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Reverse Transcriptase of RNA Tumor Viruses

V. In Vitro Proteolysis of Reverse Transcriptase from Avian Myeloblastosis Virus and Isolation of a Polypeptide Manifesting Only RNase H Activity

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Purified avian myeloblastosis virus reverse transcriptase contains two subunits that are structurally related. The large subunit, β (molecular weight, 95,000), was converted in vitro by chymotrypsin into a polypeptide of molecular weight 63,000. This polypeptide was indistinguishable from the small subunit, α (molecular weight, 65,000), in its chromatographic behavior on the phosphocellulose column and its tryptic peptide composition. During this proteolytic conversion, a polypeptide of molecular weight 32,000 (fragment B) was obtained. It was composed of tryptic peptides unique to β and appeared to be derived from the portion of the β subunit that was cleaved off during the conversion of β into α. Upon continued proteolysis, a smaller polypeptide of molecular weight 24,000 (fragment A) was generated. This polypeptide manifested only RNase H activity and shared common amino acid sequences with β and α subunits. Fragment A did not share any amino acid sequence homology with fragment B.

Retroviruses contain reverse transcriptase, which is required for the conversion of viral genetic material into complementary DNA (cDNA) (1, 2, 36-39, 42). Purified reverse transcriptase manifests both synthetic and degradative activities. The synthetic activities are characterized by RNA-directed synthesis of cDNA and DNA-directed synthesis of double-stranded DNA (34, 42). The degradative activity is represented by RNase H activity, which degrades specifically the RNA moiety of DNA · RNA hybrid (23, 26, 28, 42). Reverse transcriptase purified from virions of avian retroviruses has a molecular weight of 170,000 and consists of two subunits (in equimolar ratio) of molecular weights 95,000 (β) and 65,000 (α) (17, 22, 42). The subunits are structurally related (15, 33). Enzymatically active α subunit has been isolated and shown to possess both DNA polymerase and RNase H activities (17, 40). Recently, an enzymatically active dimer of the large subunit β has been isolated from avian sarcoma virus B77 grown in duck cells (21). Although both the synthetic and the degradative activities reside on the same polypeptide and are coded for by the viral RNA, all available data indicate that they have different functional sites (5, 42).

The function of RNase H during the synthesis of proviral DNA remains obscure. It has been proposed that during the synthesis of cDNA, RNase H degrades the RNA template and provides a primer with a 3'-hydroxyl group to initiate the synthesis of the second strand of DNA (3, 23, 26). Studies involving inhibitors specific for RNase H activity (e.g., NaF, $\geq$150 mM KCl), however, do not seem to support this notion (5, 7). The size of cDNA and the amounts of double-stranded DNA synthesized in these studies are relatively small and thus may not reflect the true picture. More satisfactory answers can be provided from studies involving mutants that have lesions in only RNase H activity. Another approach would be to use a reconstituted system containing DNA polymerase free of RNase H activity. The latter approach, however, requires physical separation of the two enzymatic activities.

Several investigators have reported the conversion of the $\alpha\beta$ form of the avian myeloblastosis virus (AMV) reverse transcriptase to α and smaller polypeptides by using either proteolytic enzymes (27, 32) or organic solvents (16). We were interested in physically separating RNase H activity from DNA polymerase activity. In this communication, we report our studies involving the use of proteolytic enzyme, chymotrypsin, to generate various polypeptides from the $\alpha\beta$ dimer. The results show: (i) the large subunit β was converted into a polypeptide virtually indistinguishable from the small subunit α; (ii) a 32,000-dalton fragment was generated during proteolysis, which possessed amino acid

sequences only present in subunit β; and (iii) a smaller polypeptide of molecular weight 24,000 was isolated, which contained only RNase H activity.

MATERIALS AND METHODS

AMV in plasma was provided by the Office of Program Resources and Logistics of the Virus Cancer Program of the National Cancer Institute, Bethesda, Md. Tritium-labeled and unlabeled deoxyribonucleoside triphosphates and ribonucleoside triphosphates were purchased from Schwarz/Mann, Orangeburg, N.Y., and New England Nuclear Corp., Boston, Mass. Polynucleotides were obtained from Miles Laboratories, Inc., Elkhart, Ind., and P-L Biochemicals, Milwaukee, Wis. Oligonucleotides were obtained from Collaborative Research, Waltham, Mass. Carrier-free $Na^{125}I$ was purchased from ICN Corp., Irvine, Calif. Immobilized (bound to Sepharose 4B) lactoperoxidase, α-chymotrypsin, and L-(1-tosylamino-2-phenyl)ethyl chloromethyl ketone (TPCK)-trypsin were purchased from Worthington Biochemicals Corp., Freehold, N.J. *Escherichia coli* RNA polymerase and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, Mo. Phage fd DNA was a gift of G. Bourguignon of the University of California, San Diego.

Purification of AMV and its reverse transcriptase. AMV and reverse transcriptase were purified as described previously (43).

Synthesis of fd-DNA · [^{3}H]RNA hybrid. The procedure described by Stavrianopoulos et al. (35) to synthesize f1 DNA · RNA hybrid was followed with some modifications. Briefly, the reaction mixture in 0.5 ml contained 80 mM Tris-hydrochloride (pH 8.3), 50 mM KCl, 10 mM dithiothreitol, 12 mM $MnCl_2$, 120 μg of fd DNA per ml, 10 mM each ATP, CTP, and GTP, 28 nmol of [^{3}H]UTP (36 Ci/mmol), and 50 μg of *E. coli* RNA polymerase (395 U/mg). After 5 h of incubation at 37°C, the reaction was stopped by addition of a solution containing 10 mM EDTA–0.3 M NaCl. The hybrid was extracted with phenol-chloroform and ethanol precipitated. It was separated from the unincorporated material by chromatography on a Sephadex G-50 column (1.1 by 20 cm) with an elution buffer containing 0.1 M Tris-hydrochloride (pH 7.5), 1 mM EDTA, and 0.3 M NaCl. The hybrid came through in the void volume. Over 95% of the hybrid was resistant to digestion by pancreatic RNase A (20 μg/ml) and RNase T_1 (4 μg/ml) in 0.3 M NaCl and 0.03 M sodium citrate and became sensitive (>98%) to the RNases after boiling and quick cooling. It was over 85% sensitive to RNase H associated with AMV reverse transcriptase.

DNA polymerase and RNase H assays. The enzymatic activities were assayed as described previously (41), except that 1 mM Mn^{2+} was replaced with 6 mM Mg^{2+} in polymerase assay and 10 mM Mg^{2+} in RNase H assay using fd DNA · [^{3}H]RNA as substrate.

Enzymatic iodination by lactoperoxidase bound to Sepharose 4B (LP-4B). The procedure described by David and Reisfeld (10) was followed with modifications. Approximately 50 μg of purified reverse transcriptase in 0.5 to 1.0 ml of buffer B containing 20 mM Tris-hydrochloride (pH 7.5), 0.1 mM EDTA, 0.15% Nonidet P-40, 15% glycerol, 0.3 M KCl, and 20 mM 2-mercaptoethanol was dialyzed extensively against the same buffer without mercaptoethanol. It is important that the concentration of 2-mercaptoethanol does not exceed 10^{-8} M. To this sample, 20 μl of extensively washed LP-4B beads and 1 mCi of carrier-free $Na^{125}I$ (17 Ci/mg) were added. The reaction was initiated with 4 μl of 0.003% H_2O_2 and continued at room temperature for 20 min. The beads were spun down in a clinical centrifuge, and the iodinated protein in the supernatant was separated from free iodine by chromatography on a Sephadex G-25 column (0.8 by 20 cm). The peak radioactive fractions eluting in the void volume were pooled, and mercaptoethanol (20 mM) and carrier protein bovine serum albumin (100 μg/ml) were added. The radiolabeled enzyme was stored at −90°C. Under these mild conditions of iodination, AMV reverse transcriptase can be labeled to a specific activity of 5 μCi/μg of protein and retains 30 to 40% of its original enzymatic activity.

Proteolysis by chymotrypsin. Unlabeled or ^{125}I-labeled reverse transcriptase in buffer B was digested with 2.5 to 20 μg of α-chymotrypsin per ml at 2°C (ice) or 37°C. The reaction was stopped by 2 mM PMSF. Portions withdrawn at different times were kept in ice and assayed for residual DNA polymerase and RNase H activities.

Sephadex G-75 column chromatography. A sample was layered on a column (1.1 by 50 cm) equilibrated with buffer B containing 5% glycerol (instead of standard 15%). The column was eluted with the same buffer at a flow rate of 10 ml/h, and 1-ml fractions were collected. A total of 20 μl from each fraction was assayed for DNA polymerase and RNase H activities.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ten percent polyacrylamide gels cross-linked with methylenebisacrylamide were prepared essentially as described by Laemmli (25) and poured as slabs (14 by 9 by 0.1 cm). Protein samples were solubilized in a buffer containing 50 mM Tris-hydrochloride (pH 6.8), 1% SDS, 2% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue. After boiling for 3 min, samples were subjected to electrophoresis at 12.5 mA for 4 to 4.5 h. Unlabeled proteins were stained with 0.03% Coomassie brilliant blue as described by Fairbanks et al. (12). Autoradiograms of analytical gels containing labeled proteins were made by the procedure of Fairbanks et al. (11), using Kodak RP-14 or NS-54T X-ray film.

Extraction of ^{125}I-labeled proteins from SDS gels. At the end of electrophoresis, the wet, preparative gels were wrapped in Saran Wrap and pressed against a sheet of Kodak NS-54T X-ray film. The exposure was carried out at 4°C for approximately 30 to 40 min. Using the developed film as a guide, the radioactive protein bands were excised from the bis-linked gels, transferred to individual test tubes, and covered with a protein extraction buffer containing 0.05 M NH_4HCO_3, 0.1% SDS, and 2% 2-mercaptoethanol. The samples were boiled for 10 min and further incubated at 37°C for 6 to 15 h. The eluates, together with 50 to 100 μg of bovine immunoglobulin, were

precipitated by trichloroacetic acid and washed twice with 95% ethanol and once with acetone at −10°C. After complete evaporation of acetone, the samples were used for peptide analyses and further proteolytic studies.

Peptide analysis. Proteins extracted from SDS gels were oxidized by performic acid according to the method of Hirs (20). The oxidized proteins were digested with TPCK trypsin and analyzed on thin-layer cellulose plates by the two-dimensional fingerprinting technique of Gibson (4, 14, 15). First-dimensional electrophoresis was performed at 1 kV for 0.7 h. Second-dimensional ascending chromatography was performed at room temperature for 6 to 7 h. The electrophoresis buffer (pH 4.7) contained butanol-pyridine-acetic acid-water in a ratio of 2:1:1:36 (vol/vol). The chromatography buffer (pH 5.3) also contained butanol-pyridine-acetic acid-water, but in a ratio of 97:75:15:60 (vol/vol). The distribution of ^{125}I-labeled tryptic peptides (mainly those containing tyrosine residues) was examined by autoradiography. The highly efficient transfer (60 to 80%) of ^{125}I to carrier proteins during performic acid oxidation, as reported by Montelaro et al. (29), did not seem to occur in these experiments. If this occurred, tryptic peptide maps of all ^{125}I-labeled proteins using the same carrier should look alike. This was not the case, as evidenced by the results shown in Fig. 8 and the data obtained with other viral proteins in this laboratory (R. Bosselman, personal communication).

RESULTS

Kinetics of inactivation of DNA polymerase and RNase H activities by chymotrypsin. Figure 1 demonstrates the effects of incubation of purified reverse transcriptase with α-chymotrypsin at a concentration of 20 μg/ml. It was noted that DNA polymerase activity was more rapidly inactivated than RNase H activity. The $t_{1/2}$ (time required to inactivate 50% of activity) of inactivation of DNA polymerase activity was 15 min, as compared to over 45 min for inactivation of RNase H activity. No significant loss of either DNA polymerase or RNase H activities was observed when the enzyme was incubated with 2.5 μg of chymotrypsin per ml for less than 30 min.

The differential inactivation of synthetic and degradative activities offered an opportunity to physically separate the two activities by controlled digestion with α-chymotrypsin.

Alterations in polypeptide patterns after chymotrypsin digestion. To examine any alterations in polypeptide structure of reverse transcriptase, samples were withdrawn at zero time, 6, 12, and 20 min after the addition of 10 μg of chymotrypsin per ml, and proteolysis was terminated by addition of PMSF. A small portion from each sample was assayed for the residual DNA polymerase and RNase H activities. The remainder was precipitated by trichloroacetic acid and analyzed by SDS-PAGE (as described above). The results of SDS-PAGE analysis are shown in Fig. 2. Lane a represents the zero-time sample. The amount of subunit β had decreased slightly, thus reducing the molar ratio of subunits β to α to less than 1. This reduction in the ratio of the two subunits indicated that proteolysis had already begun during the time lapse between the additions of chymotrypsin and PMSF. The additional band of molecular weight 38,000 was probably a contaminant of this particular preparation of reverse transcriptase because it was not seen in the other instances. A very faint band of polypeptides with an average molecular weight of 32,000 (31,000 to 34,000) was also noted. After 6 min of digestion (lane b), more than 50% of subunit β was

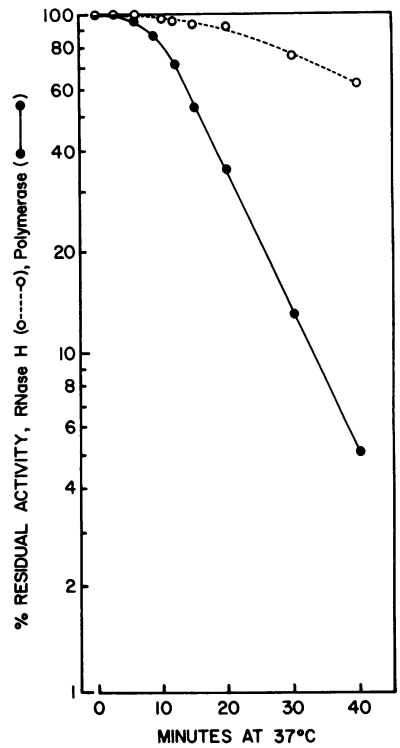

FIG. 1. *Inactivation of DNA polymerase and RNase H activities of AMV reverse transcriptase by chymotrypsin. Purified AMV reverse transcriptase (100 μg/ml) was digested with α-chymotrypsin (20 μg/ml) at 37°C. At times indicated, samples were withdrawn, and proteolysis was terminated by the addition of 2 mM PMSF. Residual DNA polymerase activity and RNase H activity were assayed, using poly(C) · oligo(dG)$_{15}$ as template-primer and fd DNA · [^{3}H]RNA as substrate, respectively. The amount of [^{3}H]dGMP incorporated and the amount of [^{3}H]RNA digested in fd DNA · [^{3}H]RNA hybrid by untreated reverse transcriptase were taken as 100% of DNA polymerase and RNase H activities, respectively.*

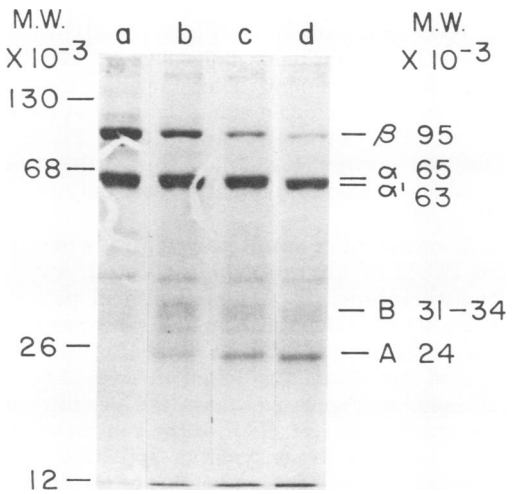

FIG. 2. *Coomassie brilliant blue-stained PAGE patterns of AMV reverse transcriptase after digestion with chymotrypsin. Portions of 15 to 20 μg of reverse transcriptase were withdrawn at (a) 0 min, (b) 6 min, (c) 12 min, and (d) 20 min after addition of 10 μg of chymotrypsin per ml, followed by incubation at 37°C. Samples were precipitated by trichloroacetic acid, washed twice with acetone, and analyzed by SDS-PAGE as described in the text. The protein markers used were β-galactosidase (molecular weight, 130,000), bovine serum albumin (molecular weight, 68,000), chymotrypsinogen (molecular weight, 25,700), and cytochrome c (molecular weight, 12,200). The estimated molecular weights of β, α, α', and fragments A and B are given in the right-hand side of the figure.*

digested, with the appearance of a polypeptide of molecular weight 63,000 (designated as α'). It was obscured by the thick band of subunit α in this blown-up photograph. The 32,000 band (designated fragment B) became darker. In addition, another polypeptide of molecular weight 24,000 (fragment A) had also appeared.

Table 1 compares the losses in DNA polymerase and RNase H activities in the samples depicted in Fig. 2. It was observed again that RNase H activity was more resistant to chymotryptic digestion than was DNA polymerase activity. After 20 min of digestion, more than 40% of DNA polymerase was inactivated, while RNase H activity remained virtually unchanged. When these differences were compared with the alterations in polypeptide structure, it appeared that DNA polymerase activity was more dependent on the physical integrity of the holoenzyme αβ than was RNase H activity. This observation offered a partial explanation as to why digestion with chymotrypsin led to a more rapid inactivation of DNA polymerase than RNase H.

Phosphocellulose column chromatographic patterns of chymotrypsin-digested reverse transcriptase. We wanted to study whether the limited proteolysis of reverse transcriptase also affected its chromatographic behavior. A partially digested sample of reverse transcriptase retaining 50% of DNA polymerase activity and over 90% of RNase H activity was analyzed on a phosphocellulose column with a salt gradient of 0 to 0.6 M KCl. As a control, an undigested sample was also analyzed. In the undigested sample (Fig. 3a), more than 90% of both polymerase and RNase H activities coeluted at a salt concentration of 0.32 M KCl (PC II). Less than 5% of either activity eluted at 0.13 M KCl (PC I). The small peak that eluted behind PC II was not further analyzed. When the chymotrypsin-treated sample was examined, a very different picture emerged (Fig. 3b). Over 80% of polymerase activity eluted at a salt concentration of 0.13 M KCl (corresponding to PC I), and the remainder eluted at a salt concentration of 0.32 M (corresponding to PC II). Furthermore, the proportion of RNase H activity eluting at these salt concentrations was different. About 60% of RNase H activity eluted in PC I, and the remainder eluted in PC II. Two small peaks of nuclease activity between PC I and PC II and to the right of PC II were found to be RNase activities and not those of RNase H.

When the peak fractions of enzymatic activities were analyzed by SDS-PAGE, the following observations were made. PC I of the control sample (Fig. 4, lane a) did not contain any polypeptides detectable by the gel system used. In this system, 0.5 μg of bovine serum albumin can be readily visualized by Coomassie brilliant blue staining. PC II of the control sample, on the other hand, consisted of β and α subunits in an

TABLE 1. *Relative loss of enzymatic activities under the condition of chymotryptic digestion as described in the legend to Fig. 2*

Gel column	Min at 37°C	% Residual activity[a]	
		DNA polymerase	RNase H
a	0	100	100
b	6	98	102
c	12	79	103
d	20	58	93

[a] AMV reverse transcriptase was digested as described in the legend to Fig. 2. A 10-μl portion from each sample was assayed for residual DNA polymerase activity, using poly(C) · oligo(dG)$_{15}$ as template-primer and [^{3}H]dGTP as substrate. Another portion was assayed for residual RNase H activity, using fd DNA · [^{3}H]RNA as substrate. The amount of [^{3}H]-dGMP incorporated and the amount of fd DNA · [^{3}H]RNA degraded by the zero-time sample were taken as 100% of DNA polymerase activity and RNase H activity, respectively.

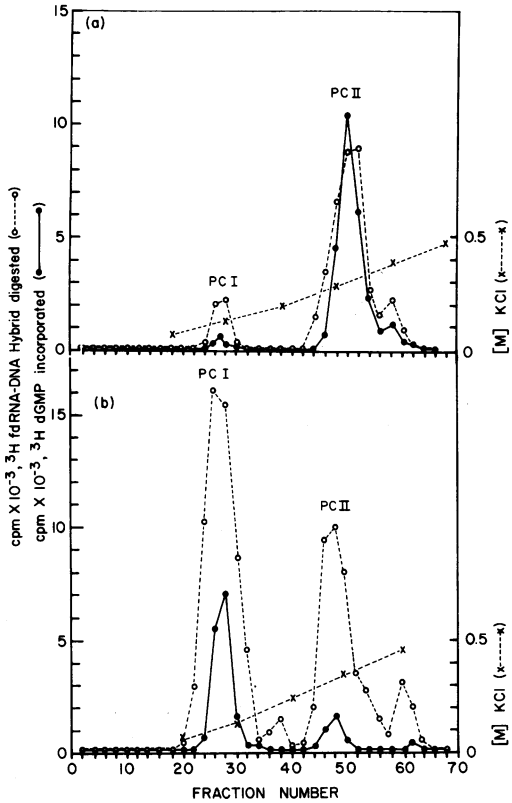

FIG. 3. *Phosphocellulose chromatography of AMV reverse transcriptase before and after limited digestion with chymotrypsin. The enzyme was eluted from the column with a 0 to 0.6 M KCl gradient in buffer B (20 mM Tris-hydrochloride [pH 7.5], 0.1 mM EDTA, 20 mM 2-mercaptoethanol, 0.15% Nonidet P-40, and 15% glycerol). Ten microliters from each fraction was assayed for DNA polymerase and RNase H activities as described in the legend to Fig. 1. (a) Control, 30 μg of reverse transcriptase; (b) 70 μg of reverse transcriptase after digestion with chymotrypsin (10 μg/ml, 37°C, 15 min).*

approximately equimolar ratio (Fig. 4, lane b). In the case of the chymotrypsin-digested sample, PC I was found to be composed of two polypeptides of molecular weights 65,000 and 63,000 (Fig. 4, lane c). The 65,000-dalton polypeptide was presumably the α subunit originally present in the holoenzyme $\alpha\beta$, and the 63,000-dalton polypeptide (α') probably originated from the β subunit during chymotrypsin digestion. However, there were other possibilities regarding the origin of α and α'. They will be discussed later in detail in the Discussion. The polypeptide composition of PC II of digested reverse transcriptase showed that it contained β, α, and α' in greatly reduced quantities (Fig. 4, lane d). In addition, the presence of smaller polypeptides of molecular weights ranging from 13,000 to 24,000 was also observed (Fig. 4, lane d). Among them, a polypeptide of molecular weight 24,000 was most prominent. These results, together with those showing an enhanced ratio of RNase H to DNA polymerase activities in PC II of digested enzyme (Fig. 3b), suggested that these smaller polypeptides might possess only RNase H activity.

Separation of small polypeptides present in PC II of chymotrypsin-treated reverse transcriptase. To isolate the small polypeptides in the undenatured state for further enzymatic studies, materials in PC II (Fig. 3b) were pooled and chromatographed on a Sephadex G-75 column. Figure 5a shows the gel filtration chromatography of PC II of the control sample (Fig. 3a). Both enzymatic activities came through in the exclusion volume of the column, and the ratio of DNA polymerase to RNase H activities remained unchanged. In the chymotrypsin-treated sample, all the DNA polymerase activity eluted in the void volume, whereas only 40% of the RNase H activity coeluted with the bulk of DNA polymerase. The remainder eluted immediately after the void volume. Although the peaks of the two enzymatic activities were separated by an elution volume of 3 to 4 ml, the overlap of the two activities was considerable. The incomplete separation suggested that the smaller polypeptides might still be bound to subunit α. Dissociating agents such as urea,

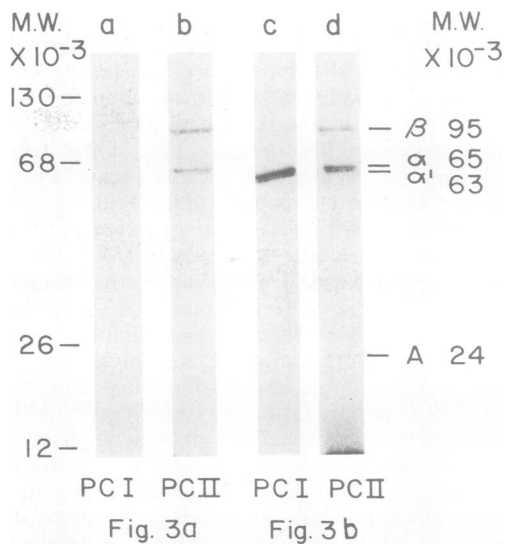

FIG. 4. *Polypeptide patterns of PC I and PC II from data of Fig. 3a and b. Peak fractions were pooled, and one-fifth from each peak was precipitated with trichloroacetic acid and analyzed by SDS-PAGE as described in the legend to Fig. 2. (a) PC I, Fig. 3a; (b) PC II, Fig. 3a; (c) PC I, Fig. 3b; (d) PC II, Fig. 3b.*

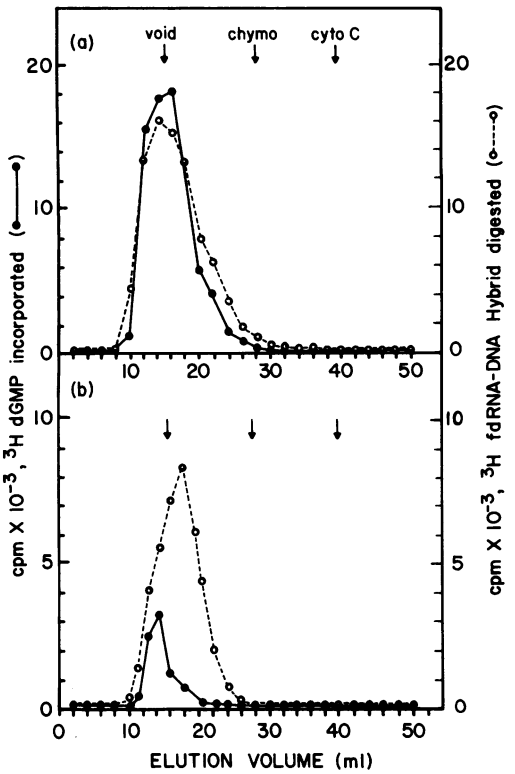

FIG. 5. *Sephadex G-75 gel filtration chromatography of control and chymotrypsin-digested PC II obtained from data of Fig. 3a and b. PC II fractions were pooled and applied to a Sephadex G-75 column. The column was eluted with a buffer containing 20 mM Tris-hydrochloride (pH 7.5), 0.1 mM EDTA, 20 mM 2-mercaptoethanol, 0.15% Nonidet P-40, and 0.3 M KCl. Twenty microliters from each fraction was assayed for DNA polymerase and RNase H activities. (a) Control, (b) chymotrypsin-digested sample. Chymotrypsinogen (molecular weight, 25,700) and cytochrome c (molecular weight, 12,200) were chromatographed in a parallel column as standards. Their elution positions are indicated by arrows.*

guanidine-hydrochloride, and SDS inactivated the enzymatic activities even at relatively low concentrations, and attempts to recover the activities after removal of urea and guanidine-hydrochloride by dialysis and SDS by Dowex 1-X2 resins as described by Weber and Kuter (45) had not been successful (data not shown).

Further proteolysis of α and α′ generated from holoenzyme αβ. It appeared that PC I of chymotrypsin-treated reverse transcriptase (Fig. 4, lane c) consisted of two polypeptides with very similar electrophoretic mobilities. This PC I also manifested both polymerase activity and RNase H activity. PC I fractions were pooled and further digested with 4 μg of chymotrypsin per ml at 2°C (ice) for 10 min. The reaction was stopped by 2 mM PMSF and analyzed on a Sephadex G-75 column. Both enzymatic activities were assayed. All of the DNA polymerase activity came through in the void volume (Fig. 6a). The distribution of RNase H activity was different. While approximately 80% of the activity coeluted with DNA polymerase in the void volume (Seph I), a minor peak of RNase H activity was included in the column with an estimated molecular weight of 26,000

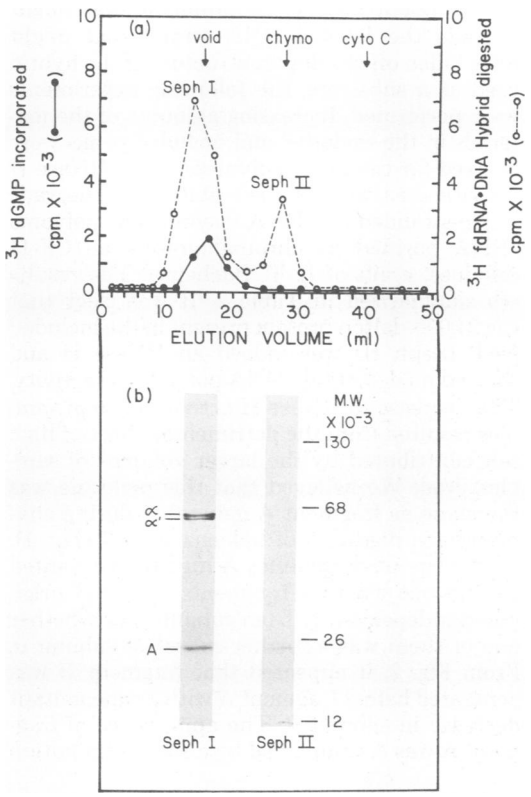

FIG. 6. *Isolation of a polypeptide containing RNase H activity. (a) Separation of RNase H activity by gel filtration. Peak fractions in PC I of Fig. 3b (α, α′) were further digested with 4 μg of chymotrypsin per ml at 2°C for 10 min. The samples were applied to a Sephadex G-75 column. The chromatography was performed as described in the legend to Fig. 5. Seph I refers to the enzymatic activities excluded in the void volume, and Seph II refers to the enzymatic activity included in the column. The protein standards included for the molecular weight estimation are chymotrypsinogen (molecular weight, 25,700) and cytochrome c (molecular weight, 12,200). (b) Polypeptide compositions in Seph I and II of Fig. 6a. Fractions in Seph I and II were pooled separately and analyzed by SDS-polyacrylamide gels. The protein markers included for the molecular weight determination were β-galactosidase (molecular weight, 130,000), bovine serum albumin (molecular weight, 68,000), chymotrypsinogen (molecular weight, 25,700), and cytochrome c (molecular weight, 12,200).*

(Seph II). SDS-PAGE (Fig. 6b) revealed that the excluded peak (Seph I) was composed of three polypeptides: α (molecular weight, 65,000), α' (molecular weight, 63,000), and a polypeptide of molecular weight 24,000, whereas the included peak (Seph II) consisted of a single polypeptide of molecular weight 24,000. Thus it appeared that we had obtained a fragment of reverse transcriptase that possessed only RNase H activity. To ascertain that it was indeed an RNase H and not a nuclear contaminant that might degrade the labeled RNA strand that might have fallen off the duplex structure of the hybrid used as a substrate, the following experiments were performed. Increasing amounts of the materials in the excluded and included peaks were assayed for the DNA polymerase and RNase H activities, as well as their abilities to degrade single-stranded [^{3}H]RNA (asymmetric polyoma cRNA purified by chromatography on CF-11 cellulose, a gift of L. T. Bacheler). The results are summarized in Table 2. It was clear that the 24,000-dalton protein present in the included peak (Seph II) was indeed an RNase H and showed no detectable DNA polymerase activity. The decrease in RNase H activity of 30-μl samples resulted from the detrimental effect of high salt contributed by the larger volumes of samples used. We believed that this molecule was the same as fragment A generated during chymotrypsin digestion of holoenzyme $\alpha\beta$ (Fig. 1).

Origins of fragments A and B. We wanted to find out whether fragments A and B originated independently from subunit β or whether one of them was a further subset of subunit α. From Fig. 2, it appeared that fragment B was generated before fragment A with a concomitant decrease in subunit β. The appearance of fragment A was accompanied by a slight diminution of α and α'. We reasoned that if fragment B was cleaved off from subunit β to generate α and α', then the tryptic peptides of fragment B and subunit α should show no structural homology. Furthermore, if fragment A was generated by cleavage of α or α', then the tryptic peptides of fragment A should share sequence homology with α, α', and β, but not with fragment B.

TABLE 2. *DNA polymerase, RNase H, and RNase activities associated with the excluded and included peaks of Sephadex G-75 column*[a]

Sample	μl in assay	cpm		
		[^{3}H]dGMP incorporated	fd DNA · [^{3}H]RNA hybrid digested	Single-stranded [^{3}H]cRNA digested
(1) Excluded peak	8	1,525	7,552	93
	15	2,786	11,429	0
	30	4,674	8,748	106
(2) Included peak	8	70	1,340	0
	15	56	2,198	0
	30	105	1,160	57

[a] The reactions in 0.1 ml were carried out at 37°C for 60 min. The specific activity of [^{3}H]dGTP was 50 cpm per pmol. The amounts of fd DNA · [^{3}H]RNA hybrid and single-stranded [^{3}H]cRNA added per reaction were approximately 20,240 and 2,760 cpm, respectively.

To test these predictions, purified reverse transcriptase was labeled in vitro with ^{125}I, using immobilized lactoperoxidase, and digested with 2.5 μg of chymotrypsin per ml at 2°C (ice). Both the temperature and the protease concentration were lowered to detect stepwise appearance of the fragments. Samples were withdrawn at 1, 5, 10, 15, 20, 30, 45, and 60 min and analyzed by SDS-PAGE. The results are presented in Fig. 7. The overall polypeptide patterns were very similar to those obtained with unlabeled reverse transcriptase (Fig. 2), with two exceptions. The ratio of fragment B to A increased, indicating that perhaps fragment B contained more tyrosine residues than did fragment A. In addition, protein bands of molecular weights 73,000 and 53,000 to 43,000 were also noted in relatively small quantities. The nature of these polypeptides had not been characterized. The polypeptide (molecular weight, 12,000) migrating near the front of the gel and present in all the samples was probably a contaminant. Peptide maps of this polypeptide did not show any sequence homology with either α or β subunits (data not shown). Preparations of enzyme purified on glycerol gradients did not contain this polypeptide. The temporal sequence of digestion appeared to be as follows: (i) subunit β was first digested, with the generation of fragment B. The decrease in the amount of subunit β seemed to correlate with the increase in the quantity of fragment B up to 5 min of digestion with chymotrypsin. Polypeptide α' could not be distinguished from subunit α in this intensely dark band. Nevertheless, the increase in the amount of radioactivity in this area could be seen. (ii) Fragment A appeared with a concomitant reduction in the amount of subunit α. As proteolysis proceeded, the amount of subunits α and β continued to decline, while the quantities of fragments A and B remained unchanged for up to 60 min of digestion, suggesting that these two fragments were further digested into polypeptides with molecular weights smaller than 12,000.

These observations also pointed to the possible relationship between subunit β and fragment B (32,000 daltons) and subunit α and fragment A (24,000 daltons). To investigate these possibilities, both subunits α and β of the undigested ^{125}I-labeled reverse transcriptase, polypeptides

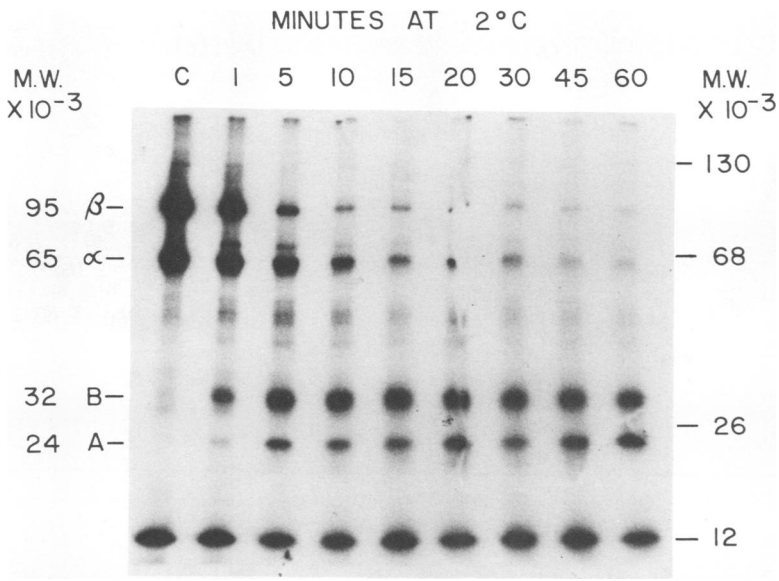

FIG. 7. *Autoradiograph of the PAGE pattern of chymotrypsin-digested ^{125}I-labeled AMV reverse transcriptase. The ^{125}I-labeled enzyme was digested with 2.5 μg of chymotrypsin per ml and incubated at 2°C for the indicated periods of time. Samples were analyzed by SDS-PAGE. After the electrophoresis, the gel was dried and exposed to a Kodak RP14 X-ray film for 15 h. Approximately 40,000 cpm (4 ng) of the samples were applied into each well. C, Undigested enzyme.*

α and α′, and fragments A and B generated by proteolysis (2.5 μg of chymotrypsin per ml, 2°C, 10 min) were excised from wet SDS gels. Due to some diffusion of protein bands during exposure of the wet gels to X-ray films and the intense images exerted on the X-ray films by ^{125}I, it was not possible to distinguish α (65,000 daltons) and α′ (63,000 daltons). Therefore, we did not attempt to separate these two protein bands. They were excised together. The isolated proteins were oxidized with performic acid and digested with TPCK-trypsin. The distributions of the tryptic peptides were determined by two-dimensional fingerprint analyses. When heavily iodinated polypeptides are separated by SDS-polyacrylamide gels, contamination of smaller polypeptides by the nonspecific breakdown products of larger polypeptides is unavoidable. The tryptic peptides having high tyrosine content from these contaminating polypeptides often show up in lesser and various amounts. Such contaminating spots in our studies will be pointed out in the summary of the results depicted in Fig. 8.

(i) Figure 8a, b, and c show the tryptic peptides of subunits α, β, and a mixture of α and β. As shown before (15), the two subunits appear to be structurally related. All the peptides present in α were found in β, except peptide spot no. 15, which appeared to be unique to subunit α and not β. Subunit α, however, lacked peptide no. 3, 4, 6, 7, and 10, which were present in β. The darkest spots, 8, 9, and 11 of subunit β were also present in subunit α, but in markedly lower levels. The relative intensities of these three spots varied from one experiment to another with regard to the two major spots, 12 and 13. Therefore, spots 8, 9, and 11 in the map of subunit α were probably contaminants derived from subunit β or unspecific breakdown products of subunit β. Figure 8c shows the map derived from a mixture of α and β tryptic peptides. Due to smaller samples used, the intensities of the majority of the spots were weaker, yet all 15 spots present in α and β were accounted for.

(ii) Figure 8d shows the tryptic peptides of α and α′ obtained after proteolysis. All the tryptic peptides present in α before digestion (Fig. 8a) were also present in the polypeptides α and α′ of the digested sample. There was an additional peptide, no. 16, which was not present in α. This could be readily explained if one assumed that α′, like α, was a cleavage product of β, but was slightly smaller than α. Thus an extra spot of peptide could arise if the cleavage occurred at a different place during the generation of α. We concluded that α and α′ had nearly complete amino acid sequence homology.

(iii) Figure 8e shows the distribution of tryptic peptides of fragment B. All five unique spots of subunit β (no. 3, 4, 6, 7, and 10) were present,

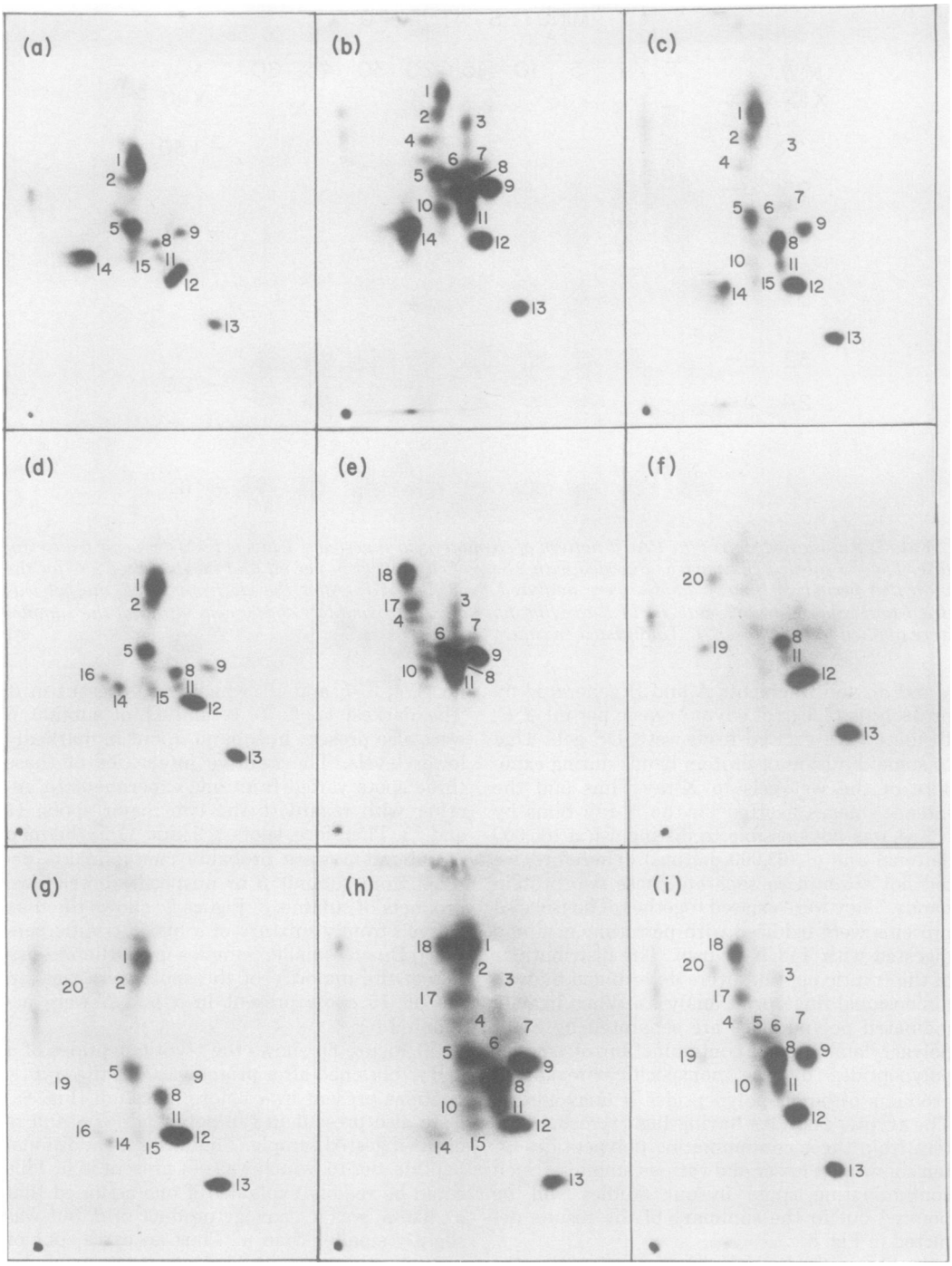

FIG. 8. *Autoradiograms prepared from the two-dimensional fingerprints of tryptic digests of subunits α and β and fragments A and B of ^{125}I-labeled AMV reverse transcriptase. (a) Subunit α, (b) subunit β, (c) mixture of α and β, (d) α and α′, (e) fragment B, (f) fragment A, (g) mixture of α and α′ and fragment A, (h) mixture of subunit α and fragment B, (i) mixture of fragments A and B. Electrophoresis was from left to right, and chromatography was from bottom to top. The spot on the lower left-hand corner was the origin of the sample application.*

suggesting a sequence homology between subunit β and fragment B. Peptides 8, 9, and 11 were present in the quantities characteristic of subunit β. The new spots, no. 17 and 18, present in fragment B, probably resulted from the processing of fragment B from β at the N-terminal, C-terminal, or at both ends.

(iv) The tryptic peptide distribution of fragment A is shown in Fig. 8f. It had a very simple peptide pattern and showed the prominent spots, 8, 12, and 13. Spots 12 and 13 were present in α (Fig. 8a), β (Fig. 8b), and α' (Fig. 8d), but were absent in fragment B (Fig. 8e). Peptide spot 8 in Fig. 8f (fragment A) probably resulted from contamination with fragment B or randomly degraded products of subunit β because its intensity, as compared to spots 12 and 13, also varied from one experiment to another. In addition, fragment A contained two new spots (no. 19 and 20) not present in either α or β. These could be the new peptides, if fragment A was cleaved from the N-terminal, C-terminal, or both ends of the α subunit. It appeared that fragment B was derived from the region of the polypeptide unique to subunit β. Fragment A, on the other hand, was derived from a region common to both subunits α and β and did not appear to be a subset of fragment B.

To further ensure the similarities or differences in the peptide maps of α, β, and fragments A and B, we compared the peptide maps of mixtures of subunit α and fragment A, subunit β and fragment B, and fragments A and B. Figure 8g shows the peptide map of a mixture of α and fragment A. The mixture showed no new peptides that were not present in both fragment A and α. Figure 8h shows the tryptic peptides of a mixture of polypeptides α and α' and fragment B. This mixture resulted in a tryptic peptide map resembling that of subunit β (Fig. 8b), indicating that fragment B and polypeptide α' and subunit α were derived from nonoverlapping regions of subunit β. The distributions of the tryptic peptides of a mixture of fragments A and B are shown in Fig. 8i. All the peptides present in fragments A and B were seen, suggesting that these two fragments shared no sequence homology.

Thus it was concluded that fragments A and B had unique amino acid sequences. Furthermore, it appeared that fragment B was generated from subunit β and shared no sequence homology with α. On the other hand, fragment A appeared to be generated from subunit α and shared sequence homology with α and β.

DISCUSSION

The DNA polymerase and RNase H activities associated with reverse transcriptase of avian or murine RNA tumor viruses are present in the same polypeptide (17, 41). However, the two activities appear to bear different functional sites (5, 42). We wanted to physically separate the two activities to study their properties individually. We found that limited proteolysis of purified AMV reverse transcriptase led to substantial inactivation of DNA polymerase activity without significantly affecting the RNase H activity (Fig. 1). Analysis of α-chymotrypsin-treated AMV reverse transcriptase by SDS-PAGE showed that three classes of polypeptides with molecular weights of 63,000 (α'), 32,000 (fragment B), and 24,000 (fragment A) were generated. Aside from slightly faster mobility in SDS-polyacrylamide gels, polypeptide α' was indistinguishable from α in the chromatographic behavior and the composition of all the tyrosine-containing tryptic peptides except peptide spot 16. It was apparently generated by the proteolysis of subunit β with concomitant production of fragment B. It was not clear whether any intermediates were involved during the production of α' and fragment B. Upon further digestion, a polypeptide of molecular wieght 24,000, fragment A, was generated. This fragment appeared to be generated from subunit α and showed amino acid sequence homology to subunits α and β. It had no structural similarities with fragment B.

There were four possible pathways by which polypeptide α' could be generated: (i) $\alpha \rightarrow \alpha'$; (ii) $\beta \rightarrow \alpha'$; (iii) $\alpha \rightarrow \alpha'$ and $\beta \rightarrow \alpha'$; and (iv) $\beta \rightarrow \alpha \rightarrow \alpha'$. Pathway (i) ($\alpha \rightarrow \alpha'$) was possible, but unlikely in our experiment. If α' was solely derived from subunit α, which was further degraded to form fragment A, one should expect to observe a decrease and not an increase in the total amount of α and α'. As demonstrated in Fig. 2 (lane b, 6-min sample) and data previously published (42; Fig. 4), one observed quite the contrary. In pathway (iii) ($\alpha \rightarrow \alpha'$ and $\beta \rightarrow \alpha'$), if α' originated from both subunits β and α, one should expect to see the amount of α' quickly exceeded that of α, a phenomenon never observed in all of our experiments. Therefore, concurrent conversion of β and α into α' did not seem to occur. Our results could be explained by either pathway (ii) ($\beta \rightarrow \alpha'$) or pathway (iv) ($\beta \rightarrow \alpha \rightarrow \alpha'$). The data presented did not really distinguish between these two pathways.

The production of a small polypeptide, fragment A, manifesting only RNase H activity suggested that the DNA polymerase and RNase H activities were perhaps located at different functional sites on subunit α. Different functional sites for DNA polymerase and RNase H activities have been suggested previously (5, 42). The DNA polymerase activity, however, appears to be more sensitive to changes in the conformations of the enzyme molecule. Thus, a milder

condition of proteolysis may be required to obtain a polypeptide fragment containing enzymatically active DNA polymerase. The *E. coli* DNA polymerase I can be cleaved with subtilisin to generate a large polypeptide fragment manifesting DNA polymerase and 3′ → 5′ exonuclease activities and a smaller fragment demonstrating 5′ → 3′ exonuclease activity (6, 24).

Although a small amount of the $\beta\beta$ form of the enzyme has been shown to exist in the virions obtained from duck cells transformed by B77 virus (21), the most abundant form of the enzyme appears to be $\alpha\beta$. The $\alpha\beta$ form of the enzyme shows greater binding affinity to templates and substrates as compared to subunit α (18, 31). Furthermore, it has been shown that $tRNA^{trp}$, required to initiate cDNA synthesis in avian retroviruses (8, 9, 13), can bind to the $\alpha\beta$ form of the enzyme and not the α subunit alone (19, 30). The physical difference between the subunit α and $\alpha\beta$ forms of reverse transcriptase appears to be fragment B. It is possible that $tRNA^{trp}$ and polynucleotides (functioning either as templates for DNA polymerase or the substrates for RNase H) bind to fragment B. It is not clear whether fragment B was generated from subunit β by cleavage at the N- or C-terminal. We are now determining the N- and C-terminals of subunits α and β and fragments A and B to locate their positions on the β molecule.

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