 and p19 proteins of RAV-0. Note that the p19 of RAV-60[RAV-2/NY1] had the same electrophoretic properties as the RAV-2 p19 in (B), and the p27 of RAV-60[RAV-2/NY1] comigrated with the RAV-0 p27 in (C). Similarly, the p27 of RAV-60[BH-RSV/NY1] migrated with the p27 of BH-RSV in (E). However, the p19 doublet of RAV-60[BH-RSV/NY1] could be distinguished from the corresponding proteins of both BH-RSV in (E) and RAV-0 in (F).*

The p27 of exogenous ALSV often focused poorly in the two-dimensional gel system, presumably because the pH gradient at the basic end of the isoelectric focusing gel approached a plateau near the pI of that protein. We sometimes observed a minor spot that had a lower isoelectric point than did the bulk of the virion p27. This was most clearly seen for the RAV-2 p27 shown in Fig. 5 and the RAV-0 p27 shown in Fig. 6. Although we do not know for certain that these spots represent two forms of the same protein, it is possible that the ALSV p27 undergoes some form of post-translational modification similar to that reported for the major capsid

TABLE 2. *Genetic origin of the internal structural proteins in several RAV-60 isolates*

RAV-60 isolate	SPAFAS chicken cells used in the production of the isolate	gs Protein[a]		
		p19	p27	p15
[BH-RSV/NY1]	gs^+h_H	?[b]	BH-RSV	NM[c]
[RAV-1/NY1]	gs^+h_H	?[b]	RAV-1	NM
[RAV-1/NY2]	gs_Lh_E	RAV-1	RAV-1	NM
[RAV-2/NY1]	gs^+h_H	RAV-2	RAV-0	NM
[RAV-2/NY2]	gs_Lh_E	RAV-2	RAV-2	NM
[RAV-7/NY1]	gs^+h_H	RAV-7	RAV-7	RAV-7

[a] The gs proteins are listed in their relative order from the amino to carboxyl terminus in the Pr76 *gag* gene product (49). The determination of the genetic origin of an individual protein was based on its mobility in the two-dimensional gel system relative to the corresponding proteins of the exogenous virus and RAV-0.

[b] These proteins did not migrate with the corresponding protein of either the exogenous virus or RAV-0. They had the same isoelectric point as the exogenous viral p19, but their electrophoretic mobility in SDS gels was similar to the RAV-0 p19.

[c] NM, No electrophoretic marker in this protein was available to distinguish between the exogenous virus and RAV-0.

protein of simian virus 40 (33). Additional studies are required to define the nature and relationship between these proteins.

There have been previous reports of type-specific antigenic determinants in ALSV internal proteins. Bolognesi and co-workers (5) found what they termed subgroup-specific determinants in the p19 proteins of PR-RSV-A, PR-RSV-B, and PR-RSV-C. Stephenson et al. (43) observed type-specific determinants in all of the gs proteins except p27. Hayman and Vogt (21) used subgroup-specific antigenic determinants in p19 and p15 as markers for studies on genetic recombination in ALSV. In the present experiments we observed altered forms of p27, p19, and p15 for certain members of ALSV. The electrophoretic variants were characteristic of the individual virus strain and not of its subgroup specificity. For example, RAV-7 of subgroup C had a p15 protein that could be distinguished from that of other ALSV, including PR-RSV-C. The subgroup A viruses, SR-RSV-A and RAV-1, had different forms of p19. Studies with the subgroup E viruses demonstrated that some RAV-60s carried structural proteins that were derived from the exogenous virus used in their isolation. Therefore, no clear correlation could be made between the virus subgroup and electrophoretic markers in the internal structural proteins. It is also of interest to note that the p27 of RAV-0 had a lower isoelectric point and altered mobility in SDS gels, but type-specific antigenic determinants in this protein were not detected in previous studies (43).

The present experiments show that electrophoretic variants can be used as unselected markers in genetic studies with RNA tumor viruses. We have used structural protein markers to determine the genetic origin of gs proteins in RAV-60 produced after infection of chicken cells by exogenous ALSV. The subgroup E viruses, RAV-0 and RAV-60, could initially be distinguished only by their relative rates of growth on permissive cells (18). Two mechanisms were postulated for the formation of RAV-60 (19): (i) activation of a latent endogenous chicken virus by infection with exogenous ALSV or (ii) recombination between endogenous and exogenous viral genes. Hybridization studies using complementary DNA probes have established that RAV-60[RAV-2/NY1] has nucleic acid sequences specific for both the endogenous and exogenous viruses (23). Moreover, infection by exogenous ALSV does not significantly alter the expression of endogenous viral glycoprotein genes (24). These results support the recombination model for RAV-60 production.

We have demonstrated that some isolates of RAV-60 carry structural protein markers supplied by the exogenous virus. However, in some of our RAV-60s from gs^+h_H cells, at least one gs protein was apparently derived from endogenous viral information because it did not migrate with the corresponding protein of the exogenous virus. RAV-60[RAV-2/NY1] had an altered p27 protein that comigrated with the p27 of RAV-0 obtained from line 7 × 15 cells. We interpret this result as direct evidence that RAV-0 is, at least in part, related to endogenous viral information in SPAFAS chickens. RAV-60[BH-RSV/NY1] and RAV-60[RAV-1/NY1] had p19 proteins that did not migrate with p19's of either the exogenous virus or RAV-0. These may be hybrid polypeptides with sequences derived from both the exogenous virus and endogenous RAV-0. Alternatively, RAV-0 may be only partially homologous to the endogenous oncovirus in SPAFAS chickens, and the p19's of these two RAV-60 isolates may be specified by endogenous viral information that codes for a p19 different

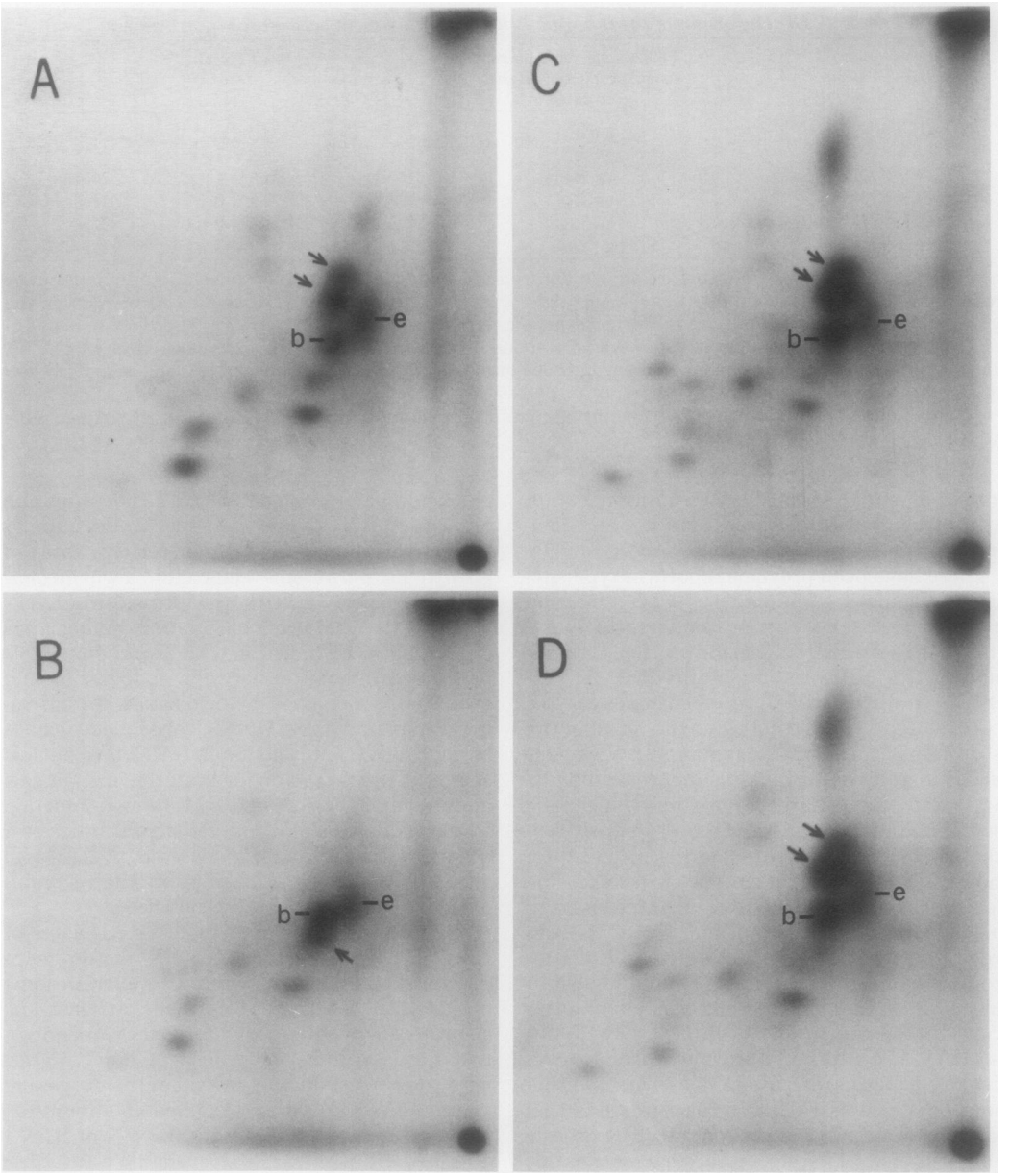

FIG. 9. *Autoradiograms of ^{125}I-labeled tryptic peptides of viral p19 proteins from two isolates of RAV-60[RAV-2]. The orientation of the panels is the same as shown in Fig. 4. (A) p19 peptides of RAV-2, (B) p19 peptides of RAV-0, (C) p19 peptides of RAV-60[RAV-2/NY1], and (D) p19 peptides of RAV-60[RAV-2/NY2]. The major ^{125}I-labeled peptides of radioiodinated p19's are marked as follows. The common b- and e-peptides are labeled in each panel. The c- and d-peptides specific to the RAV-2 p19 are indicated with arrows in (A), (C), and (D). The a-peptide of the RAV-0 p19 is also marked with an arrow in (B).*

from that of RAV-0. Analysis of the primary structures of these proteins is currently underway to distinguish between these possibilities.

Nontransforming avian oncoviruses have three genes in which markers are currently available (2): (i) *env,* which defines the subgroup properties of the viral glycoprotein; (ii) *pol,* which codes for the RNA-dependent DNA polymerase; and (iii) *gag,* which specifies the Pr76 precursor to the internal structural proteins. The phenotypic selection of RAV-60 is based on its subgroup E host range properties, and therefore

the *env* gene of these viruses is apparently derived from the endogenous oncovirus in chicken cells. There is recent evidence that the *pol* gene of RAV-60 is obtained from the exogenous virus (R. C. Sawyer and H. Hanafusa, unpublished data). It is possible that the p15 of RAV-60 is also supplied by the exogenous virus. We examined eight independent isolates of RAV-60[RAV-7] from gs^+h_H cells and found that all carried the p15 marker of RAV-7. Radioimmunoassay techniques have established that uninfected gs^+h_H chicken cells contain viral p27 and p19 proteins, but no detectable p15 (42; J. H. Chen, personal communication), suggesting that the latter protein may be defective or deleted in the endogenous virus. This is supported by the findings that the largest size class of cytoplasmic viral RNA in gs^+h_H cells has only about 80% of the genetic information found in the complete RAV-0 genome (51) and that viral gs proteins present in these cells are in the form of an unprocessed precursor that lacks p15-specific tryptic peptides (R. Eisenman, personal communication). We have shown that the p27 and p19 proteins of RAV-60s are sometimes combinations of the respective proteins from exogenous and endogenous viruses. We assume that this is due to crossing-over within the *gag* genes of the parental genomes during the formation of RAV-60. These studies will be combined with other techniques in an effort to determine the point in the viral life cycle at which this recombination takes place.

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