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Biosynthesis of reverse transcriptase from Rauscher murine leukemia virus by synthesis and cleavage of a *gag-pol* read-through viral precursor polyprotein

(RNA-dependent DNA nucleotidyltransferase/processing of viral proteins/oncornaviral protein synthesis)

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ABSTRACT **Reverse transcriptase (RT; RNA-dependent DNA nucleotidyltransferase) from Rauscher leukemia virus is synthesized in infected cells by way of a read-through polyrotein of 200,000 molecular weight. This polyprotein (Pr200$^{gag\text{-}pol}$) was precipitated by antiserum to RT; in a previous study all the monospecific antisera to *gag* proteins recognized Pr200$^{gag\text{-}pol}$. Pr200$^{gag\text{-}pol}$ contains both p30 and RT peptide sequences. Intermediate RT-related precursors of 145,000 (Pr145^{pol}), 135,000 (Pr135^{pol}), and 125,000 (Pr125^{pol}) molecular weights were specifically recognized by precipitation from infected cell extracts by antiserum to RT. These proteins shared methionine-containing tryptic peptide sequences with a virion polypeptide of 80,000 molecular weight (p80^{pol}) precipitable by antiserum to RT. Purification of active RT enzyme from virions labeled with [^{3}H]methionine showed that p80^{pol} was the major component, based on analysis by gel electrophoresis and tryptic peptide mapping experiments. A polypeptide (Pr80^{pol}), similar in size to mature viral p80^{pol}, was also precipitated from infected cells by antiserum to RT. Its peptide map was nearly identical to that of virion p80^{pol}. Pulse-chase studies showed that Pr80^{pol}, Pr125^{pol}, and Pr135^{pol} were stable polypeptides, whereas Pr200$^{gag\text{-}pol}$ and Pr145^{pol} were unstable precursors. Pulse-chase studies with the protein synthesis inhibitor, cycloheximide, showed that the processing of Pr200$^{gag\text{-}pol}$ occurred for a short time in the absence of protein synthesis.**

Type-C retrovirus genomes contain genes for their internal structural proteins, reverse transcriptase (RT; RNA-dependent DNA nucleotidyltransferase), and envelope proteins.‡ These coding regions have been termed *gag* (group antigens), *pol*, and *env*, respectively (2). RT is present in much lower amounts in virus particles and in infected cells (3–5) than either the *gag* or *env* proteins. In this report we show that RT from Rauscher leukemia virus (RLV) is made in infected cells by synthesis and processing of a 200,000-dalton read-through protein containing both *gag* and *pol* determinants.

MATERIALS AND METHODS

Cells and Virus. RLV-infected NIH Swiss mouse embryo fibroblasts (JLS-V16) and BALB/C spleen-thymus (JLS-V5) cell lines were used in these studies. Virus was produced in infected JLS-V5 cells, whereas precursors were isolated from JLS-V16 cells.

Labeling of Cells and Virus. These procedures have been described (3, 6).

Immune Precipitation and Peptide Mapping. Antisera to leukemia virus RT were supplied by the Office of Program Resources and Logistics of the National Cancer Institute. The sera were absorbed before use with uninfected cell proteins and disrupted virus (3). Immune precipitation was performed by indirect immune precipitation (3); tryptic digestion of precursors and viral proteins (7) was as described.

Viral RT. RT was assayed as described (8). The active enzyme was purified as described (9).

RESULTS

Immunoprecipitation of Virion Proteins with Antiserum to RT. Absorbed antiserum to partially purified RT was used to isolate the viral enzyme from [^{3}H]methionine-labeled virus. The predominant polypeptide precipitated from detergent-treated virus by anti-RT was an 80,000 molecular weight polypeptide, termed p80^{pol} (Fig. 1, lanes B and C). Comparison of this pattern with that of total virus revealed that p80^{pol} is at most a minor component of the virion (compare lane A with lanes B and C). Minor amounts of polypeptides from the anti-RT precipitate from virions also comigrated with p30. Previous studies have indicated that p30 is specifically associated with RT precursors in infected cells (3).

The anti-RT serum was tested for its effect on the RT reaction. The results (not shown) indicated that the preabsorbed anti-RT serum inhibited the synthesis of poly(dT) from poly(rA)·oligo(dT) whereas anti-p30 serum had no inhibiting effect.

Kinetics of Synthesis and Cleavage of Intracellular RT Precursors. To identify intracellular viral proteins that are recognized by anti-RT serum, we pulse-labeled virus-infected JLS-V16 cells with [^{3}H]leucine for 20 min and immunoprecipitated the cytoplasmic extracts with absorbed anti-RT serum. The results of polyacrylamide gel electrophoresis (Fig. 2 lane A) showed that the most prominent polypeptides formed in 20-min pulse labeling were two polypeptides (labeled *a* and *b*) termed Pr200$^{gag\text{-}pol}$. Smaller amounts of polypeptides identified as Pr145^{pol}, Pr135^{pol}, Pr125^{pol}, and Pr80^{pol} were also detected, as well as the *gag* precursor Pr65^{gag} (3).

In order to determine the stability of these rapidly labeled polypeptides, we pulse-labeled replicate cultures for 20 min with [^{3}H]leucine and "chased" the radioisotope by incubating the cultures in complete growth medium for various amounts

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Abbreviations: RLV, Rauscher leukemia virus; RT, reverse transcriptase (RNA-dependent DNA nucleotidyltransferase); $NaDodSO_4$, sodium dodecyl sulfate.

‡ We are using a retrovirus protein nomenclature agreed upon at a meeting on March 8–9, 1977, in Arlington, VA, at the Tumor Viral Immunology Workshop sponsored by the Collaborative Research Branch, RNA Tumor Virus Studies Section, of the National Cancer Institute (see ref. 1).

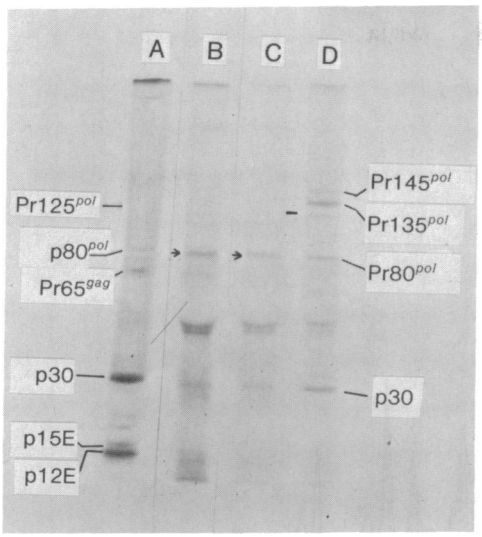

FIG. 1. Fluorography of a sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide slab gel of detergent-disrupted virus precipitated with anti-RT serum. [^{3}H]Methionine-labeled RLV was disrupted by homogenization in a Dounce homogenizer with 0.5% Nonidet P-40/0.5% sodium deoxycholate/0.2% $NaDodSO_4$ in 2 ml of either 10 mM Tris·HCl, pH 7.2 (lane B) or 10 mM sodium phosphate, pH 7.2 (lane C). After a 10-min incubation at room temperature, the disrupted virus was clarified by centrifugation at 10,000 × *g* for 10 min at 4°. The supernatant fluid was mixed with 5 μl of absorbed goat anti-RT serum (3) for 10 min at room temperature and overnight at 4°. The immunoprecipitate was collected by indirect immune precipitation with rabbit anti-goat IgG serum (3). The washed precipitates were analyzed by electrophoresis on a 6–12% linear gradient $NaDodSO_4$/polyacrylamide slab gel. One-fourth of the immunoprecipitate from 4×10^6 cpm of virus was applied to the gel (lane B, 24,710 cpm; lane C, 18,170 cpm). In lane A, 60,000 cpm of [^{3}H]methionine-labeled virus was applied; in lane D, 20,000 cpm of [^{3}H]methionine-labeled intracellular viral specific proteins precipitable with anti-RT serum was applied. In this case, RLV-infected JLS-V16 cells were pulse-labeled for 20 min with [^{3}H]methionine in Hanks' buffer and chased in complete growth medium for 2 hr at 37°. The cytoplasmic extract was treated with 5 μl of absorbed anti-RT serum. The gel was processed for fluorography as described (3). The exposure time was 7 days.

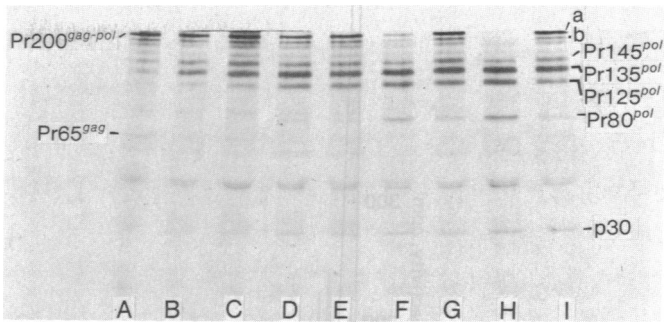

FIG. 2. Kinetics of formation and disappearance of RT-related viral specific precursor polypeptides in the presence and absence of cycloheximide. RLV-infected JLS-V16 cells were pulse-labeled for 20 min (lane A) with [^{3}H]leucine in Hanks' buffer at 37°. Parallel cultures were pulse-labeled as above and then incubated in complete growth medium for 15 min (lanes B and C), 30 min (lanes D and E), 60 min (lanes F and G), and 2 hr (lanes H and I). In lanes C, E, G, and I, cycloheximide was added to the chase medium at 100 μg/ml. After the incubations, the cultures were processed for cell lysis in detergent and the cytoplasmic extract was treated with absorbed anti-RT serum in an indirect immunoprecipitation reaction as in Fig. 1. The immunoprecipitates were analyzed on a 6–12% gel and processed for fluorography as in Fig. 1. Equal amounts of radioactivity (≃7500 cpm) were applied to each sample well. Exposure was about 7 days.

of time (Fig. 2). Approximately equal amounts of radioactivity from each anti-RT immune precipitate were applied to each gel track. The amount of radioactivity incorporated in a 20-min pulse was similar to that found after a 30-min chase. After longer chase periods, anti-RT-precipitable radioactivity began to decrease. About a 50% reduction of radioactivity was observed after a 2-hr chase, while a 75% reduction was seen after 4 hr of chase (results not shown). This decrease in anti-RT-precipitable proteins is most likely due to export of newly formed virus particles and because anti-RT serum will recognize only the RT sequences and not the non-RT sequences (e.g., $Pr65^{gag}$) that are present in $Pr200^{gag\text{-}pol}$. Based on the apparent molecular weights of $Pr200^{gag\text{-}pol}$ and $p80^{pol}$, only 40% of the mass of $Pr200^{gag\text{-}pol}$ can be accounted for by $p80^{pol}$.

The results of this pulse-chase experiment (Fig. 2 lanes A, B, D, F, and H) clearly show that $Pr200^{gag\text{-}pol}$ is a rapidly labeled but unstable protein. The polypeptides in the $Pr200^{gag\text{-}pol}$ region disappeared during the chase. As $Pr200^{gag\text{-}pol}$ decreased, $Pr135^{pol}$, $Pr125^{pol}$, and $Pr80^{pol}$ steadily increased. $Pr145^{pol}$ decreased during the chase period, but it often was transiently increased (slightly) early in the chase (after 30 min) (Fig. 2 lanes B, D, and F). When chases were carried out for up to 4 hr, $Pr145^{pol}$ was nearly gone whereas $Pr135^{pol}$, $Pr125^{pol}$ and $Pr80^{pol}$ remained as major components (results not shown). Also, $Pr125^{pol}$ comigrates with a protein that is nonspecifically precipitated by anti-bovine serum albumin (3).

In another experiment, formation of $Pr145^{pol}$, $Pr135^{pol}$, $Pr125^{pol}$, and $Pr80^{pol}$ during a brief chase incubation of 30 min (Fig. 2, lanes D and E) was not prevented by cycloheximide treatment (100 μg/ml). Thus, protein synthesis is not required initially for the processing of $Pr200^{gag\text{-}pol}$ to $Pr80^{pol}$. However, longer chase periods (1–2 hr) in the presence of cycloheximide did significantly interfere with the post-translational processing of $Pr200^{gag\text{-}pol}$ (Fig. 2, lanes F–I). Similar effects have been observed with cyloheximide or puromycin in the processing of the *gag* protein precursor $Pr65^{gag}$ as well as of $Pr200^{gag\text{-}pol}$ (see figure 3 of ref. 10).

Peptide Maps of Intracellular RT Precursors. To prove that $Pr200^{gag\text{-}pol}$ is in fact structurally related to $Pr145^{pol}$, $Pr135^{pol}$, $Pr125^{pol}$, and $Pr80^{pol}$, we compared tryptic peptide maps of radioactive methionine-labeled proteins. The tryptic maps of $Pr200^{gag\text{-}pol}$ compared to those of $Pr135^{pol}$ and $Pr80^{pol}$ are shown in Figs. 3 and 4. The results clearly showed that these polypeptides share at least nine tryptic peptide fractions. The profiles of $Pr145^{pol}$ and $Pr125^{pol}$ were very similar to that of $Pr135^{pol}$ (results not shown). The complexity of the peptide maps also decreased with decreasing apparent molecular weight.

The *gag* proteins that contain methionine are p30 and p12 (11). The latter contains an acidic methionine tryptic peptide that does not bind to the cation-exchange column (12) used to generate the results shown in Figs. 3–6. The p30 methionine tryptic peptides elute at fractions 95 and 105 (Figs. 3 and 4). Fraction 95 is seen in $Pr200^{gag\text{-}pol}$ but it was not present in $Pr135^{pol}$, $Pr125^{pol}$ (Fig. 3), $Pr80^{pol}$ (Fig. 4), and virion $p80^{pol}$ (Fig. 5). Fraction 105, however, is found in $Pr200^{gag\text{-}pol}$ and all the RT-related polypeptides. One interpretation of these results is that both a p30-specific and an RT-specific tryptic peptide elute in the same peak fraction (fraction 105). Two-dimensional peptide maps of these proteins labeled with [^{3}H]tyrosine failed to show any evidence of *gag* proteins in $Pr145^{pol}$, $Pr135^{pol}$, $Pr125^{pol}$, or $Pr80^{pol}$ (Kopchick and Arlinghaus, unpublished results). These results are supported by

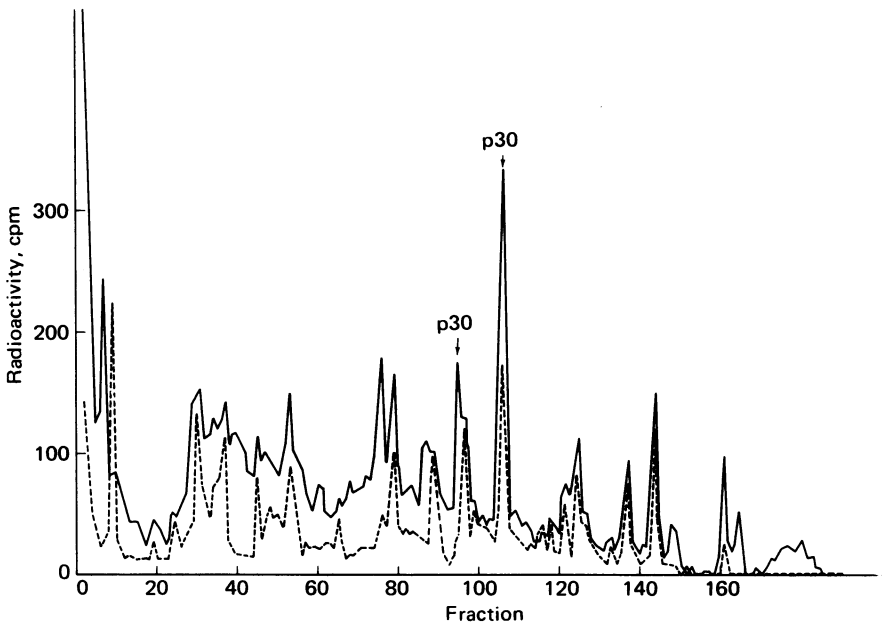

FIG. 3. Ion-exchange chromatography of tryptic digests of Pr200$^{gag\text{-}pol}$ and Pr135^{pol} labeled with methionine. Pr200$^{gag\text{-}pol}$ was isolated from infected cells pulse-labeled for 20 min with [^{35}S]methionine. The cytoplasmic extract was treated with rabbit anti-RLV serum (7) and Pr200$^{gag\text{-}pol}$ polypeptides (formerly Pr1*a*+*b*) were isolated by NaDodSO$_4$/polyacrylamide gel electrophoresis on a 6–12% gradient gel. The *a* and *b* polypeptides of Pr200$^{gag\text{-}pol}$ have identical methionine-labeled tryptic digest profiles under reducing conditions (L. J. Arcement and R. B. Arlinghaus, unpublished results). However, some minor differences in these polypeptides can be detected in the profiles of performic acid-oxidized tryptic peptides labeled with [^{35}S]methionine (7). Pr135^{pol} was isolated from cells pulse-labeled with [^{3}H]methionine for 20 min followed by a 2.5-hr chase. The anti-RT precipitate was fractionated on a 6–12% linear gradient gel. The gel bands were digested with trypsin as described (11). Approximately 28,000 cpm of [^{35}S]methionine-labeled Pr200$^{gag\text{-}pol}$ (—) and 20,000 cpm of [^{3}H]methionine-labeled Pr135^{pol} (----) were applied to the Chromo Bead Type P (Technicon) ion-exchange column as described (11). Recovery of radioactivity from the column eluate and flow-through was 71%.

our previous studies with monospecific antisera (3) in which Pr200$^{gag\text{-}pol}$ is recognized by anti-*gag* and anti-RT sera but not by anti-gp69/71 serum, while Pr145^{pol}, Pr135^{pol}, Pr125^{pol}, and Pr80^{pol} are recognized only by anti-RT serum.

Peptide Maps of Intracellular Pr80^{pol} and p80^{pol}. To determine the relationship of intracellular peptides recognized by anti-RT serum to the virion RT enzyme, we isolated the p80^{pol} from disrupted virus by immunoprecipitation with anti-RT and compared its peptide map to intracellular Pr80^{pol}. Comparison of the tryptic maps (Fig. 5) indicated that Pr80^{pol}

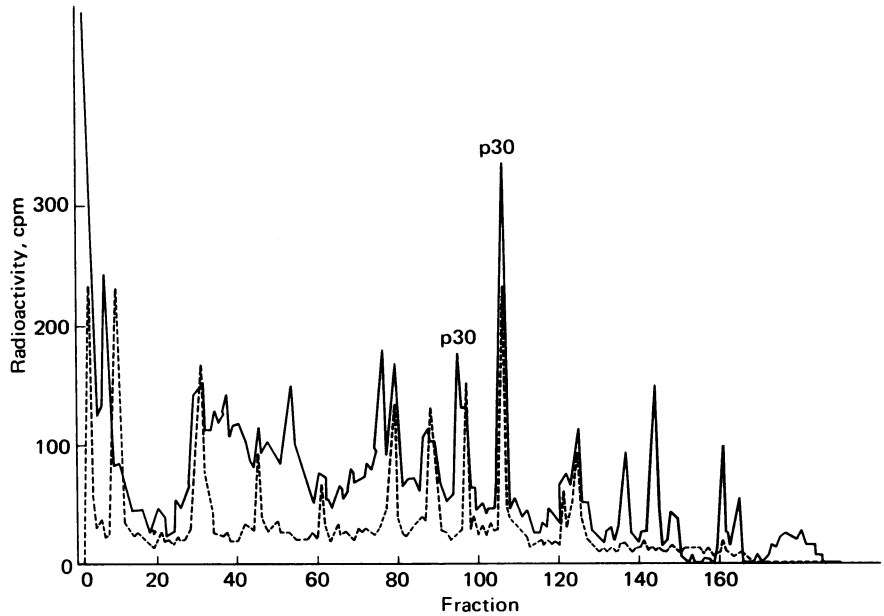

FIG. 4. Ion-exchange chromatography of tryptic digests of Pr200$^{gag\text{-}pol}$ and Pr80^{pol} labeled with methionine. Pr200$^{gag\text{-}pol}$ was labeled with [^{35}S]methionine and isolated as in the legend to Fig. 3. Pr80^{pol} was obtained in the same manner as Pr135^{pol}, as described in the legend to Fig. 3. The samples were digested with trypsin and applied to the column. About 25,000 cpm of [^{35}S]methionine-labeled Pr200$^{gag\text{-}pol}$ (—) and 21,500 cpm of [^{3}H]methionine-labeled Pr80^{pol} (----) were applied to the column. About 70% recovery was obtained.

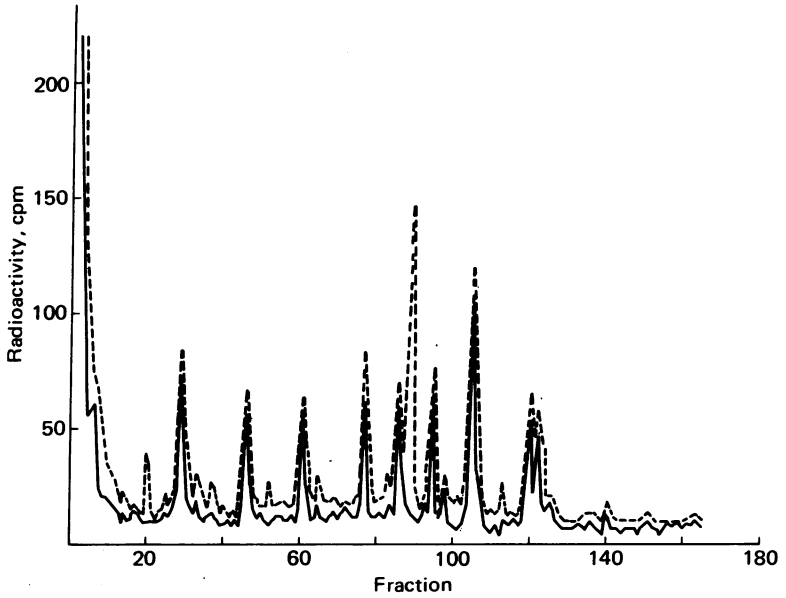

FIG. 5. Ion-exchange chromatography of tryptic digests of Pr80^{pol} from virus-infected cells and p80^{pol} from virions obtained by anti-RT precipitation. Pr80^{pol} was isolated from infected cells in the same manner as Pr135^{pol} described in the legend to Fig. 3. p80^{pol} was isolated as in the legend to Fig. 1, lane C, with anti-RT and [^{3}H]methionine-labeled virus. Tryptic digestion and ion-exchange chromatography were as described in the legend to Fig. 3. About 11,500 cpm of [^{35}S]methionine-labeled Pr80^{pol} (—) and 10,000 cpm of [^{3}H]methionine-labeled p80^{pol} (----) were applied to the column. Recovery was 68%.

and p80^{pol} share nine tryptic peptide fractions. At least one additional peak (Fig. 5, fraction 85) was present in the virion p80^{pol}. The nature of the extra peak is unknown.

We also purified the RT enzyme from virus labeled with [^{3}H]methionine (9). The active enzyme contained as its major constituent a polypeptide that comigrated with p80^{pol} (1). This

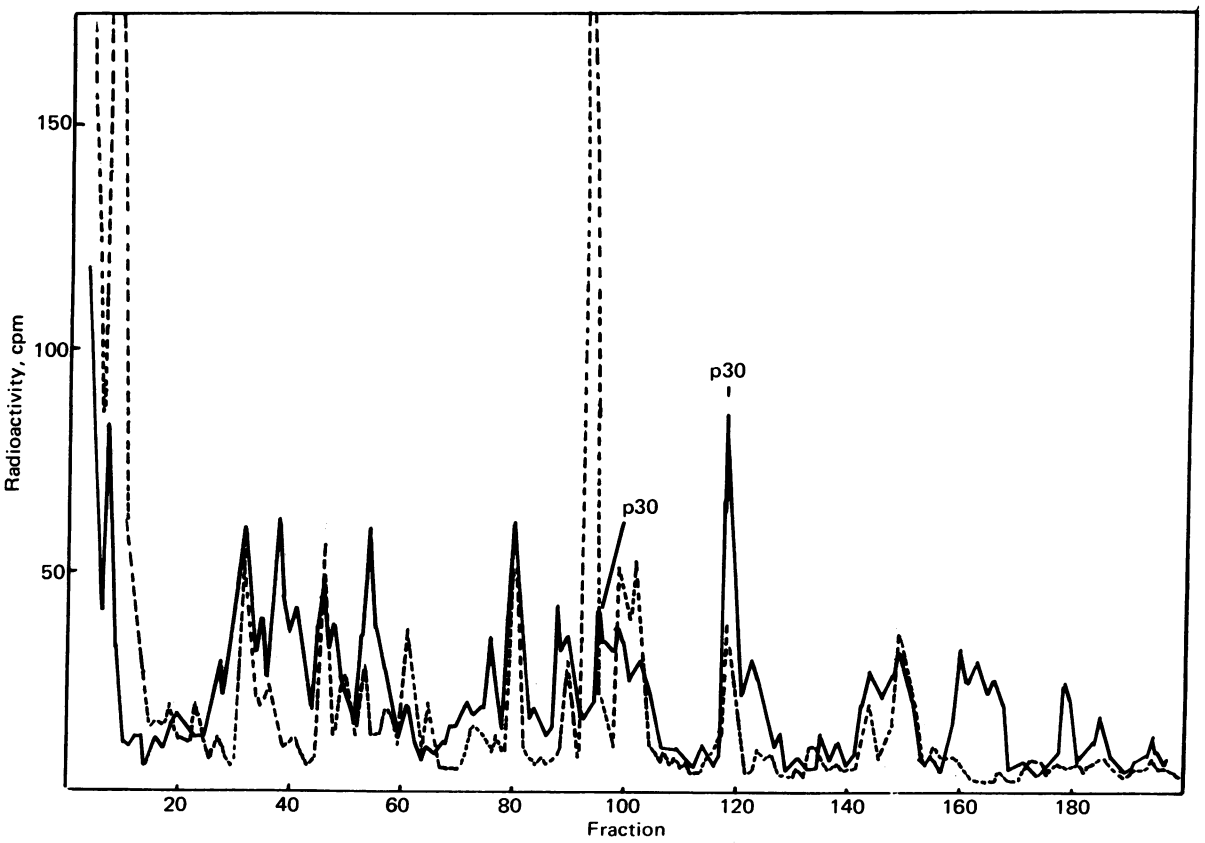

FIG. 6. Comparison of tryptic peptides of virion p80^{pol} obtained by RT enzyme purification to tryptic peptides of intracellular Pr200$^{gag\text{-}pol}$. Pr200$^{gag\text{-}pol}$ was labeled with [^{35}S]methionine and isolated as in the legend to Fig. 3. Virion RT labeled with [^{3}H]methionine was purified as described (9). Approximately 100 × 10^6 cpm of [^{3}H]methionine-labeled RLV produced in infected JLS-V5 cells was used as a source of the labeled RT (1). About 8000 cpm of [^{35}S]methionine-labeled Pr200$^{gag\text{-}pol}$ (—) and 12,000 cpm of [^{3}H]methionine-labeled p80^{pol} (----) obtained from a 6–12% gradient gel of NaDodSO$_4$/polyacrylamide (1) were applied to the column. Recovery was about 75%. This column analysis was completed in 200 fractions instead of the usual 180 due to an inadvertent change in the pump rate.

80,000-dalton polypeptide from the active enzyme was digested with trypsin and its tryptic peptides were compared to those of $Pr200^{gag\text{-}pol}$ (Fig. 6). The results clearly showed that these two proteins shared at least nine tryptic peptide fractions. Furthermore, anti-RT-precipitable cellular $Pr80^{pol}$ and virion $p80^{pol}$, as well as $p80^{pol}$ obtained by enzyme purification, had very similar tryptic peptide profiles (compare Figs. 4, 5, and 6).

DISCUSSION

The results presented here provide strong evidence that the RT from RLV ($\simeq$80,000 molecular weight) is made by synthesis and cleavage of a high molecular weight polyprotein precursor ($\simeq$200,000 daltons), designated $Pr200^{gag\text{-}pol}$, that contains gene products of the viral *gag* and *pol* genes. Anti-RT serum (treated to remove antibodies to *gag* proteins) specifically recognized $Pr200^{gag\text{-}pol}$ and three intermediate polypeptides, $Pr145^{pol}$, $Pr135^{pol}$, and $Pr125^{pol}$. Another polypeptide, $Pr80^{pol}$, which is similar in size to the mature viral enzyme, $p80^{pol}$, is found in infected cells. The absorbed antisera inhibited the *in vitro* RT reaction and it mainly precipitated an 80,000 molecular weight polypeptide ($p80^{pol}$) from virions. The latter is similar in size to the purified polymerase protein (1, 5, 13, 14).

Pulse-chase studies (Fig. 2) have established that $Pr200^{gag\text{-}pol}$ is the initial precursor of the viral RT. This polyprotein is rapidly cleaved to produce polypeptides $Pr145^{pol}$, $Pr135^{pol}$, $Pr125^{pol}$, and $Pr80^{pol}$. This post-translational processing of $Pr200^{gag\text{-}pol}$ does take place in the absence of further protein synthesis for at least 30 min. However, continued processing does require synthesis of some protein (Fig. 2 and ref. 10). $Pr135^{pol}$, $Pr125^{pol}$, and $Pr80^{pol}$ are relatively stable polypeptides. It is quite possible that some or all intracellular $Pr80^{pol}$ is in fact present in viral particles. Such viral particles may be in the process of budding from the cell or they may represent cell-associated, entrapped virus.

Peptide mapping studies support both the immunoprecipitation results with anti-RT and the pulse-chase studies. Tryptic peptide sequences of a virion polypeptide of 80,000 molecular weight, recognized by anti-RT and present as the major constituent in purified RT preparations (1), are contained in $Pr200^{gag\text{-}pol}$ and the lower molecular weight RT precursors $Pr145^{pol}$, $Pr135^{pol}$, and $Pr125^{pol}$. These studies are consistent with the following cleavage scheme for the production of RT: $Pr200^{gag\text{-}pol} \rightarrow Pr145^{pol} \rightarrow Pr135^{pol} \rightarrow Pr125^{pol} \rightarrow Pr80^{pol}$.

It remains to be determined whether or not $Pr80^{pol}$ is further processed to virion $p80^{pol}$. Comparisons of the tryptic maps of these two proteins indicate that they are very similar in structure. However, one additional tryptic peptide was detected in virion $p80^{pol}$ that was not observed in cellular $Pr80^{pol}$. The significance of this difference is not known, but it could be a manifestation of further processing of $Pr80^{pol}$ to $p80^{pol}$.

Our studies, at this point, have not clarified whether the *gag* portion of $Pr200^{gag\text{-}pol}$ gives rise to mature *gag* proteins. The difficulty in determining this is due to the fact that the mature *gag* proteins are mainly derived from $Pr80^{gag}$ (3).

Another point of interest concerns the extra sequences in $Pr125^{pol}$ that cannot be accounted for by the mature polymerase alone. As discussed above, $Pr125^{pol}$ is cleaved to generate $Pr80^{pol}$ plus a polypeptide of about 40,000–50,000 daltons. The evidence for the existence of such a polypeptide stems from the finding of 13 methionine-containing tryptic peptides in $Pr125^{pol}$, only 9 of which are accounted for by mature RT. Furthermore, none of these "extra" tryptic peptides are found in *gag* precursors $Pr80^{gag}$ and $Pr65^{gag}$. The nature of this extra protein material is not known. It may be degraded as waste or it may represent an additional viral gene product(s).

Our previous studies (1, 3) have provided evidence that $Pr200^{gag\text{-}pol}$ is the result of an occasional read-through mechanismiin which ribosomes are able to translate through some sort of a termination signal about 5% of the time. It is an intriguing possibility that such read-through events may be involved in the synthesis of certain normal mammalian cell proteins similar to that seen in bacterial systems (15).

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