

Synthesis and Processing of Polymerase Proteins of Wild-Type and Mutant Avian Retroviruses

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We have studied the biosynthesis of avian retrovirus proteins related to reverse transcriptase in permissive avian embryonic cells. Analysis of immune precipitates from avian sarcoma virus (ASV)-infected cells demonstrated the presence of the 180,000-dalton *gag-pol* "read-through" protein (Pr180^{*gag-pol*}) and a 130,000-dalton polypeptide (Pr130^{*gag-pol*}). Pr130^{*gag-pol*} was found, in serological and peptide mapping studies, to consist primarily of sequences related to reverse transcriptase and the *gag*-encoded protein p15. Pr180^{*gag-pol*} was found to be phosphorylated, whereas Pr130^{*gag-pol*} was not. In addition, only Pr180^{*gag-pol*} but not Pr130^{*gag-pol*} was susceptible to cleavage with the virion protease p15. Although the structure of Pr130^{*gag-pol*} would suggest that it is generated by removal of a portion of the *gag* region from Pr180^{*gag-pol*}, an analysis of labeling kinetics has failed to demonstrate unequivocally whether Pr130^{*gag-pol*} is a cleavage product of Pr180^{*gag-pol*} or a primary translation product. We were repeatedly unable to detect either Pr180^{*gag-pol*} or Pr130^{*gag-pol*} in virus particles released from the cell, whereas both β and α subunits were readily observed. Several presumed intermediates between Pr130^{*gag-pol*} and the β subunit of reverse transcriptase were also observed in virions. These studies indicate cleavage of polymerase precursors at the time of virus budding. On the basis of these data, we present a processing scheme for the generation of reverse transcriptase subunits. We have also examined reverse transcriptase biosynthesis in cells producing two mutants that fail to package the enzyme. Previous work showed that integrated proviruses of both mutants are missing DNA sequences in *pol*: one mutant, PH9 (Mason et al., *J. Virol.* **30**:132-140, 1979), contains a deletion near the 3' end of *pol*, whereas the other, SE52d (Linial et al., *Virology* **87**:130-141, 1978), may have inserted a host cell sequence near the 5' end of *pol*. Neither mutant synthesized Pr180^{*gag-pol*} or Pr130^{*gag-pol*}, but instead produced novel proteins comprised of sequences shared with *gag* proteins plus a region antigenically related to reverse transcriptase. Both proteins were defective as precursors to reverse transcriptase. Whereas Pr180^{*gag-pol*} and Pr130^{*gag-pol*} were precipitated by an antiserum raised against p32 (a virion protein derived from the portion of the β subunit removed during processing of β to α [Schiff and Grandgenett, *J. Virol.* **28**:279-291, 1978]), the novel protein synthesized by PH9 was not precipitated. This suggests that the α subunit is generated by a COOH-terminal cleavage of the β subunit.

The group of enzymatic activities known as reverse transcriptase is found within the core structure of retrovirus particles (48). This enzyme complex possesses RNA- and DNA-dependent DNA polymerase activities as well as a processive hybrid-specific RNase activity (RNase H). All three appear to be localized on one polypeptide chain, and the two polymerase activities probably share the same active site (48, 50). In the avian sarcoma and leukemia viruses (ASLV), reverse transcriptase is isolated from virions as a two-subunit complex. Both the larger β (95,000-dalton) and the smaller α (65,000-dalton) subunits possess polymerase and RNase H activities (21, 50).

Reverse transcriptase is encoded by the retrovirus gene termed *pol*. Evidence for its viral origin rests on the existence of conditional viral mutants possessing temperature-sensitive polymerase activity (29, 49) as well as on deletion or substitution mutants in *pol* (15, 28, 31, 35). Furthermore, genomic 39S RNA isolated from avian and murine retroviruses can program the *in vitro* synthesis of a polyprotein containing reverse transcriptase (2, 23, 39, 40).

Studies on the biosynthesis of reverse transcriptase in avian and mammalian retrovirus systems have led to similar conclusions. In both systems, infected cells synthesize a polyprotein in the size range of 180,000 to 200,000 daltons

which contains reverse transcriptase and the viral core proteins (16, 20, 37). The core proteins are the products of the *gag* gene which lies on the 5' side of *pol*. Thus, reverse transcriptase is synthesized as part of a large polyprotein that is the common product of the *gag* and *pol* genes ($Pr^{gag-pol}$). The *gag*-specified portion of $Pr^{gag-pol}$ is also synthesized as a separate product which, in ASLV, is a 76,000-dalton polyprotein ($Pr76^{gag}$). $Pr76^{gag}$ is the direct precursor to the four virion core proteins, which it contains in the order NH₂-p19-p27-p12-p15-COOH (47). Kinetic experiments suggest that the 180,000-dalton ASLV protein ($Pr180^{gag-pol}$) is not a precursor to $Pr76^{gag}$ (16, 37). Moreover, the amount of $Pr180^{gag-pol}$ found in infected cells is 20- to 50-fold lower than the amount of $Pr76^{gag}$. These data are consistent with the relative levels of core proteins and reverse transcriptase in virus particles (4, 38).

Relatively little is known concerning the actual processing of $Pr180^{gag-pol}$ except that the intracellular half-life of ASLV- $Pr180^{gag-pol}$ is significantly longer than for $Pr76^{gag}$ (16, 37). It is known that at least a portion of the β subunit is cleaved to generate the 65,000-dalton α subunit (12, 33) and a 32,000-dalton fragment (p32) which possesses endonuclease activity (14, 44).

In this communication, we examine the biosynthesis of reverse transcriptase-related polypeptides in cells infected with wild-type ASLV and in two types of cells shedding nonconditional mutants in *pol*. Both of these mutants had been initially characterized as lacking virion-associated reverse transcriptase activity. The integrated genome of the mutant cdSE52d contained a substitution near the 5' end of *pol* (8, 28), whereas the mutant rdPH9PR-A contained a deletion near the 3' end of *pol* (31). We show that cells harboring these viral genomes synthesize altered forms of $Pr^{gag-pol}$ which are not processed to generate reverse transcriptase. In addition, the position of the 3' *pol* deletion in rdPH9PR-A has allowed us to determine that the location of the polypeptide segment unique to the β subunit (i.e., p32 cleaved from β during generation of α) is at the COOH terminus of the subunit. We also describe a 130,000-dalton protein, present in cells infected with wild-type virus, that possesses reverse transcriptase and at least a portion of p15, the COOH-terminal protein of $Pr76^{gag}$. This species seems likely to be an intermediate derived from cleavage of a portion of the *gag* region of $Pr180^{gag-pol}$.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblast cells (CEF) were cultured from 10-day embryos of White

Leghorn chickens obtained from H and N (Redmond, Wash.). Quail embryo fibroblasts (QEF) were cultured from 9-day embryos obtained from Life Sciences, Inc., St. Petersburg, Fla. C/E embryos of line 6, subline 3, homozygous at the I locus for group-specific antigen expression (6), were provided by L. B. Crittenden.

The standard laboratory strain of the Prague C strain of Rous sarcoma virus (PR-RSV-C) was used. The polymerase mutant cdSE52dPR-C (SE52d) was isolated from a transformed quail cell clone producing noninfectious virus after coinfection of the quail cells by PR-RSV-C and Rous-associated virus-0 (RAV-0). Its isolation has been described in detail by Linial et al. (28). The virus designated PR-E 52+ is a wild-type sister clone of SE52d.

The transformed quail cell clone designated Q-PRA-9 was originally derived after infection with the PR-A strain and shown to shed the polymerase mutant rdPH9PR-A (PH9). The isolation and characterization of PH9 have been described by Mason et al. (31). All transformed cultures were grown in Ham F10 containing 10% tryptose phosphate broth, 5% calf serum, and 1% dimethyl sulfoxide (GM+D), and cells were passaged whenever confluent.

Radioactive labeling of cells and viruses. For radioactive labeling, confluent monolayers were washed three times with phosphate-buffered saline (PBS) (37°C) and incubated at 37°C for various amounts of time in Earle basal salt solution containing the indicated concentrations of [³⁵S]methionine (specific activity >100 Ci/mmol). For labeling periods exceeding 20 min, the cells were labeled in minimal essential medium lacking methionine. The cells were either lysed immediately or, for chase experiments, further incubated in growth medium containing a fivefold excess of unlabeled methionine at 37°C for various time periods. For lysis, the plates were transferred onto ice, washed once with ice-cold 0.02 M Tris-hydrochloride (pH 7.5)-0.05 M NaCl, and lysed by the addition of the same buffer containing 0.5% sodium deoxycholate and 0.5% Nonidet P-40 (NP-40) (Particle Data) (Ab buffer) plus 1% trasyloyl (Aprotinin; Sigma Chemical Co.), a protease inhibitor. The resulting lysate was scraped into a tube, blended for 30 s, and centrifuged at 10,000 × *g* for 10 min. The supernatant was used for immunoprecipitation.

[³H]methionine-labeled virus was prepared by growth of confluent cells with 250 μ Ci of [³H]methionine (Amersham Corp.) for 16 h at 37°C. The labeled medium was then replaced with GM+D, and the incubation was continued for 6 h. The unlabeled and labeled media were combined and centrifuged at 10,000 × *g* for 10 min, and the supernatant was layered over 2 ml of 25% sucrose in 0.02 M Tris-hydrochloride-1 mM EDTA (pH 7.5). After centrifugation in an SW41 rotor at 35,000 rpm for 90 min, the supernatant fluids were aspirated and the pellet was resuspended in Ab buffer plus 0.3% sodium dodecyl sulfate (SDS) and 1% trasyloyl.

Immune reagents. The antiserum referred to as anti-*gag* serum (A-180) was prepared in a New Zealand white rabbit by subcutaneous injection of 50 μ g of p19, p27, p12, and p15 (*gag* proteins). The *gag* proteins, from disrupted avian myeloblastosis virus (AMV), were purified by chromatography on phos-

phocellulose. When tested on labeled disrupted virions, this serum precipitated only p27, p19, p12, and p15 but not polymerase subunits nor envelope glycoprotein components (not shown). The antiserum referred to as anti-AMV was prepared by using whole disrupted AMV virions as previously described (9).

Rabbit monospecific anti-p27, anti-p19, anti-p12, and anti-p15 sera were gifts from V. M. Vogt and were prepared using AMV proteins purified by gel filtration in 6 M guanidine-hydrochloride. Rabbit anti-*pol* serum was a gift from H. Oppermann and was raised against purified reverse transcriptase from PR-RSV-C (37). Rabbit antiserum against the p32 protein was a gift from D. Grandgenett and was prepared as described by Schiff and Grandgenett (44).

To block antibodies against *gag* proteins, we took advantage of the fact that virus particles contain more than 50-fold-higher quantities of *gag* proteins than reverse transcriptase. Ten microliters of antiserum was incubated with 120 μ g of disrupted purified AMV for 30 min at room temperature. Appropriate volumes of the blocked sera were used directly for immunoprecipitation.

Immunoprecipitation. All immunoprecipitation experiments were carried out with Formalin-treated *Staphylococcus aureus* as previously described (9). Briefly, to cell lysates or resuspended viral pellets, SDS was added to 0.3% followed by the indicated amount of antiserum. The mixture was incubated at 4°C for 30 min. Twenty volumes per volume of serum of a 100-mg/ml solution of Formalin-fixed *S. aureus* was added, and the lysate was incubated for a further 30 min at 4°C. The fixed bacterial suspension had been previously washed in 0.5% NP-40 and a 1-mg/ml concentration of bovine serum albumin in PBS and suspended in Ab buffer plus bovine serum albumin (1 mg/ml). The immune complexes were centrifuged at 4°C at 1,000 $\times g$ for 10 min and washed once in Ab buffer plus 0.1% SDS and 1% trasyol, once in PBS containing 0.1% SDS and 0.5% NP-40, and finally in 0.01 M Tris-hydrochloride (pH 7.5).

Immune complexes were resuspended in 75 μ l of electrophoresis sample buffer (24) containing 5% SDS and 4% 2-mercaptoethanol. The mixture was boiled for 3 min and centrifuged at 12,000 $\times g$ for 5 min at room temperature. The supernatant was carefully removed and stored at -20°C.

Gel electrophoresis. Discontinuous SDS-polyacrylamide slab gels were prepared as described by Laemmli (24) and modified by Blattler et al. (3). Electrophoresis was carried out at 20 mA. Gels were stained in 0.25% Coomassie brilliant blue dye in 50% methanol-7% acetic acid and destained by diffusion in the same buffer without dye. For autoradiography, the gels were extensively washed with water and dried on a sheet of Whatman no. 3 filter paper in vacuo before exposure to X-OMAT R film (Eastman Kodak). Fluorography was as described by Laskey and Mills (26) using X-OMAT R film preexposed to an optical density of 0.1.

The apparent molecular weight of relevant proteins was determined by electrophoresis with mixtures of ¹⁴C-dansylated polypeptides of known molecular weight in an adjacent well. The distances of migration of the polypeptides were measured relative to the

bromophenol blue dye and plotted against the logarithm of molecular weight of the polypeptide standards.

For scanning densitometry of autoradiographs, a Zeineh Soft Laser scanning densitometer, equipped with an automatic integrator, was used (Biomed Instruments Inc.).

Tryptic digestion and ion-exchange chromatography. Preparative gels for tryptic mapping were run as described above, but were not stained or destained before drying. The paper edges of the dried gel were spotted with radioactive ink, and the resulting spots on the autoradiograph were used to orient the film with the dried gel. The regions of the dried gel corresponding to the relevant labeled bands on the autoradiograph were located and excised. Each gel slice was placed in 1 ml of 0.01 M NH₄HCO₃ (pH 8.5) containing 100 μ g of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin per ml (Worthington Biochemicals Corp.) and digested for 24 h at 37°C. The solution was then replaced, and the incubation was continued for an additional 24 h. The digests were lyophilized several times to remove salts and taken up in 0.2 ml of performic acid. After 1 h at 0°C, the oxidation was terminated by the addition of 1 ml of water and subsequent lyophilization.

The labeled tryptic peptides were mixed in buffer A (140 ml of acetic acid-1.5 ml of pyridine-360 ml of water, pH 2.5) and rinsed into a 50°C water-jacketed column (30 by 1 cm) of Chromobeads type P (Technicon Corp., Inc.) cation-exchange resin which had been previously equilibrated with buffer A. Peptides were eluted from the column with a quadratic gradient to pH 4.2. Mixing chambers 1 and 2 each contained 180 ml of buffer A, and chamber 3 contained 60 ml of buffer A and 120 ml of buffer B (71.5 ml of acetic acid-80.5 ml of pyridine-350 ml of water, pH 4.5). The column was finally rinsed with buffer B. The flow rate was 18 ml/h. Fractions of 3 ml were dried overnight at 120°C, redissolved in 50 mM HCl, diluted 20-fold with water-solubilizing scintillation fluid (Biofluor, New England Nuclear Corp.), and counted in a liquid scintillation counter. Recoveries from the column were generally >90%.

RESULTS

Reverse transcriptase-related proteins synthesized by wild-type viruses. Figure 1 shows the results of SDS-polyacrylamide gel (PAGE) analysis of immune complexes formed by addition of anti-p27^{gag} (lanes A and C) and anti-*pol* (lanes B and D) sera to lysates of ASV-infected CEF or QEF. Cultures of CEF producing PR-RSV-C (lanes C and D) and QEF producing the recombinant virus PR-E 52+ (lanes A and B) were labeled for 2 h with [³⁵S]methionine before lysis in a detergent mixture. Both viruses are wild type for reverse transcriptase as well as other replication and transformation functions. Anti-p27^{gag} serum precipitated the *gag*-related proteins Pr180^{gag-pol}, Pr76^{gag}, and p27 and some cleavage intermediates from PR-RSV-C-infected cells (lane C), whereas anti-*pol*

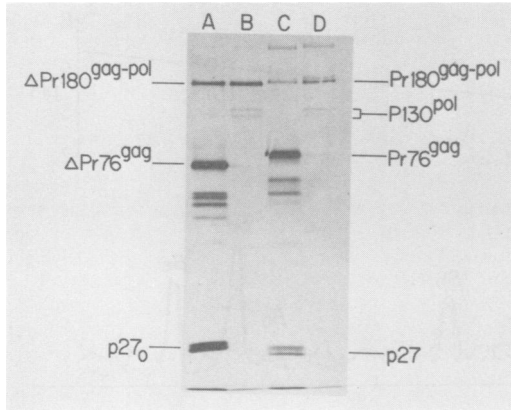


FIG. 1. Analysis of anti-p27 and anti-pol immunoprecipitates from ASV-infected cells. CEF producing PR-RSV-C and QEF producing the wild-type recombinant virus PR-E 52+ were labeled for 2 h with 100 μ Ci of [35 S]methionine in methionine-depleted medium. The cells were lysed, and the lysates were divided in half and subjected to immunoprecipitation with antiserum raised against p27 (2 μ l) or reverse transcriptase (2 μ l) (anti-pol) as described. The anti-pol serum was absorbed with 120 μ g of unlabeled purified AMV to remove contaminating antibodies against gag proteins. The washed precipitates were dissolved in electrophoresis sample buffer and fractionated by 10% PAGE in SDS. (A) QEF infected with PR-E 52+ precipitated with anti-p27; (B) QEF infected with PR-E 52+ precipitated with anti-pol; (C) CEF infected with PR-RSV-C precipitated with anti-p27; (D) CEF infected with PR-RSV-C precipitated with anti-pol. The band designated p27₀ is a higher-molecular-weight form of p27 found in isolates of the endogenous virus RAV-0 and its recombinants.

serum precipitated Pr180^{gag-pol} and a faint doublet with an apparent molecular weight of 130,000 (P130) (lane D). The recombinant virus PR-E 52+ produced the P130 doublet as well as gag-related precursors migrating faster than wild-type virus proteins because of a deletion of several thousand daltons in gag generated by a recombinational event (Δ Pr76^{gag}, Δ Pr180^{gag-pol}; 46).

Although the amount of P130 precipitated with anti-pol serum was low in Fig. 1, other experiments showed relatively higher levels of this protein (see Fig. 3, 7, and 10). The reason for this variability is unknown at present, but does not appear to be dependent on whether the virus is grown in chick or quail cells (not shown). In addition, P130 is unlikely to be a product of nonspecific proteolysis of Pr180^{gag-pol} occurring in the lysate because the ratio of P130 to Pr180^{gag-pol} remained constant regardless of whether (i) labeled cells were lysed and immediately subjected to immunoprecipitation, (ii)

labeled cells were frozen and thawed before lysis, or (iii) the lysate was allowed to stand at room temperature for 30 min (data not shown).

To further study the composition of P130, the [35 S]methionine-labeled protein was eluted from a gel slice in the presence of TPCK-trypsin, oxidized, and subjected to cation-exchange chromatography as previously described (9). Figure 2A shows the elution profile of tryptic peptides of [35 S]methionine P130 from PR-RSV-C cochromatographed with the [3 H]methionine-labeled tryptic peptides of the β subunit of reverse transcriptase (immunoprecipitated from virions of PR-RSV-C). It is evident that nearly all the [35 S]methionine-labeled peptides of P130 were coincident with those of reverse transcriptase. A group of peptides lying between fractions 60 and 80, however, did not appear to be present in the β subunit (Fig. 2A). Figure 2B shows the results of cochromatography of peptides of [35 S]methionine-labeled P130 of the recombinant PR-E 52+ with the tryptic peptides of the [3 H]methionine-labeled virion gag proteins p27, p19, and p15 from PR-RSV-C virions. The origin of each of the gag protein peptides, as assigned in the figure, was determined in previous studies by mapping of each of the individual proteins (51). The data here demonstrate that P130 does not possess extensive gag-related sequences. One ambiguity concerns the coelution of peaks in the region of the p27 peptides (27b, c). Since P130 is not precipitated with anti-p27 serum (Fig. 1), it seems unlikely that P130 contains sequences related to p27. In addition, there is another P130 peak, eluting between the 19b and 19c gag marker peptides in Fig. 2B, which does not appear to be present in the β subunit. This peptide could be derived from a region cleaved from P130 to generate β . Alternatively, it could be part of the COOH terminus of Pr76. Indeed, its position corresponds to the 15a gag peptide (which was not recovered in this marker preparation but whose position is known from many other mapping experiments). Since the 15b marker is located within the shoulder of the β peak and the 15c peptide (the most COOH terminal of the p15 methionine peptides, as determined by pactamycin ordering [51]) is not found in either Pr180^{gag-pol} (not shown) or P130, these mapping data do not eliminate the possibility that p15 is contained in P130.

To determine whether P130 contains the COOH-terminal region of Pr76^{gag}, we performed immunoprecipitation experiments using monospecific antisera raised against individual gag proteins. As shown in Fig. 3A (lane A), anti-pol serum (absorbed with disrupted AMV virions to block antibodies against gag proteins) precipitated P130 and Pr180^{gag-pol} from PR-E 52+ cells

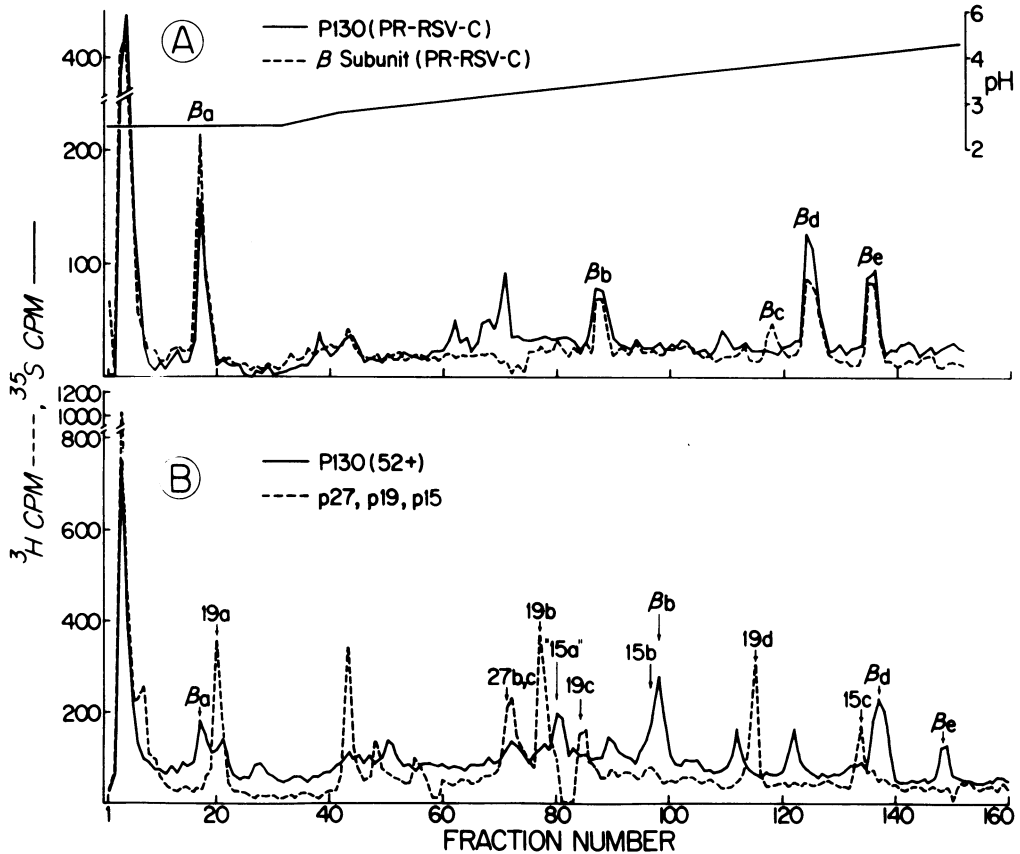


FIG. 2. Tryptic maps of P130, the β subunit of reverse transcriptase, and virion gag proteins. [^{35}S]-methionine-labeled p130 was prepared by labeling three 100-mm plate of CEF or QEF infected with PR-RSV-C or 52+, respectively, with 600 μCi per plate for 2 h. Lysates were precipitated with anti-pol serum and fractionated on SDS-PAGE. Virion [^{35}H]methionine-labeled $\alpha\beta$ subunits and gag proteins were prepared by labeling of CEF infected with PR-RSV-C for 16 h with 250 μCi per plate. The supernatant medium was harvested, and the virus was pelleted through 20% sucrose and disrupted in lysis buffer. Immunoprecipitations were carried out with anti-pol and anti-gag sera. Precipitates were dissolved in SDS electrophoresis sample buffer and fractionated by SDS-PAGE. Labeled bands were excised from the gel, eluted in 0.05 M NH_4HCO_3 plus TPCK-trypsin (100 $\mu\text{g}/\text{ml}$), and oxidized as described. Appropriate mixtures of tryptic digests were absorbed onto a Chromobead type P cation-exchange column at 50°C and eluted with a 540-ml gradient of pyridine-acetate. Fractions of 3 ml were dried, redissolved in 0.05 M HCl, and counted in scintillation fluid. Radioactivity has been corrected for 5.2% crossover from the ^{35}S to the ^3H channel and 1% crossover from the ^3H to the ^{35}S channel. The designations β_a , 19a, etc., refer to individual tryptic peptides of the β subunit, p19, etc. Although the 15a peptide was not recovered in this preparation its position, as judged from other experiments, is indicated by "15a" in panel B.

labeled for 2 h with [^{35}S]methionine. When a portion of the same cell lysate was treated with anti-p12 serum, only Pr76^{gag} and Pr180^{gag-pol} were precipitated (Fig. 3A, lane C), whereas anti-p15 serum precipitated P130 in addition to Pr76^{gag} and Pr180^{gag-pol} (Fig. 3A, lane B). When unlabeled virions were added to the anti-p15 serum to the same level as used to absorb gag antibodies in the anti-pol serum, the anti-p15 serum failed to precipitate any of the proteins shown in Fig. 3A, confirming that anti-p15 is precipitating P130 by virtue of gag determinants

(not shown). Thus, whereas neither anti-p19 (not shown), anti-p27 (Fig. 1), nor anti-p12 (Fig. 3) serum precipitated P130, the anti-p15 serum efficiently did so. Taken together, our immunoprecipitation experiments and tryptic peptide mapping data demonstrate that P130 contains the amino acid sequences of reverse transcriptase and at least a portion of p15. Data presented below suggest that P130 is a precursor to the β and α subunits of reverse transcriptase. According to convention, we designate this protein Pr130^{gag-pol}.

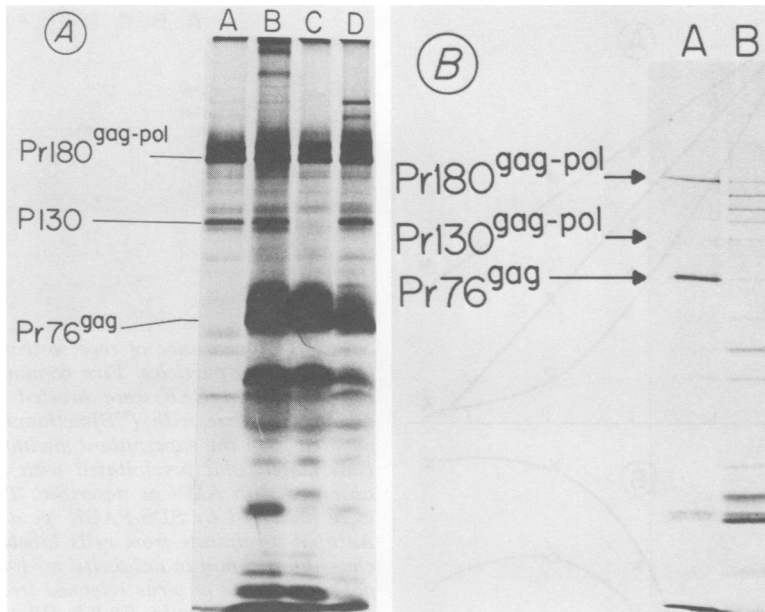


FIG. 3. Analysis of immunoprecipitates, using different antisera and after *in vitro* cleavage with virion-associated protease. (A) A 100-mm dish of PR-E 52+-infected QEF was labeled for 2 h with 500 μ Ci of [35 S]-methionine. The lysate prepared from the labeled cells was divided equally, and each portion was immunoprecipitated with the indicated antiserum as described. The washed precipitates were dissolved in SDS electrophoresis buffer and fractionated on a 10% polyacrylamide gel. Lane A, 4 μ l (equivalent) of anti-pol serum absorbed with 56 μ g of disrupted AMV virions; B, 20 μ l of anti-p15 serum; C, 20 μ l of anti-p12 serum; D, 4 μ l of anti-pol serum, unabsorbed. (B) *In vitro* cleavage with virion protease. An anti-gag plus anti-pol immunoprecipitate from a 2-h labeling of PR-RSV-C CEF was resuspended in 0.05 M Tris-acetate (pH 7.0)-0.15 M NaCl-0.1% NP-40 and divided into two portions. To one (lane A), no additions were made; to the other (lane B), 10 μ g of AMV was added. After a 60-min incubation at 37°C, the samples were dissolved in SDS electrophoresis sample buffer and fractionated on a 10% polyacrylamide slab gel.

Kinetic analysis and distribution of *pol*-related proteins. We next sought to determine whether the labeling kinetics and turnover rates of Pr130^{*gag-pol*} and Pr180^{*gag-pol*} are consistent with their putative roles in the generation of reverse transcriptase. PR-RSV-C-infected CEF were labeled with [35 S]methionine for increasing lengths of time, and viral proteins were precipitated by using antiserum having both anti-*gag* and anti-*pol* activities. We found that Pr180^{*gag-pol*}, Pr130^{*gag-pol*}, and Pr76^{*gag*} were labeled at equal rates for times up to at least 25 min (data not shown). In a series of pulse-chase experiments, we labeled infected cells for 2 h and then allowed the cells to grow for different lengths of time in unlabeled medium (chase). After immunoprecipitation and SDS-PAGE, the relative amount of radioactivity in each polypeptide was determined by scanning densitometry (Fig. 4A). The data show that Pr180^{*gag-pol*} turned over less rapidly than Pr130^{*gag-pol*} (Pr180^{*gag-pol*} $t_{1/2}$ = 2.25 hours; Pr130^{*gag-pol*} $t_{1/2}$ = 1.25 h). Half-lives of 3 h (37) and 1.2 h (16) have been reported previously for Pr180^{*gag-pol*}.

The kinetics of appearance of reverse transcriptase subunits in virions is shown in Fig. 4B. The presence of the β subunit was detected first, whereas the α subunit appeared after a lag period, in agreement with reports that α is derived by cleavage from β (12, 33). The turnover rates of both Pr180^{*gag-pol*} and Pr130^{*gag-pol*} can be interpreted as being consistent with both or either of these polyproteins as direct precursors to virion reverse transcriptase (compare Fig. 4A and 4B).

An electrophoretic analysis of the appearance of polymerase-related proteins in virus particles is shown in Fig. 5. In this experiment, virus was harvested after different intervals of continuous labeling of PR-RSV-C-infected cells. The virus preparations were disrupted and subjected to immune precipitation with anti-*pol* serum. Although relatively high levels of the β and α subunits appeared with increasing time, neither Pr180^{*gag-pol*} nor Pr130^{*gag-pol*} was present in significant amounts (Fig. 5A-F). The presence of a stack of bands just above β was reproducibly observed (Fig. 5C-F). These may represent a series of cleavage products of Pr130^{*gag-pol*} and

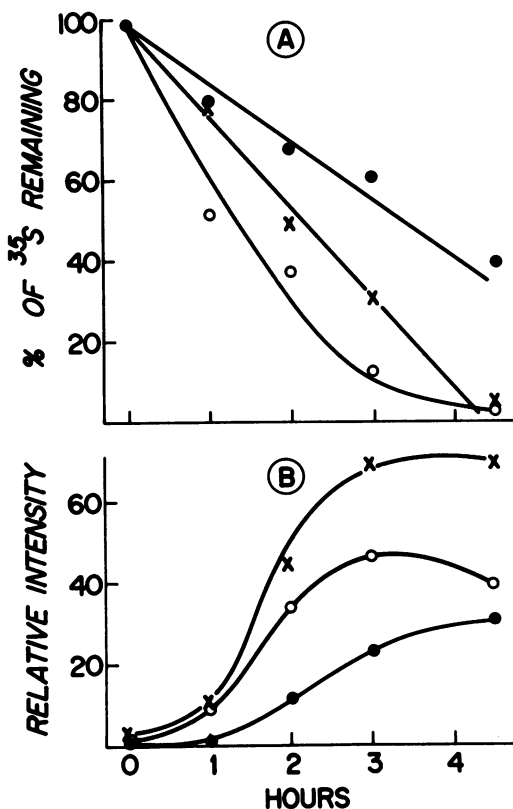


FIG. 4. Metabolism of reverse transcriptase-related proteins. (A) Levels of intracellular PR-RSV-C Pr180^{gag-pol} (x), PR-RSV-C Pr130^{gag-pol} (O), and SE52d P125 (●) after a 2-h labeling period. Five 65-mm plates of CEF infected with PR-RSV-C or QEF shedding the mutant SE52d were each labeled for 2 h with 100 μ Ci of [³⁵S]methionine. The plates were then washed; one plate was lysed immediately, and the others were allowed to grow in medium supplemented with five times the usual concentration of unlabeled methionine. At the indicated times after the pulse-label, the medium was removed and the cells were lysed. The lysates and virus purified from the supernatant medium were precipitated with anti-pol serum, and the immune precipitates were fractionated on a 12.5% polyacrylamide gel. The relative amounts of radioactivity in the different bands were determined by scanning densitometry and expressed as a percentage of the intensity of the band from the pulse-labeled cells. (B) Levels of reverse transcriptase subunits in virus released from PR-RSV-C-infected CEF. Symbols: ●, α subunit; O, β subunit; x, $\alpha + \beta$. Virus was purified from the supernatant medium of the pulse-labeled and chased cells, dissolved in lysis buffer, precipitated with anti-pol serum, and fractionated on a 12.5% polyacrylamide gel. The relative amounts of radioactivity in each band were determined by scanning densitometry.

Pr180^{gag-pol} formed during virus budding. Oppermann and co-workers (37) have previously reported bands of much higher molecular weight

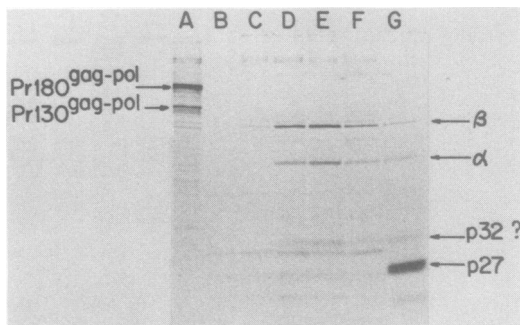


FIG. 5. Appearance of reverse transcriptase subunits in virus particles. Five 65-mm plates of PR-RSV-C-infected CEF were labeled for increasing amounts of time with [³⁵S]methionine. Virus was purified from the supernatant medium, dissolved in lysis buffer, and precipitated with anti-pol serum absorbed with AMV as described. The precipitates were analyzed by SDS-PAGE on a 12.5% gel. (A) Anti-pol precipitate from cells labeled for 2 h and chased for 60 min in unlabeled medium. (B-F) Anti-pol precipitates of virus released from cells after a labeling for (B) 60 min, (C) 2 h, (D) 4 h, (E) 6 h, and (F) 2 h, followed by a 60-min chase. Lane G contains purified PR-RSV-C virus labeled for 16 h.

in total released virus which they have suggested might be Pr180^{gag-pol}. Because we were unable to detect Pr180^{gag-pol} in our experiments, we performed a pulse-chase experiment under their conditions, except that we precipitated proteins from the released virus with anti-pol serum. We labeled cells for 15 min and sampled viral supernatants at 30-s intervals by precipitation with anti-pol serum. Again, no polypeptides were detected in the 130,000- or 180,000-dalton region, although some contaminating host bands were seen in the region above 200,000 daltons (data not shown). These data suggest that neither Pr180^{gag-pol} nor Pr130^{gag-pol} was incorporated into virions as a precursor to β and α . Even in a 60-min chase after a 2-h labeling, Pr180^{gag-pol} and Pr130^{gag-pol} were present in cells but absent from budded virus (compare Fig. 5A and 5F). In other experiments, we have failed to detect either α or β in cells (not shown). Thus, polymerase precursors are probably cleaved concomitant with virus budding, and only the products of these cleavages are actually incorporated into virus particles.

Cleavage of Pr180^{gag-pol} with virion protease. A number of gag protein cleavages appear to be mediated by the gag protein p15 (7, 53). This protein can be found in infected cells and in virus particles. Addition of lysed virions or purified p15 has been shown to result in the *in vitro* cleavage of a number of gag-related polyproteins including Pr180^{gag-pol} (52). To determine whether p15 could directly generate Pr130^{gag-pol} from Pr180^{gag-pol}, we added 10 μ g of

detergent-lysed virus to immune complexes containing these two proteins and Pr76 (Fig. 3B, lanes A and B). The proteolytic activity appeared to cleave primarily Pr76^{gag} and Pr180^{gag-pol}, with the major products of Pr180^{gag-pol} cleavage being a series of polypeptides in the range of 140,000 to 170,000 daltons. It is probable that these resulted from successive cleavages within the *gag* region of Pr180^{gag-pol}.

Phosphorylation of *pol*-related proteins. The *gag* proteins p12 and p19 have been previously shown to be phosphorylated (25). In the case of reverse transcriptase, the phosphorylation of the β subunit, but not its cleavage product, the α subunit, has been reported (18). To determine which of the polymerase-related polypeptides might be phosphorylated, we labeled cells infected with the wild-type virus PR-E 52+ for 2.5 h with 2.5 mCi of ³²PO₄ or 150 μ Ci of [³⁵S]methionine and precipitated the lysates with anti-*gag* or anti-*pol* serum. As shown in Fig. 6, both Pr76^{gag} and Pr180^{gag-pol} were phosphorylated. However, Pr130^{gag-pol} could not be detected with ³²P labeling, whereas it was easily observed when labeled with [³⁵S]methionine (Fig. 6, lanes D and E). This result would suggest that significant phosphorylation of reverse transcriptase does not occur until formation of the β subunit.

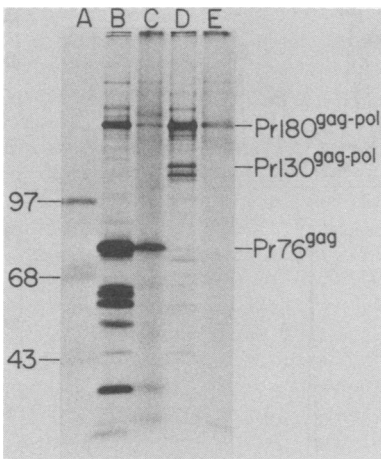


FIG. 6. Phosphorylation of intracellular polymerase-related proteins. Single plates of QEF infected with the wild-type virus PR-E 52+ were labeled for 2.5 h with either 2.5 mCi of ³²PO₄ or 150 μ Ci of [³⁵S]methionine. Each plate was lysed and divided into two portions; one was precipitated with anti-*gag* serum, and the other was precipitated with anti-*pol* serum absorbed with disrupted AMV. Precipitates were analyzed by SDS-PAGE on a 10% gel. (A) Molecular weight marker proteins; (B) [³⁵S]methionine-labeled cells, anti-*gag* precipitate; (C) ³²PO₄, anti-*gag* precipitate; (D) [³⁵S]methionine, anti-*pol* precipitate; (E) ³²PO₄, anti-*pol* precipitate.

Polypeptides synthesized by the mutant cdSE52d: an alteration near the NH₂ terminus of polymerase. The nonconditional mutant cdSE52dPR-C (SE52d) is shed from a line of transformed quail cells. Initial characterization of this virus (28) indicated the following: (i) SE52d virions are noninfectious although they possess internal structural proteins and functional envelope glycoproteins; (ii) virions do not possess reverse transcriptase, as judged by standard exogenous or endogenous assays and by the absence of polypeptides comigrating with β or α subunits; and (iii) SE52d genomic RNA lacks a region of about 1,000 nucleotides present in wild-type RSV near the 5' end of *pol* as determined by hybridization competition experiments. More recent studies have shown that a segment of quail cell genetic information has been incorporated into genomic RNA although the quail cell sequence has not been directly shown to lie within *pol* (8; P. Neiman, personal communication).

We were interested in determining what sort of polymerase-related polypeptides might be synthesized intracellularly in SE52d-producing cells. Figure 7 shows the results of an electrophoretic analysis of anti-*pol* immunoprecipitates formed from lysates of cells infected with wild-type virus and cells producing SE52d. Both PR-RSV-C (Fig. 7C) and a wild-type sister clone of SE52d termed PR-E 52+ which, like SE52d, is a recombinant (Fig. 7A) produced Pr180^{gag-pol} and Pr130^{gag-pol}. By contrast, SE52d-producing cells did not synthesize Pr180^{gag-pol}, but instead synthesized a polypeptide (P125) migrating slightly faster than Pr130^{gag-pol} (Fig. 7B). Another polypeptide of about 40,000 daltons was variably precipitated from SE52d-producing cells (Fig. 7B). Anti-*gag* serum also failed to precipitate Pr180^{gag-pol} in SE52d cells, whereas the P125 protein was efficiently precipitated (data not shown).

A tryptic peptide analysis of P125 from SE52d-producing cells is shown in Fig. 8. Panel A shows the results of cochromatography with a tryptic digest of the β subunit of reverse transcriptase from RAV-0. It is evident from the figure that none of the resolved methionine peptides from the β subunit were present in the P125 protein. Panel B shows the results of cochromatography with a tryptic digest of the *gag* proteins of RAV-0. The data indicate that P125 contained *gag* sequences. At least three peptides related to p19 were present: 19a, 19d, and 19f. The presence of the 19f peptide and the lack of the 19b peptide is expected for a certain class of viruses which have undergone recombination within *gag* (8, 46). In addition, the p27 peptides 27b,c and the p12 and p15 peptides 15a and 15b

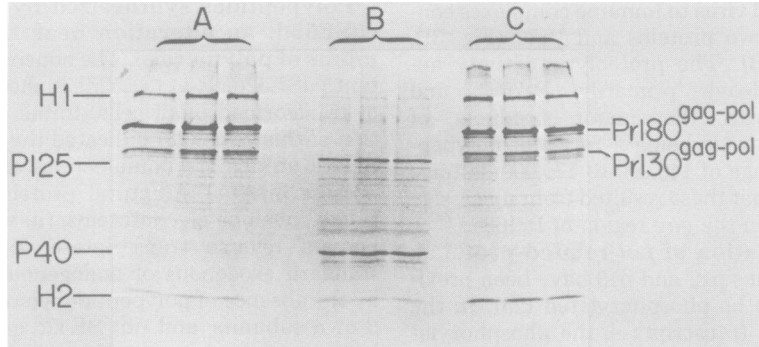


FIG. 7. Analysis of anti-pol immunoprecipitates from cells producing wild-type viruses and the mutant SE52d. Four 100-mm plates each of CEF infected with PR-RSV-C, QEF infected with wild-type virus PR-E 52+, and QEF producing the mutant SE52d were each labeled with 500 μ Ci of [35 S]methionine for 2.5 h. The cells were lysed and precipitated with anti-pol serum. The precipitates were fractionated by SDS-PAGE on a 12.5% polyacrylamide gel. A radioautograph of the dried gel is shown. (A) PR-RSV-C CEF; (B) SE52d QEF; (C) 52+ QEF; H1 and H2 are host cell protein contaminants.

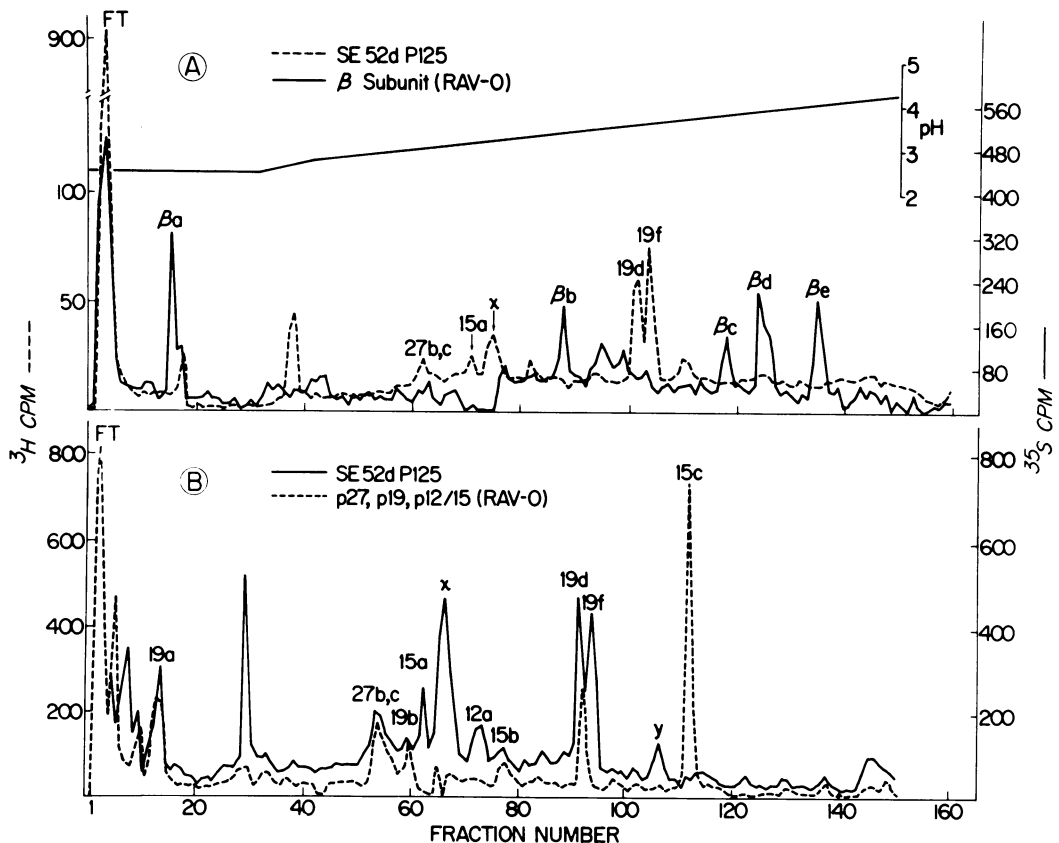


FIG. 8. Tryptic peptide maps of SE52d-P125, the β subunit of reverse transcriptase, and the gag proteins of RAV-0. The P125 polypeptide of SE52d was prepared from two 150-mm plates of confluent QEF producing SE52d which were labeled for 2 h with 1 mCi of [35 S]methionine. [3 H]methionine-labeled β subunit and gag proteins were prepared from RAV-0 virions. Immunoprecipitation, trypsinization, oxidation, and cochromatography are described in Materials and Methods and the legend to Fig. 2.

were present. The 12a, 15a, and 15b peptides were poorly recovered in the RAV-0 marker, and their presence in P125 was largely deduced by comparison with many other elution profiles (9, 51). The absence of the 15c peptide in P125 is not surprising since this peptide was also missing in wild-type Pr180^{gag-pol} and Pr130^{gag-pol} (Fig. 2). The 15c peptide is the most COOH terminal of the three methionine-containing p15 peptides (51) although it is not the COOH-terminal tryptic peptide of p15 (R. Sauer, personal communication), and its absence may indicate that the COOH-terminal region of p15 is lacking in these proteins. The origin of the x and y peptides, which are not related to known *gag* and *pol* peptides (Fig. 8B), is unknown, but they could derive from translational read-through into the host nucleotide sequences apparently substituted into the SE52d genome. In any case, SE52d-P125 should not be precipitated with anti-*pol* serum (absorbed extensively with *gag* proteins) unless some sequences related to reverse transcriptase are present. Either there are such sequences present in P125 whose peptides we have failed to resolve, or the substituted regions (represented by the x and y peptides) themselves are antigenically related to reverse transcriptase.

We also examined the turnover of SE52d-P125 under the same conditions of pulse and chase used for determining the metabolism of Pr180^{gag-pol} and Pr130^{gag-pol}. The results (Fig. 4A) indicate that P125 was more slowly metabolized than either of the other *pol*-related proteins. The half-life of P125 was approximately 3.25 h. This is also considerably longer than the 45- to 60-min half-life measured for Pr76^{gag} (37, 51). Thus it is difficult to determine whether SE52d-P125 is specifically processed in cells shedding SE52d, since the products of Pr76^{gag} cleavage have already accumulated. However, addition of virion protease p15 to SE52d-P125 immune precipitates, as described in the preceding section, led to disappearance of the P125 band and the appearance of a band several thousand daltons larger than Pr76^{gag}. Small amounts of p27 may also be generated (data not shown). Taken together, our data indicate that SE52d-P125 is a functionally defective polypeptide containing predominantly *gag*-related sequences that is unlikely to serve in vivo as a precursor to *gag*- or *pol*-related proteins.

Because 52d contains no functional polymerase and yet appears to have polymerase-related antigenic determinants, we were interested in determining whether the 52d genome could rescue by recombination several temperature-sensitive (ts) polymerase mutants. We tested five

such mutants, tsLA335 (29), tsPH553, tsPH620, tsPH568, and tsPH1045 (32). In all cases, growth of these mutants on SE52d cells resulted in production in wild-type virus in at least 10-fold excess over the spontaneous rate of phenotypically wild-type virus production from normal quail cells (data not shown). It has been previously shown (32) that PH553, PH568, PH620, and PH1045 are rescued by LA337 (29), whereas LA335 is not rescued. This indicates that at least the LA335 mutation maps at a position different from the four PH *pol* mutants. Taken together, these data indicate that SE52d contains genetic information able to rescue at least two classes of ts polymerase mutants. Whether or not these sequences are encoded in the *pol*-related P125 is at present unknown.

Polypeptides synthesized by the mutant PH9: an alteration near the COOH terminus of *pol*. A transformed quail cell clone designated Q-PRA-9, originally derived after infection with the PR-A strain of ASV, previously was found to produce noninfectious virions devoid of reverse transcriptase activity (31). The defective virus carried by these cells has been termed rdPH9PR-A (PH9). Figure 9 depicts a radioautograph of SDS-PAGE fractionation of [³⁵S]methionine-labeled proteins from wild-type and PH9 virions. As expected, polypeptides in the position of the β and α polymerase subunits were present in the preparation of wild-type virus (Fig. 9F) and in immunoprecipitates formed with anti-*pol* (Fig. 9, lane B) and anti-whole virus (Fig. 9, lane E) sera. In contrast, these polypeptides were not detectable in similar analysis of the PH9 virions released from Q-PRA-9 cells (Fig. 9, lanes A, C, and D). Thus virus released from Q-PRA-9 cells did not contain processed reverse transcriptase.

In immunoprecipitation experiments using antiserum containing both anti-*pol* and anti-*gag* activity, both Pr76^{gag} and a novel 140,000-dalton protein (P140) were observed in PH9 cells, whereas no Pr180^{gag-pol} was evident (31). Figure 10 shows a kinetic analysis of intracellular *pol*-related proteins from cells producing wild-type PR-A (wild type), Q-PRA-9 cells producing PH9, and cells harboring the genome of rdPH2B77-C, a mutant which has deleted *gag*, *pol*, and *env* genes (31). In cells producing wild-type virus, Pr180^{gag-pol} and Pr130^{gag-pol} were metabolized in a series of chases after a 20-min pulse-label. The half-life of Pr180^{gag-pol} was approximately 2.5 h and the half-life of Pr130^{gag-pol} was approximately 1.5 h in this experiment. By contrast, the P140 polypeptide synthesized by PH9 had a half-life of approximately 5 h. The higher-molecular-weight bands above

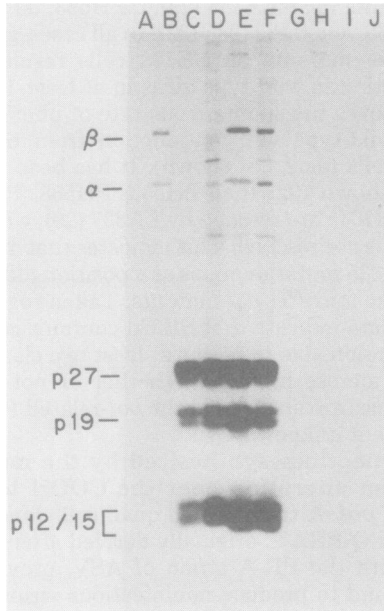


FIG. 9. Virion proteins of PR-RSV-C and the mutant PH9. The quail cell lines QPR-A-4 and QPR-A-9 carrying wild-type virus and the mutant virus rdPH9PR-A (PH9), respectively (31), were grown to confluency in 100-mm dishes. Cells were labeled with 100 μ Ci of [35 S]methionine for 14 h in medium containing 2% the usual level of methionine. The supernatant medium was first clarified by low-speed centrifugation and used to pellet released virus, which was then banded to equilibrium in a 15 to 55% sucrose gradient. Purified virus preparations were either electrophoresed directly or disrupted in Ab buffer and immunoprecipitated with the indicated antiserum. All samples were dissolved in electrophoresis sample buffer and subjected to electrophoresis on an 18% polyacrylamide gel. (A) PH9, anti-*pol* serum; (B) wild type, anti-*pol* serum; (C) PH9, anti-whole virus serum (anti-AMV); (D) PH9 virus; (E) wild type, anti-whole virus serum; (F) wild-type; (G) PH9, rabbit anti-human serum albumin; (H) wild type, rabbit anti-human serum albumin; (I) PH9, normal rabbit serum; (J) wild type, normal rabbit serum.

Pr180^{*gag-pol*} are presumed to be host cell contaminants. No significant intracellular accumulation of α or β subunits was observed in any of the cells used.

Comparative tryptic peptide mapping of the PH9 P140 protein with *gag* proteins from PR-RSV-C demonstrated mostly *gag* peptides from p19, p27, p12, and p15, whereas only two putative polymerase-related peptides (β _a and β _c of Fig. 2) were present (data not shown). The presence of these peptides and the precipitation with anti-*pol* serum (Fig. 10) suggest that PH9-P140 is also a *gag-pol* read-through product partially

lacking sequences related to polymerase.

Precipitation of polymerase-related proteins with antiserum against the unique portion of the β subunit. Grandgenett and co-workers (13, 14, 44) have presented evidence suggesting that a 32,000-dalton virion-associated polypeptide (p32) is structurally related to the β , but not the α , subunit of reverse transcriptase and is derived from cleavage of β to generate α . We have obtained an antiserum prepared against p32 (14) and analyzed its ability to precipitate a number of polymerase-related polypeptides. In the first experiment, we precipitated proteins from [35 S]methionine-labeled wild-type virions with anti-*pol* and anti-p32 sera in the presence of SDS so that association between the β and α subunits would be minimized. Our results supported the conclusions of Schiff and Grandgenett (44), since anti-p32 precipitated only the β subunit and p32 whereas anti-*pol* precipitated both the β and α subunits (data not shown).

We next tested the anti-p32 serum on a series of polypeptides possessing different deletions of polymerase amino acid sequence. Figure 11 shows the results of precipitations from extracts of cells producing wild-type ASV, using anti-*pol*, anti-p32, and normal rabbit sera. Both the anti-*pol* (Fig. 11A, lane A) and the anti-p32 (Fig. 11A, lane B) sera precipitated Pr180^{*gag-pol*} and Pr130^{*gag-pol*} as expected, whereas the serum from the nonimmunized rabbit precipitated neither polypeptide (Fig. 11A, lane C). The same sera were used to form immunoprecipitates from the cytoplasm of cells derived from embryos of inbred line 6 chickens. Line 6 chickens harbor the *ev-3* locus (1), which appears to be the structural gene for P120^{GS}. The latter is a 120,000-dalton polyprotein containing the *gag* proteins p19, p12, and p27 as well as a portion of reverse transcriptase, but lacking p15 and at least half of reverse transcriptase (9). Both the anti-*pol* and anti-p32 sera precipitated P120^{GS} from line 6 cells (Fig. 11A, lanes G-I), suggesting that the p32 portion of the β subunit is retained in P120^{GS}.

We then tested the anti-p32 serum on the two polymerase mutants described in this paper, SE52d and PH9. Figure 11A (lane D-F) indicates that the P140 protein synthesized in PH9-producing Q-PRA-9 cells was precipitated only by anti-p32 serum (lane D) but not by anti-p32 serum (lane E). When the experiment was repeated with SE52d-producing cells, we observed that the SE52d P125 protein was also precipitated only by anti-*pol* serum (Fig. 11B, lane D) but not by anti-p32 serum (Fig. 11B, lane E). We conclude that both PH9-P140 and SE52d-P125,

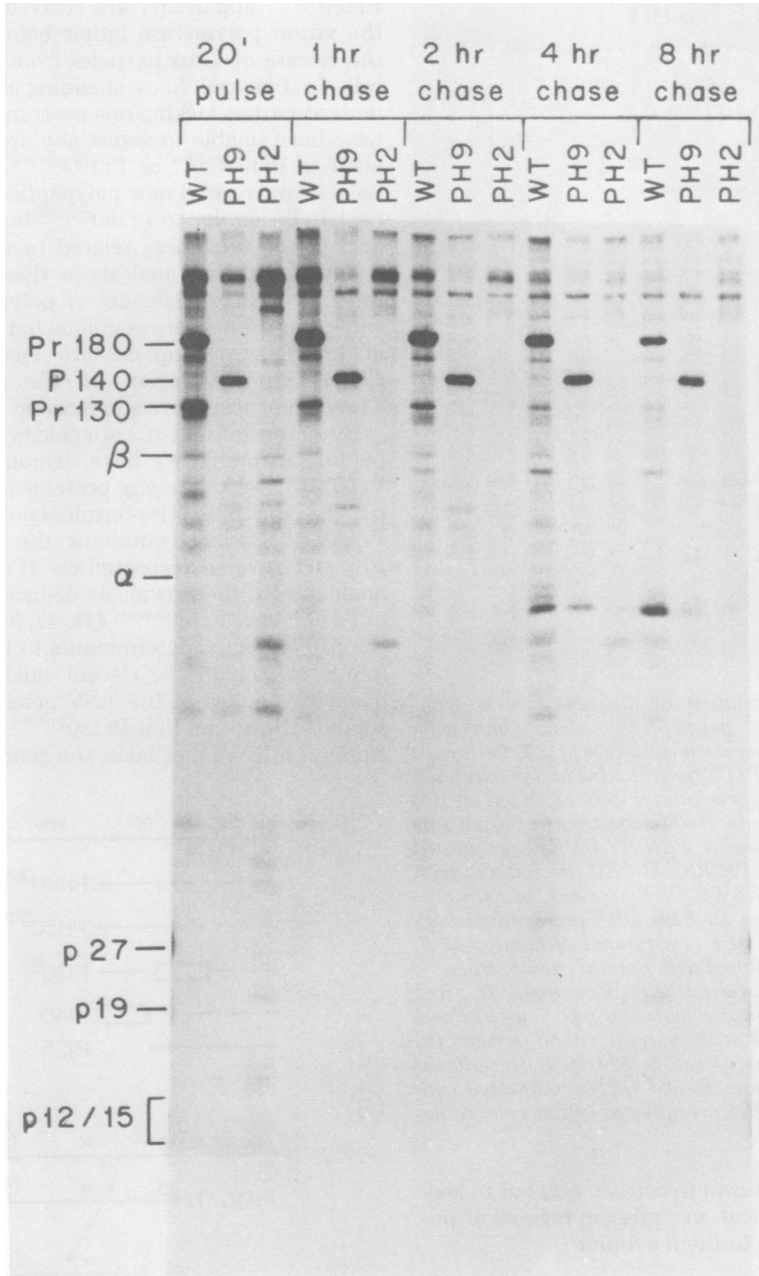


FIG. 10. Analysis of anti-pol immunoprecipitates from cells infected with PR-RSV-C and the mutants PH9 and PH2. The cells and viruses were as described in the legend to Fig. 9. Q-B77-2 is a quail cell line producing rdPH2B77-C (PH2) which contains deletions spanning the gag, pol, and env genes (31). Cells were pulse-labeled for 20 min in 60-mm dishes with 37.5 μ Ci of [35 S]methionine in Earle saline. After the pulse labeling, cells were either frozen for later lysis or rinsed twice and chased for the indicated times in growth medium containing 180 μ M unlabeled methionine before freezing. Immunoprecipitations were carried out with anti-pol serum, and the washed precipitates were analyzed on a 12.5% polyacrylamide gel. The positions of the β and α subunits were determined from disrupted virus analyzed on the same gel.

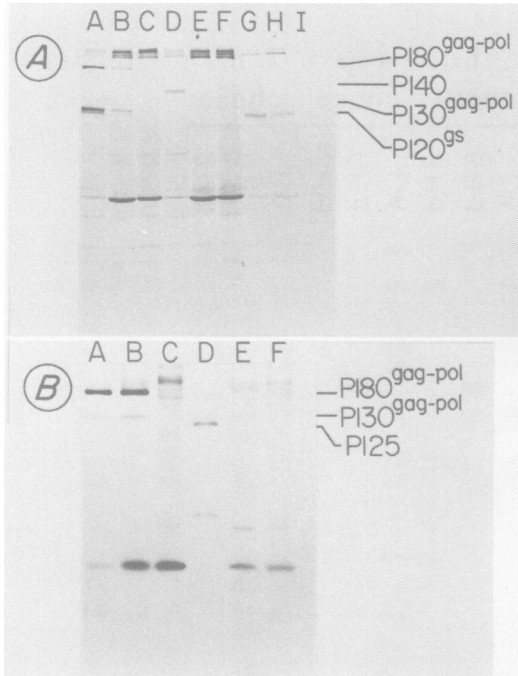


FIG. 11. Precipitation of different reverse transcriptase-related polypeptides with antiserum against either reverse transcriptase or p32. Cells were labeled for 2 h with [35 S]methionine and precipitated as described. The precipitates were analyzed on 10% polyacrylamide gels. Radioautographs of dried gels are shown. (A) Lanes A, PR-RSV-C CEF precipitated with anti-pol; B, PR-RSV-C CEF precipitated with anti-p32; C, PR-RSV-C CEF precipitated with normal rabbit serum; D, PH9 QEF precipitated with anti-pol; E, PH9 QEF precipitated with anti-p32; F, PH9 QEF precipitated with normal rabbit serum; G, *gs*(+) line 6 cells precipitated with anti-pol; H, *gs*(+) line 6 cells precipitated with anti-p32; I, *gs*(+) line 6 cells precipitated with normal rabbit serum. (B) Lanes A-C are as above; D, 52d QEF precipitated with anti-pol serum; E, 52d QEF precipitated with anti-p32; F, 52d QEF precipitated with normal rabbit serum.

which we have shown to contain *gag* but to lack some *pol* sequences, are missing regions of polymerase unique to the β subunit.

DISCUSSION

In this paper we have reported our observations on the synthesis and organization of reverse transcriptase-related polypeptides specified by wild-type and mutant avian retroviruses. We have shown that cells infected by wild-type viruses synthesize a nonphosphorylated 130,000-dalton polypeptide (Pr130^{*gag-pol*}) composed of sequences related to virion reverse transcriptase and the *gag* protein p15. Both Pr130^{*gag-pol*} and

Pr180^{*gag-pol*} apparently are cleaved to generate the virion polymerase either before or during the release of virus particles from the infected cell. In two cell lines shedding noninfectious mutant viruses lacking reverse transcriptase, we have been unable to detect any proteins equivalent to Pr130^{*gag-pol*} or Pr180^{*gag-pol*}. Instead, we have characterized new polypeptides which appear to be similar to Pr180^{*gag-pol*} but which lack part of the sequences related to reverse transcriptase. Further analysis of these and other proteins lacking segments of polymerase indicates that the sequences unique to the β -subunit of reverse transcriptase are located in the COOH-terminal region of the polypeptide. These conclusions are summarized in Fig. 12.

By the criterion of antigenicity and tryptic peptide mapping, we have demonstrated that Pr180^{*gag-pol*} lacks the *gag* proteins p19, p27, and p12 (i.e., the three NH₂-terminal polypeptides of Pr180^{*gag-pol*}) while containing the *gag* protein p15 and reverse transcriptase (Fig. 12B). By analogy with the previously deduced structures of Pr76^{*gag*} and Pr180^{*gag-pol*} (41, 47, 51), we assign the p15 antigenic determinants to the NH₂ terminus of Pr130^{*gag-pol*}. Recent nucleic acid sequencing studies of the ASV genome strongly support the notion that Pr180^{*gag-pol*} is translated from an mRNA that lacks the genomic nucleo-

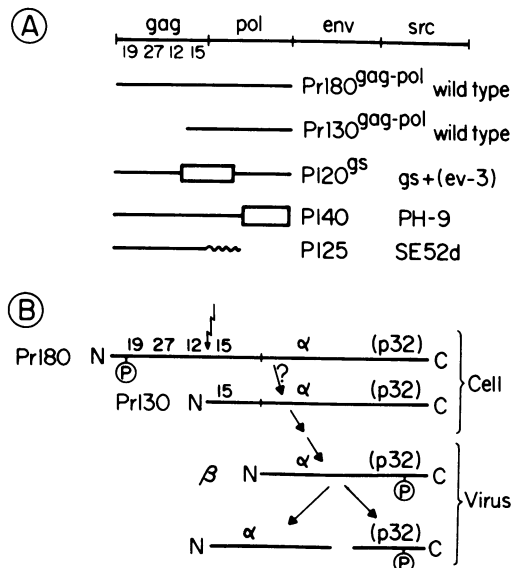


FIG. 12. (A) Proposed structure for reverse transcriptase-related polypeptides. Open boxes indicate deleted regions. Wavy line indicates possible substitution of quail cell information in SE52d-P125. (B) Proposed scheme for the generation of reverse transcriptase. Wavy arrow indicates possible cleavage site. See text for details.

tide sequences which lie between *gag* and *pol* and possibly even a portion of the COOH terminus of p15 (D. Schwartz, personal communication). Thus we cannot be certain how much of p15 is actually present in Pr130^{*gag-pol*}.

The apparent molecular weight of Pr130^{*gag-pol*} indicates that it probably also contains about 15,000 to 20,000 daltons not present in either p15 or the 95,000-dalton β subunit of reverse transcriptase. Our inability to precipitate Pr130 with antiserum against p12 (the adjacent *gag* protein) makes it unlikely that the additional information is at the NH₂ terminus of Pr130. In addition, since (i) we cannot precipitate Pr130 with anti-*env* serum and (ii) two ASV mutants [NY8 and BH RSV(-)] which contain large deletions spanning *env* (22, 43) produce unaltered Pr180 and Pr130 (data not shown), we do not believe that any information derived from *env* is present in these proteins. Thus, the remaining amino acid sequences probably arise from regions at the 3' or 5' border of *pol*.

A polypeptide with properties somewhat analogous to those of Pr130^{*gag-pol*} has been described previously in cells infected by murine leukemia viruses (20, 27, 36, 55), supporting the notion that this type of polypeptide may generally be involved in the metabolism of reverse transcriptase. However, no *gag* polypeptide segments have been identified in the murine virus protein (36). This may be related to the fact that no murine virus *gag* protein having the proteolytic properties of ASLV p15 has been detected. It seems possible, given the analogies between the murine and avian virus systems, that the murine virus Pr130^{*pol*} does contain an as yet unidentified *gag* protease.

In pulse-chase experiments, we have observed the disappearance of both Pr180^{*gag-pol*} and Pr130^{*gag-pol*} from the cell (Fig. 4A), although little or no mature β or α subunits can be observed intracellularly. This would suggest either that the polyproteins are exported and cleaved in virions, or that intracellular cleavage is followed by rapid export of the products. Analysis of polymerase-related proteins in virus released from prelabeled cells showed that no polymerase precursors were detected in virus particles, although both subunits appeared in virus harvested after a 30-min chase period (Fig. 4B and 5). Thus, we believe that cleavage of *pol* polyproteins precedes release of virus particles in much the same way as Pr76^{*gag*} cleavage precedes export (51). This interpretation differs from that of Oppermann et al. (37), who, although observing rapid appearance of polymerase subunits in virions, observed a decrease in the level of a high-molecular-weight protein also incorporated into virus (see Fig. 6 of Oppermann et al. [37]).

Because these investigators did not use antipolymerase serum in their analysis and could not positively identify the high-molecular-weight band as Pr180^{*gag-pol*}, we believe that the polypeptide they observed may have been a host cell contaminant. However, we cannot rule out that polymerase precursors would be packaged into virions under certain, probably restrictive, conditions. Interestingly, neither of the defective *pol*-related proteins produced by the two polymerase mutants, SE52d and PH9, can be detected in virus particles.

The proposed structure of Pr130^{*gag-pol*} has implications relating to the processing pathway for reverse transcriptase. In the generation of the virion *gag* proteins, the first processing event for Pr76^{*gag*} is removal of the COOH-terminal p15 to form Pr66^{*gag*} by what we presume to be a host cell-specified protease (51). If Pr180^{*gag-pol*} is directly cleaved to generate Pr130^{*gag-pol*}, then the same protease which cleaves at the p12-p15 junction to generate Pr66^{*gag*} could also give rise to Pr130^{*gag-pol*} (Fig. 12B). The conformation of the polypeptide as well as the p12-p15 junction may be important for this cleavage step since the two mutant proteins which presumably retain the junction but lack portions of polymerase are cleaved poorly if at all. The remaining processing events, removal of p15 and other polypeptide segments, would occur in several steps in the virus particle (Fig. 5). None of these enzymatic activities has been positively identified, although recent data have indicated that p15 may play a role in the cleavage of β (34).

In Fig. 12B we propose a processing scheme for the production of reverse transcriptase based on a single cleavage of Pr180^{*gag-pol*} to give Pr130^{*gag-pol*}. This would seem to be the most attractive model in the sense that all the polypeptides under discussion would be utilized in polymerase metabolism. Consistent with this interpretation are the results of Witte and Baltimore (55), who demonstrated, in ts mutants of murine leukemia virus unable to cleave Pr180^{*gag-pol*}, that active reverse transcriptase and Pr130^{*pol*} could be detected only after shift to permissive temperature and subsequent cleavage of Pr180^{*gag-pol*}. On the other hand, certain problems with this model arise when the kinetics of synthesis and turnover of the two proteins are considered. In a series of short continuous labelings, both Pr180^{*gag-pol*} and Pr130^{*gag-pol*} appeared at the same rate. When a pulse-label was followed by a series of chases in unlabeled medium, the $t_{1/2}$ of Pr180^{*gag-pol*} (3.25 h) was about 1 h longer than that of Pr130^{*gag-pol*} (2.25 h) (Fig. 4). The results of both experiments are apparently inconsistent with Pr180^{*gag-pol*} being a direct precursor to Pr130^{*gag-pol*}. These results could be

reconciled if only a fraction of Pr180^{gag-pol} was rapidly metabolized to Pr130^{gag-pol}. Alternatively, Pr130^{gag-pol} might be a primary translational product. If so, one would predict the existence of an mRNA possessing *pol* close to its 5' end, in analogy with the mRNA's detected for the *gag*, *env*, and *src* gene products (17, 54). No such mRNA has been identified yet, although its detection would be expected to be difficult because of its low amount and presumed similarity in size to 39S genomic RNA.

We do not know what role, if any, the differential phosphorylation of polymerase-related proteins plays in cleavage (Fig. 6). Our results indicate that although reverse transcriptase amino acid sequences are present within Pr180^{gag-pol} and Pr130^{gag-pol}, phosphorylation of these sequences probably does not occur until formation of the β subunit (Fig. 12B). This would suggest that the phosphorylated region is inaccessible to kinase when part of the polyprotein. In contrast, at least one of the two *gag* phosphoproteins is phosphorylated as part of Pr76^{gag} (10, 25). Interestingly, all three phosphoproteins (pp19, pp12, and β) are known to be nucleic acid-binding proteins. Moreover, the fate of the phosphorylated segment of β is to be cleaved from the COOH terminus of β to form a 32,000-dalton virion protein possessing DNA endonuclease activity (13, 14, 44). It will be important to determine whether phosphorylation of these proteins represents signals for export or cleavage and if nucleic acid binding is related to these processes.

We have extended our studies of reverse transcriptase biosynthesis to two nonconditional mutants: SE52d and PH9. Both of these mutants appear to lack significant regions of *pol* and both synthesize deleted forms of Pr180^{gag-pol} and, at least phenotypically, resemble the murine leukemia virus mutant described by Gerwin et al. (11). In the case of PH9, detailed restriction enzyme analysis of the integrated PH9 provirus in the Q-PrA-9 clone indicated a major deletion of 0.41 ± 0.004 megadaltons to the left of the *Kpn*I site at 2.71 megadaltons from the 3' end of the genome (31). This would place the deletion close to the 3' end of *pol*. We have demonstrated that the 140,000-dalton protein synthesized in these cells contains both *gag*- and *pol*-related sequences and lacks antigenic determinants unique to the β subunit of reverse transcriptase (Fig. 11). Since both the organization and size of P140 appear to reflect the structure of the PH9 provirus as deduced by restriction mapping, we have assigned the portion of polymerase unique to the β subunit to the COOH-terminal region of reverse transcriptase and have incorporated this into the processing scheme outlined in Fig.

12B. Our conclusions are supported by pactamycin mapping of tryptic peptides recently reported by Rettenmier et al. (41). The endogenous virus protein P120^{GS}, which also lacks a significant portion of polymerase believed to be at the *gag-pol* junction (9), contains the β -specific determinants, as do Pr180^{gag-pol} and Pr130^{gag-pol}.

The P125 polypeptide synthesized by the mutant SE52d also lacks the β -specific determinants. P125 is composed mostly of *gag*-related amino acid sequences with an additional polypeptide region of unknown origin. One possibility is that the non-*gag* sequences derive from a segment of viral polymerase. Alternatively, it is possible that the additional sequences arise from the substitution of quail cell genetic information for part of the viral *pol* gene (8). Since the SE52d-P125 protein is precipitated with anti-*pol* serum, it is clear that, whatever the origin of the non-*gag* region, it is probably related to reverse transcriptase. Indeed, our recombination data indicate that 52d contains sequences capable of rescuing the *ts* lesions in five different polymerase mutants. The marker rescue experiments of Cooper (5) demonstrate that uninfected quail cells contain genetic information capable of complementing defects in viral polymerase. Further studies will be required to ascertain whether the SE52d substitution is derived from such information.

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ADDENDUM IN PROOF

Copeland et al. (T. D. Copeland, D. P. Grandgenett, and S. Oroszlan, *J. Virol.* **36**:115-119, 1980) have recently shown by amino acid sequencing that the NH₂ termini of both subunits of reverse transcriptase are identical. These data lend further support to the notion that the α subunit is generated by a COOH terminal cleavage of the β subunit.

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