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Type C Viral *gag* Gene Expression in Chicken Embryo Fibroblasts and Avian Sarcoma Virus-Transformed Mammalian Cells

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Sensitive radioimmunoassays were developed for avian type C viral *gag* gene-coded proteins. These assays were used to examine the restriction to virus production by avian embryo cells and mammalian cells transformed by avian sarcoma viruses. The results indicate that although a high-molecular-weight primary translational product of the *gag* gene is expressed, its cleavage and processing are incomplete. Furthermore, analysis of intermediate cleavage products provided information regarding the order of sequences coding for the individual viral proteins within the avian type C viral *gag* gene.

Studies of avian RNA tumor viruses have led to the identification of four distinct regions of the viral genome. These include genes coding for the RNA-dependent DNA polymerase, the envelope glycoprotein, and several lower-molecular-weight proteins synthesized in the form of a common precursor (7, 28). These regions of the viral genome have been designated *pol*, *env*, and *gag*, respectively (2). A fourth viral gene, *src*, has been implicated in malignant transformation (8, 16, 19). The relative positions of the known structural genes from the 5′ to 3′ end of the avian sarcoma virus genome have tentatively been established as *gag-pol-env-src* (15, 30 to 32).

Several approaches have been used to map regions of the *gag* gene of type C RNA viruses coding for individual viral structural proteins. One technique that has been applied to studies of avian type C viruses has been the use of pulse-labeling experiments with pactamycin-treated, virus-infected embryo cultures (9, 28, 29). The results of these studies have provided suggestive evidence for the presence of a 19,000-molecular-weight protein (p19) at the amino-terminal end of the avian type C viral *gag* gene product and a 15,000-molecular-weight protein (p15) at its carboxy terminus.

An approach used in mapping the mammalian type C viral *gag* gene has involved the application of competition immunoassays, specific for individual viral proteins, to the analysis of the components of the *gag* gene precursor protein and its intermediate cleavage products (3). The possibility that the *gag* gene of avian type C viruses might be mapped by an approach analogous to that used for the mammalian type C viral genome was suggested by the demonstration that certain chicken embryo cells (5, 6, 25), as well as several avian tumor virus-transformed mammalian cell lines (9, 22), express *gag* gene translational products in the absence of infectious virus production. In the present study, such cells were analyzed by competition immunoassay for the presence of *gag* gene precursor polypeptides and their intermediate cleavage products in an attempt to order genetic sequences coding for individual viral proteins within the avian type C viral *gag* gene.

MATERIALS AND METHODS

Viruses. Avian myeloblastosis virus (AMV) in chicken plasma, supplied by J. Beard, and the Prague strain of Rous sarcoma virus (Prague RSV), from University Laboratories, were obtained through the courtesy of J. Gruber, Office of Resources and Logistics, National Cancer Institute.

Chicken embryos and avian sarcoma virus-transformed cell lines. Leukosis-free chicken embryos were obtained from SPAFAS Inc., Norwich, Conn. Several RAV-0-positive chicken embryos (line 100) were a gift of L. B. Crittenden, U.S. Department of Agriculture Regional Poultry Research Laboratory, East Lansing, Mich. A clonal Schmidt-Ruppin RSV-transformed rat kidney cell line, SR-NRK, and the XC cell line, originally derived from a Wistar rat tumor induced with Prague RSV, have been described (23, 27).

Preparation of cell extracts. Embryo cell extracts were prepared by homogenization of minced 12-day

whole embryos for 30 s in an equal volume of 0.01 M Tris-hydrochloride (pH 7.8), 1 mM EDTA, 0.01 M NaCl, 1 mM dithiothreitol, and 10% glycerol. Embryo cell homogenates and tissue culture fibroblasts resuspended in the same buffer were subjected to sonic treatment for 30 s (Biosonic II sonic oscillator) and centrifuged at 20,000 rpm for 30 min in a Beckman type 30 rotor. Post-microsomal supernatants were tested for protein by the method of Lowry et al. (18), divided into equal portions, and stored at −70°C.

Purification of viral proteins. Viral proteins were purified from density gradient-purified AMV. Virus was disrupted in 0.1 M Tris-hydrochloride (pH 9.0) buffer containing 1.0% Triton X-100, sonically treated for 30 s, and centrifuged at 20,000 rpm in a Beckman type 30 rotor for 30 min. The process was repeated, and the pooled supernatants were dialyzed overnight against 200 volumes of BET buffer [0.01 M *N*,*N*-bis-(2-hydroxyethyl)-2-amino-ethanesulfonic acid (pH 6.5), 0.001 M EDTA, and 0.5% Triton X-100]. Solubilized proteins were applied to a phosphocellulose column (1.5 by 5 cm; Whatman P11, H. Reeve Angel and Co., Clifton, N.J.) equilibrated with BET. The column was washed with 50 ml of BET, and 2-ml fractions of a 0.0 to 0.5 M linear KCl gradient were collected. Individual viral structural proteins were localized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or competition radioimmunoassay as previously described (4, 5, 13, 17, 20, 22, 26).

Unbound proteins in the wash containing predominantly p12 were lyophilized, resuspended in 0.2 ml of 0.05 M Tris-hydrochloride buffer (pH 8.5) containing 0.01 M dithiothreitol, 0.002 M EDTA, and 8 M guanidine hydrochloride (GuHCl), applied to an agarose A-0.5 (100 to 200 mesh) (Bio-Rad Products, Richmond, Calif.) column (1.5 by 90 cm), and subjected to gel filtration in the presence of sodium phosphate buffer (pH 6.5) containing 6 M GuHCl and 0.01 M dithiothreitol. Fractions eluting at the same molecular-weight regions as ^{125}I-labeled AMV p12 marker were dialyzed against Tris-hydrochloride buffer (pH 7.5) containing 0.01 M NaCl and 0.1% Triton X-100, divided into equal portions, and stored under liquid nitrogen.

Proteins eluting from phosphocellulose between 0.01 and 0.05 M KCl were concentrated with a hollow fiber device (Bio-Rad Products) and subjected to further purification by gel filtration on an Ultrogel AcA 54 column (1.5 by 90 cm; LKB Products, Rockville, Md.) in 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 0.1 M NaCl and 0.1% Triton X-100. Fractions co-chromatographing with an ^{125}I-labeled AMV p27 marker were divided into equal portions and stored under liquid nitrogen. Viral p19 eluting from the phosphocellulose column between 0.1 and 0.2 M KCl was further purified by agarose gel filtration in the presence of 6 M GuHCl as described above for p12. Viral p15, eluting from phosphocellulose between 0.2 and 0.4 M KCl, was dialyzed against 0.01 M Tris-hydrochloride buffer (pH 8.5) containing 0.001 M EDTA and 0.1% Triton X-100 and applied to a DEAE-cellulose column (1.5 by 5 cm; Whatman DE52) previously equilibrated with the same buffer. The column was washed with the same buffer, and p15 eluted in the wash fraction while the major contaminants adhered to the column.

Radioimmunoassays. Viral proteins were labeled with ^{125}I (14 mCi/mol; Amersham/Searle, Arlington Heights, Ill.) by the chloramine T method (12). Double-antibody competition radioimmunoassays were performed as described previously (23). Serial twofold dilutions of purified proteins, detergent-disrupted virus, or tissue homogenates were tested for the ability to compete with ^{125}I-labeled viral proteins for binding limiting amounts of porcine antisera prepared against AMV proteins. Reaction mixtures (0.2 ml) contained 0.01 M Tris-hydrochloride (pH 7.8), 0.4% Triton X-100, 0.001 M EDTA, 1% bovine serum albumin, and either 0.05 M NaCl (p27 and p12 immunoassays) or 0.3 M NaCl (p19 and p15 immunoassays). Unlabeled antigen and antisera were incubated at 37°C for 1 h. ^{125}I-labeled antigen was added with incubation at 37°C for 3 h and then at 4°C for 18 h. Goat antiserum against porcine immunoglobulin G (0.025 ml) was then added to each reaction. After incubation for 1 h at 37°C and an additional 6 h at 4°C, the precipitate, obtained by centrifugation at 2,500 × *g* for 15 min, was counted in a gamma scintillation counter. All antisera were generously provided by R. Wilsnack through the Office of Resources and Logistics, National Cancer Institute.

Agarose gel column chromatography. Cell extracts were analyzed by agarose gel filtration in the presence of 6 M GuHCl according to previously described procedures (10). Briefly, around 25 mg of cell extract was lyophilized, resuspended in 0.2 ml of 0.05 M Tris-hydrochloride (pH 8.5), 0.01 M dithiothreitol, 0.002 M EDTA, and 8 M GuHCl, and applied to an agarose A-5 column (1.5 by 90 cm) in the presence of sodium phosphate (pH 6.5)-6 M GuHCl-0.01 M dithiothreitol buffer. Fractions (0.5 ml) were dialyzed for 18 h against 0.01 M Tris-hydrochloride buffer (pH 7.8) containing 0.1% Triton X-100 and tested at serial twofold dilutions in competition immunoassays for individual viral proteins.

RESULTS

Molecular-size analysis of type C viral antigens expressed in RAV-0-positive chicken embryo cells. The four avian tumor virus structural proteins isolated in the present study were ^{125}I-labeled by the chloramine T procedure as described in Materials and Methods. Each was shown by SDS-PAGE molecular-size analysis to exhibit a high degree of radiochemical purity. Further, all four labeled antigens could be over 90% precipitated by antibody to AMV, but not by control sera prepared against type C viruses of mammalian origin. Competition immunoassays developed by use of these ^{125}I-labeled proteins were highly specific for each individual protein (data not shown). In initial studies, embryo cells of line 100 chickens, known to be constitutively positive for RAV-0

TABLE 1. *Type C viral antigen expression in chicken embryos and avian tumor virus-transformed mammalian cells*[a]

Cells tested	Level of viral protein (ng of antigen/mg of cell extract)			
	p27	p19	p15	p12
RAV-0-positive chicken embryos				
6091	1,230	1,040	800	700
6092	1,650	1,170	910	740
6093	995	700	560	450
SPAFAS embryo cells				
7320	307	250	10	150
7321	40	35	<2	20
7322	250	155	<2	110
7324	490	340	<2	230
Mammalian fibroblast cultures				
XC	85	60	50	40
SR-NRK	65	45	40	30
NRK	<2	<3	<2	<0.5

[a] Extracts were prepared and assayed by serial twofold dilutions in competition immunoassays for viral protein as described in the text. The results represent mean values from three separate determinations.

production, were tested by competition immunoassay for avian type C viral antigen expression. Each of three individual embryos examined expressed p27, p19, p15, and p12 at relatively high levels (Table 1). Several extracts were pooled and subjected to molecular-size analysis by agarose gel filtration. The main peaks of p27, p19, p15, and p12 antigenic reactivity were observed at molecular weights of 27,000, 19,000, 15,000, and 12,000, respectively (Fig. 1). A minor portion of the p19 antigenic reactivity chromatographed at a molecular weight of 15,000, consistent with an earlier report of the breakdown of p19 into a 15,000-dalton moiety (11).

Expression and molecular-size analysis of type C virus-coded proteins in virus-negative chicken (SPAFAS) embryo cells. In an attempt to internally map the avian RNA tumor virus *gag* gene, a number of randomly selected virus-negative SPAFAS chicken embryos were tested for viral antigen expression. The majority of the embryos examined were characterized by relatively high levels of p27, p19, and p12 (Table 1). In contrast, despite the similar sensitivity of the immunoassay for p15, three of the four representative embryos shown lacked detectable reactivity, whereas in the fourth embryo the level of p15 expression was at least 15-fold lower than that of the remaining *gag* gene proteins. The detection of p15 in one embryo was consistent with previous findings that some SPAFAS embryos contain low levels of immunologically reactive p15 (6). These findings confirm and extend the previous results of Smith et al. (22) demonstrating coordinate expression of p27 and p19, in the absence of detectable p15, in embryo cells of selected inbred chickens.

In an effort to characterize intermediate cleavage products of the *gag* gene-coded precursor, several SPAFAS embryo extracts containing high levels of p27, p19, and p12 were pooled and subjected to molecular-size analysis (Fig. 2). The absence of detectable p15 immunological reactivity in partially purified column fractions confirms the results obtained with crude extracts, indicating noncoordinate *gag* gene

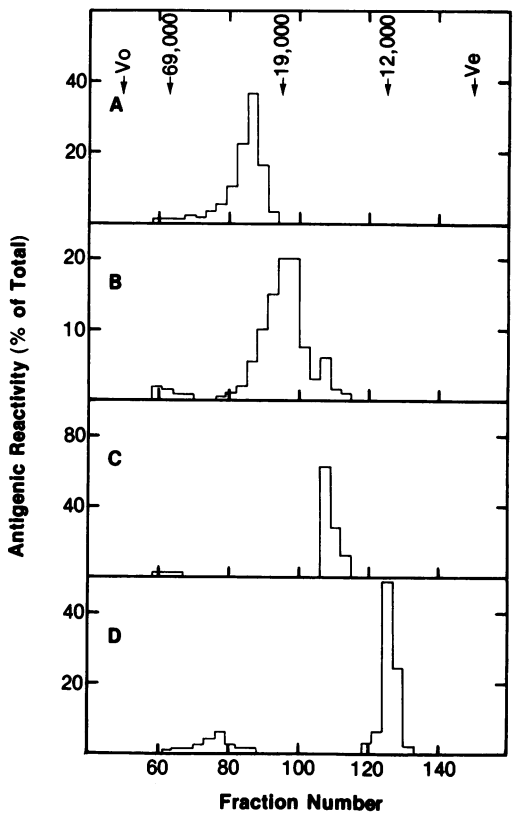

FIG. 1. *Molecular-size analysis of avian type C viral antigen expressed in the RAV-0-positive line 100 chicken embryo cells. Twenty-five milligrams of cell extract, prepared as described in the text, was subjected to agarose gel filtration in the presence of 6 M GuHCl (10). Fractions were tested at serial twofold dilutions as described in the text in competition immunoassays for the following avian type C viral proteins: (A) p27; (B) p19; (C) p15; and (D) p12. Results are expressed as the percentages of total antigenic reactivity per fraction. Molecular-weight standards included tracer amounts of ^{125}I-labeled bovine serum albumin (69,000), AMV p19 (19,000), and AMV p12 (12,000).*

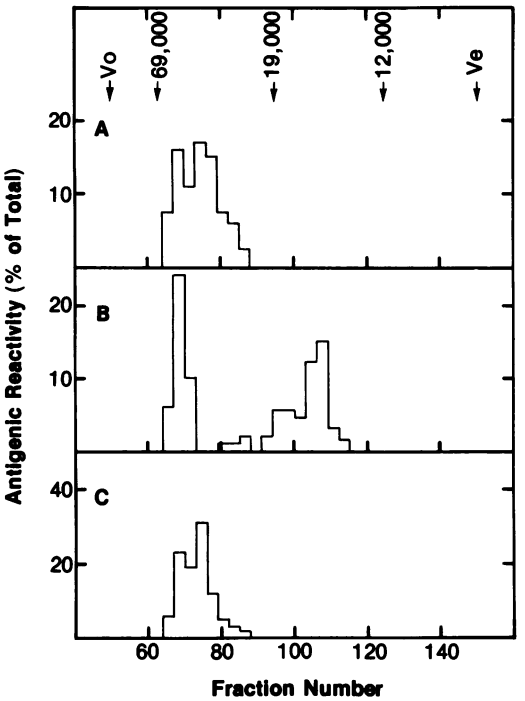

FIG. 2. *Molecular-size analysis of avian type C viral antigen expression in SPAFAS chicken embryo cells. Extracts were prepared, chromatographed, and tested in competition immunoassays for (A) p27, (B) p19, and (C) p12 as described in the legend to Fig. 1.*

expression in SPAFAS embryo cells. The remaining three *gag*-coded antigens were primarily detected in fractions eluting at high molecular weight relative to standards. The highest-molecular-weight component (60,000) competed in immunoassays for p27, p19, and p12. In addition, a peak containing p12 and p27 in the absence of p19 was detected at a molecular weight of 35,000 to 40,000. These results suggest the terminal location of p15 within the *gag* gene product and, in addition, indicate that p12 and p27 occupy adjacent positions.

Analysis of virus-coded proteins and intermediate cleavage products in avian sarcoma virus-transformed mammalian cells. To further study expression and to define the relative order of the viral structural proteins within the precursor protein, the above studies were extended to analysis of type C viral antigen expression in mammalian cells nonproductively transformed by avian sarcoma viruses. Analysis of the XC and SR-NRK cell lines indicated relatively high levels of expression of all four *gag* gene products (Table 1). In contrast, a control culture of normal rat kidney cells lacked detectable reactivity in any of these assays.

Molecular-size analysis of viral protein expression in SR-NRK cells indicated that the extent of precursor protein cleavage was limited. A 75,000-molecular-weight precursor containing antigenic reactivities of all four *gag* proteins was detected (Fig. 3). In addition, intermediate precursor cleavage products of approximately 35,000 to 40,000 daltons containing reactivity for p12 and p27 and a 60,000-molecular-weight intermediate cleavage product containing p27, p19, and p12 were observed. A less pronounced peak containing p12 and p19 was detected at a molecular weight of about 30,000. These findings are consistent with the results obtained by analysis of viral antigen expression in SPAFAS chicken embryo cultures and, in addition, provide evidence for the possibility that p19 and p12 occupy adjacent positions within the primary translational product of the *gag* gene. However, because of the minor na-

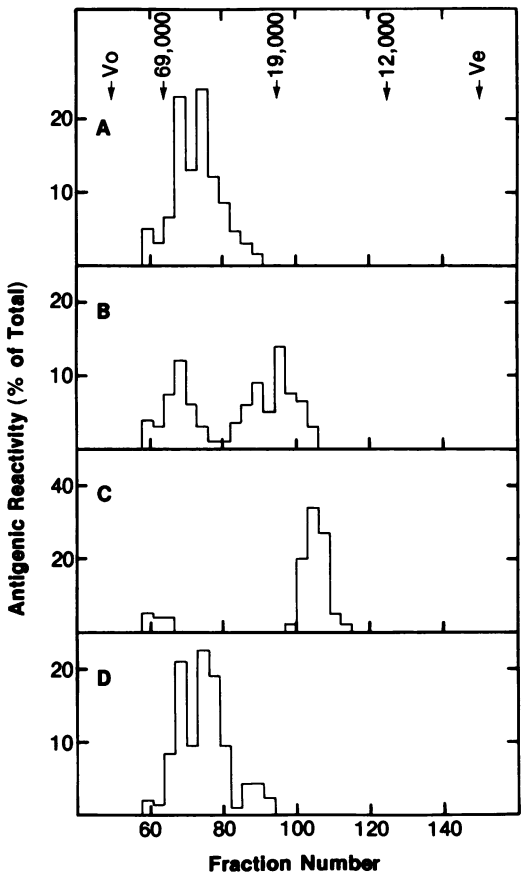

FIG. 3. *Molecular-size analysis of avian type C viral antigen expression in SR-NRK cells. Extracts were prepared, chromatographed, and tested in competition immunoassays for (A) p27, (B) p19, (C) p15, and (D) p12 as described in the legend to Fig. 1.*

ture of this latter peak, it is not possible on the basis of this evidence alone to conclusively establish this portion of the sequence.

DISCUSSION

It has previously been established that several nonglycosylated avian type C viral structural proteins, synthesized in the form of a high-molecular-weight precursor protein, are coded by a viral gene designated as *gag* (2, 21, 28). In the present study, the *gag* gene-coded precursor polypeptide was shown to include antigenic reactivities corresponding to each of four avian type C viral structural proteins, p27, p19, p15, and p12. The virus-coded nature of these proteins was indicated by the fact that each was demonstrable in avian tumor virus-transformed mammalian cells and by the finding that sera from hamsters bearing avian sarcoma virus-induced tumors contain antibodies directed against all four proteins (unpublished observations). The possible existence of a fifth low-molecular-weight avian type C virus-coded structural protein (p10) of 8,000 to 10,000 daltons has been proposed (10). In the present studies, the isolation of a 10,000-molecular-weight protein immunochemically distinct from the other viral proteins was not achieved using either AMV or the Prague RSV. Moreover, the experimentally determined molecular weights of the *gag* gene precursor and each of the intermediate cleavage products detected could be accounted for by their known *gag* gene components.

The use of molecular-size analysis and radioimmunoassays provides an approach for the localization of individual virus structural proteins within precursor polypeptides (3, 24). The location of p15 was deduced from an examination of its expression in avian cells. Embryo cells of one particular line of inbred chickens (line 6) were previously reported to lack p15 while expressing p27 and p19 (22). In the present study, a number of randomly bred SPAFAS embryos also were shown to express *gag* gene proteins p27, p19, and p12 in the absence of detectable p15. This situation would most likely arise from a mutation located toward the 3' end of the *gag* gene and hence the carboxy-terminal end of the precursor protein. A terminal position for p15 within the *gag* gene translational product is further indicated by the isolation of a precursor from SPAFAS embryo cells containing p27, p19, and p12 in the absence of p15.

Immunological analysis of the avian *gag* gene-coded 76,000-dalton precursor and its intermediate cleavage products provides a means of further ordering the genetic sequences coding for viral structural proteins within the avian RNA tumor virus *gag* gene. The isolation from several types of avian virus antigen containing cells of an approximately 40,000-molecular-weight protein immunologically reactive in competition immunoassays for both p12 and p27 indicates that these two proteins occupy adjacent positions within the *gag* gene product. In addition, there was evidence of a less pronounced peak containing immunological reactivity of p19 and p12 at approximately 25,000 to 30,000 daltons, suggesting that p19 and p12 are also adjacent. Earlier mapping studies, in which immunoprecipitates prepared from pulse-labeled and pactamycin-treated avian type C virus-infected cells were analyzed, suggested the localization of p19 at the amino-terminal end, with p27 in the center and p15 at the carboxy terminus of a 76,000-dalton precursor (9, 28). The demonstration that, of the major internal proteins of AMV and Prague strain RSV, only p19 was found to have a blocked amino terminus further suggests its location at the amino-terminal end of the *gag* gene primary translational product (14). The avian type C viral *gag* gene-coded precursor sequence most consistent with the present as well as previous (9, 29) results is NH_2-p19-p12-p27-p15-COOH. However, conclusive demonstration of this sequence will require the isolation and detailed characterization of each of the *gag* gene-coded intermediate cleavage products.

The present findings have implications regarding the restriction to endogenous avian leukosis virus expression in both virus antigen-positive chicken embryo cells and avian sarcoma virus-transformed mammalian cells. In both cell types, post-translational cleavage of the primary *gag* gene product was incomplete. Moreover, leukosis virus-negative, SPAFAS chicken embryo cells were shown to express p19, p12, and p27 in the absence of p15. The lack of p15 immunological reactivity could be attributable either to a partial deletion of this region of the genome or to a mutation in the viral genome causing premature termination of translation. The finding of defective cleavage of RSV *gag* gene-coded polyprotein precursors in nonproductively infected mammalian fibroblasts is consistent with a previous report by Eisenman et al. (9) and argues for a cell-coded origin of the cleavage enzymes. Furthermore, the fact that the *gag* gene precursor in cells productively infected with RAV-0 undergoes complete post-translational cleavage argues that the endogenous RAV-0 *gag* gene may code for a defective precursor polyprotein that is not recognized by the cellular cleavage enzymes. This could result from amino acid substitutions

in the primary structure of the precursor or, alternatively, may be a consequence of conformational changes resulting from the lack of p15.

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