JOURNAL OF VIROLOGY, July 1975, p. 27-33

Vol. 16, No. 1
Printed in U.S.A.

Analysis of Antigenic Determinants of Structural Polypeptides of Avian Type C Tumor Viruses

JOHN R. STEPHENSON, EUGENE J. SMITH, LYMAN B. CRITTENDEN, AND STUART A. AARONSON*

National Cancer Institute, Bethesda, Maryland 20014, and Animal Physiology and Genetics Institute, Beltsville, Maryland 20705*

Received for publication 4 March 1975

Radioimmunoassays were developed for the 19,000, 15,000, and 12,000 molecular weight polypeptides of avian myeloblastosis virus and for the 19,000 and 12,000 polypeptides of RAV-0, a subgroup E avian tumor virus. Each polypeptide was shown to possess both group- and type-specific antigenic determinants, in contrast to the 27,000 mol wt polypeptide, which contained only group-specific determinants. The corresponding low-molecular-weight polypeptides of subgroup A, B, and E viruses were shown to be immunologically indistinguishable. The findings that low-molecular-weight polypeptides of subgroup C and D viruses reacted very differently in immunoassays for the respective polypeptides of avian myeloblastosis virus or RAV-0 suggest that subgroups C and D may have evolved differently from subgroups A, B, and E.

The development in recent years of radioimmunoassays for the structural components of type C RNA viruses has greatly increased the sensitivity with which these viruses can be detected. For example, in chicken embryo cultures that are negative by standard complement fixation techniques, the 27,000 mol wt polypeptide of the avian type C RNA virus can be readily quantitated by radioimmunoassays (3, 16, 17). More recently, immunoassays for two additional avian type C viral polypeptides of mol wt 19,000 and 15,000 have been used to demonstrate their coordinate expression with the 27,000 mol wt polypeptide in cells of chicken embryos that lack any evidence of virus release (4).

Immunoassays for type C viral polypeptides have also proven very useful in the identification of new virus isolates. In particular, immunoassays for low-molecular-weight polypeptides of several mammalian type C viruses have been shown to be highly type specific and thus to provide specific markers for different strains of the same virus species (14, 15, 18). In the present studies, the immunological relatedness of different avian type C viruses was investigated. Competition immunoassays for structural components of two prototype avian type C virus strains were developed and used to compare the reactivities of representatives of major avian virus subgroups.

MATERIALS AND METHODS

Viruses. Avian myeloblastosis virus (AMV), obtained from plasma of infected chickens (16), was kindly provided by J. W. Beard through the Resources and Logistics Segment, National Cancer Institute. Representative avian leukosis viruses of subgroups A and B were provided by P. K. Vogt, as pseudotypes of the Bryan high-titer Rous sarcoma virus (BH-RSV) (23). These viruses, designated BH-RSV(RAV-1) and BH-RSV(RAV-2), were grown in line 100 C/B and line 100 C/A, respectively. Clonal stocks of the Prague strain of RSV belonging to subgroups A (Pr-RSV-A) and C (Pr-RSV-C), obtained from R. E. Smith (13), and a Carr-Zilber stock of subgroup D (CZ-RSV-D), supplied by P. K. Vogt (6), were grown to high titer in fibroblasts of specific pathogen-free embryos (SPAFAS, Inc). RAV-0, an endogenous subgroup E avian tumor virus, was grown in line 100 C/A fibroblasts (5). Tissue culture-propagated virus stocks were shown to be negative for the presence of virus of other subgroups by standard host-range and interference assays (23).

Isolation of avian type C viral polypeptides. Plasma from AMV-infected chickens was clarified by centrifugation at 10,000 rpm for 15 min, and virus was pelleted from the supernatant by centrifugation at 25,000 rpm for 1 h. The pellet was resuspended in 1 ml of 0.01 M Tris-hydrochloride, 1 mM EDTA, pH 7.8, buffer and the virus was purified by density gradient centrifugation as previously described (16). A sample (2 mg) of purified virus was dialyzed against 0.2 M borate buffer, pH 9.0, and labeled with [^{3}H]formaldehyde at a specific activity of approximately 1 μCi/mg as described by Velicer and Graves (20). After dialysis and lyophilization, the labeled preparation was mixed with 7 mg of unlabeled AMV, disrupted by exposure to 6 M guanidine hydrochloride (GuHCl) for 30 min at 45 C, and chromatographed on an agarose column (1.5 by 90 cm) (Bio Gel A-5m, 200 to 400 mesh, BioRad Laboratories, Richmond, Va.) at room temperature at

a flow rate of 1.5 ml/h in the presence of 6 M GuHCl according to previously published methods (7). Fractions corresponding to the 19,000, 15,000, and 12,000 mol wt radioactive peaks were dialyzed against 0.01 M Tris, pH 8.0. RAV-0 polypeptides were isolated from virus obtained from tissue culture fluids using procedures described above. Molecular weights relative to standards including *Escherichia coli* alkaline phosphatase (40,000 mol wt), carbonic anhydrase (29,000 mol wt), β-lactoglobulin (18,400 mol wt), lysozyme (14,300 mol wt) and cytochrome *c* (12,400 mol wt) were determined as previously described (15).

Iodination. Purified viral polypeptides were labeled with ^{125}I at specific activities of 10 to 20 μCi/μg according to the chloramine T procedure of Greenwood et al. (8).

Radioimmunoassays. A double antibody radioimmunoassay for the AMV 27,000 mol wt major structural polypeptide, p27, has been described previously (16). By using the same methods, competition immunoassays were developed in the present study for the 19,000, 15,000, and 12,000 mol wt polypeptides of AMV and the 19,000 and 12,000 mol wt RAV-0 polypeptides. Pig antisera against detergent-disrupted AMV and goat antisera against RAV-0 and Pr-RSV-V as well as goat anti-pig immunoglobulin G and pig anti-goat immunoglobulin G were generously provided by R. Wilsnack through the Resources and Logistics Segment, National Cancer Institute.

RESULTS

Reactivities of avian tumor viruses of different subgroups in homologous and heterologous AMV p27 immunoassays. Studies were performed to determine whether viruses of different avian tumor virus subgroups could be distinguished on the basis of type-specific antigenic determinants of their p27 polypeptides. For this purpose, the reactivities of AMV and representative viruses of each of five avian tumor virus subgroups were tested in a homologous immunoassay for AMV p27. This assay measures the ability of unlabeled antigen to compete with a limiting concentration of anti-AMV serum for binding ^{125}I-labeled AMV p27 (16). In addition, each virus isolate was tested in a heterologous p27 immunoassay in which antiserum prepared against RAV-0 was used to precipitate AMV ^{125}I-labeled p27. Each of the viruses tested, including Pr-RSV-A, BH-RSV(RAV-2), Pr-RSV-C, CZ-RSV-D, and RAV-0, reacted comparably to AMV in the homologous AMV p27 immunoassay (Fig. 1). Further, no significant differences were detected in the reactivities of any of the viruses in the heterologous p27 immunoassay (Fig. 1B). These results indicate a lack of significant type-specific differences between the p27 polypeptides of five major subgroups of avian tumor viruses.

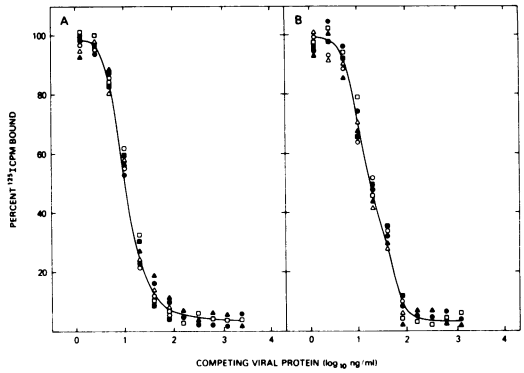

FIG. 1. *Comparison of immunological reactivities of representative avian tumor viruses in AMV p27 immunoassays. Detergent-disrupted viruses were assayed at twofold serial dilutions by measuring their capacity to compete with ^{125}I-labeled p27 for limiting antibody (16). The results are expressed as percentage of the total ^{125}I counts per minute in the antigen-antibody precipitate standardized to 100% in the absence of competing antigen. Reactivity was tested in (A) a homologous AMV p27 immunoassay utilizing anti-AMV serum with ^{125}I-labeled AMV p27; (B) a heterologous avian p27 assay in which antiserum to RAV-0 was used with ^{125}I-labeled AMV p27. The viruses included: AMV (■); RAV-0 (□); Pr-RSV-A (○); BH-RSV (RAV-2) (△); Pr-RSV-C (●); CZ-RSV-D (▲).*

Isolation and physical analysis of three low-molecular-weight AMV structural polypeptides. In an attempt to isolate three of the low-molecular-weight AMV structural polypeptides, approximately 9 mg of AMV was subjected to agarose gel filtration in 6.0 M GuHCl as described in Materials and Methods. Fractions corresponding to the 19,000, 15,000, and 12,000 mol wt peaks were pooled, dialyzed against 0.01 M Tris, pH 8.0, and labeled with ^{125}I at high specific activity (5 to 20 μCi/μg). To determine the degree of radiochemical purification achieved, each ^{125}I-labeled polypeptide was analyzed by agarose gel filtration in 6.0 M GuHCl (Fig. 2) and by sodium dodecyl sulfate gel electrophoresis (data not shown). By each procedure all three ^{125}I-labeled polypeptides were found to migrate as single peaks comprising at least 95% of the total radioactivity. The molecular weights, relative to standards, were 19,000, 15,000 and 12,000. The isolated polypeptides were, thus, designated p19, p15, and p12, respectively, according to convention (1).

Lack of immunological cross-reactivity between four major AMV structural polypeptides. Competition immunoassays were developed for each of the three newly isolated polypeptides analogous to that previously described for AMV p27 (16). To examine the possibility of

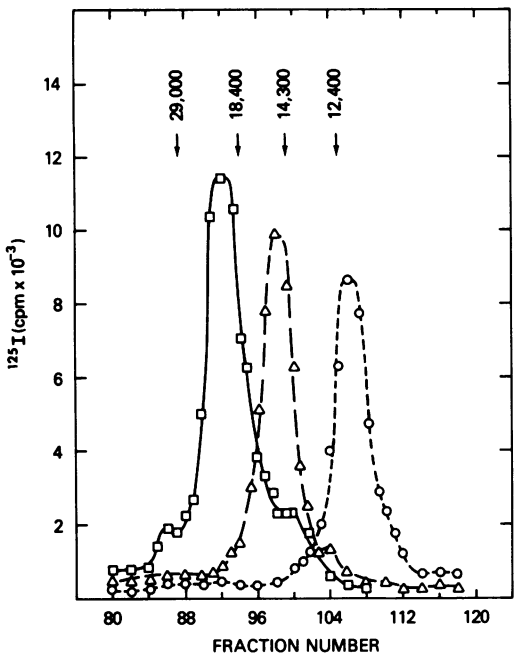

FIG. 2. *Analysis of AMV p19, p15, and p12 by gel filtration chromatography in the presence of 6 M GuHCl. Around 100,000 counts/min of ^{125}I-labeled AMV p19 (□), p15 (Δ), and p12 (○) were individually analyzed on the same agarose column. The results from all these experiments are superimposed. Molecular weights relative to standards were determined as described in Materials and Methods.*

antigenic cross-reactivity between different molecular weight AMV polypeptides, all four unlabeled polypeptides were assayed for their abilities to compete limiting anti-AMV sera for binding each ^{125}I-labeled viral polypeptide. The results summarized in Fig. 3 show that each polypeptide reacted with greatest efficiency in its respective homologous assay. The p27 and p15 assays were sufficiently sensitive to detect their homologous unlabeled viral antigens at concentrations as low as 0.5 to 1.0 ng/ml. Around 2.0 to 5.0 ng of p19 or p12 per ml was required for detection in their respective homologous assays. The differences in sensitivities of the four homologous AMV assays were due to differences in the affinities of the antiserum for each polypeptide (unpublished observations).

Unlabeled p27 failed to compete in homologous AMV p19, p15, or p12 assays, indicating its high degree of immunogical purity and lack of antigenic cross-reactivity with any of the other polypeptides. In contrast, at high concentrations unlabeled p19 competed in both p27 and p15 immunoassays. Similarly, unlabeled p15 exhibited a low level of reactivity in both p19 and p12 assays, and p12 reacted very slightly in the p15 immunoassay (Fig. 3). Wherever cross-reactivity at a low level was detected, the slope of the competition was found to be parallel to that exhibited by the homologous competing antigen. This argues that cross-reactivity was due to minor contamination (less than 2 to 3%) by other viral polypeptides rather than to shared immunological determinants among the different polypeptides.

Immunological type specificities of AMV low-molecular-weight polypeptides. To test for possible immunological type specificity of p19, p15, and p12, the reactivities of AMV and representative viruses of five avian tumor virus subgroups were tested in homologous immunoassays for each polypeptide. The results (Fig. 4) demonstrate no detectable difference between the reactivities of AMV, Pr-RSV-A, BH-RSV-(RAV-2), or RAV-0 in each assay. The slopes of the competition curves as well as the extent of competition achieved with each virus were indistinguishable. Similar results were obtained with BH-RSV(RAV-1), another subgroup A

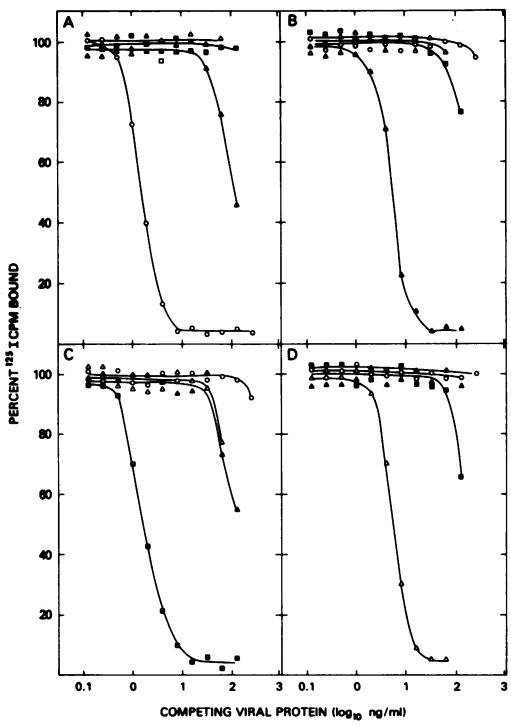

FIG. 3. *Immunological cross-reactivity between AMV structural polypeptides. Immunoassays measured the ability of unlabeled antigen to compete with a limiting concentration of anti-AMV serum for binding ^{125}I-labeled AMV (A) p27; (B) p19, (C) p15, and (D) p12. The unlabeled viral antigens tested included: AMV p27 (○); p19 (▲); p15 (■); and p12 (Δ).*

virus (data not shown). In contrast, Pr-RSV-C, a representative subgroup C virus, and CZ-RSV-D, a subgroup D virus, reacted less efficiently in immunoassays for all three polypeptides.

Heterologous immunoassay for avian tumor virus low-molecular-weight polypeptides. It was possible that the lower degree of reactivity of Pr-RSV-C and CZ-RSV-D in the homologous AMV p19, p15, and p12 immunoassays reflected either a lower concentration of these polypeptides in subgroup C and D viruses or loss of antigenicity during preparation or storage. To test these possibilities, heterologous competition immunoassays were developed by utilizing antiserum prepared against Pr-RSV-C to precipitate AMV ^{125}I-labeled p19, p15, and p12. The antiserum would be expected to recognize antigenic determinants shared by the corresponding polypeptides of Pr-RSV-C and AMV. The results in Fig. 5 demonstrate that viruses of each subgroup tested reacted similarly in heterologous immunoassays for p19, p15, and p12. These results, thus, argue that the low reactivities of Pr-RSV-C and CZ-RSV-D in the homologous immunoassays for AMV p19, p15, and p12 were due to differences in their type-specific antigenic determinants rather than to differences in the concentration of the polypeptides. These findings do not resolve how closely the p19, p15, and p12 antigens of subgroup C virus are immunologically related to the respective viral polypeptides of subgroup D.

Reactivities of avian tumor viruses in ho-

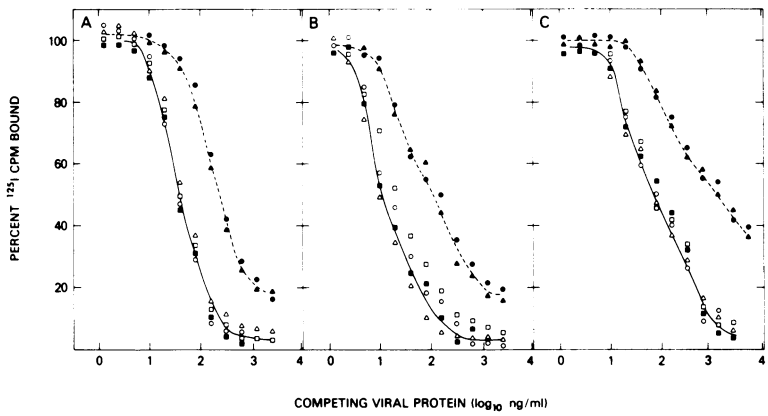

FIG. 4. *Analysis of low-molecular-weight AMV polypeptides for type-specific antigenic determinants. Detergent-disrupted viruses were tested in a homologous AMV immunoassay by utilizing anti-AMV serum in combination with ^{125}I-labeled AMV (A) p19; (B) p15; and (C) p12. The viruses tested and symbols used are as described in the legend to Fig. 1.*

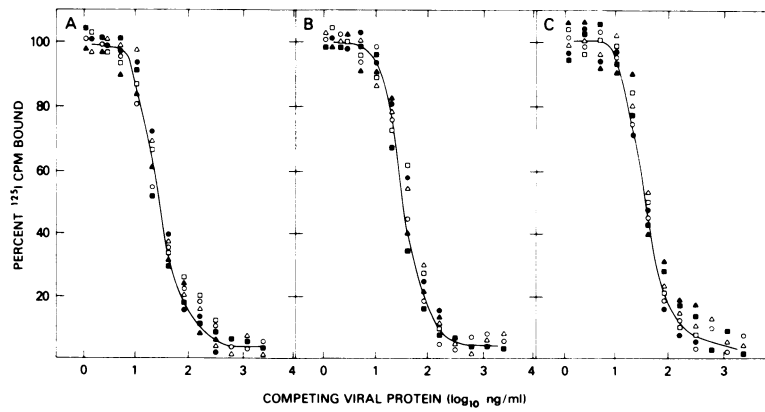

FIG. 5. *Heterologous group-specific immunoassays for avian tumor virus p19, p15, and p12 polypeptides. Detergent-disrupted viruses were tested in heterologous immunoassays by utilizing anti-Pr-RSV-C serum in combination with ^{125}I-labeled AMV (A) p19; (B) p15; and (C) p12. Viruses tested and symbols used are described in the legend to Fig. 1.*

mologous RAV-0 p19 and p12 immunoassays. There is evidence that viruses of subgroups A and B can be isolated from in vivo-propagated stocks of AMV (10). If these viruses were present in the AMV stock used here, it might account for the cross-reactivity subgroup of A and B viruses in homologous immunoassays for AMV polypeptides. Since, as shown above, subgroup E virus, an endogenous virus of chicken cells (5, 24), was also immunologically indistinguishable from AMV, immunoassays for polypeptides of this virus were developed to further examine the relatedness of subgroups A, B, E, and AMV. RAV-0, a prototype subgroup E virus, was first screened by the phenotypic mixing test (W. Okazaki, H. G. Purchase, and B. R. Burmester, submitted for publication) and found to be negative for contamination by viruses of other subgroups. From around 10 mg of RAV-0, purified by sucrose density gradient centrifugation, two polypeptides, p19 and p12, were isolated by agarose gel filtration as described in Materials and Methods. These were labeled with ^{125}I and shown to be more than 95% radiochemically pure (Fig. 6) when analyzed by agarose gel filtration in the presence of 6 M GuHCl.

Representative viruses of each of the five avian virus subgroups, as well as AMV, were tested for their reactivities in homologous RAV-0 p19 and p12 immunoassays. The results, summarized in Fig. 7, were similar to those observed with homologous immunoassays utilizing the corresponding AMV polypeptides. The reactivities of AMV, Pr-RSV-A, BH-RSV(RAV-2), and RAV-0 were indistinguishable, whereas Pr-RSV-C and CZ-RSV-D competed less efficiently in both RAV-0 p19 and p12 assays. Additional isolates of subgroups A, C, and E were examined and shown to have immunological reactivities indistinguishable from those of the prototype A, C, and E viruses tested above (data not shown). These findings support the conclusion that the type-specific determinants detected with the prototype viruses were representative of their respective subgroups.

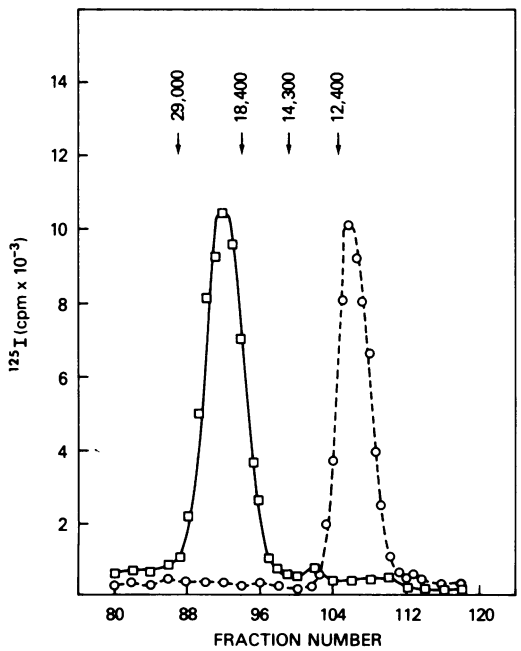

FIG. 6. *Analysis by gel filtration chromatography of ^{125}I-labeled RAV-0 p19 (□) and p12 (○) polypeptides. Molecular weights were determined as described in the legend to Fig. 2.*

DISCUSSION

The immunological reactivities of different subgroups of avian type C RNA viruses have been compared in homologous competition immunoassays for the p27, p19, p15, and p12 antigens of two prototype viruses, AMV and RAV-0. The results indicate that p27 is the most group specific of the four viral polypeptides examined. Respresentatives of five major subgroups were immunologically indistinguishable in the homologous assay for this AMV polypeptide. In contrast, three lower weight viral polypeptides, p19, p15, and p12, each demonstrated type-specific determinants. In these characteristics, the avian RNA viruses appear analogous to previously studied mammalian type-C viruses, where the 27,000 to 30,000 mol wt poly-

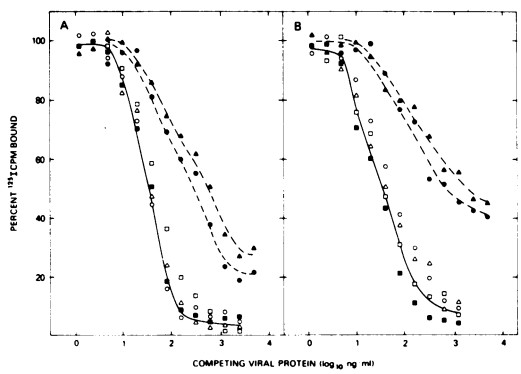

FIG. 7. *Analysis of RAV-0 p19 and p12 type-specific antigenic determinants. Detergent-disrupted viruses were tested for immunological reactivity in homologous RAV-0 immunoassays by utilizing anti-RAV-0 serum in combination with ^{125}I-labeled RAV-0 (A) p19; (B) p12. Viruses tested and symbols used are described in the legend to Fig. 1.*

peptides have also been shown to be much less type specific than the lower-molecular-weight structural polypeptides (15, 18).

Among the avian type C viruses analyzed, the reactivities of representative subgroup C and D viruses differed markedly from viruses of subgroups A, B, and E in homologous typing immunoassays utilizing either AMV or subgroup E ^{125}I-labeled viral antigens. The present findings are consistent with molecular hybridization studies indicating a lack of complete genetic homology between RAV-0 and Pr-RSV-C (11). The fact that the respective p19, p15, and p12 polypeptides of viruses of A, B, and E subgroups were immunologically indistinguishable indicates that subgroups A and B share with subgroup E virus genetic information coding for identical or at least very similar low-molecular-weight polypeptides. These subgroups do differ markedly from one another in several characteristics, including host range, neutralization, and interference (6, 23). All of these properties, however, could simply reflect differences between the glycoproteins of their viral envelope.

Subgroup E virus has been reported to be chemically inducible from virus-negative chicken cells (24), and molecular hybridization studies indicate homology of this virus with normal chicken cellular DNA (2, 12, 19, 26). Whereas these findings have provided evidence for the genetic transmission of this virus, the origin of avian tumor viruses of the other subgroups is not as yet known. Subgroup A and B viruses, whose lower-molecular-weight viral polypeptides closely resemble those of subgroup E, could have originated as a result of genetic change affecting the viral glycoprotein. It is possible that viruses of subgroups C and D, because they differ from subgroup E in several of their polypeptides, either evolved differently through horizontal transmission, or originated from different classes of endogenous virus not as yet indentified. Determination of the degree of sequence homology between representatives of each of the major subgroups, both with each other and with cellular DNA, should help to resolve these possibilities.

The type-specific determinants present on low-molecular-weight polypeptides of avian type C viruses provide useful markers for analysis of genetic recombinants (9, 21, 25). Appropriate recombinants could be isolated from selected parental viruses possessing immunologically distinguishable low-molecular-weight polypeptides as well as host range and neutralization properties. Determination of the markers in such recombinants should lead to information concerning genetic linkage between different avian tumor viral structural genes.

ACKNOWLEDGMENTS

We thank Jeanne M. LeBon for excellent technical assistance.

This work was supported in part by contracts no. NCI-E-73-3213 and YOI-CP-40214 of the Virus Cancer Program of the National Cancer Institute.

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. Baluda, M. A. 1972. Widespread presence, in chickens, of DNA complementary to the RNA genome of avian leukosis viruses. Proc. Natl. Acad. Sci. U.S.A. **60**:576–580.
3. Chen, J. H., and H. Hanafusa. 1974. Detection of a protein of avian leukoviruses in uninfected chick cells by radioimmunoassay. J. Virol. **13**:340–346.
4. Chen, J. H., W. S. Hayward, and H. Hanafusa. 1974. Avian tumor virus proteins and RNA in uninfected chicken embryo cells. J. Virol. **14**:1419–1429.
5. Crittenden, L. B., E. J. Smith, R. A. Weiss, and P. S. Sarma. 1974. Host gene control of endogenous avian leukosis virus production. Virology **57**:128–138.
6. Duff, R. G., and P. K. Vogt. 1969. Characteristics of two new avian tumor virus subgroups. Virology **39**:18–30.
7. Fleissner, E. 1971. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. I. Avian leukemia sarcoma viruses. J. Virol. **8**:778–785.
8. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labeled human growth hormone of high specific activity. Biochem. J. **89**:114–123.
9. Kawai, S., and H. Hanafusa. 1972. Genetic recombination with avian tumor viruses. Virology **49**:37–44.
10. Moscovici, C., and P. K. Vogt. 1968. Effects of genetic cellular resistance on cell transformation and virus replication in chicken hematopoietic cell cultures infected with avian myeloblastosis virus (BAI-A). Virology **35**:487–497.
11. Neiman, P. E., S. E. Wright, C. McMillin, and D. MacDonnell. 1974. Nucleotide sequence relationships of avian RNA tumor viruses: measurement of the deletion in a transformation-defective mutant of Rous sarcoma virus. J. Virol. **13**:837–846.
12. Rosenthal, P. N., H. L. Robinson, W. S. Robinson, T. Hanafusa, and H. Hanafusa. 1971. DNA in uninfected and virus-infected cells complementary to avian tumor virus RNA. Proc. Natl. Acad. Sci. U.S.A. **68**:2336–2340.
13. Smith, R. E., and E. H. Bernstein. 1973. Production and purification of large amounts of Rous sarcoma virus. Appl. Microbiol. **25**:346–353.
14. Strand, M., R. Wilsnack, and J. T. August. 1974. Structural proteins of mammalian oncogenic RNA viruses: immunological characterization of the p15 polypeptide of Rauscher murine virus. J. Virol. **14**:1575–1583.
15. Stephenson, J. R., S. R. Tronick, and S. A. Aaronson. 1974. Analysis of type specific antigenic determinants of two structural polypeptides of mouse RNA C-type viruses. Virology **58**:1–8.
16. Stephenson, J. R., R. Wilsnack, and S. A. Aaronson. 1973. Radioimmunoassays for avian C-type virus group-specific antigen: detection in normal and virus-transformed cells. J. Virol. **11**:893–899.

17. Suni, J., A. Vaheri, and E. Ruoslahti. 1973. Radioimmunoassay of avian RNA tumor virus group-specific antigens. Intervirology 1:119–126.
18. Tronick, S. R., J. R. Stephenson, and S. A. Aaronson. 1974. Comparative immunological studies of primate RNA C-type viruses. Radioimmunoassay for a low molecular weight polypeptide of woolly monkey leukemia virus. Virology 57:347–356.
19. Varmus, H. E., R. A. Weiss, R. R. Friis, W. Levinson, and J. M. Bishop, 1972. Detection of avian tumor virus-specific nucleotide sequences in avian cell DNAs. Proc. Natl. Acad. Sci. U.S.A. 69:20–28.
20. Velicer, L. F., and D. C. Graves. 1974. Properties of feline leukemia virus: in vitro labeling of the polypeptides. J. Virol. 14:700–703.
21. Vogt, P. K., and P. H. Duesberg. 1973. On the mechanism of recombination between avian RNA tumor viruses, p. 505–511. *In* F. C. Fox and W. S. Robinson (ed.), Virus research. Academic Press Inc., New York.
22. Vogt, P. K., and R. R. Friis. 1971. An avian leukosis virus related to RSV(0): properties and evidence for helper activity. Virology 44:223–234.
23. Vogt, P. K., and R. Ishizaki, 1966. Patterns of virus interference in the avian leukosis and sarcoma complex. Virology 30:368–374.
24. Weiss, R. A., R. R. Friis, E. Katz, and P. K. Vogt. 1971. Induction of avian tumor viruses in normal cells by physical and chemical carcinogens. Virology 46:920–938.
25. Weiss, R. A., W. S. Mason, and P. K. Vogt. 1973. Genetic recombinants and heterozygotes derived from endogenous and exogenous avian RNA tumor viruses. Virology 52:535–552.
26. Yoshikawa-Fukada, M., and J. D. Ebert. 1969. Hybridization of RNA from Rous sarcoma virus with cellular and viral DNA's. Proc. Natl. Acad. Sci. U.S.A. 64:870–877.