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Radioimmunological Comparison of the DNA Polymerases of Avian Retroviruses

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^{125}I-labeled DNA polymerases of avian myeloblastosis virus and spleen necrosis virus were used in a radioimmunological characterization of avian retrovirus DNA polymerases. It was shown that avian leukosis virus and reticuloendotheliosis virus DNA polymerases do not cross-react in radioimmunoassays. Within the avian leukosis virus species, species-specific and type-specific antigenic determinants of the DNA polymerase were defined. The previous finding of genus-specific antigenic determinants in avian myeloblastosis virus and Amherst pheasant virus DNA polymerases was confirmed and extended to members of all subgroups of avian leukosis virus. It was shown that there is little immunological variation between the DNA polymerases of the four members of the reticuloendotheliosis virus species. Particles with RNA-dependent DNA polymerase activity from the allantoic fluid of normal chicken eggs and from the medium of a goose cell culture did not compete for the antibodies directed against any of the sets of antigenic determinants defined in this study.

There are three species of avian retroviruses; these are avian leukosis-sarcoma viruses (ALV), avian reticuloendotheliosis viruses (REV), and pheasant viruses (PV) (8, 23, 26). Viruses of each of these species contain a characteristic RNA-dependent DNA polymerase, which is required for the synthesis of viral DNA.

DNA polymerases isolated from different strains of the same avian retrovirus species have the same size and subunit composition. In the case of ALV, the mature form of the enzyme is composed of two subunits, α (molecular weight, 60,000) and β (molecular weight, 90,000), with β being the precursor to α (6, 17, 21, 24). The REV DNA polymerase is a single polypeptide with a molecular weight of 70,000 to 80,000 (16, 18). The DNA polymerase of Amherst PV (APV), a member of the PV species, is an enzyme with a molecular weight of 150,000 and is composed of subunits. Its active site is antigenically related to the active site of avian myeloblastosis virus (AMV) DNA polymerase (5).

Previous comparisons of RNA-dependent DNA polymerases within the ALV species, within the REV species, and between these two species have been based on immunoglobulin G (IgG) inhibition tests or blocking assays. In all cases, the nature of the tests restricted the analysis to determinants at the active sites of the enzymes (14, 15, 19, 22).

DNA polymerases of several strains of ALV were indistinguishable in IgG inhibition tests, indicating a close relationship of these DNA polymerases, at least at the active site. With the exception of a radioimmunological comparison of Prague strain Rous sarcoma virus (subgroup A) (Pr-RSV-A) and AMV DNA polymerases, no immunological characterization of the entire ALV DNA polymerases has been published (20). The comparison of Pr-RSV-A and AMV DNA polymerases demonstrated a type-specific difference between the two DNA polymerase molecules. Other indications of differences among ALV DNA polymerases came from a comparison of the tryptic peptides of Pr-RSV-C and AMV DNA polymerases (6) and from an enzymological comparison of AMV and Rous-associated virus-0 (RAV-0) DNA polymerases (3). No systematic comparison of ALV DNA polymerases has been published.

In the case of REV DNA polymerases, a strong inhibition of activity of DNA polymerases of all members of the species indicated a close serological relationship (14).

Serological relationships between REV and ALV DNA polymerases were demonstrated in IgG inhibition tests in which antiserum to AMV DNA polymerase was used and in IgG absorption studies (15). The finding of serological relationships between REV and ALV DNA polymerases was, however, contrasted to the findings that antisera to ALV DNA polymerases other than AMV did not inhibit the activity of spleen necrosis virus (SNV) DNA polymerase and antiserum to SNV DNA polymerase did not inhibit

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the activity of ALV DNA polymerases (5, 15). The degree of cross-reaction between REV and ALV DNA polymerases, therefore, remained unclear.

We have recently shown that, in the case of the DNA polymerases of the related viruses ALV and PV, the conservation of common antigenic sites is much greater at the active site than in the rest of the molecule (5). Therefore, the IgG inhibition tests previously used do not seem to be the best method to estimate the degree of variability of DNA polymerases within a species or to give a good estimate of the relationships among DNA polymerases of different species. Therefore, we performed a systematic comparison of avian retrovirus DNA polymerases, using radioimmunological techniques.

Radioimmunoassays have been successfully used to demonstrate relatedness among mammalian retrovirus DNA polymerases (1, 10, 11). Although a radioimmunoassay for AMV DNA polymerase has been described previously (20), it was used only for quantitation of DNA polymerase content in AMV and for a comparison of Schmidt-Ruppin RSV (subgroup A) (SR-RSV-A) and AMV DNA polymerases.

In this study we describe a radioimmunological characterization of avian retrovirus DNA polymerases. Our data show that ALV and REV DNA polymerases do not cross-react in radioimmunoassays. Within the ALV species, we define species-specific and type-specific antigenic determinants of the DNA polymerases. We also confirm the finding of genus-specific antigenic determinants in AMV and APV DNA polymerases (5), and, in this study, extend the finding of genus-specific determinants to members of all subgroups of ALV. We also present data which show that within the REV species the DNA polymerases are immunologically homologous.

It has been shown recently that allantoic fluids of embryonated chicken eggs contain particle-associated RNA-dependent DNA polymerase activity (2). A similar phenomenon has been described for cells of uninfected goose embryos (4). In both cases the significance and origin of the enzyme was unclear. The availability of sensitive radioimmunoassays prompted us to test for immunological relationships between particle-associated RNA-dependent DNA polymerase activities and avian retrovirus DNA polymerases. None of the antigenic determinants defined during this study was shared by the particle-associated RNA-dependent DNA polymerase activities.

MATERIALS AND METHODS

Cells and viruses. Avian retroviruses (except RAV-0 and AMV) were grown in chicken cells from C/E embryos obtained from SPAFAS, Norwich, Conn. Cells were negative for ALV group-specific antigen, sedimentable DNA polymerase activity, and chick helper factor. Cell cultures were prepared according to standard procedures. They were grown in Temin-modified Eagle minimal essential medium containing 20% tryptose phosphate broth, 2% calf serum, and 2% fetal bovine serum. Medium from virus-infected cells was harvested daily and stored at −20°C.

(i) ALVs. The following ALVs were used: from subgroup A, SR-RSV-A; from subgroup B, Pr-RSV-B and RAV-2; from subgroup C, Pr-RSV-C and the Bratislava 77 strain of avian sarcoma virus (B77-ASV-C); from subgroup D, SR-RSV-D and RAV-50; from subgroup E, RAV-$0_{\text{line 100}}$; and from subgroup F, RAV-61 and RAV-F.

SR-RSV-A, Pr-RSV-B, Pr-RSV-C, RAV-2, RAV-49, RAV-50, and B77-ASV-C were generous gifts from P. Vogt, University of Southern California. RAV-61 was a gift from T. Hanafusa, Rockefeller University. Chicken eggs from line 100 (spontaneously producing RAV-0) were a gift from L. A. Crittenden, Regional Poultry Research Laboratory, East Lansing, Mich. SR-RSV-D was a gift from C. G. Ahlstrom, University of Lund. RAV-F has been described previously (9). Purified AMV was obtained from J. Beard through the courtesy of the Office of Program Resources and Logistics, National Cancer Institute.

(ii) PVs. APV was a gift from T. Hanafusa. PVs have been shown to belong to an independent species of avian retroviruses (8) which is evolutionarily linked to ALV (5). APV was grown in chicken cells.

(iii) REVs. SNV, duck infectious anemia virus (DIAV), chick syncytial virus (CSV), and REV-T have been described previously (27).

Mammalian retroviruses (types B, C, and D) were obtained as gradient-purified stocks from Charles Pfizer & Co., Electro Nucleonics Inc., and the Frederick Cancer Research Center through the courtesy of the Office of Program Resources and Logistics, National Cancer Institute. The type C retroviruses used were Rauscher murine leukemia virus (R-MuLV), Gross-MuLV, Moloney-MuLV, RD-114 virus, and baboon endogenous virus. The type B retrovirus used was mouse mammary tumor viruses, and the type D retrovirus used was Mason-Pfizer monkey virus.

Particles with reverse transcriptase activity from the allantoic fluids of virus-negative embryonated chicken eggs and from the supernatant of cells from a goose embryo were obtained and purified as previously described (2, 4).

Purification of avian retroviruses. Large-scale purification by membrane ultrafiltration has been described previously (5). For small-scale purification (liter amounts of virus-containing medium), the protocol was changed such that membrane filtration was omitted and the fluid was subjected to ultracentrifugation directly after low-speed centrifugation. Purified viruses from the density gradient were kept in portions at −70°C.

Purification of DNA polymerase. The purification of AMV DNA polymerase and SNV DNA polymerase by ion-exchange chromatography for immunization and iodinization has been described previously (5).

Antisera. Antiserum to purified SNV DNA polymerase was prepared in a male New Zealand rabbit. A 40-μg amount of DNA polymerase was mixed with complete Freund adjuvant and injected into the footpads at 7- to 10-day intervals (first four immunizations) and at monthly intervals (fifth and sixth immunizations). Antisera used in this study were obtained 1 week after the fourth or sixth immunization. Rabbit antiserum to purified AMV DNA polymerase was obtained by injecting 40 μg of AMV DNA polymerase (plus complete Freund adjuvant) three times at biweekly intervals into the footpads of a male New Zealand rabbit.

Rabbit antiserum to APV DNA polymerase has been described previously (5). Rabbit antiserum to RAV-61 DNA polymerase was a kind gift from S. Mizutani, McArdle Laboratory. Goat antisera to the DNA polymerases of AMV, R-MuLV, and baboon endogenous virus were obtained from L. Wilsnack, Huntingdon Research Center, through the courtesy of the Office of Program Resources and Logistics, National Cancer Institute.

Protein determination. The method of Lowry et al. (13) was used; crystalline bovine serum albumin was used as a standard.

Gel electrophoresis. The method of Laemmli was used (12).

Iodination of DNA polymerases. We used the chloramine T method, which was originally described by Greenwood et al. (7) and modified by Krakower et al. (10, 11). A total reaction volume of 60 μl contained 1 μg of SNV DNA polymerase or 2.5 μg of AMV DNA polymerase. Reactions were carried out in the presence of a solution containing 50 mM Tris-hydrochloride (pH 7.8), 1 mCi of $Na^{125}I$, 200 mM KCl, and 0.08 mg of chloramine T per ml (in the case of SNV DNA polymerase) or 0.11 mg of chloramine T per ml (in the case of AMV DNA polymerase) for 25 min in an ice bath. The reactions were terminated by the addition of 25 μl of sodium metabisulfite (3 mg/ml). Labeled protein was separated from free iodine by using P10 polyacrylamide beads (Bio-Rad Laboratories; column, 0.9 by 30 cm) equilibrated with a solution containing 50 mM Tris-hydrochloride (pH 7.8), 200 mM KCl, 10% glycerol, and 0.4% Triton X-100.

The peak fractions from the P10 chromatography were brought to 1 mg of bovine serum albumin per ml and further run on a 10 to 30% glycerol gradient in a solution containing 50 mM Tris-hydrochloride (pH 7.8), 150 mM KCl, and 0.4% Triton X-100 (SW41 rotor; 39,000 rpm; 4°C; 18 h). The gradients were fractionated from the bottom, and the peak fractions of full-sized material were pooled, divided into samples, and stored at −70°C.

Typical specific radioactivities were 2×10^7 to 4×10^7 cpm/μg of AMV DNA polymerase and 4×10^7 to 8×10^7 cpm/μg of SNV DNA polymerase.

In the case of AMV DNA polymerase, a specific breakdown of the β subunit to the size of the α subunit was observed when 0.08 to 0.1 mg of chloramine T per ml was present (data not shown). Also, the enzyme was separated into subunits. To obtain similar labeled DNA polymerases from independent iodinations, the reaction was carried out at 0.11 mg of chloramine T per ml (where 90% of β was converted to α). On glycerol gradients 60 to 70% of the material was of α size. This material was pooled and used throughout this study. As Fig. 1A shows, the labeled AMV DNA polymerase was quite homogenous. When iodinated SNV DNA polymerase was analyzed on a glycerol gradient, about 50% sedimented at the position of full-sized molecules. A total of 10 to 20% of the radioactivity was found in faster-sedimenting material. As the original SNV DNA polymerase had been more than 95% pure, the faster-sedimenting material may either have been aggregates formed during the iodination reaction or denatured protein with changed sedimentation properties. A total of 30 to 40% of the material from the P10 column sedimented as a discrete peak, slower than full-sized SNV DNA polymerase. The

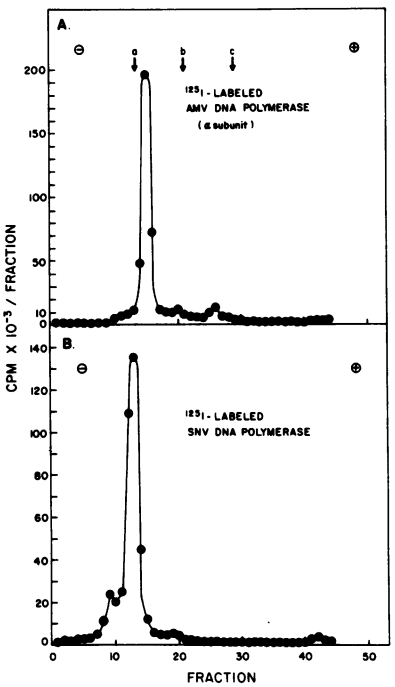

FIG. 1. *Analysis of ^{125}I-labeled DNA polymerases by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. AMV DNA polymerase (A) and SNV DNA polymerase (B) were iodinated, passed through a column of P10 polyacrylamide beads (Bio-Rad Laboratories), and further purified by glycerol gradient centrifugation as described in the text. The peak fractions of the gradients were pooled, and a sample of each of the pools was dissolved in sample buffer and loaded directly (without previous heating) onto a sodium dodecyl sulfate-polyacrylamide gel (10%; 0.6 by 9.5 cm). Electrophoresis was performed at 5 mA/gel. Molecular weight markers in a parallel gel were (a) bovine serum albumin (molecular weight, 68,000), (b) ovalbumin (45,000), and (c) chymotrypsinogen A (25,000). Gels with ^{125}I-labeled proteins were sliced, and the individual slices were counted directly in a gamma counter. Positions of the marker proteins were determined by staining the gel with Coomassie brilliant blue.*

peak of full-sized SNV DNA polymerase was used for the experiments described below. As judged by polyacrylamide gel electrophoresis, this material was relatively homogenous and free of major impurities (Fig. 1B).

Immunoprecipitation. Reactions were carried out in radioimmunoassay buffer (10), which contained 50 mM Tris-hydrochloride (pH 7.8), 200 mM KCl, 10% glycerol, 0.4% Triton X-100, and 5 mg of bovine serum albumin (A grade; Calbiochem-Behring Corp.) per ml. ^{125}I-labeled DNA polymerase (10,000 to 30,000 cpm) and antiserum were preincubated for 18 h at 4°C in a total volume of 200 μl. Then carrier normal serum (if necessary for the formation of a visible precipitate) and a second antibody (goat antiserum to rabbit IgG or rabbit antiserum to goat IgG [Calbiochem-Behring Corp.]) were added, and the incubation at 4°C was continued for another 4 to 5 h. The immunoprecipitates were collected by low-speed centrifugation (Sorvall RC-3; 2,500 rpm; 20 min; 4°C), and the supernatants were aspirated. The exact amounts of carrier normal serum and second antibody used are detailed in the figure legends.

Competition radioimmunoassay. An amount of antiserum sufficient to precipitate 30 to 50% of the labeled DNA polymerase was preincubated with serial dilutions of competing material at 37°C for 1 h in a total volume of 190 μl in radioimmunoassay buffer. The assays were cooled to 4°C, 20 μl of radioimmunoassay buffer containing 20,000 to 30,000 cpm of ^{125}I-labeled DNA polymerase was added, and incubation was continued for 18 h at 4°C. Immunoprecipitation by the double-antibody technique was as described above.

RESULTS

Immunoprecipitation studies. Immunoprecipitations of labeled AMV or SNV DNA polymerase by antisera against the DNA polymerases of members of the three avian retrovirus species were carried out to check for possible immunological cross-reactions among the DNA polymerases of the three retrovirus species and to find optimal conditions for later competition radioimmunoassays (limiting amounts of antibody).

Figure 2A shows that ^{125}I-labeled AMV DNA polymerase was precipitated by antisera against the DNA polymerases of AMV and RAV-61. In the case of AMV, antisera raised in both the goat and the rabbit were used. As reported earlier (5), antiserum to the DNA polymerase of APV could also precipitate ^{125}I-labeled AMV DNA polymerase. In this case, however, for comparable precipitation higher concentrations of antiserum were needed compared with anti-RAV-61 or anti-AMV sera. Antisera against SNV DNA polymerase or R-MuLV DNA polymerase, as well as normal rabbit or goat sera, did not precipitate AMV DNA polymerase.

^{125}I-labeled SNV DNA polymerase was very efficiently precipitated by antiserum against

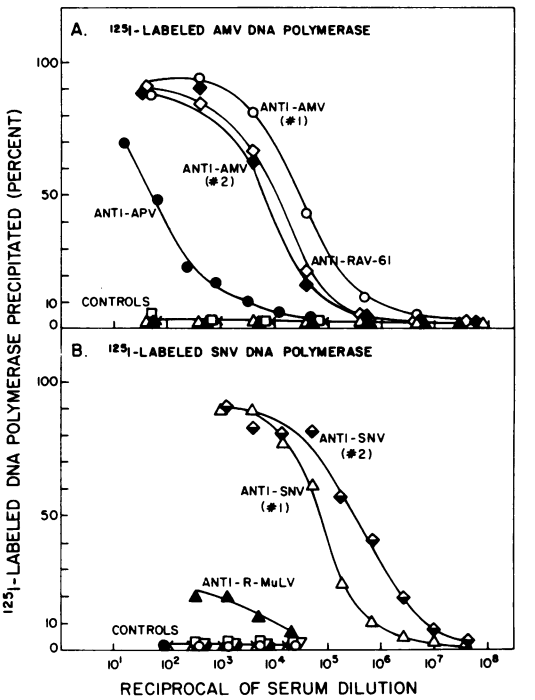

FIG. 2. *Double-antibody immunoprecipitation of ^{125}I-labeled DNA polymerases. Serial dilutions of various preimmune sera and antisera to viral DNA polymerases were incubated with 22,000 cpm of ^{125}I-labeled AMV DNA polymerase (A) or 16,000 cpm of ^{125}I-labeled SNV DNA polymerase (B) for 18 h at 4°C in the presence of a solution containing 50 mM Tris-hydrochloride (pH 7.8), 200 mM KCl, 0.4% Triton X-100, 5 mg of bovine serum albumin per ml, and 10% glycerol in a total volume of 200 μl. Normal serum as a carrier was added to samples containing a 10^{-3} dilution of serum or less. The amount of carrier normal serum was 50 μl of a 1:10 dilution in the case of rabbit serum and 25 μl of a 1:50 dilution in the case of goat serum. The corresponding second antibody was goat anti-rabbit serum (150 μl, corresponding to 1.5 U as defined by Calbiochem) or rabbit anti-goat serum (150 μl). Samples with serum concentrations greater than 10^{-3} did not receive additional normal serum, and the optimal amount of second antibody was determined for each serum and each concentration. It was in the range of 300 to 400 μl for both groups of serum. (A) Symbols: ○, goat antiserum to AMV DNA polymerase (serum 1); ◆, rabbit antiserum to AMV DNA polymerase (serum 2); ◇, rabbit antiserum to RAV-61 DNA polymerase; ●, rabbit antiserum to APV DNA polymerase; △, rabbit antiserum to SNV DNA polymerase (sera after fourth and sixth immunizations were used); ×, normal rabbit serum; ▲, goat antiserum to R-MuLV DNA polymerase; □, normal goat serum. (B) Symbols: △, rabbit antiserum to SNV DNA polymerase (after third immunization) (serum 1); ◆, rabbit antiserum to SNV DNA polymerase (after 6th immunization) (serum 2); ▽, goat antiserum to baboon endogenous virus DNA polymerase; all other symbols as in (A).*

SNV DNA polymerase (Fig. 2B). Antisera 1 and 2 were obtained from the same animal after different numbers of immunizations. Antisera 1 and 2 were similar in ability to neutralize SNV DNA polymerase in an inhibition test (data not shown), but differed in the titer of IgG binding to SNV DNA polymerase.

Antiserum to the DNA polymerase of R-MuLV gave some precipitation of SNV DNA polymerase. This cross-reaction was variable from experiment to experiment, probably due to the presence of proteases in the serum. This cross-reaction has been studied further by using a different approach (Bauer and Temin, J. Virol., in press). Antisera to the DNA polymerases of ALVs and PVs, as well as normal sera, did not cause precipitation of SNV DNA polymerase.

These immunoprecipitation studies did not reveal any relationship between SNV DNA polymerase and ALV and PV DNA polymerases. There seems to be a strong immunological relationship among the DNA polymerases within the ALV species. A moderate relationship between ALV and PV DNA polymerases, which has been recently described (5), was confirmed.

Competition radioimmunoassays. The results shown in Fig. 2 enabled us to determine the concentrations of the antisera required for 30 to 50% immunoprecipitation. These concentrations were used for competition radioimmunoassays. In this type of study, disrupted virions were preincubated with antiserum, and then the remaining immunoprecipitation of labeled DNA polymerase was measured. This assay, therefore, allowed an estimate of the amounts of determinants shared between the DNA polymerase of the tested virus and the labeled DNA polymerase. It measured the degree of relationship and determined the specificity of cross-reactions in immunoprecipitation reactions.

(i) Species-specific antigenic determinants of AMV DNA polymerase. The combination of antiserum to RAV-61 DNA polymerase with ^{125}I-labeled AMV DNA polymerase was likely to be a test for antigenic determinants common to all members of the ALV species. As Fig. 3 shows, disrupted viruses from several members of the ALV species (subgroups A through F) competed to the same degree in this competitive radioimmunoassay. In contrast, PV, all four members of the REV species, and mammalian type B, C, and D viruses, as well as the particle-associated RNA-dependent DNA polymerase activities from the allantoic fluids of normal chickens or from normal goose cells, did not compete significantly. This result defines ALV as a discrete species.

A comparison of the amount of purified AMV DNA polymerase required versus whole AMV virions allowed quantitation of the DNA polymerase content of AMV virions. This value was 3 to 4%, which is in agreement with a previous estimate by Panet et al. (20).

(ii) Type-specific antigenic determinants of AMV DNA polymerase. Competition for the binding of ^{125}I-labeled AMV DNA polymerase to antibody directed against the DNA polymerase of this virus should test whether other ALV DNA polymerases share all of the antigenic sites of AMV DNA polymerase. As Fig. 4 shows, all ALVs compete with a different slope and to a lesser degree than AMV does. This result indicates that none of the viruses tested shares all of the antigenic sites of AMV DNA polymerase; that is, there is some type specificity within the ALV species. This experiment also confirms the result of the quantification shown in Fig. 3B and C and again demonstrates the nature of ALV as a discrete species, since none of the control viruses competed in this assay. None of the determinants of AMV DNA polymerase, which may have been missed in the previous species-specific assay but were detected in the type-specific assay, seemed to be shared by any of the control viruses and particles. The demonstration of type-specific differences was not restricted to the combination of labeled DNA polymerase and antiserum used in the experiment described above, but was also clearly demonstrated by using an antiserum against AMV DNA polymerase produced in a rabbit (Fig. 2A, serum 2; data not shown).

Figure 4 shows that all ALV DNA polymerases tested lacked some of the antigenic determinants of AMV DNA polymerase. To test whether the same or different determinants are missing throughout the group, combinations of two viruses were allowed to compete for the binding of antibody against AMV DNA polymerase. To allow a precise measurement of the type-specific differences in these experiments, a fivefold-higher amount of ^{125}I-labeled DNA polymerase was used. Parallel experiments showed that the equilibrium of the immune reaction was such that the percentage of ^{125}I-labeled DNA polymerase precipitated by a given antibody concentration was not changed when the amount of labeled AMV DNA polymerase was changed over a 10-fold range (data not shown). Therefore, the results obtained under the modified conditions should be comparable to those obtained in Fig. 4.

Figure 5 shows how combinations of two viruses competed for antibody against AMV DNA polymerase. It can be seen that some viruses lack the same determinants, so that a combina-

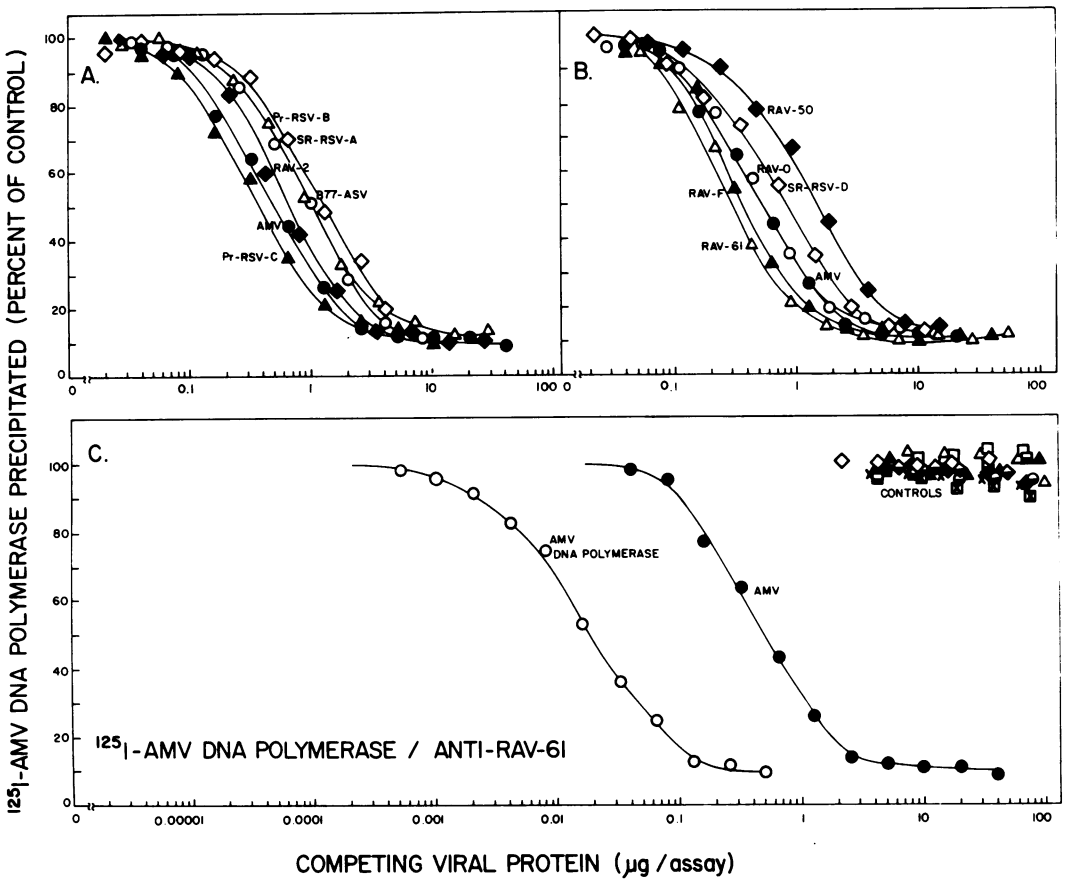

FIG. 3. *Species-specific antigenic determinants of the ALV species. Serial twofold dilutions of competing material (in radioimmunoassay buffer) were preincubated with a 2.5 × 10^{-4} dilution of rabbit antiserum to RAV-61 DNA polymerase in 190 μl for 1 h at 37°C. The assays were cooled to 4°C, 20,000 cpm of ^{125}I-labeled AMV DNA polymerase (in 20 μl of radioimmunoassay buffer) was added, and incubation at 4°C was continued for 18 h. After the addition of 50 μl of a 1:10 dilution of normal rabbit serum and 150 μl of goat antiserum to rabbit IgG, incubation was continued for another 4 to 5 h at 4°C. The immunoprecipitates were collected by low-speed centrifugation. Samples were counted for 5 min in a gamma counter. The 100% value (no competing material present) was calculated from 12 independent samples and was 48,500 counts per 5 min. The ALV curves are labeled in the figure. The symbols for the controls are as follows: ◒, APV; △, SNV; ▲, DIAV; ◇, CSV; ◆, REV-T; ×, chicken particles with RNA-dependent DNA polymerase activity; ⊠, goose particles with RNA-dependent DNA polymerase activity; ⬓, R-MuLV and baboon endogenous virus; □, mouse mammary tumor virus; ◈, Mason-Pfizer monkey virus. The data from one experiment are shown in three panels to allow clearer presentation.*

tion of two of them does not increase competition. For example, RAV-61, RAV-F, and RAV-2 cannot reach higher competition when any two or all three of them are combined.

In contrast, Pr-RSV-B or SR-RSV-D combined with RAV-F or RAV-61 resulted in higher competition. The same is true for the combination of SR-RSV-D with RAV-50, Pr-RSV-B, or B77-ASV or the combination of RAV-50 with B77-ASV, RAV-F, RAV-61, or Pr-RSV-B. These data show that some viruses lack different determinants relative to AMV DNA polymerase and can complement each other for competition.

In no case was it possible to reach the same degree of competition as AMV by a combination of only two viruses. However, a combination of four viruses (SR-RSV-D, B77-ASV, RAV-50, and RAV-61) could reach the same plateau as AMV alone (data not shown).

(iii) Genus-specific antigenic determinants of ALV DNA polymerases. We have shown previously that ALV and PV DNA polymerases are grossly different from each other, but share some antigenic determinants at their

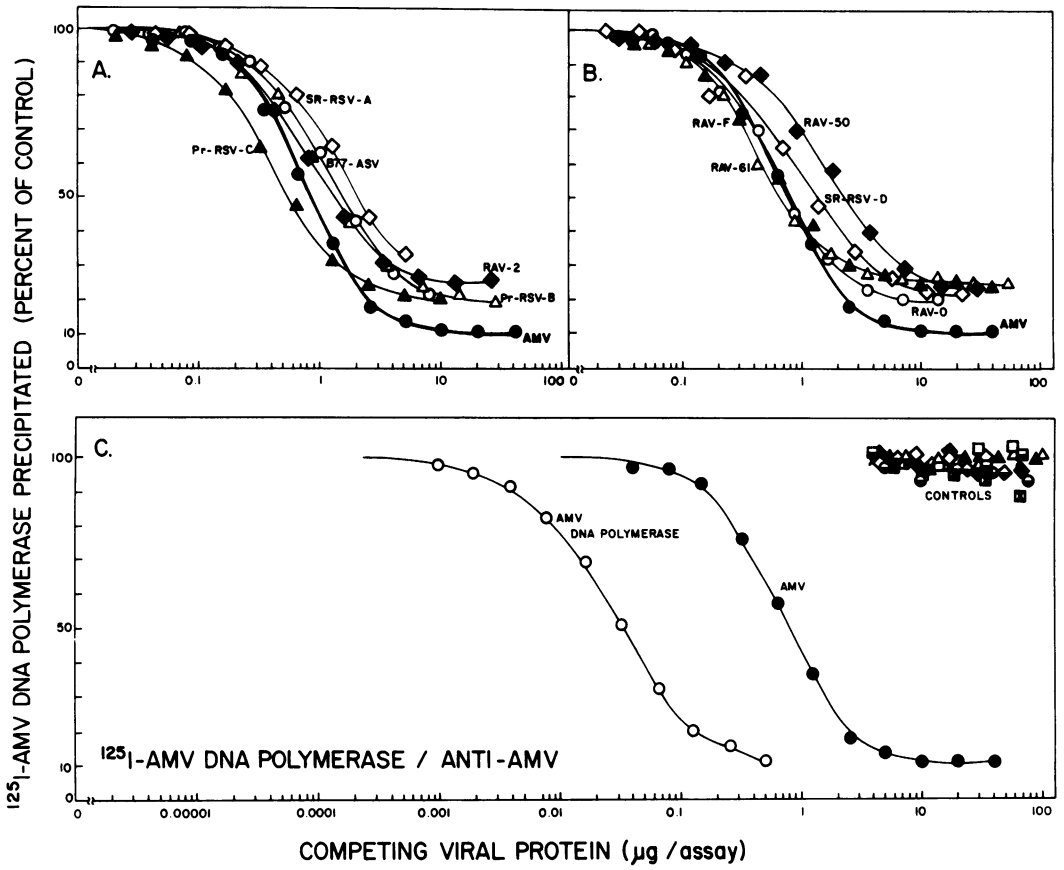

FIG. 4. *Type-specific antigenic determinants of ALV DNA polymerases. The experiment was performed as described in the legend to Fig. 3, except that goat antiserum to AMV DNA polymerase was used (final dilution, 5×10^{-5}). The second antibody reaction was performed by adding 75 μl of a 1:100 dilution of normal goat serum and 75 μl of rabbit antiserum to goat IgG. The 100% value was 38,000 counts per 5 min. Symbols are as described in the legend to Fig. 3. The data are from a single experiment and are in three panels to allow clearer presentation of the results.*

active sites (5). In a genus-specific competition radioimmunoassay, the specificity of the cross-reaction between AMV and APV DNA polymerases was established. SNV DNA polymerase did not share antigenic determinants common to AMV and APV.

We extended the previous experiment by testing whether members of all subgroups of ALV shared the genus-specific antigenic determinants to the same degree and whether all members of the REV species and other controls lacked those determinants. We extended the previous competition immunoassay (^{125}I-labeled AMV DNA polymerase and antibody to APV DNA polymerase) by including several members of the ALV species (subgroups A through F), REV species, particle-associated RNA-dependent DNA polymerase activities, and mammalian retroviruses. As Fig. 6 shows, APV, as well as all members of the ALV species tested, competed with the same characteristics in this assay. This result demonstrates that the determinants shared between AMV and APV DNA polymerases are common determinants of the ALV DNA polymerase rather than a peculiarity of AMV DNA polymerase. None of the control DNA polymerases, including those of all four REVs, particles with reverse transcriptase activity from chicken and goose cells, and mammalian type B, C, and D retroviruses, competed significantly. These results define the genus-specific antigenic determinants as a population of determinants unique for ALV and PV. The fact that all ALV tested could compete completely, (that is, each one possesses the whole set of genus-specific antigenic determinants) indicates that the genus-specific antigenic determinants are a subset of the species-specific determinants.

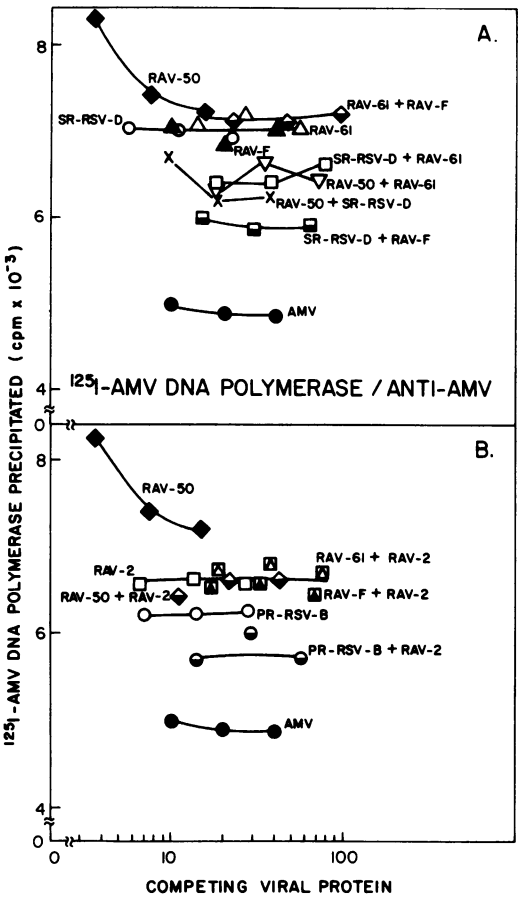

FIG. 5. *Competition of mixtures of ALV for antibody directed against type-specific determinants of AMV DNA polymerase. Amounts of ALV sufficient to reach or come close to a plateau (see Fig. 3) were preincubated individually or in various combinations (as indicated below) in a volume of 190 μl with goat antiserum to AMV DNA polymerase (final dilution, 5×10^{-5}) for 1 h at 37°C. After cooling, 100,000 cpm of ^{125}I-labeled AMV DNA polymerase was added, and incubation was continued at 4°C for 18 h. The amount of labeled AMV DNA polymerase bound to antibody was determined by using the double-antibody technique. Conditions were as described in the legend to Fig. 4. (A) Symbols: ◆, RAV-50; ●, AMV; ○, SR-RSV-D; △, RAV-61; ▲, RAV-F; ▽, RAV-50 + RAV-61; □, SR-RSV-D + RAV-61; ×, RAV-50 + SR-RSV-D; ◇, RAV-61 + RAV-F; ⬓, SR-RSV-D + RAV-F. (B) Symbols: ◆, RAV-50; □, RAV-2; ●, AMV; ○, Pr-RSV-B; ◒, Pr-RSV-B + RAV-2; ⧄, RAV-2 + RAV-F; ⧄, RAV-61 + RAV-2; ◆, RAV-50 + RAV-2. The data shown are from a single experiment and are in two panels for clearer presentation of the results.*

Antigenic determinants of SNV DNA polymerase. A competition radioimmunoassay using ^{125}I-labeled SNV DNA polymerase and antiserum to SNV DNA polymerase was used to determine the degree of variability among DNA polymerases within the REV species, to quantify the DNA polymerase content in REV virions, and to test for competition for antibody against SNV DNA polymerase by DNA polymerases of other retrovirus species or of particles with RNA-dependent DNA polymerase activity from cells. As Fig. 7 shows, all four REVs competed for the antibody, demonstrating a high degree of homology of DNA polymerases within the REV group. Only CSV did not compete completely. Although this type-specific difference was minor, it was reproducible with the particular antiserum used. When an antiserum obtained after additional immunization (Fig. 2B, serum 2) was used, the type-specific difference between CSV and SNV DNA polymerases was no longer detectable (data not shown).

None of the controls, including members of the ALV species, APV, mammalian type B, C, and D retroviruses, and particles with reverse transcriptase activity from chicken and goose cells, competed for antibody against SNV DNA polymerase. These results again demonstrate that REV DNA polymerase is grossly different from the DNA polymerases of ALV, PV, mammalian type B, C, and D retroviruses, and particles with RNA-dependent DNA polymerase activity from chicken or goose cells. A specific relationship between REV DNA polymerase and mammalian type C virus DNA polymerase will be presented elsewhere (Bauer and Temin, J. Virol., in press).

A comparison of the competition by purified SNV DNA polymerase versus the competition by disrupted viruses allowed quantitation of the DNA polymerase content of REVs. For SNV and DIAV the relative amount of DNA polymerase seems to be 2% of the virus protein; the values for REV and CSV are 0.5 and 0.4%, respectively. These values are of the same order of magnitude as those for ALV.

DISCUSSION

Iodination of purified DNA polymerases of AMV and SNV by the chloramine T method, combined with subsequent glycerol gradient centrifugation, allowed preparation of radioactive DNA polymerases which could be used in radioimmunoassays. In the case of AMV DNA polymerase, conditions of iodination and repurification were chosen such that purified subunit α was recovered. The use of this approach circumvented the problem of having labeled DNA polymerase preparations varying in their α/β ratios after different iodinations. The use of labeled α also prevented competition by p32, an ALV virion protein which carries β-specific antigenic determinants (25).

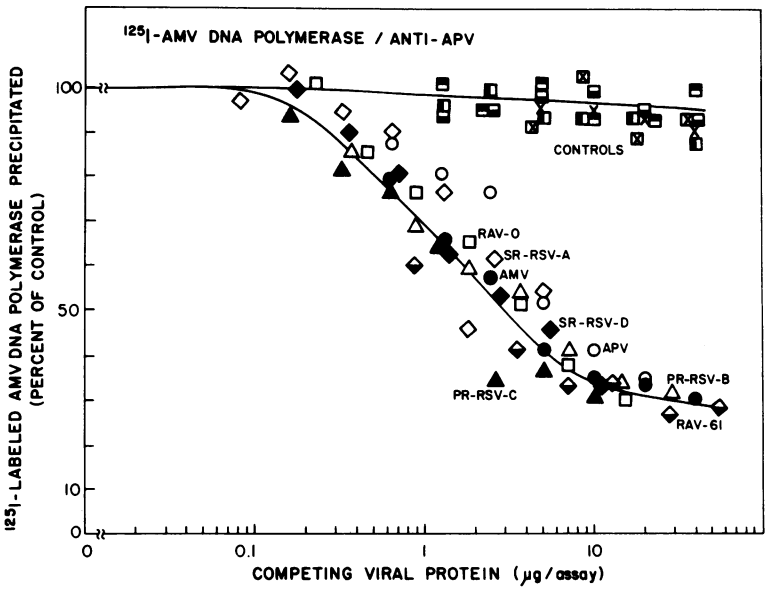

FIG. 6. *Demonstration of genus-specific antigenic determinants of avian retroviruses (ALV and PV species). The competition experiment was performed as described in the legend to Fig. 3, except that rabbit antiserum to APV DNA polymerase (final dilution, 4×10^{-3}) was used. In this experiment 50 μl of a 1:50 dilution of normal rabbit serum and 150 μl of goat antiserum to rabbit IgG were used for immunoprecipitation; 100% precipitation was 30,000 counts per 5 min. The ALV and APV curves are labeled on the figure. The symbols for the controls are as follows: ■, SNV; ▤, DIAV; ◧, REV; ×, chicken particles with RNA-dependent DNA polymerase activity; ⊠, goose particles with RNA-dependent DNA polymerase activity. Not indicated in the figure are CSV, R-MuLV, baboon endogenous virus, mouse mammary tumor virus, and Mason-Pfizer monkey virus, which competed less than 5% at up to 40 μg of viral protein per assay.*

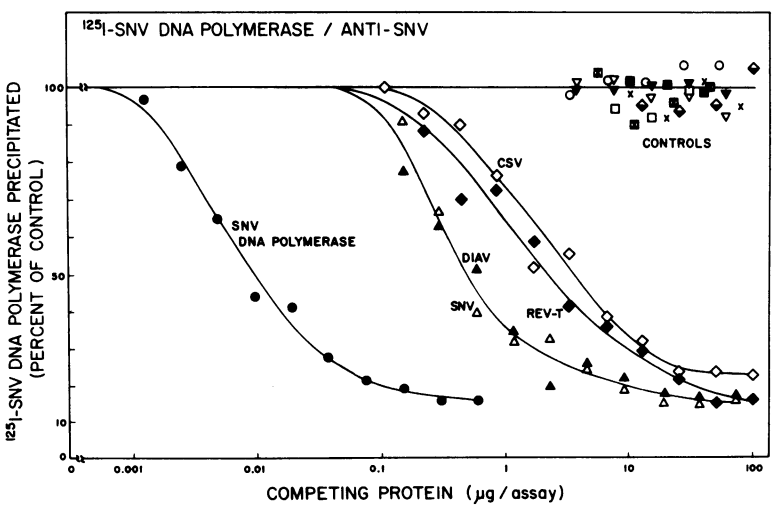

FIG. 7. *Homologous radioimmunoassay for SNV DNA polymerase. The experiment was performed as described in the legend to Fig. 3, except that rabbit antiserum to SNV DNA polymerase (final dilution of antiserum obtained after fourth immunization of a rabbit with SNV DNA polymerase, 10^{-4}; see Fig. 2B) and ^{125}I-labeled SNV DNA polymerase (16,000 cpm/assay) were used. The 100% value (no competing material present) was 38,000 counts per 5 min. The REV curves are labeled on the figure. The symbols for the controls are as follows: ×, goose particles with RNA-dependent DNA polymerase activity; ▽, AMV; ▼, SR-RSV-D; ◆, APV; ■, mouse mammary tumor virus; ○, R-MuLV; ⊠, chicken particles with RNA-dependent DNA polymerase activity. Not indicated on the figure are Moloney-MuLV and Gross-MuLV, which competed less than 5% at up to 50 μg of viral protein per assay.*

The iodinated DNA polymerases could be precipitated by homologous antiserum almost completely and, thus, allowed us to set up conditions for radioimmunoassays.

AMV DNA polymerase could be efficiently precipitated by antisera against ALV DNA polymerases (such as RAV-61 and AMV), and it could not be precipitated by antiserum to SNV DNA polymerase.

In a complementary experiment, ^{125}I-labeled SNV DNA polymerase could not be precipitated by any of the antisera against ALV DNA polymerase, but was efficiently precipitated by antisera against SNV DNA polymerase.

Antiserum to APV DNA polymerase could precipitate ^{125}I-labeled AMV DNA polymerase, but not ^{125}I-labeled SNV DNA polymerase. The precipitation of AMV DNA polymerase by anti-APV serum required higher concentrations of serum than precipitation by anti-ALV serum. As previously shown, this result reflects the fact that AMV and APV DNA polymerases are grossly different, but share a limited number of antigenic determinants. As previously shown (5), these common antigenic determinants are not shared with SNV DNA polymerase. Other sets of antigenic determinants are not shared between APV and SNV, since anti-APV serum could not precipitate SNV DNA polymerase.

None of the preimmune sera allowed precipitation of ^{125}I-labeled DNA polymerases, indicating the specificity of the immunoprecipitation.

The precipitation of ^{125}I-labeled AMV DNA polymerase by various antisera allowed the establishment of different types of competition radioimmunoassays: (i) species specific (AMV/anti-RAV-61), (ii) type specific (AMV/anti-AMV), and (iii) genus specific (AMV/anti-APV). The species-specific radioimmunoassay showed the existence of a set of antigenic determinants shared by all members of the ALV species to the same extent; in each case the competition curves ran parallel and to the same plateau valve. None of the REVs, mammalian retroviruses, or particles with RNA-dependent DNA polymerase activity from normal chicken or goose cells possesses a DNA polymerase with sufficient homology to allow detectable competition. The type-specific assay (AMV/anti-AMV) showed that none of the ALVs tested can reach the same slope and plateau as AMV; that is, there are AMV-specific determinants missing on each one of the other ALV DNA polymerases. Although the type-specific differences lead to only a 10 to 15% difference in competition, they are real, as can be demonstrated by using higher amounts of labeled DNA polymerase. Mixing experiments show that viral DNA polymerases may lack the same or different antigenic determinants relative to AMV DNA polymerase.

The genus-specific assay (AMV/anti-APV) focused on the determinants common to AMV and APV DNA polymerases. As reported earlier, most, if not all, of the common determinants seem to be located at the active centers of both enzymes. Here we extended the original finding by demonstrating that the common determinants are shared by ALVs of different strains and subgroups. These genus-specific determinants, therefore, seem to be a subset of the species-specific determinants of ALV. However, antibody against the common determinants seems to be a relatively small population within the antibodies against RAV-61 (or AMV) DNA polymerase, since APV did not compete significantly in the species-specific or type-specific radioimmunoassays of ALV. The result of the genus-specific assay again shows the specificity of the cross-reaction between ALV and APV since none of the control DNA polymerases competed.

The SNV/anti-SNV competition radioimmunoassay showed that there was not much variation within the REV species; SNV, REV-T, and DIAV could compete completely in this assay, and only CSV showed a minor type-specific difference. This difference was no longer demonstrated when antisera obtained after additional immunizations were used (data not shown).

The data presented here and elsewhere (5) do not indicate any cross-reaction between REV polymerases and ALV or PV DNA polymerases in radioimmunoassays. In addition, we demonstrated a specific cross-reaction between REV and mammalian type C retrovirus DNA polymerases (Bauer and Temin, J. Virol., in press). Therefore, we favor the idea that REVs originated from mammalian type C retroviruses. Thus, the ability of REV DNA polymerase to absorb antibody directed against the active site of ALV DNA polymerase (15) seems to reflect a similar feature at a definite site which is not prominent enough to allow cross-reactions in precipitations. Therefore, it seems unlikely that it reflects a common origin of REV and ALV, as originally proposed.

The comparison of the amounts of DNA polymerase versus the amounts of whole virions necessary for the same degree of competition allows an estimation of the DNA polymerase content of virions. For AMV we estimate that the relative concentration is 4% DNA polymerase per virion, and the value for SNV is about 2%. The value of 4% for AMV is in agreement with a previously published quantification, in which the same method was used (20). The

apparent variation in the DNA polymerase contents of different members of the ALV or REV species probably reflects the difficulty in determining the exact amount of virion protein in not completely pure virus preparations. As the different viruses grow to different titers, the relative amounts of contaminating proteins from the media are different.

Particles isolated from the allantoic fluid of embryonated chicken eggs and from the supernatant medium of cells from a goose embryo, which contain RNA-dependent DNA polymerase activity, did not compete significantly for antibodies directed against any of the determinants defined in this study. Therefore, they seem not be closely related to ALV, PV, or REV. However, in the case of particles from the allantoic fluid, DNA polymerase could not be used in excess in the radioimmunoassays since the DNA polymerase content estimated by enzyme activity per amount of protein is only 5% of that of ALV and the material is hard to prepare in large quantities. In the case of goose particles, the DNA polymerase content is of the same order of magnitude as REV (based on enzyme activity). Since particle-associated DNA polymerases cannot be obtained in large enough quantities for immunization or as purified enzymes for iodination, heterologous radioimmunoassays which might detect cross-reactions at antigenic sites different from those defined in this study cannot be established at this time. Therefore, the origin and significance of particle-associated DNA polymerase activities remain unclear.

Finally, the fact that RAV-61 and RAV-F could compete for the majority of antibodies directed against AMV DNA polymerase (comparable to the competition by other members of the ALV species) directly proves that the parental DNA polymerase gene was retained when the viruses were formed by recombination between ALV and pheasant cells. As previously reported, only the envelope gene was exchanged during this recombination (9).

Although radioimmunoassay is a well-established method to detect relationships between proteins, the degrees of relationships obtained are mainly determined by the relative concentration of IgG against certain determinants within the total IgG. We are aware that the relative concentrations of various IgG's are not necessarily correlated with the significance of a certain antigenic determinant with respect to the entire molecule. Therefore, results obtained with radioimmunological assays should be looked at as demonstration of the presence of common antigenic determinants between different DNA polymerases or a demonstration of a lack of defined determinants, but not as a precise measure of homology.

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