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Virus-Coded Origin of a 32,000-Dalton Protein from Avian Retrovirus Cores: Structural Relatedness of p32 and the β Polypeptide of the Avian Retrovirus DNA Polymerase

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A 32,000-dalton protein (p32) located in avian retrovirus cores was immunoprecipitated from [^{35}S]methionine-labeled avian myeloblastosis virus (AMV) propagated in cultured chicken embryo fibroblast cells by an antiserum preparation (sarc III) derived from tumor-bearing hamsters injected with cloned and passaged cells from an avian sarcoma virus-induced primary hamster tumor. Since sarc III serum apparently contained antibodies only to virus-coded proteins and not to chicken cellular proteins, the immunoprecipitation of p32 from AMV by sarc III serum strongly suggested that p32 is virus coded. The origin of p32 was more definitively established by demonstrating the existence of a structural relationship between p32 and the AMV DNA polymerase. AMV p32 cross-reacted with the β polypeptide of AMV αβ DNA polymerase in radioimmunoprecipitation and radioimmunoprecipitation inhibition assays, indicating that p32 and β share common antigenic determinants. This relationship was clarified by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the peptides generated by limited proteolysis of ^{125}I-labeled AMV DNA polymerase polypeptides and of ^{125}I-labeled AMV p32 by chymotrypsin or *Staphylococcus aureus* V-8 protease. The peptides which appeared during proteolytic digestion of p32 were a subset of those produced by digestion of the β polypeptide; however, p32 had no discernible peptides in common with the α polypeptide. Further, all of the peptides produced by limited proteolysis of β were present in the digests of either p32 or α. Our findings suggest that p32 is apparently derived by cleavage of the β polypeptide of AMV DNA polymerase, presumably at a site near or identical to that at which α is generated from β by proteolytic cleavage.

The avian retrovirus genome contains four known genes, which encode the "gag" internal virion proteins, the RNA-directed DNA polymerase, the envelope glycoproteins, and a possible sarcoma ("src") gene product (1). Avian retrovirus cores contain five to six major polypeptides, which include the DNA polymerase α and β polypeptides, with molecular weights of 62,000 and 92,000, respectively, and the internal proteins with molecular weights of 27,000 (p27) and 12,000 (p12) (2, 42, 43). A polypeptide of hitherto undefined origin, with a molecular weight of 32,000 (p32), is also present in isolated cores and in ribonucleoprotein particles derived from cores (2, 7, 10, 40, 42, 43). We have determined immunochemically that p32 is antigenically related to the β polypeptide of the αβ form of avian myeloblastosis virus (AMV) DNA polymerase. Peptide mapping of ^{125}I-labeled polypeptides has revealed that p32 is derived from the β polypeptide of AMV DNA polymerase by proteolytic cleavage, but is structurally unrelated to the α polypeptide, which is itself derived from β by proteolytic cleavage (13, 30, 36, 41).

MATERIALS AND METHODS

Materials. Chicken cells from C/E embryos were obtained from SPAFAS, Inc., Norwich, Conn. All chicken embryo fibroblast (CEF) cells were negative for sedimentable DNA polymerase activity and, according to complement-fixation tests, for avian leukosis virus group-specific antigens and chick helper factor. AMV, BAI strain A, was purified from leukemic chicken plasma kindly provided by Joseph Beard of Life Sciences, Inc., St. Petersburg, Fla., through the Virus Cancer Program of the National Cancer Institute. Goat antiserum directed against AMV αβ DNA polymerase was also provided by the National Cancer Institute. Medium 199 and Eagle minimal essential medium were obtained from Grand Island Biological Co., Grand Island, N.Y. Fetal bovine serum was purchased from Flow Laboratories, Inc., Rockville, Md., and dialyzed fetal bovine serum was obtained from KC Biological, Inc. L-[^{35}S]methionine (specific activity in excess of 400 Ci/mmol), iodine-125 (specific activity approximately 17 Ci/mg), and [^{3}H]TTP (specific ac-

tivity 40 to 60 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Chymotrypsin was purchased from Worthington Biochemical Corp., Freehold, N.J., and *Staphylococcus aureus* V-8 protease was obtained from Miles Laboratories, Inc., Elkhart, Ind. RP Royal X-Omat film was bought from Eastman Kodak Co., Rochester, N.Y. Normal and tumored sera from Syrian hamsters were generous gifts from J. Brugge and E. Erikson, University of Colorado Medical Center, Denver. Goat antiserum directed against the p19 and p27 proteins of AMV was a kind gift of D. Bolognesi, Duke University Medical Center, Durham, N.C. L-[^{3}H]leucine-labeled virion proteins from the Moloney strain of murine leukemia virus (MuLV) were kindly provided by G. Shanmugam, Saint Louis University Medical Center, St. Louis, Mo.

Growth of cells and virus. Primary and later cultures of CEF cells were propagated at 37°C in plastic tissue culture flasks (75-cm^2) using medium 199 containing 5% fetal bovine serum and supplemented with 10% tryptose phosphate broth.

To prepare infectious AMV from leukemic chicken plasma, frozen (−70°C) samples of plasma were thawed, diluted fourfold with the complete growth medium used for the propagation of cells, and passed through a 0.45-μm pore diameter nitrocellulose membrane filter. The undiluted plasma contained 10^{11} to 10^{12} virus particles per ml, as shown by adenosine triphosphatase assays (31).

Cell monolayers containing approximately 10^6 cells each were infected with 1-ml samples of diluted and filtered virus 5 to 6 h after subculturing. After incubation and occasional gentle agitation at 37°C for 1 h, the newly infected cell monolayers were fed with growth medium of the same composition as that used for the propagation of uninfected cells. The monolayers were then purged with carbon dioxide and incubated at 37°C. Infected cell monolayers received a change of medium 2 or 3 days after infection; the supernatant media from certain monolayers were harvested at 24-h intervals and assayed for the presence of RNA-directed DNA polymerase activity using $(A)_n \cdot (dT)_{12-18}$ (14) to demonstrate that infection of the cell monolayers had indeed been established.

Purification of AMV virions and core structures. AMV virions were purified from leukemic chicken plasma as described elsewhere (18a) for use in immunizing animals and in purifying the p32 protein and DNA polymerase. AMV cores for immunization were prepared by Sterox SL detergent disruption of intact virions and isopycnic sucrose gradient centrifugation (42, 43).

Purification of the p32 protein from AMV. The p32 protein was purified from Nonidet P-40 (NP-40) detergent-lysed AMV whole virions by phosphocellulose and polyuridylic acid [poly(U)]-Sepharose 4B chromatography (18a). The purification procedure was monitored by DNA binding assays (14), which measured the retention of ^{3}H-labeled polydeoxyadenylic acid-polydeoxythymidylic acid to nitrocellulose membrane filters by the p32 protein.

Purification of AMV DNA polymerase. The αβ DNA polymerase was purified from AMV by DEAE-cellulose and phosphocellulose chromatography (16) and either Sephadex G-150 (14) or poly(U)-Sepharose 4B (15) chromatography.

Preparation of antisera. The following immunizing antigens were prepared: AMV virions, disrupted by treatment with 0.5% NP-40 for 15 min at 0°C; AMV core structures; AMV αβ DNA polymerase purified to homogeneity through poly(U)-Sepharose chromatography (15); and the AMV p32 protein. The p32 protein was purified through poly(U)-Sepharose chromatography and then subjected to electrophoresis on 6% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) (Schiff and Grandgenett, manuscript in preparation). The electrophoretic band containing the p32 protein was visualized by Coomassie brilliant blue staining, excised directly from the gel, lyophilized, and ground into a fine powder with the SDS-denatured p32 antigen still present within the polyacrylamide matrix (45); this preparative technique ensured that the samples of the AMV p32 protein employed to immunize animals were purified to homogeneity. Each of these antigen preparations was emulsified with an equal volume of Freund complete adjuvant (final volume, 1 to 4 ml) and injected at several sites subcutaneously into the footpads or dorsal body surface of female New Zealand white rabbits, in quantities ranging from 20 μg of protein for the p32 protein to 920 μg of protein for the virion preparation. After the initial inoculations, the rabbits received two booster injections of the immunizing antigens at intervals of at least 14 days. The ability of all antisera prepared from the immunized rabbits to immunoprecipitate the antigens for which they were specific was demonstrated by agar immunodiffusion assays.

Labeling of cellular and viral proteins in vivo. To label cellular proteins in vivo, nearly confluent monolayer cultures of uninfected and AMV-infected CEF cells were starved for methionine by incubation for 1 h at 37°C with methionine-free Eagle minimal essential medium containing 5% dialyzed fetal bovine serum; the cultures were then labeled for 30 min at 37°C with 200 μCi of [^{35}S]methionine per ml in methionine-free minimal essential medium containing 5% dialyzed fetal bovine serum. After labeling, the cells were washed twice with warm (37°C) phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and were scraped from their flasks with a rubber policeman. The cells were then pelleted by centrifugation at 1,000 rpm for 7 min at 4°C, and the cell pellets were washed three times with cold (0°C) phosphate-buffered saline containing 1 mM PMSF. After the final washing, the cell pellets were resuspended in 5 volumes of 50 mM NaCl, 20 mM Tris-hydrochloride (pH 7.5), and 1 mM PMSF, and were stored frozen at −70°C.

To label AMV proteins in vivo, nearly confluent monolayer cultures of AMV-infected CEF cells were labeled for 24 h at 37°C with 50 μCi of [^{35}S]methionine per ml in methionine-free Eagle minimal essential medium containing 5% dialyzed fetal bovine serum, 0.25% nondialyzed fetal bovine serum, and 5% medium 199; there was no starvation period prior to labeling. After labeling, these cells were processed as described above, and AMV was harvested from their supernatant media. Virions present in the clarified supernatants were pelleted by centrifugation at 36,000 rpm for

1 h at 4°C in a Spinco SW41 rotor through a 1-ml layer of 20% (wt/vol) sucrose in NTE-PMSF buffer (100 mM NaCl, 10 mM Tris-hydrochloride at pH 7.5, 1 mM EDTA, and 1 mM PMSF). The pelleted AMV was resuspended in a minimum volume of 20% (wt/vol) sucrose in NTE-PMSF, and the quantity of trichloracetic acid-precipitable radioactivity present in the resuspended virus was determined by liquid scintillation counting. These suspensions of radioactively labeled AMV virions were then stored frozen at −70°C.

Radioiodination of AMV p32 for radioimmunoprecipitation assays. Samples of the AMV p32 protein, purified through poly(U)-Sepharose 4B chromatography, were radioiodinated by the chloramine T-oxidation procedure (19). Approximately 6 μg (5 μl) of purified p32 was diluted with 55 μl of 0.25 M potassium phosphate buffer (pH 7.5) to which was added 2 μl of $Na^{125}I$ (1 mCi). Three microliters of chloramine T (1.38 mg/ml in 0.25 M potassium phosphate buffer) was added to the p32 preparations, followed by incubation at 0°C for 30 s. Four microliters of sodium metabisulfite (1.19 mg/ml in 0.25 M potassium phosphate buffer) was added to stop the reaction, and 10 μl of KI (24 mg/ml in 0.25 M potassium phosphate buffer) was added to decrease nonspecific binding of labeled iodine. The radioiodinated protein was purified by gel filtration on a Sephadex G-50 column (0.9 by 30 cm) pre-equilibrated with 50 mM potassium phosphate (pH 8.0). The fractions in the void volume which contained the radioactivity peak were pooled and stored at 4°C after the addition of 0.02% sodium azide.

Extraction of labeled proteins and radioimmunoprecipitation assays. The frozen suspensions of uninfected and AMV-infected cells were thawed, and to each sample were added the following substances at the final concentrations shown: 10% glycerol, 500 mM urea, 5 mM EDTA, 1 mM 2-mercaptoethanol, 0.5% NP-40, and 1.0% sodium deoxycholate. The samples were placed in an ice bath for 10 min with occasional Vortex mixing. The samples were then sonically disrupted in a Raytheon DF-101 sonic oscillator (10 min at full power) and were clarified of nuclei and debris by centrifugation at 15,000 rpm for 20 min at 4°C. The quantity of trichloroacetic acid-precipitable radioactivity present in the supernatant fraction of each sample was determined by liquid scintillation counting. Approximately 80% of the trichloroacetic acid-precipitable radioactivity was routinely found in the supernatants.

Virus-specific proteins present in the labeled cell extracts or in the preparations of in vivo-labeled AMV virions were immunoprecipitated by a modification of the staphylococcal protein A-antibody adsorbent method of Kessler (24, 25). All steps were carried out at 0°C, except where indicated. Suspensions of Formalin-fixed and heat-killed *S. aureus* of the protein A-bearing Cowan I strain were washed once with NTE-PMSF buffer containing 0.5% NP-40 and once with NTE-PMSF buffer containing 0.05% NP-40. The bacteria were then resuspended to a concentration of 10% by volume in the NTE-PMSF-0.05% NP-40 buffer. Samples of the labeled cell extracts or labeled virions, containing 0.75×10^6 or 1.0×10^6 cpm of trichloroacetic acid-precipitable ^{35}S, were diluted to 450 μl with the NTE-PMSF-0.05% NP-40 buffer; samples of the radioiodinated p32 protein, containing approximately 27,000 cpm of trichloroacetic acid-precipitable ^{125}I, were similarly diluted. Each diluted sample was preadsorbed for 1 h with 0.1 volume (50 μl) of the washed 10% (vol/vol) suspension of *S. aureus* to remove nonspecific material. The samples were then centrifuged at 25,000 rpm for 20 min. The pellets, containing the bacterial adsorbent, were discarded, and the supernatant fractions were incubated overnight with 10 or 15 μl of the appropriate antiserum or immunoglobulin G preparation. In certain experiments, the antisera and immunoglobulin G preparations were pretreated for 1 h with 1% (vol/vol) Trasylol, a general inhibitor of proteases (4), which was also present in the antigen-antibody reaction mixtures at a concentration of 1% by volume. In radioimmunoprecipitation inhibition assay experiments, certain antiserum samples which had already been pretreated with Trasylol were subsequently incubated for 2 h with either 5 μg of poly(U)-Sepharose-purified AMV p32 or 10 μg of poly(U)-Sepharose-purified AMV αβ DNA polymerase, before the addition of the antisera to the labeled protein preparations which had been preadsorbed with *S. aureus*. Finally, bovine serum albumin at a final concentration of 1 mg/ml was present in all antigen-antibody reaction mixtures as a carrier for the immunoprecipitation of proteins.

After overnight incubation, the antigen-antibody complexes were bound by the addition to each sample of 100 μl of the 10% (vol/vol) suspension of *S. aureus*, which had been washed once again with the NTE-PMSF-0.05% NP-40 buffer. The samples were incubated with the bacteria for 1 h, after which the adsorbent was collected from each sample by centrifugation for 90 s in a Beckman Microfuge B. The adsorbents were each washed three times with 1 ml of buffer containing 500 mM NaCl, 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, 1 mM PMSF, 0.5% NP-40, and 1.0% sodium deoxycholate, with thorough resuspension between washes. The adsorbents were washed a fourth time with 0.5 ml of the same buffer. The antigen-antibody complexes in each sample were eluted from the *S. aureus* adsorbent by resuspension of the pellet from the final microfuge centrifugation in 40 μl of 6 M urea containing 4% SDS and incubation of the samples in a boiling water bath for 3 min. The samples were then centrifuged in the microfuge for 4 min to remove inert adsorbent; the supernatant fraction from each sample was reduced and prepared for electrophoresis by the addition of one-fifth volume (10 μl) of 50% glycerol, 250 mM dithiothreitol, and 10% saturated bromophenol blue solution. The samples were heated at 100°C for 4 min, and the quantity of trichloroacetic acid-precipitable radioactivity present in each sample was determined by liquid scintillation counting.

SDS-polyacrylamide gel electrophoresis, fluorography, and autoradiography of immunoprecipitated proteins. The immunoprecipitated proteins (2,000 to 10,000 trichloroacetic acid-precipitable cpm per sample) were subjected to polyacrylamide gel electrophoresis in the presence of 0.1% (wt/vol) SDS, using the discontinuous buffer system of Laemmli (26)

and a slab gel apparatus similar to that described by Studier (44). Resolving gels contained a linear gradient of 8 to 22% acrylamide (30:8, acrylamide-*N,N'*-methylenebisacrylamide, by weight); stacking gels contained 4.65% acrylamide. The thickness of the gels was 1.5 mm. Molecular weight markers for electrophoresis were [^{35}S]methionine-labeled AMV virion proteins (5,000 or 10,000 trichloroacetic acid-precipitable cpm), [^{3}H]leucine-labeled Moloney MuLV virion proteins (5,000 to 10,000 trichloroacetic acid-precipitable cpm), and poly(U)-Sepharose-purified and radioiodinated AMV p32 (3,600 to 5,000 trichloroacetic acid-precipitable cpm). Electrophoresis was at a constant current of 25 mA for 4.25 to 5.5 h, after which the gels were processed for fluorography by the method of Bonner and Laskey (3), dried under vacuum, and exposed to Kodak RP Royal X-Omat film for 6 to 28 days.

Radioiodination of proteins for peptide mapping. Three to five micrograms of the AMV p32 protein, purified through poly(U)-Sepharose 4B chromatography, or of AMV $\alpha\beta$ DNA polymerase was precipitated from solution with trichloroacetic acid and washed twice with acetone. The protein was then resuspended in 90 μl of 225 mM Tris-hydrochloride (pH 7.5) containing 2 M urea and 1% SDS, and was iodinated by the addition of 10 μl (100 μCi) of Na^{125}I and 5 μl of chloramine T (5 mg/ml in 50 mM Tris-hydrochloride at pH 7.5). After either 3 or 10 min at 25°C, the reaction was stopped by the addition of 3 μl of 100% 2-mercaptoethanol and 5 μl of unlabeled NaI (20 mg/ml in 50 mM Tris-hydrochloride at pH 7.5). Each sample was then layered onto a Sephadex G-50 column (0.9 by 30 cm) pre-equilibrated with 10 mM Tris-hydrochloride (pH 7.5) and eluted at a flow rate of approximately 5 ml/h. The purified radioiodinated proteins eluting in the void volume were pooled and subjected to preparative electrophoresis on 6% polyacrylamide gels in the presence of SDS. The $\alpha\beta$ DNA polymerase samples were subjected to electrophoresis at 10 mA per gel for 7 h; the gel was cut into 2-mm slices, and the radioactivity in each slice was counted by a gamma counter. The fractions containing the radioactivity peak corresponding to the β polypeptide in different gels were combined, as were the peak α fractions from different gels. The labeled protein was eluted from the gel slices by overnight electrophoresis into dialysis tubing. The radioiodinated p32 protein was purified by preparative SDS-polyacrylamide gel electrophoresis as described above, except that the gels were subjected to electrophoresis for 4 h. The specific activity of these labeled proteins was approximately 10^7 cpm/μg.

Proteolytic digestion of eluted proteins. After electrophoretic elution from the gel slices, purified and radioiodinated AMV p32, α, or β was precipitated by the addition of 12% trichloroacetic acid in the presence of 300 μg of bovine serum albumin carrier per ml at 0°C. The precipitated samples were washed with ether at 0°C and were then dissolved in digestion buffer, consisting of 125 mM Tris-hydrochloride (pH 6.8), 0.5% SDS, and 10% glycerol, according to the method of Cleveland et al. (5). The samples were heated at 100°C for 2 min. The quantities of the radioiodinated proteins utilized for proteolytic digestion were varied as indicated in the figure legends. The final concentration of bovine serum albumin in the digestion mixtures varied between 196 and 320 μg/ml, but was held constant within each set of experiments. The protein samples were partially digested by treatment with either chymotrypsin or the *S. aureus* V-8 protease; the latter proteolytic enzyme cleaves peptide bonds carboxy-terminal to aspartic acid and glutamic acid residues (23). The concentrations of the proteases in the hydrolytic reaction mixtures and the duration of incubation of the radioiodinated protein samples with protease at 37°C were also varied as indicated in the figure legends. The reactions were stopped by denaturing the samples at 100°C for 2 min in the presence of 3% 2-mercaptoethanol, 1% SDS, and 2% saturated bromophenol blue solution.

SDS-polyacrylamide gel electrophoretic analysis of proteolytic digests of eluted proteins. Electrophoresis in polyacrylamide gels in the presence of 0.1% (wt/vol) SDS was performed in a slab gel apparatus, utilizing the discontinuous buffer system described by Laemmli (26). Resolving gels contained 15% acrylamide (30:0.8, acrylamide-*N,N'*-methylenebisacrylamide, by weight); stacking gels contained 4.65% acrylamide. The thickness of the gels was 1.5 mm. After electrophoresis of the samples at a constant current of 30 mA for approximately 220 min, the gels were dried under vacuum and exposed for 66 to 168 h to Kodak RP Royal X-Omat film for autoradiography.

RESULTS

Purification of AMV 32. The AMV p32 protein was purified by sequential chromatography of NP-40-lysed AMV on phosphocellulose and poly(U)-Sepharose 4B (Grandgenett et al., in press). The p32 protein eluted from phosphocellulose at 0.36 M potassium phosphate and from poly(U)-Sepharose at 0.66 M KCl. A typical DNA-binding elution profile of AMV p32 from poly(U)-Sepharose is shown in Fig. 1. The AMV p32 protein was purified to near homogeneity as demonstrated by SDS-polyacrylamide slab gel electrophoresis (Fig. 1, insert). The protein electrophoresed as a doublet at a molecular weight of approximately 32,000, although AMV p32 migrated as a single band with a molecular weight of 32,000 on SDS-6% polyacrylamide cylindrical gels (see Fig. 6; also Grandgenett et al., in press). An extremely small amount of DNA polymerase activity coeluted with p32, but neither the α nor the β polypeptide of AMV $\alpha\beta$ DNA polymerase was visible upon electrophoresis of these samples (Fig. 1).

Immunochemical identification of AMV p32. Radioimmunoprecipitation and radioimmunoprecipitation inhibition experiments were utilized to determine if the AMV p32 protein was virus coded or was instead a cellular protein incorporated into virions in small quantities (approximately 1% of the total virion protein) (7, 42). [^{35}S]methionine-labeled AMV virions were immunoprecipitated with an antiserum (sarc III)

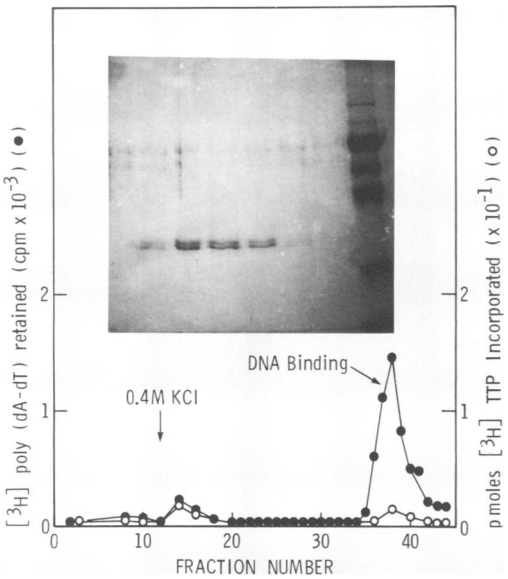

FIG. 1. *Purification of AMV p32. Phosphocellulose-purified p32 was adsorbed to poly(U)-Sepharose (0.6 by 10 cm), washed with 0.4 M KCl, and eluted with a 70-ml gradient from 0.1 M to 1.0 M KCl. Aliquots (3 μl) of various fractions (2.0 ml) were assayed for DNA polymerase activity* (○) *using* $(A)_n \cdot (dT)_{12-18}$, *and for DNA-binding activity* (●) *using* ^3H*-labeled polydeoxyadenylic acid-polydeoxythymidylic acid. The protein concentration at the peak of DNA-binding activity was 22 μg/ml, and the salt concentration was 0.66 M KCl (fraction 38). The proteins in 200-μl samples of fractions 35 to 41 (see insert, left to right) were trichloroacetic acid precipitated, denatured, and subjected to electrophoresis in a 0.75-mm-thick SDS-10% discontinuous polyacrylamide slab gel. The protein bands were stained with Coomassie brilliant blue. The molecular weight markers are phosphorylase A, bovine serum albumin, human gamma globulin, ovalbumin, and chymotrypsinogen.*

derived from tumor-bearing Syrian hamsters which had been injected with cloned and passaged cells from an ASV-induced primary hamster tumor. This antiserum thus contained antibodies to avian retrovirus antigens and to other antigens expressed in hamster tumor cells, but presumably not to chicken cellular antigens (9). Normal Syrian hamster serum and hamster sera derived from animals with primary ASV-induced tumors (LSH 3 or LSH 6) were used as controls. Figure 2 shows the immunoprecipitation of the β (p92) and α (p62) DNA polymerase polypeptides, a doublet at 32,000 daltons, and the low-molecular-weight virion proteins p27, p19, p10, and p15 from a preparation of in vivo-labeled AMV by the sarc III serum (lane 6). The LSH 3 serum immunoprecipitated only p27, p19, p10, and p15 from labeled AMV (lane 9); the β and α polypeptides and the doublet at 32,000 daltons appeared to be absent. No virus-specific proteins were immunoprecipitated from labeled AMV by normal Syrian hamster serum (lane 3). These results suggested that the doublet at 32,000 daltons in immunoprecipitates of AMV virions is encoded by the viral genome, as are the DNA polymerase polypeptides and the low-molecular-weight internal virion proteins.

The tumored hamster sera were also tested for their ability to immunoprecipitate AMV p32 that had been purified directly from plasma virus. For this purpose, poly(U)-Sepharose-purified p32 was radioiodinated and subjected to immunoprecipitation analysis as described above. Figure 3 shows that purified and ^{125}I-labeled AMV p32 was immunoprecipitated by the sarc III (lane 2) and LSH 6 (lane 3) tumored hamster sera but not by normal Syrian hamster serum (lane 1). A non-immunoprecipitated marker preparation of purified and radioiodinated AMV p32 is shown in lane 5. It should be noted that one or more electrophoretic bands migrating faster than the p32 protein proper were present in all immunoprecipitated or marker preparations of ^{125}I-labeled AMV p32. These bands, which were not eliminated by the presence of 1 mM PMSF and 1% Trasylol in the antigen-antibody reaction mixtures nor by pretreatment of antisera and immunoglobulin G preparations with 1% Trasylol (see Materials and Methods), probably represent spontaneous breakdown products of the p32 protein formed during storage of radioiodinated p32 at 4°C.

The sarc III serum (Fig. 2, lane 6; Fig. 3, lane 2) and rabbit antisera directed against AMV cores or detergent-disrupted whole virions (data not shown) were able to immunoprecipitate both (i) a doublet at 32,000 daltons from preparations of labeled AMV virions, and (ii) purified and radioiodinated AMV p32. Radioimmunoprecipitation inhibition assays were utilized to examine the relationship between these two species. In this experiment, 5 μg of poly(U)-Sepharose-purified, unlabeled AMV p32 (Fig. 1) was competed with the p32 protein in preparations of labeled AMV virion proteins for immunoprecipitation by the sarc III serum. Figure 4 demonstrates that both bands of the electrophoretic doublet at 32,000 daltons, which was immunoprecipitated from a preparation of labeled virions by sarc III antiserum (lane 1), were competed out of the immunoprecipitation reaction almost entirely by pretreatment of the antiserum with 5 μg of purified, unlabeled AMV p32 (lane 2). Similar radioimmunoprecipitation inhibition results were obtained with labeled virions that were immunoprecipitated by rabbit antisera directed against AMV p32 (data not shown). These results offer evidence that the electropho-

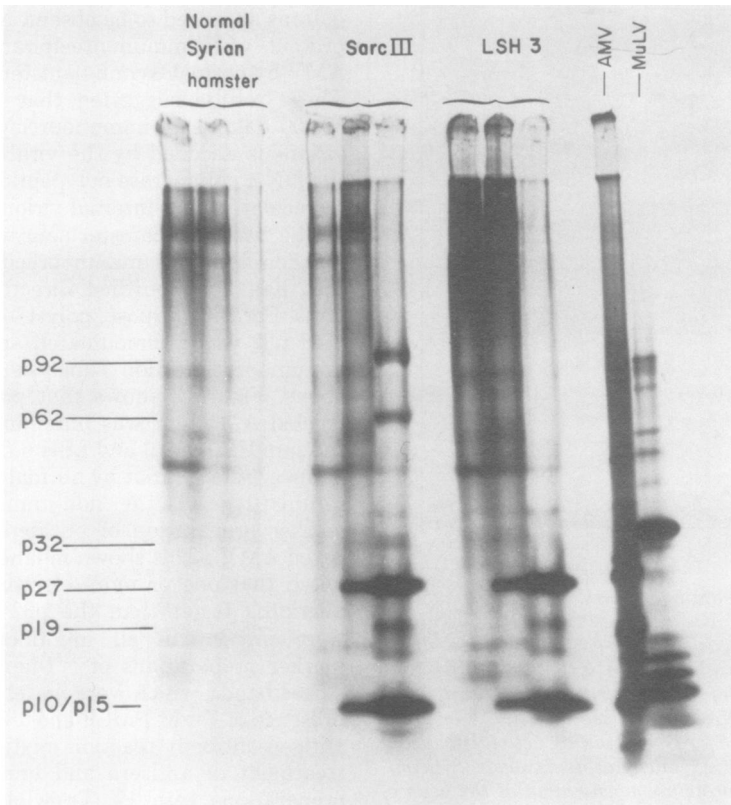

FIG. 2. *Immunoprecipitation of virus-coded proteins. Autoradiogram of SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins immunoprecipitated from 30-min-labeled uninfected (lanes 1, 4, and 7) and 30-min-labeled AMV-infected (lanes 2, 5, and 8) CEF cells and from in vivo labeled AMV virions (lanes 3, 6, and 9) by normal Syrian hamster serum (lanes 1 to 3), sarc III serum (lanes 4 to 6), or LSH 3 serum (lanes 7 to 9). The origins of these antisera are described in the text. Markers are [^{35}S]methionine-labeled AMV virion proteins (lane 10) and [^{3}H]leucine-labeled Moloney MuLV virion proteins (lane 11).*

retic doublet at 32,000 daltons that was immunoprecipitated from labeled virion preparations was indeed identical to the p32 protein from AMV.

Antigenic relationship of the p32 protein to the DNA polymerase β polypeptide. To determine whether the avian retrovirus p32 protein is structurally related to any of the known virus-coded proteins, we attempted to immunoprecipitate AMV p32 with antisera directed specifically against either viral DNA polymerase or certain viral structural proteins. Figure 3 shows that poly(U)-Sepharose-purified and radioiodinated AMV p32 was immunoprecipitated by goat immunoglobulin G directed against AMV αβ DNA polymerase (lane 4); in contrast, ^{125}I-labeled AMV p32 was not immunoprecipitated by goat antiserum directed against the p19 and p27 proteins of AMV (data not shown). Further, the p32 doublet as well as the DNA polymerase α and β polypeptides was immunoprecipitated from in vivo labeled AMV by rabbit antiserum directed against AMV αβ DNA polymerase purified to homogeneity through poly(U)-Sepharose chromatography (Fig. 5, lane 1). The α and β polypeptides and the doublet representing p32 were also immunoprecipitated from labeled virions by rabbit antiserum directed against the AMV p32 protein purified to homogeneity as described in Materials and Methods (Fig. 5, lane 2). Figure 5 also shows that the ratio of the DNA polymerase α polypeptide to the β polypeptide in immunoprecipitates from labeled AMV was reduced substantially with the anti-p32 antiserum (lane 2), compared to the relative amounts of these polypeptides immunoprecipitated by the anti-αβ antiserum (lane 1). This finding suggests that p32 and β were specifically immunoprecipitated by the anti-p32 antiserum, while α was present among the immunoprecipitated proteins primarily because of its close physical association with the β polypeptide in the αβ form

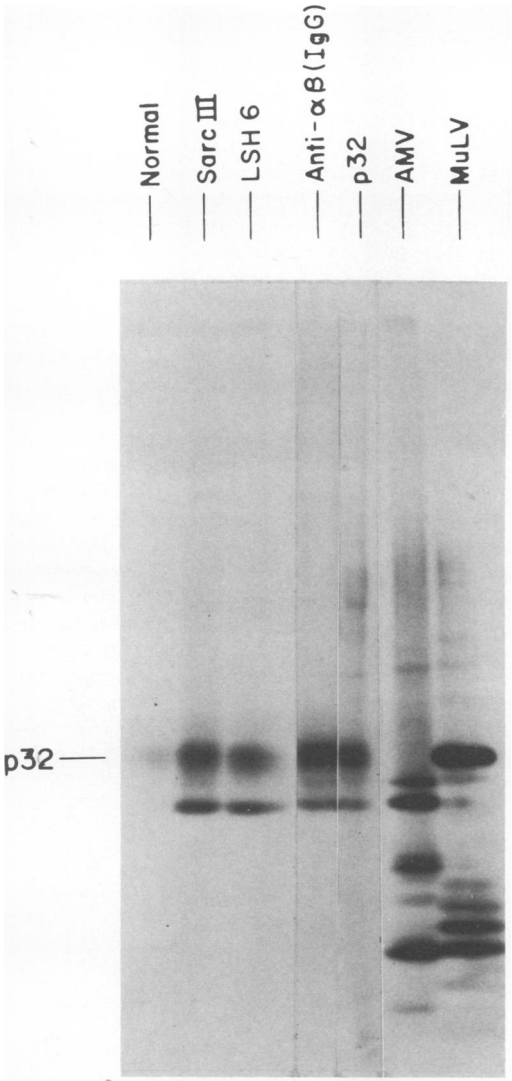

FIG. 3. *Immunoprecipitation of ^{125}I-labeled AMV p32. Autoradiogram of SDS-polyacrylamide gel electrophoresis of purified and ^{125}I-labeled AMV p32 immunoprecipitated by normal Syrian hamster serum (lane 1), sarc III serum (lane 2), LSH 6 serum (lane 3), or goat immunoglobulin G directed against the αβ form of AMV DNA polymerase (lane 4). Markers are ^{125}I-labeled AMV p32 (lane 5), [^{35}S]methionine-labeled AMV virion proteins (lane 6), and [^{3}H]leucine-labeled Moloney MuLV virion proteins (lane 7).*

of the DNA polymerase molecule.

Radioimmunoprecipitation inhibition assays also demonstrated that pretreatment of the sarc III serum with purified, unlabeled αβ DNA polymerase inhibited the immunoprecipitation of the p32 protein as well as that of the α and β polypeptides of in vivo-labeled virions (Fig. 4, lanes 1 and 3). Careful examination of Fig. 4 (lanes 1 and 2) also indicates that pretreatment of the sarc III serum with purified, unlabeled p32 reduced slightly the immunoprecipitation of the DNA polymerase β polypeptide, but not the α; this inhibitory effect was considerably more pronounced with the antisera directed against AMV αβ DNA polymerase or p32 (data not shown). The AMV p32 protein thus appears to share common antigenic determinants with the β polypeptide of the AMV αβ DNA polymerase molecule.

Sequence relatedness of the p32 protein

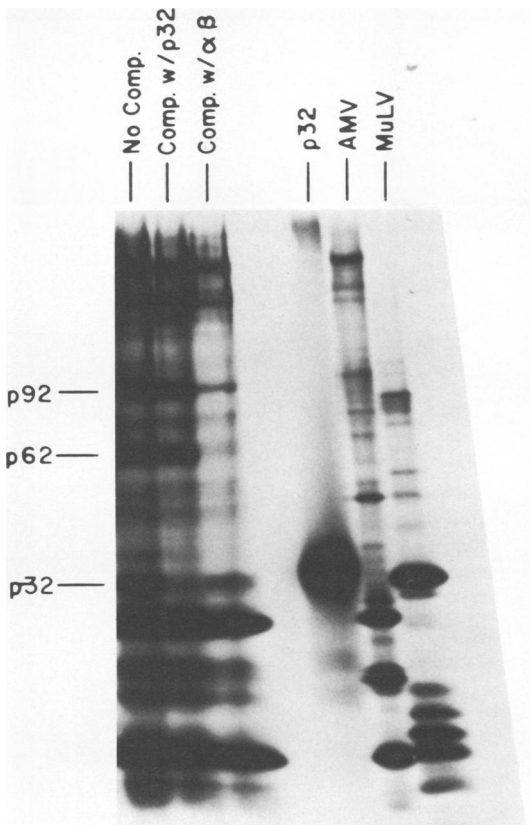

FIG. 4. *Competition of in vivo-labeled p32 with purified AMV p32 and αβ DNA polymerase. Autoradiogram of SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins immunoprecipitated from in vivo-labeled AMV virions by sarc III serum (lanes 1 to 3). Certain samples of this antiserum were preadsorbed for 2 h at 0°C with either 5 μg of poly(U)-Sepharose-purified (see Fig. 1, fraction no. 38) AMV p32 (lane 2) or 10 μg of poly(U)-Sepharose-purified (ref. 15) AMV αβ DNA polymerase (lane 3). Markers are ^{125}I-labeled AMV p32 (lane 4), [^{35}S]methionine-labeled AMV virion proteins (lane 5), and [^{3}H]leucine-labeled Moloney MuLV virion proteins (lane 6).*

FIG. 5. *Immunological cross-reactivity of AMV p32 and the β polypeptide of AMV DNA polymerase. Autoradiogram of SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins immunoprecipitated from in vivo-labeled AMV virions by rabbit antisera directed against the αβ form of AMV DNA polymerase (lane 1) or the AMV p32 protein (lane 2). Markers are [^{35}S]methionine-labeled AMV virion proteins (lane 3) and [^{3}H]leucine-labeled Moloney MuLV virion proteins (lane 4).*

and the DNA polymerase β polypeptide. To characterize the structural relationship between the polypeptides of AMV αβ DNA polymerase and the AMV p32 protein, we radioiodinated the α and β DNA polymerase polypeptides and the p32 protein to subject them to peptide mapping by the method of Cleveland et al. (5). Figure 6 presents the electrophoretic profiles of radioiodinated, Sephadex G-50–purified, and denatured αβ DNA polymerase and p32. Only the fractions containing the radioactivity peaks corresponding to β (fractions 20 and 21, top), to α (fractions 30–33, top), or to the single p32 band resolved by this electrophoretic system (fractions 35–38, bottom) were combined from six gels and enzymatically digested for peptide mapping.

High-resolution, one-dimensional peptide maps produced by SDS-polyacrylamide gel electrophoresis of partial chymotryptic or *S. aureus* V-8 protease digests of ^{125}I-labeled AMV p32, α, and β are shown in Fig. 7 and 8, respectively. With each protease, a unique pattern of proteolytic peptides was obtained from each labeled polypeptide, either by varying the concentration of the enzyme used for a constant digestion period or by varying the duration of hydrolysis with a constant amount of protease. Figure 7 reveals numerous similarities between the profiles of the peptides produced by limited chymotryptic hydrolysis of ^{125}I-labeled p32 and β. In Fig. 7A, the first set of three lanes represents undigested polypeptides (no treatment); at a chymotrypsin concentration of 75 μg/ml, the β polypeptide was completely degraded (lane 12)

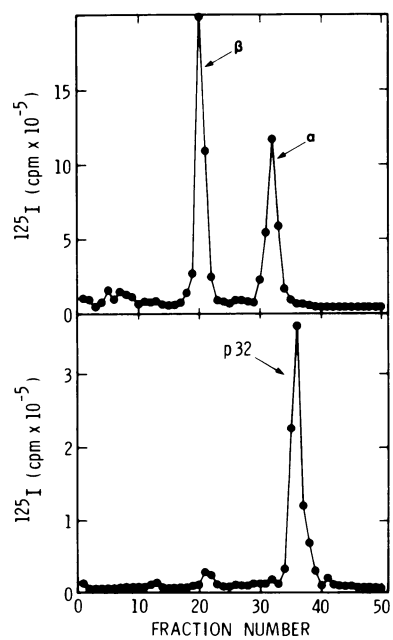

FIG. 6. *Preparative SDS-polyacrylamide gel electrophoresis of AMV αβ DNA polymerase (top) and AMV p32 (bottom) following radioiodination and Sephadex G-50 column chromatography. Migration was from left to right in both profiles.*

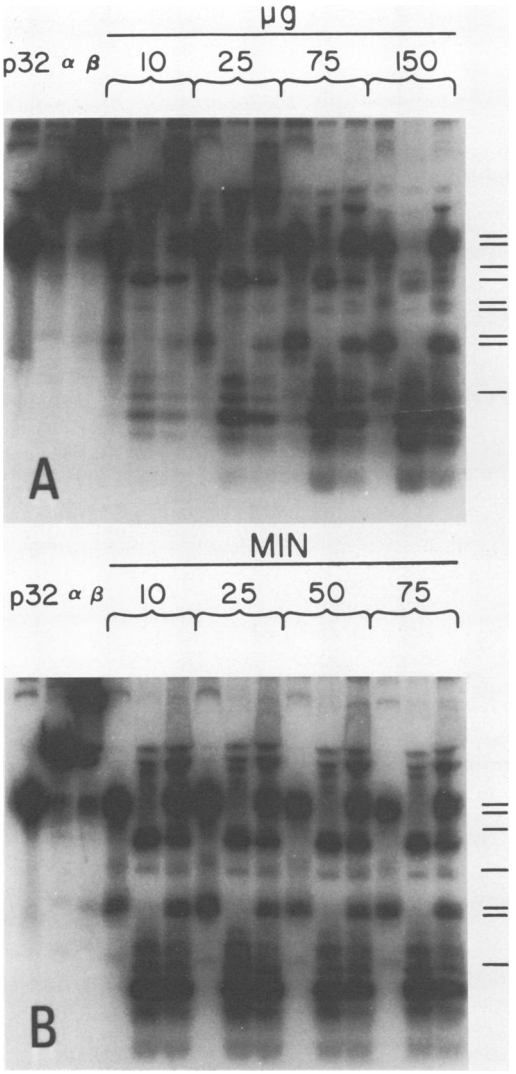

FIG. 7. *Chymotryptic peptide maps of AMV p32 and the α and β polypeptides of AMV DNA polymerase. (A) A total of 40,000 cpm each of ^{125}I-labeled p32, α, β, all purified by Sephadex G-50 column chromatography and preparative SDS-polyacrylamide gel electrophoresis (see Fig. 6), were digested with 0 to 150 μg of chymotrypsin per ml for 30 min at 37°C, as described in the text. Each group of three peptide maps, viewed from left to right, corresponds to p32, α, and β, respectively. (B) The same procedure was carried out except that a constant concentration of chymotrypsin (25 μg/ml) and a varying incubation period (indicated on the figure in minutes) were utilized.*

to yield a number of peptides, including a species which migrated as a doublet at the same molecular weight as the intact p32 protein (lane 1). With 150 μg of chymotrypsin per ml, nine peptide fragments of AMV p32 were produced (lane 13), all comigrating with peptides derived from β (lane 15; see indicator lines). It should be noted that the fastest-migrating peptide in this digest of the p32 protein is discernible in the β hydrolysate in autoradiograms exposed for shorter intervals.

Similar results were obtained when labeled p32 and β were hydrolyzed for varying periods

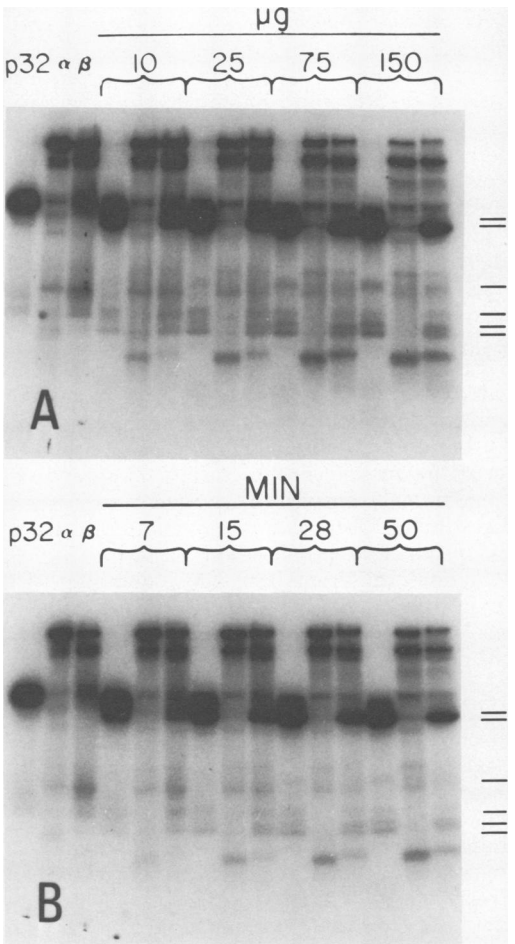

FIG. 8. *S. aureus V-8 protease digestion of AMV p32 and the α and β polypeptides of AMV DNA polymerase. (A) Approximately 50,000 cpm each of ^{125}I-labeled p32, α, and β, all purified by Sephadex G-50 column chromatography and preparative SDS-polyacrylamide gel electrophoresis (see Fig. 6), were digested with 0 to 150 μg of the S. aureus V-8 protease per ml for 30 min at 37°C, as described in the text. Each group of three peptide maps, viewed from left to right, corresponds to p32, α, and β, respectively. (B) The same procedure was carried out except that a constant concentration of V-8 protease (25 μg/ml) and a varying incubation period (indicated on the figure in minutes) were utilized.*

with a constant amount (25 μg/ml) of chymotrypsin (Fig. 7B). Over the full range of hydrolysis conditions employed (the concentration of chymotrypsin or the duration of the exposure of the radioiodinated polypeptides to the proteolytic enzyme), our results indicate that all of the peptides that were electrophoretically demonstrable in chymotryptic digests of labeled AMV p32 were also present in digests of the β polypeptide of AMV DNA polymerase. In contrast, chymotryptic digests of p32 and the α polypeptide appeared to share no common peptide sequences (Fig. 7), suggesting the existence of little or no structural homology between these polypeptides. Consistent with the established derivation of the DNA polymerase α polypeptide from β by proteolytic cleavage (13, 30, 36, 41), our peptide maps demonstrate that approximately 9 to 11 common peptides were shared by chymotryptic digests of radioiodinated α and β (Fig. 7). In fact, hydrolysates of ^{125}I-labeled β seemed to contain no peptides that were not present in digests of either α or p32.

Peptide mapping of purified and radioiodinated AMV p32, α, and β was also carried out using a second proteolytic enzyme, *S. aureus* V-8 protease, to minimize the likelihood of misinterpreting instances of fortuitous comigration of nonidentical peptides. *S. aureus* V-8 protease digestion of ^{125}I-labeled p32, α, and β resulted in the production of digestion patterns (Fig. 8) completely different from those obtained with chymotrypsin (Fig. 7). Six peptide species were resolved in *S. aureus* V-8 protease digests of the p32 protein, and all six comigrated with peptides found in V-8 protease digests of the β polypeptide (Fig. 8; see indicator lines). A peptide species represented by a doublet obtained from intact p32 was also present in V-8 protease digests of ^{125}I-labeled β (top two indicator lines in Fig. 8A or B), but was not clearly resolved in these digests of the p32 protein itself because of the relatively long autoradiographic exposure. Treatment of labeled p32 or β with 200 μg of *S. aureus* V-8 protease per ml for 60 min resulted in the complete degradation of each of these polypeptides, with all four of the peptides present in the digest of p32 also present in the β hydrolysate (see indicator lines 2, 3, 5, and 6 in Fig. 8A or B). As in the case of chymotryptic hydrolysis, no common peptides were shared by the *S. aureus* V-8 protease digests of ^{125}I-labeled p32 and α, while six or seven peptides were common to V-8 protease digests of both α and β. The results from both the chymotryptic and *S. aureus* V-8 protease peptide maps thus indicate that p32 is derived from the DNA polymerase β polypeptide, but not from the α-specific moiety of β.

Expression of AMV p32 in virus-infected cells. Radioimmunoprecipitation and radioimmunoprecipitation inhibition experiments were also used to study the expression of the p32 protein in AMV-infected CEF cells. Figure 2 shows that both the sarc III (lane 5) and LSH 3 (lane 8) sera immunoprecipitated virion proteins p27, p10, and p15, and the 33,000-dalton precursor (light band migrating slightly slower than the p32 doublet in lane 6) to the viral p19 protein (8, 12, 46), and a single electrophoretic band at 32,000 daltons from extracts of 30-min-labeled AMV-infected CEF cells. The α and β polypeptides of the AMV DNA polymerase were not apparent among the proteins immunoprecipitated from extracts of labeled AMV-infected CEF cells, due to the extremely limited expression of these polypeptides in infected cells (33); nevertheless, minor bands which comigrated with the α and β DNA polymerase polypeptides have been immunoprecipitated by rabbit anti-DNA polymerase antiserum from 2-h-labeled ASV-infected cells (32). In control assays, the sarc III (lane 4) and LSH 3 (lane 7) sera were unable to precipitate either the known virus-coded proteins or the single or double band at 32,000 daltons from extracts of 30-min-labeled uninfected cells; also, normal Syrian hamster serum precipitated no virus-specific proteins from labeled cell extracts (lanes 1 and 2).

Radioimmunoprecipitation assays were conducted utilizing the rabbit antisera directed only against AMV αβ DNA polymerase or p32 as more specific probes for the intracellular expression of the p32 protein; rabbit antisera directed against AMV cores or detergent-disrupted whole virions were also used. Although all of these antisera immunoprecipitated the DNA polymerase polypeptides and the p32 protein from labeled virions (see Fig. 5), no antiserum was able to immunoprecipitate them in discernible quantities from 30-min-labeled or 24-h-labeled AMV-infected cells (data not shown). Therefore, even if the p32 protein was expressed in these virus-infected cells, its expression, like that of the viral DNA polymerase polypeptides, was extremely limited compared to the expression of the gag internal virion proteins.

DISCUSSION

We have demonstrated that a polypeptide species with a molecular weight of 32,000, which migrated as an electrophoretic doublet, can be selectively immunoprecipitated (along with other virus-coded proteins) from AMV propagated in CEF cells by a hamster antiserum (sarc III) directed against antigens of virus-coded origin but not against CEF cellular antigens. Radioimmunoprecipitation inhibition assays

showed that the immunoprecipitated, in vivo labeled 32,000-dalton protein was identical to the p32 nucleic acid-binding protein that was purified from plasma AMV. We have also demonstrated that AMV p32 cross-reacted with the β polypeptide of AMV $\alpha\beta$ DNA polymerase in radioimmunoprecipitation and radioimmunoprecipitation inhibition assays, indicating the existence of a structural relationship between p32 and β. Peptide mapping experiments clarified the nature of the structural relationship between p32 and the β polypeptide. These experiments suggested that p32 arises by proteolytic cleavage of the β polypeptide at a site near or identical to that at which the α polypeptide is generated from β by proteolytic cleavage.

Our peptide mapping data (see Fig. 7) showed that partial chymotryptic digestion of ^{125}I-labeled β resulted in the formation of a peptide species migrating as a doublet at the same molecular weight as the doublet representing intact, labeled AMV p32; this peptide was not found in hydrolysates of ^{125}I-labeled α. Lai and Verma (27) have similarly demonstrated that chymotryptic digestion of radioiodinated AMV $\alpha\beta$ DNA polymerase yielded a peptide with an apparent molecular weight of 32,000; further, peptide mapping indicated that this peptide was derived from the β polypeptide but was not contained in its α-specific moiety.

The α form of the avian retrovirus DNA polymerase binds with less affinity than either $\alpha\beta$ or β_2 DNA polymerase to a variety of natural and synthetic nucleic acids, including viral 70S and 35S RNA (17, 21, 22, 35) and the tRNA^{Trp} primer for RNA-directed DNA synthesis (6, 18, 22, 34). Our data provide evidence that the p32 protein represents the portion of the β polypeptide which enables $\alpha\beta$ and β_2 to bind in vitro with greater affinity than free α to the nucleic acid substrates for their enzymatic activities. Perhaps the p32-specific portion of the β polypeptide enhances the affinity with which β binds to nucleic acids by directly providing additional high-affinity nucleic acid-binding sites; alternatively, the p32 portion may modify the conformation of the β polypeptide with respect to the nucleic acid-binding sites on the α-specific part of the molecule.

Using radioimmunoprecipitation assays and peptide mapping techniques, a polypeptide with a molecular weight of approximately 180,000 has been identified in cell-free translation systems (37, 39) and in ASV-transformed CEF cells (32, 39) as a precursor to the avian retrovirus group-specific antigenic (gag) proteins and the DNA polymerase. The 180,000-dalton precursor appears to result from the uninterrupted translation of the genes coding for the gag proteins (*gag* gene) and the DNA polymerase molecule (*pol* gene), which occupy the 5′ end of the avian retrovirus genome (32, 37, 39). The findings presented in this report are consistent with previously proposed models for the posttranslational processing of this precursor to yield the $\alpha\beta$, β_2, and α DNA polymerase species (20, 37). It appears that the p32 protein may be derived in vivo from proteolytic cleavage of the β_2 form of the viral DNA polymerase, which has been shown to be present in relatively large quantities in ASV that had been propagated in cultured duck embryo fibroblasts (20).

The biochemical basis of the electrophoretic heterogeneity displayed by the AMV p32 protein in immunoprecipitates from virions and in purified form is not understood at present. Perhaps p32 is partially phosphorylated in vivo, as are the avian retrovirus p19 (9, 28) and p12 (28) proteins, with the phosphorylated form of the protein migrating more slowly in SDS-polyacrylamide gels than the non-phosphorylated species. Preliminary evidence from our laboratory suggests that the AMV p32 protein indeed exists in both phosphorylated and non-phosphorylated forms in vivo (R. D. Schiff and D. P. Grandgenett, manuscript in preparation). It is interesting that a polypeptide with a molecular weight of approximately 38,000 was immunoprecipitated from [^{35}S]methionine-labeled AMV by the sarc III and LSH 3 sera (Fig. 2, lanes 6 and 9) and by rabbit antisera directed against AMV $\alpha\beta$ DNA polymerase or p32 (Fig. 5, lanes 1 and 2). The immunoprecipitation of this polypeptide from labeled AMV by sarc III serum was inhibited by competition with purified unlabeled AMV p32 or $\alpha\beta$ DNA polymerase (Fig. 4, lanes 1 to 3). The origin and significance of this polypeptide are unknown. One other polypeptide, migrating at a molecular weight of approximately 47,000, was present in all immunoprecipitates examined. This species is probably a cellular protein which becomes incorporated into virions during assembly and owes its presence in immunoprecipitates simply to nonspecific precipitation or "trapping."

Other investigators have identified DNA polymerase polypeptides with a molecular weight of approximately 110,000 daltons, which were specific to RD-114 virus (11) and Rauscher MuLV (29) and were present predominantly in virus-infected cells. In addition, a high-molecular-weight DNA polymerase (approximately 110,000) was produced by cell-free translation of 35S RNA from Moloney MuLV (38). In contrast, the virion-associated polymerase of each of these mammalian retroviruses has a molecular weight between 70,000 and 84,000. It is not known whether the polypeptide fragment with a molec-

ular weight of approximately 30,000 cleaved from the high-molecular-weight DNA polymerase molecules to generate the virion-associated DNA polymerase exists as an independent protein species in cells or virions in these systems, analogous to the avian retrovirus p32 protein.

A DNA endonuclease activity is associated with the p32 protein throughout its purification from AMV (Grandgenett et al., in press). In the presence of Mg^{2+}, this p32-associated endonuclease activity is able to convert *E. coli* supercoiled ColE1 DNA to the relaxed form by means of a single-stranded nick introduced by the enzyme at a limited number of preferred sites on the DNA molecule. When Mn^{2+} is substituted for Mg^{2+} in the enzymatic reaction mixtures, the endonuclease activity of AMV p32 with supercoiled ColE1 DNA as substrate is enhanced 20-fold but site specificity is lost. While purified AMV p32 does not share the DNA polymerase and RNase H activities characteristic of the $\alpha\beta$, β_2, and α forms of the DNA polymerase enzyme, our findings and characterization of the precursor-product relationship between the β polypeptide and the p32 protein suggest that β must also possess the capacity for DNA endonuclease activity, which is encoded in its p32-specific portion. Recent evidence from our laboratory indicates that the $\alpha\beta$ form of AMV DNA polymerase, but not free α, is capable of nicking supercoiled ColE1 plasmid DNA in the presence of Mn^{2+} (M. C. Golomb and D. P. Grandgenett, submitted for publication).

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