JOURNAL OF VIROLOGY, Feb. 1983, p. 727-739
0022-538X/83/020727-13$02.00/0

Vol. 45, No. 2

# Mechanism of Action of the Endonuclease Associated with the αβ and ββ Forms of Avian RNA Tumor Virus Reverse Transcriptase

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Received 9 August 1982/Accepted 10 November 1982

Preparations of the αβ and the ββ forms of reverse transcriptase from the Prague C strain of Rous sarcoma virus grown in chicken embryo fibroblasts, the αβ and the ββ forms of the enzyme from the B77 strain of Rous sarcoma virus grown in duck embryo fibroblasts, and the αβ form of reverse transcriptase from avian myeloblastosis virus have been analyzed. All these enzyme preparations contain a $Mn^{2+}$-activated endonuclease activity. The ββ form of enzyme, in addition, contains a $Mg^{2+}$-dependent endonuclease. Such an activity is barely detectable in the αβ form of enzymes. The endonuclease associated with reverse transcriptase introduces single- and double-strand breaks containing 3′ OH and 5′ P termini into RF I DNA. The conversion of RF I DNA to RF III DNA is more readily catalyzed by the ββ form of reverse transcriptase. In contrast to a recently published report by Hizi et al. (J. Virol 41:974–981, 1982), we have failed to detect the conversion of RF I DNA to covalently closed relaxed circles (RF IV DNA) by any of the αβ form of enzymes tested. RF IV DNA was not produced by the ββ form of reverse transcriptase either. We conclude that topoisomerization is not an intrinsic activity of reverse transcriptase. Although the conversion of RF I DNA to RF II DNA was found to be rapid, the endonuclease associated with reverse transcriptase acted slowly on RF II, RF III, and RF IV DNAs. Circular and linear single-stranded DNAs were also susceptible to cleavage by the endonuclease at a rate comparable to nicking of RF I DNA. This pattern of activity suggests that the endonuclease cleaves the RF I DNA in the single-stranded regions of the DNA induced by its supercoiling. The preference of the αβ and the ββ forms of the endonuclease for viral DNA was tested with Rous-associated virus type 2 and Rous sarcoma virus transformation-defective Schmidt-Ruppin B strain DNA molecularly cloned in plasmid pBR322 and M13 DNA vectors, respectively. The rate of nicking of RF I DNA containing viral DNA or partial sequences of viral DNA with one or two tandem long terminal repeats was the same as when these sequences were not present in the host vectors. A similar lack of preference was observed with single-stranded M13 DNAs.

RNA-directed DNA polymerase has been purified from a variety of avian RNA tumor viruses. The purified enzyme exists in three enzymatically active molecular forms referred to as α, αβ, and ββ. The α and β subunits are 68,000 and 92,000 daltons, respectively. The amino acid sequence of the α subunit is a subset of the β subunit (3, 20), suggesting that α is derived from β by a proteolytic cleavage event. Each form has several enzymatic activities associated with it, including RNA-directed DNA polymerase, DNA-dependent DNA polymerase, RNase H, and polynucleotide unwinding (for a review of these enzymatic activities see reference 25).

More recently, Golomb and co-workers (4, 5) have shown that a DNA endonuclease activity is associated with the αβ but not the α form of avian myeloblastosis virus (AMV) reverse transcriptase. In the presence of $Mn^{2+}$ ions, this activity nicks covalently closed supertwisted ColE1 DNA; in the presence of $Mg^{2+}$ ions it is inactive. These authors also reported that the endonuclease acts slowly on linear double- and single-stranded DNAs. It has been established that the endonuclease is an integral part of the polymerase since it is found in temperature-sensitive form associated with the polymerase purified from LA335 virus (5). This virus is temperature sensitive for replication and contains a reverse transcriptase that is temperature sensitive in both polymerization and RNase H activities (17, 26). Since the α subunit of the

polymerase lacks the above activity, the active site for the endonuclease probably resides on the β subunit in a portion of the polypeptide chain that is cleaved from β to form α. A 32,000-dalton polypeptide purified from AMV cores has a DNA endonuclease activity which requires either $Mg^{2+}$ or $Mn^{2+}$ ions for activity and is structurally related to the β but not the α subunit (21). These results suggest that the ββ form of reverse transcriptase should contain an endonuclease activity, a finding that has been recently reported by Hizi et al. (8). The function for this enzymatic activity in viral replication is unknown, although it has been suggested that it might play a role in integrating viral DNA into host cellular DNA. In this report, we have characterized the mechanism of action of the DNA endonuclease associated with the αβ and the ββ forms of several RNA tumor virus reverse transcriptases to assess its role. For this purpose, we have utilized a variety of substrates which include molecularly cloned DNAs containing viral and host DNA sites presumed to be recognized in the integration reaction. We have shown that the endonuclease acts preferentially on single-stranded DNA and that the presence or absence of retroviral specific sequences does not alter the apparent rate of cleavage.

(Part of this work will be submitted by G.D. in a dissertation to the Department of Biochemistry, Case Western Reserve University, for the degree of Doctor of Philosophy.)

## MATERIALS AND METHODS

**Reagents:** [$^3$H]thymidine (12 Ci/mmol) and [$^3$H]dTTP (18.6 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. [γ-$^{32}$P]ATP (5,000 Ci/mmol) was from ICN, Irvine, Calif. Cesium chloride was from the Harshaw Chemical Co., Solon, Ohio. Ethidium bromide and propidium diiodide were purchased from Calbiochem-Behring. La Jolla, Calif. The agarose (SeaKem; Rockland, Maine) was from Marine Colloids Division of FMC Corp. DNA restriction enzymes *Sal*I (3,000 U/0.045 ml). *Bam*HI (4,000 U/0.13 ml), *Pvu*II (2,500 U/0.1 ml), and *Pst*I (1,000 U/0.03 ml) were from New England Biolabs, Beverly, Mass.; *Cla*I (4,000 U/0.058 ml), *Hin*dIII (10,000 U/ml), and *Eco*RI (10,000 U/ml) were from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Pancreatic DNase and bacterial alkaline phosphatase (BAP) were from Worthington Diagnostics, Freehold, N.J. T4 DNA ligase and *Escherichia coli* DNA exonuclease III were from P-L Biochemicals, Inc., Milwaukee, Wis. DNA polymerase I of *E. coli* was fraction VII prepared by the method of Jovin et al. (11). T4 polynucleotide kinase was prepared as described by Muster et al. (19).

**Growth of viruses.** The B77 strain of Rous sarcoma virus (RSV) was grown in primary Peking duck embryo fibroblasts as previously described (9). The Prague C strain of RSV was grown in primary chicken embryo fibroblasts as described by Smith et al. (23). AMV was derived from citrate-treated plasma from leukemic chickens as previously described (14).

**Purification of reverse transcriptase.** The αβ form of AMV reverse transcriptase (30,000 U/mg of protein) was purified as described by Leis (14). The αβ and the ββ forms of reverse transcriptase from the Prague C and B77 strains of RSV were purified as described by Hizi and Joklik (9). A polymerizing unit is defined as the amount of enzyme which incorporates 1 nmol of nucleotide in 30 min with polyadenylic acid-oligodeoxythymidylic acid 14 as the primer template.

**Preparation of cloned viral DNA.** Subclones of plasmids pBR322 and M13 were prepared from segments of retroviral DNA originally cloned in phage λ vectors with covalently closed circular viral DNAs extracted from infected cells (12). The plasmid p13 (a pBR322 derivative of λSRBtd-2.2 prepared by Bryan Cullen) contains a complete equivalent of viral DNA with two tandem copies of the long terminal repeat (LTR) and is joined to the vector via the single *Sal*I site in the *env* gene. Procedures for cloning and preparing plasmid DNA were described previously (7). $^3$H-labeled plasmid DNA was obtained from cultures exposed to [*methyl*-$^3$H]thymidine (80 Ci/mmol) at 200 μCi/100 ml of culture added 3 h after the addition of chloramphenicol. Cells were harvested, and DNA was extracted 10 to 12 h later. The M13 clones utilized the vector mp9 obtained from Bethesda Research Laboratories and prepared according to the directions of the supplier. The viral DNAs in these subclones were derived from λRAV-2. They contain *Sal*I-*Bam*HI viral DNA inserts of 1.85 and 2.2 kilobases spanning the ends of the viral genome and including one or two tandemly repeated LTRs, respectively. RF I DNA and single-strand circular phage DNA were prepared from the M13 recombinants by protocols generously provided by J. Messing (personal communication, University of Minnesota, St. Paul).

**Restriction digestion of DNA.** RF M13 mp9 2-LTR DNA (2 μg) was incubated in a 50-μl volume with a final concentration of 10 mM $MgCl_2$, 20 mM Tris-hydrochloride (pH 8), and *Cla*I restriction enzyme (13.8 U) for 60 min at 38°C. In a second reaction vessel, 2 μg of the same DNA was incubated in a 50-μl volume containing a final concentration of 6 mM $MgCl_2$, 0.14 M NaCl, 20 mM Tris-hydrochloride (pH 7.5), *Bam*HI (4 U), and *Sal*I (3 U) for 60 min at 38°C. The reaction was stopped by bringing the volume to 0.2 ml and adding 5 μmol of EDTA, 20 μmol of NaCl, and 1 mg of sodium dodecyl sulfate (SDS). The protein was removed by extraction twice with phenol, and the DNA was precipitated with the addition of two volumes of 95% ethanol.

**Preparation of different topoisomer forms of pBR322 DNA. RF II DNA.** RF I plasmid pBR322 DNA (125 μg) was incubated for 15 min at 37°C in a 50-μl volume containing 5.6 mM 2-mercaptoethanol, 20 mM Tris-hydrochloride (pH 8), 10 mM $MgCl_2$, and serial dilutions of pancreatic DNase (5 to 0.05 ng). The reaction was stopped by heating at 65°C for 4 min, and the extent of nicking of the DNA was determined by agarose gel electrophoresis.

**RF III DNA.** RF I plasmid pBR322 DNA (10 μg) was incubated with *Sal*I (33 U) as described above for 60 min at 37°C. The reaction was stopped with the addition of 2.5 μmol of EDTA and brought to a final concentration of 0.5% SDS in a volume of 0.2 ml. The

mixture was extracted two times with an equal volume of phenol and then was alcohol precipitated in 0.1 M NaCl with two volumes of 95% ethanol.

**RF IV DNA.** A mixture of 1 μmol of Tris-hydrochloride (pH 7.5), 0.14 μmol of 2-mercaptoethanol, 0.0725 μmol of ATP, and 40 U of T4 DNA ligase was added to the reaction vessel containing RF II DNA prepared above and was incubated for 30 min at 37°C. Reactions were stopped by heating to 65°C for 2 min and adding 5 μmol of EDTA. The incubation mixture was brought to a final volume of 2.2 ml to which were added 0.005 ml of 10% Sarkosyl, 2.3 g of CsCl, and 0.2 ml of propidium diiodide (2 mg/ml). This mixture was spun at 38,000 rpm for 40 h in a Spinco SW50.1 rotor at 20°C. Fractions were collected from a hole pierced in the bottom of the centrifuge tube, and 0.5 μl of each fraction was diluted 18-fold with 5% glycerol and layered onto a 0.7% agarose gel containing 50 mM Tris-borate (pH 8.3), 1 mM EDTA, and 0.5 μg of ethidium bromide per ml. Electrophoresis was carried out as described below. The fractions containing RF IV DNA were pooled, extracted 10 times with an equal volume of isopropanol equilibrated with 3 M NaCl, diluted threefold with water, and concentrated by alcohol precipitation with two volumes of 95% ethanol.

**Agarose gel electrophoresis.** Neutral agarose gel electrophoresis of DNAs was carried out in 0.7 to 1% agarose minigels (9 by 5 cm) containing 50 mM Tris-borate (pH 8.3) as buffer. EDTA (1 mM) was added to some gels as indicated. DNA samples were layered into wells 2 mm wide in the horizontal gel, and electrophoresis was carried out for 45 min to 2 h at 150 V at room temperature. Ethidium bromide (0.5 μg/ml) or propidium diiodide (0.5 μg/ml) was included in the gel and buffer where indicated. In some gels, 36 mM Tris–30 mM sodium phosphate (pH 7.7)–1 mM EDTA was used instead of the above buffer. DNA was detected by fluorescence upon exposure to UV light (short wavelengths). When denaturing conditions were used, gels were run with 30 mM NaOH–1 mM EDTA. In this case, gels were soaked for 30 min with 0.1 M ammonium acetate–0.22 μg of ethidium bromide per ml before exposure to UV light.

**Endonuclease assay.** The endonuclease assay was carried out in a 10-μl incubation volume in 1.0- or 1.5-ml Eppendorf centrifuge tubes with tight sealing caps. The reaction mixture contained 20 mM Tris-hydrochloride (pH 8.5); or 20 mM Tris-hydrochloride (pH 9.0), 5 mM 2-mercaptoethanol, and 2 mM $MnCl_2$; or 5 mM $MgCl_2$, 0.3 to 0.5 nmol of single- or double-stranded DNA, and various amounts of reverse transcriptase as indicated. After incubation at 37°C for indicated lengths of time, the reaction was stopped by removing a 4-μl sample and adding it to 1 μl of 25% glycerol–5% SDS. The sample was then placed onto an agarose gel, and electrophoresis carried out as described above. In some assays, the DNA was treated with 0.1 mg of proteinase K in the presence of 0.2% SDS for 5 min at 37°C before analyzing the DNA on an agarose gel.

**Labeling of 5′ termini of RFI pBR322 DNA treated with reverse transcriptase.** RF I plasmid pBR322 DNA was treated with the αβ form of reverse transcriptase as described above. Reactions were stopped by heating the incubation mixture at 65°C for 5 min. BAP (0.05 U) was added to each reaction mixture and incubated for 15 min at 37 or 65°C. The 5′ OH termini were phosphorylated by T4 polynucleotide kinase (0.20 U) with [$\gamma$-$^{32}$P]ATP ($1.5 \times 10^5$ cpm/pmol) in the presence of 5 mM sodium phosphate (pH 7) and 5 mM dithioeryitol as described by Leis et al. (15).

## RESULTS

Golomb and co-workers (4, 5) have reported a $Mn^{2+}$-activated DNA endonuclease activity that is an integral part of the avian tumor virus reverse transcriptase. We have confirmed and extended this observation. Preparations of the αβ form of AMV, B77 RSV, and Prague C RSV reverse transcriptase contain a $Mn^{2+}$-activated endonuclease that nicks supertwisted RF I forms of DNA. The effect of the AMV polymerase on RF I plasmid pBR322 DNA is shown in Fig. 1. In this experiment, pBR322 DNA was incubated with AMV polymerase for 30 min, and the DNA was analyzed by agarose gel electrophoresis as described above. RF I DNA was converted into mostly RF II DNA with small amounts of RF III DNA (Fig. 1, lanes 6 and 7). When the enzyme was incubated with $MgCl_2$ or

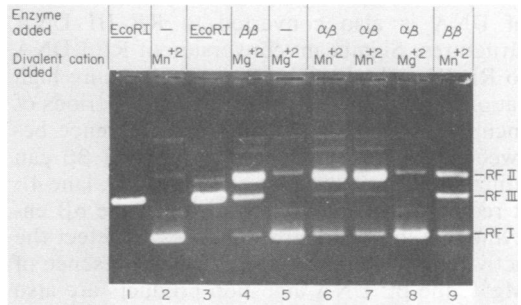


FIG. 1. Gel electrophoresis separation of plasmid pBR322 DNA treated with the αβ and ββ forms of reverse transcriptase in the presence of $Mn^{2+}$ and $Mg^{2+}$ divalent cations. The αβ form of AMV reverse transcriptase or the ββ form of B77 RSV reverse transcriptase were incubated with RF I pBR322 DNA (4.4 μg) in a 50-μl volume with 2 mM $MnCl_2$ or 10 mM $MgCl_2$ as described in the text. After 30 min at 37°C, 0.4 μmol of EDTA, 20 μg of SDS, and 20 μg of proteinase K were added, and the mixture was incubated for 5 min at 37°C. The reaction mixture was brought to 5% glycerol concentration, and a sample was layered onto a 1% agarose gel containing 40 mM Tris-acetate (pH 8.1), 2 mM EDTA, and 0.22 μg of ethidium bromide per ml and was run at 50 V for 6 h. RF I pBR322 DNA was incubated with: lane 1, *Eco*RI (10 U); lane 2, no enzyme and 2 mM $MnCl_2$; lane 3, *Eco*RI (10 U); lane 4, $3 \times 10^{-3}$ U of B77 RSV reverse transcriptase and 10 mM $MgCl_2$; lane 5, no enzyme and 10 mM $MgCl_2$; lane 6, 10 U of AMV reverse transcriptase and 2 mM $MnCl_2$; lane 7, 3.3 U of AMV reverse transcriptase and 2 mM $MnCl_2$; lane 8, 3.3 U of AMV reverse transcriptase and 10 mM $MgCl_2$; lane 9, $3 \times 10^{-3}$ U of B77 RSV reverse transcriptase and 2 mM $MnCl_2$.

$MgCl_2$ plus 2 mM ATP (Fig. 1, lane 8), no change in the migration of the RF I DNA from the controls (Fig. 1, lane 5) was observed. With longer incubation times or higher concentrations of enzyme added to the incubation, some $Mg^{2+}$-dependent activity could be observed. No activity was noted in the absence of added divalent cations (data not shown).

The finding of Golomb and co-workers (4, 5) that the αβ form of AMV polymerase, but not the α form, contains endonuclease activity suggests that the endonuclease-active site resides on the polypeptide fragment that is cleaved from the β chain to form the α chain. If this is correct, then the ββ form of reverse transcriptase should also contain an active DNA endonuclease activity. We had previously purified and characterized the ββ form of B77 RSV reverse transcriptase (10). These enzyme preparations were tested and found to have endonuclease activity in the presence of $Mn^{2+}$ (Fig. 1, lane 9). At first glance this activity appears to be similar to the αβ form of polymerase. In the presence of $Mn^{2+}$ ions, the ββ enzyme converts the RF I DNA into RF II DNA. However, in contrast to what is seen with the αβ enzyme, much of the RF I form of DNA is also converted to RF III DNA structures. Significant conversion of RF I DNA to RF III DNA by the αβ enzyme require high ratios of enzyme to DNA or extended periods of incubation (see Fig. 3). Another difference between these two enzyme forms is that ββ can utilize $Mg^{2+}$ as a divalent cation (Fig. 1, lane 4); it required higher concentrations of the αβ enzyme and longer incubation times to detect the activity in this enzyme form. In the presence of $Mg^{2+}$, the ββ DNA digestion products are also RF II DNA and RF III DNA. As with the αβ form, there is no detectable activity in the absence of added divalent cation.

**Purification and properties of the endonuclease associated with the ββ form of RSV polymerase.** The ββ forms of reverse transcriptase from Prague C and B77 RSV virions were purified by sequential chromatography on DEAE-cellulose, phosphocellulose, and polyuridylic acid-cellulose as previously described (9). In each case, the DNA endonuclease activities copurified with the DNA polymerization activity (data not shown). These results are consistent with those of Golomb et al. (5) and Hizi et al. (8), which indicate that the endonuclease is part of reverse transcriptase.

The ββ form of reverse transcriptase represented 11% of the detectable polymerase units purified from the Prague C RSV as compared with 45% for the B77 RSV. The $Mn^{2+}$-activated endonuclease could be detected in extracts of both viruses. However, the $Mg^{2+}$-dependent endonuclease was not detected until the ββ form of enzyme was separated from the other two forms of enzyme on the phosphocellulose column. When virus purified by equilibrium sucrose gradient centrifugation was used, endonuclease was not associated with the α form of enzyme, in agreement with the results of Golomb and Grandgenett (4). However, with less pure virus preparations, a second $Mg^{2+}$- and $Mn^{2+}$-dependent endonuclease activity was found to elute from the phosphocellulose column almost coincidental with the α form of enzyme. When these preparations were examined by SDS-polyacrylamide gel electrophoresis, many bands of protein were seen, including one of 32,000 daltons (data not shown). This protein may be related to the pp32 protein described by Schiff and Grandgenett (21). This fraction was not studied further.

The requirements for the endonuclease activities are summarized below. The endonucleases were active over a broad range of pH between 7.5 and 10; optimal activity was observed between pH 8.5 and 9.0. The addition of 0.1 M NaCl, up to 5 mM ATP, 1 mM $NAD^+$, 4 mM GTP, or 5 μM *S*-adenosylmethionine had no effect upon the reaction with either form of enzyme under all conditions tested. The ββ form of enzyme had a broad $Mg^{2+}$ requirement between 1 and 10 mM; optimal activity was observed with 5 mM $MgCl_2$. Higher concentrations of $MgCl_2$ were inhibitory. To assess the relative amount of DNA endonuclease associated with each of the different forms of enzyme, various amounts of the Prague C RSV enzymes (determined by polyadenylic acid-oligodeoxythymidylic acid 14-directed dTMP incorporation) were incubated with plasmid pBR322 RF I DNA (0.14 nmol) for 30 min at 37°C with either 2 mM $MnCl_2$ or 5 mM $MgCl_2$; the DNA products were analyzed by agarose gel electrophoresis. The $Mg^{2+}$-dependent endonuclease was two- to threefold more active than the $Mn^{2+}$-dependent enzyme associated with the ββ form of the enzyme. The amounts of $Mn^{2+}$-dependent endonuclease associated with the αβ and the ββ forms of RSV reverse transcriptase were about the same.

**Nature of the DNA products.** The breaks introduced into RF I DNA by the αβ and the ββ forms of reverse transcriptase contain 3′ OH and 5′ P termini. This is shown by the data summarized in Tables 1 and 2. In the experiments presented in Table 1, plasmid pBR322 DNA was treated with various enzymes and then tested as a substrate for nick translocation by purified *E. coli* DNA polymerase I. The RF I pBR322 DNA by itself did not support dTMP incorporation catalyzed by DNA polymerase I. However, if the pBR322 DNA was first nicked with pancreatic DNase (as a control), the αβ form of AMV

TABLE 1. DNA ends produced by the endonucleases associated with αβ and ββ forms of reverse transcriptase

| Enzyme for first incubation[a] | *E. coli* DNA polymerase I incubation[b] | dTMP incorporated (pmol) |
|---|---|---|
| None | + | 0.17 |
| Pancreatic DNase | + | 66.0 |
| αβ Reverse transcriptase | − | 0.63[c] |
| | + | 38.0 |
| ββ Reverse transcriptase | + | 17.5 |

[a] Plasmid pBR322 DNA (6.3 μg) was incubated in a 10-μl volume with AMV reverse transcriptase (αβ, 3.3 U), B77 RSV reverse transcriptase (ββ, $0.6 \times 10^{-3}$ U), or pancreatic DNase (0.5 ng) together with 10 mM $MgCl_2$ or 2 mM $MnCl_2$ (with the AMV enzyme) plus 20 mM Tris-hydrochloride (pH 8.0) for 30 min at 37°C as described in the text. The reaction was stopped by heating the reaction mixture to 65°C for 2 min.

[b] For the second incubation, the incubation mixture was brought to a final volume of 50 μl containing 20 mM Tris-hydrochloride (pH 8), 10 mM $MgCl_2$, dATP, dGTP, and dCTP (0.5 μmol/ml each), [$^3$H]dTTP (0.08 μmol/ml, 345 cpm/pmol), and (where indicated) 0.76 U of *E. coli* DNA polymerase I. Reaction mixtures were then incubated for 30 min at 37°C, and acid-insoluble radioactivity was determined.

[c] [$^3$H]dTTP and the three other unlabeled deoxynucleotides were added directly to a 50-μl incubation containing the AMV reverse transcriptase; dTMP incorporation was determined as above.

reverse transcriptase, or the ββ form of B77 RSV reverse transcriptase, the DNA now supported dTMP incorporation catalyzed by *E. coli* DNA polymerase I. Since this enzyme requires 3′ OH termini to catalyze DNA synthesis, both forms of reverse transcriptase must produce 3′ OH ends at the breaks.

The breaks in the DNA contain 5′ P termini as shown by the data in Table 2. RF I plasmid pBR322 DNA was incubated with the αβ form of B77 RSV reverse transcriptase for 30 min as described above until about 25% of the DNA was converted to RF II DNA as analyzed by agarose gel electrophoresis. The DNA was then incubated with T4 polynucleotide kinase and [γ-$^{32}$P]ATP. Barely detectable amounts of $^{32}$P label were incorporated into the treated DNA compared with a control DNA incubated without reverse transcriptase. In contrast, when the treated DNA was first incubated with BAP at 37 and 65°C, there were 3- and 36-fold increases, respectively, in the amount of $^{32}$P label incorporated during incubation with the T4 polynucleotide kinase. These results indicate that (i) the breaks introduced into the DNA by reverse transcriptase contain 5′ P termini, and (ii) the initial breaks introduced by the αβ form of reverse transcriptase are at nicks. This latter conclusion is based on the fact that treatment of the DNA with BAP at 65°C was required to activate the DNA for the T4 polynucleotide kinase. When RF II DNA was substituted for

TABLE 2. Phosphorylation of 5′ hydroxyl termini in DNA treated with the αβ form of B77 RSV reverse transcriptase

| DNA added | Enzyme treatment | | | Phosphorylation of 5′ OH DNA (pmol)[c] |
|---|---|---|---|---|
| | First incubation: αβ reverse transcriptase added[a] | Second incubation[b] | | |
| | | Enzyme | Temp (°C) | |
| RF I pBR322 | − | None | 37 | 0.001 |
| | − | None | 65 | 0.003 |
| | − | BAP | 37 | 0.003 |
| | − | BAP | 65 | 0.025 |
| | + | None | 37 | 0.009 |
| | + | None | 65 | 0.014 |
| | + | BAP | 37 | 0.026 |
| | + | BAP | 65 | 0.51 |
| RF II pBR322 | − | None | 37 | 0.005 |
| | − | None | 65 | 0.232 |
| | + | BAP | 37 | 0.007 |
| | + | BAP | 65 | 0.253 |

[a] Plasmid pBR322 DNA (0.224 pmol of DNA molecules) was incubated for 30 min with 9.4 U of B77 RSV reverse transcriptase (αβ) where indicated and 5 mM $MnCl_2$ as described in the text.

[b] BAP (0.05 U) was added to a reaction mixture where indicated and incubated 15 min at either 37 or 65°C.

[c] T4 polynucleotide kinase (0.2 U) and [γ-$^{32}$P]ATP (3 nmol, $1.5 \times 10^5$ cpm/pmol) were added to each reaction mixture as described in the text, and acid-insoluble radioactivity was determined after a 15-min incubation at 37°C.

TABLE 3. Characterization of plasmid pBR322 DNA products incubated with the ββ form of B77 RSV reverse transcriptase

| DNA added | Enzyme added | | RF I detected on agarose gels (%)[b] | DNA rendered acid soluble (%)[c] | DNA bound to nitrocellulose filters (%)[d] |
|---|---|---|---|---|---|
| | First incubation[a] | Second incubation | | | |
| pBR322 | None | None | 90 | ND[e] | 8 |
| | *Sal*I | None | 0 | 3 | ND |
| | *Sal*I | *Exo*III | 0 | 46 | ND |
| | ββ Reverse transcriptase | None | 60 | 3 | 42 |
| | ββ Reverse transcriptase | *Exo*III | 60 | 23 | ND |
| | ββ Reverse transcriptase | None | 5 | 3 | ND |
| | ββ Reverse transcriptase | *Exo*III | 5 | 37 | ND |
| p13 | None | None | ND | ND | 21 |
| | Pancreatic DNase | None | ND | ND | 78 |
| | ββ Reverse transcriptase | None | ND | ND | 82 |

[a] $^3$H-labeled RF I pBR322 DNA (5.8 cpm/pmol, 1.33 nmol) or RF I p13 DNA (6.8 cpm/pmol 0.78 nmol) was incubated with $0.3 \times 10^{-3}$ or $1.8 \times 10^{-3}$ U of B77 RSV reverse transcriptase (ββ), 10 U of *Sal*I, or 0.5 ng of pancreatic DNase in 10 mM $MgCl_2$ as described in the text. Reactions were stopped by heating the incubation mixture to 65°C for 4 min.

[b] A sample of the reaction mixture containing 0.1 nmol of DNA was treated with proteinase K in the presence of SDS and was analyzed on a 0.8% agarose gel containing 50 mM Tris-borate (pH 8.3)–1 mM EDTA as described in the text. The amount of RF I DNA remaining in the sample was estimated from the total ethidium bromide staining material.

[c] A sample of the reaction mixture, where indicated, containing 0.61 nmol of pBR322 DNA was brought to a final volume of 20 μl containing 25 mM Tris-hydrochloride (pH 8), 7 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 4.4 U of *E. coli* DNA exonuclease III. After incubation for 30 min at 37°C, the reaction was stopped by adding 0.1 ml of 0.1 M sodium pyrophosphate, 0.2 mg of serum albumin, and 0.5 ml of cold 5% trichloroacetic acid. The reaction vessel was spun in a Sorvall SE-12 rotor for 2 min at 2,000 rpm, and the acid-soluble radioactivity was determined by counting in 10 ml of Bray scintillation cocktail in a Beckman scintillation spectrometer. The data are presented as percentages of the total $^3$H-labeled DNA converted to acid-soluble form.

[d] A sample of the reaction mixture, where indicated, containing 0.61 nmol of pBR322 or 0.68 nmol of p13 DNA was brought to 0.5 ml volume containing 1 M KCl, 10 mM Tris-hydrochloride (pH 7.5), and 1 mM EDTA. The mixture was boiled for 10 min, cooled rapidly in ice, and passed through a nitrocellulose filter. The filters were dried, and the amounts of radioactivity retained on the filters were determined in a toluene-based scintillation cocktail as described above. The data are presented as percentages of the total $^3$H-labeled DNA retained on the nitrocellulose filters.

[e] ND, Not determined.

RF I DNA during the first incubation and then was treated as above, no increase in the incorporation of $^{32}$P label into the DNA was observed. This result indicates that the endonuclease activity of reverse transcriptase acts very slowly on RF II DNA. From the stoichiometry of the RF I DNA remaining in the reaction (75%) and the number of termini phosphorylated in the DNA product (Table 2), it is concluded that the αβ form of reverse transcriptase under these conditions introduces an average of nine breaks per RF II DNA molecule.

**Characterization of the DNA reaction products.** The data shown in Fig. 1 indicate that among the DNA products formed by the action of either form of reverse transcriptase are nicked circles (RF II DNAs). However, it seemed possible that some of the DNA product might be covalently closed relaxed circles (RF IV DNA) which migrate at a position close to the RF II DNA in this gel. Such a DNA product would result from the action of a topoisomerase which has been reported by Hizi et al. (8) to be present in their preparations of B77 RSV polymerase (αβ form). We evaluated this possibility by determining whether there was a direct correlation between the loss of supercoiled substrate and the production of DNA ends. This was done by direct examination of the DNA products with equilibrium CsCl density gradient centrifugation, by electron microscopy, and by agarose gel electrophoresis. In the first experiment (Table 3), $^3$H-labeled RF I plasmid pBR322 or p13 DNA (see above) was incubated with the ββ form of reverse transcriptase for various lengths of time, and samples of the reaction mixture were analyzed by agarose gel electrophoresis to determine the amounts of RF I DNA remaining. In the second experiment, samples of the same reaction mixture were heat denatured and then passed through nitrocellulose filters as described in the legend to Table 3 to determine the amounts of labeled DNA retained. In the third experiment, samples of the same reaction mix-

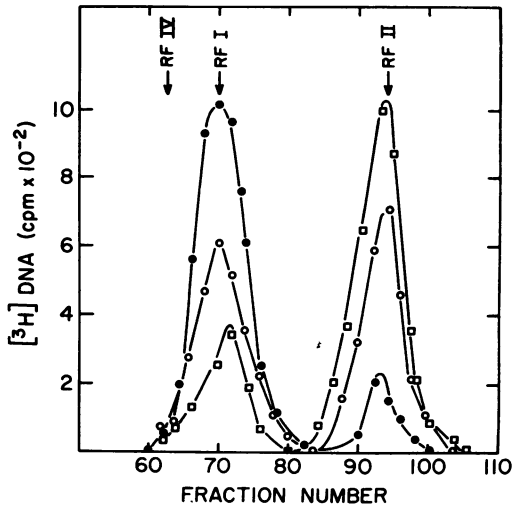


FIG. 2. Equilibrium cesium chloride density gradient centrifugation of RF I plasmid pBR322 DNA treated with the ββ form of B77 RSV reverse transcriptase. $^3$H-labeled pBR322 DNA (14.4 nmol, 1.9 cpm/pmol) was incubated with $1.55 \times 10^{-3}$ U of B77 RSV reverse transcriptase (ββ) with $MgCl_2$ for various lengths of time, and the reaction was stopped by digestion with proteinase K as described in the text. The incubation mixture was brought to a final volume of 2.2 ml containing 10 mM EDTA, 0.15 M NaCl, 0.15 M sodium citrate, 0.2% Sarkosyl; 2.2 g of CsCl and 0.2 ml of propidium diiodide (2 mg/ml) were added. $^{14}$C-labeled pBR322 DNA (2.3 nmol, 2.4 cpm/pmol) was also added as a reference marker. The gradients were formed by centrifugation for 45 h at 40,000 rpm in a Spinco SW50.1 rotor at 20°C. Fractions were collected from a hole pierced in the bottom of the tube. A cold 0.1 ml of 0.1 M sodium pyrophosphate, 0.02 ml of salmon sperm DNA (1.6 mg/ml), and 3 ml of 5% trichloroacetic acid were added to each fraction. The DNA was collected on a Gelman glass fiber filter (type E). Filters were dried and counted under double label conditions in a Beckman Scintillation Spectrometer. Plasmid pBR322 DNA was treated with B77 RSV reverse transcriptase for 0 min (●), 40 min (○), or 2 h (□). Arrows show the position of banding of the $^{14}$C-labeled RF I, RF IV, and RF II DNAs.

ture were treated with *E. coli* DNA exonuclease III, and acid-soluble radioactivity was determined, also as described in the legend to Table 3. The plasmid pBR322 DNA incubated without polymerase was found to migrate on agarose gels at the position of RF I DNA, it did not bind to nitrocellulose filters, and it was resistant to the action of DNA exonuclease III. In contrast, when the DNA was first incubated with the B77 RSV reverse transcriptase, DNA migrated on the agarose gels at the position of RF II DNA, and the labeled DNA was now retained on the nitrocellulose filter and was sensitive to the action of DNA exonuclease III. All three assay procedures indicate that DNA ends are produced in the reaction and that the percentage of DNA ends is approximately that expected from the percentage loss of form I DNA. These results suggest that little, if any, RF IV DNA was made during the reaction. Similar results were obtained with the αβ form of AMV reverse transcriptase.

A second test for the presence of RF IV DNA utilized equilibrium CsCl gradient centrifugation in the presence of propidium diiodide (Fig. 2). In these assays, which tested the same products shown in Table 3, no labeled DNA (<2%) was found to band at a density heavier than the RF I DNA marker, as would be expected of RF IV DNA. Instead, with increasing incubation time, increasing amounts of the DNA were found to shift from the density characteristic of RF I DNA to those of RF II and RF III DNAs.

In a third test, plasmid pBR322 DNA was treated with the AMV αβ or B77 RSV ββ forms of the enzyme under standard conditions and was analyzed by electron microscopy. The results (not shown) showed supercoils in the absence of enzyme, predominantly relaxed circles in the presence of the αβ enzyme, and relaxed circles and linears in the presence of the ββ enzyme. We found no evidence of the formation of catenanes or other complex structures catalyzed by various topisomerases (2).

Finally, we analyzed RF I plasmid pBR322 DNA treated with the αβ form of Prague C RSV, AMV, or B77 RSV reverse transcriptase by agarose gel electrophoresis in the presence of propidium diiodide. Under these conditions, RF I, RF II, RF III, and RF IV DNAs are resolved from one another. We did not detect any RF IV DNA among the reaction products in the incubation of any enzyme form tested in the presence of either $Mg^{2+}$ or $Mn^{2+}$ as divalent cation (Fig. 3). The only DNA products observed migrated at the position of RF II DNA and RF III DNA. Similar results were obtained when the Prague C RSV ββ reverse transcriptase was used (data not shown). Treatment of reaction products with proteinase K before loading onto agarose gels did not result in a shift in the mobilities of the major bands, suggesting that there were no detectable covalent DNA-protein complexes formed in the course of the reaction. Eucaryote topoisomerase I has been observed to form such intermediates (1). We conclude from these results that the activity associated with either structural form of reverse transcriptase is an endonuclease and not a topoisomerase.

**Test of activity of the DNA endonucleases with RF I DNA containing viral sequences.** The evidence presented above indicates that the activity associated with reverse transcriptase is an endonuclease. We therefore tested to see whether the

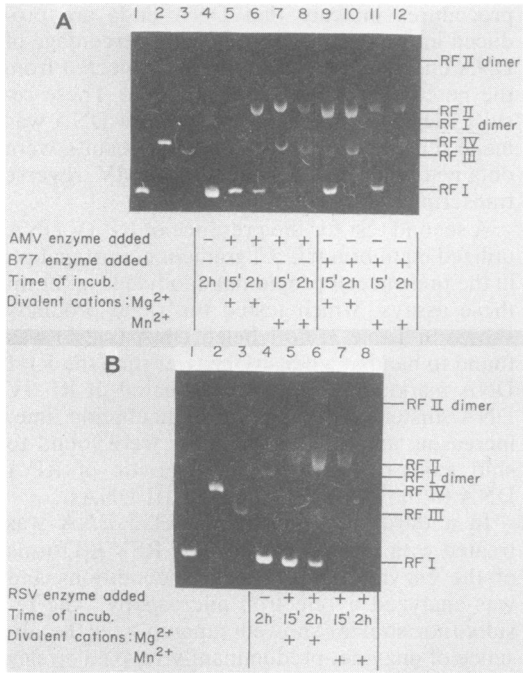


FIG. 3. Gel electrophoresis separation of plasmid pBR322 DNA treated with the αβ forms of avian retrovirus reverse transcriptases. The αβ forms of AMV, B77 RSV, and Prague C RSV reverse transcriptase (each 0.9 U) were incubated with RF I pBR322 DNA (0.09 μg) plus 5 mM $MnCl_2$ or $MgCl_2$ as indicated for various lengths of time as described in the text. The DNA products were analyzed on a 0.7% agarose gel containing 50 mM Tris-borate (pH 8.3), 1 mM EDTA, and 0.5 μg of propidium diiodide per ml. The electrophoresis was run at 150 V until the bromphenol blue dye marker migrated 9 cm. In both gels A and B, lane 1 contained RF I pBR322 DNA, lane 2 contained RF IV pBR322 DNA, lane 3 contained RF III pBR322 DNA, and lane 4 contained RF I pBR322 DNA incubated 2 h without enzyme. In gel A, AMV reverse transcriptase was added to lanes 5 through 8 and B77 RSV reverse transcriptase was added to lanes 9 through 12. Other additives and incubation times were as follows: lanes 5 and 9, 5 mM $MgCl_2$ for 15 min; lanes 6 and 10, 5 mM $MgCl_2$ for 2 h; lanes 7 and 11, 5 mM $MnCl_2$ for 15 min; lanes 8 and 12, 5 mm $MnCl_2$ for 2 h. In gel B, Prague C RSV reverse transcriptase was added to lanes 5 through 8. Other additives and incubation times were as follows: lane 5, 5 mM $MgCl_2$ for 15 min; lane 6, 5 mM $MgCl_2$ for 2 h; lane 7, 5 mM $MnCl_2$ for 15 min; lane 8, 5 mM $MnCl_2$ for 2 h. A small amount of plasmid pBR322 DNA dimer was present in the RF I DNA preparation.

enzyme would preferentially act on DNA containing viral sequences, particularly the viral LTR sequence which is presumed to be involved in integrating viral DNA into cellular DNA (7, 22, 24). Various amounts of plasmid pBR322 vector DNA, pdr-R2 DNA, or p13 DNA were incubated with different concentrations of the αβ form of AMV reverse transcriptase or the ββ form of B77 RSV reverse transcriptase. The pdr-R2 DNA contains a 350-base pair *Eco*RI fragment of the LTR sequence, whereas the p13 DNA contains the entire SRB-td viral DNA. After incubation with the different forms of reverse transcriptase, the DNA products were separated by agarose gel electrophoresis, and the relative rates of conversion of RF I DNA to RF II DNA or RF III DNA were compared. The results (Fig. 4) indicate that the plasmid pBR322 DNAs containing viral DNA sequences were nicked at about the same rate as the DNA that did not. Similar results were obtained if the Prague C RSV enzymes were used instead of the AMV and the B77 RSV enzymes or if equimolar amounts of the M13 mp9 1-LTR and 2-LTR DNAs containing a 1.85-kilobase or a 2.2-kilobase fragment subcloned from two λRAV-2 derivatives were used instead of the pBR322 DNAs (data not shown). The subcloned regions span the viral DNA termini and contain one and two tandem copies, respectively, of the LTR sequence. Thus, it appears that the presence of the viral DNA, including the LTR sequence, does not significantly enhance the sensitivity of the DNA to the endonuclease associated with either form of reverse transcriptase.

**Activity of the DNA endonuclease for topoisomeric forms of pBR322 DNA.** Although the endonuclease associated with reverse transcriptase does not require a virus-specific sequence for its action, it can select a substrate based on secondary structure. This is demonstrated by the experiment described below. RF II (nicked circle) DNA, RF III (linear) DNA, and RF IV (covalently closed relaxed circle) DNA were constructed from RF I plasmid pBR322 DNA as described above. Each form of DNA was incubated with the αβ form of AMV reverse transcriptase for various lengths of time, and the DNA products were separated by agarose gel electrophoresis (Fig. 5). We observed a rapid conversion of RF I DNA to RF II DNA: 80% in 30-min incubations (Fig. 5, lanes 1 through 3). In contrast, there was little detectable activity observed with RF II DNA (lanes 7 through 9), RF III DNA (lanes 10 through 12), or RF IV DNA (lanes 4 through 6). In the case of the RF IV DNA, where the concentration of the DNA was about half that of the RF I DNA, about 10% was converted to RF II DNA in 30 min of incubation. The gel used in this experiment to analyze the DNAs was nondenaturing, so that limited numbers of nicks introduced into the RF II DNA and RF III DNA would not be detected. We therefore repeated the above experiment with the RF II and RF III DNA forms but analyzed the DNA on alkaline agarose gels. Under these condi-

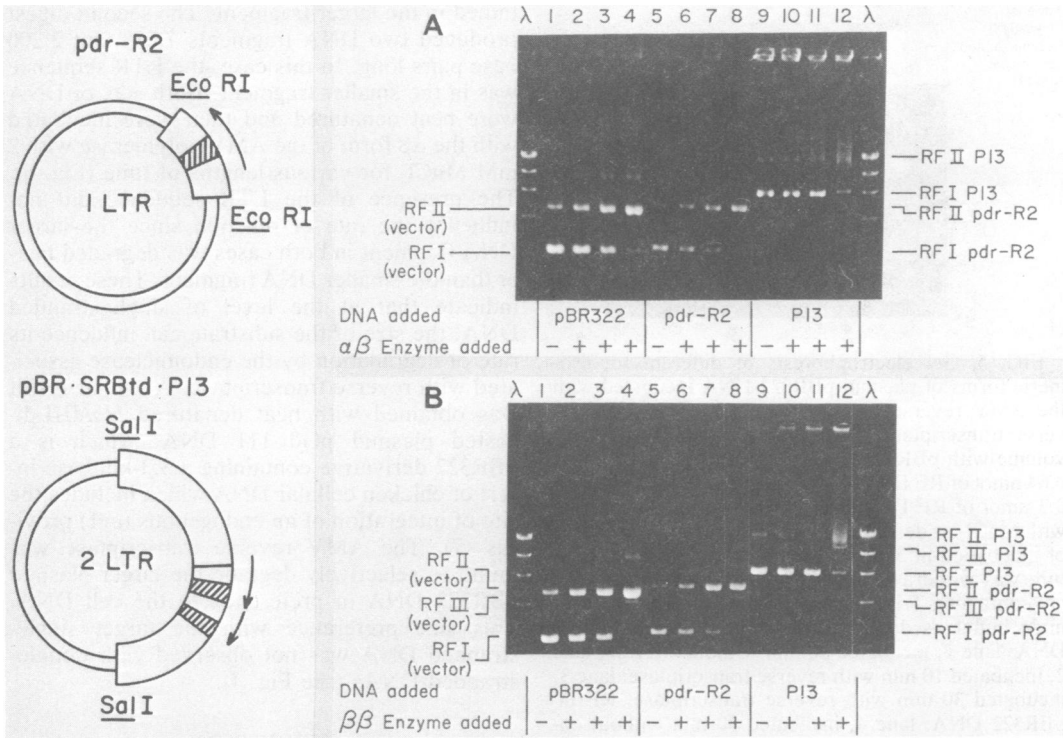


FIG. 4. Gel electrophoresis separation of RF I plasmid pBR322, pdr-R2, and p13 DNAs treated with the αβ and ββ forms of reverse transcriptase. Various concentrations of the αβ form of AMV reverse transcriptase or the ββ form of B77 RSV reverse transcriptase were incubated with RF I pBR322 DNA (0.2 μg), pdr-R2 DNA (0.35 μg), or p13 DNA (0.2 μg) as described in the text. The reactions were incubated for 30 min at 38°C, and the DNA was analyzed by electrophoresis on a 0.7% agarose minigel in Tris-borate buffer as described in the text. In the presence of the αβ form of enzyme, the final concentration of divalent cation was 2 mM $MnCl_2$; 10 mM $MgCl_2$ was used in reactions containing the ββ form of enzyme. In both gels, lanes 1 through 4 contained pBR322 DNA, lanes 5 through 8 contained pdr-R2 DNA, and lanes 9 through 12 contained p13 DNA. (A) AMV reverse transcriptase was added as follows: lanes 1, 5, and 9, 0 U; lane 10, 0.01 U; lanes 2, 6, and 11, 0.3 U; lanes 3, 7, and 12, 0.9 U; lanes 4 and 8, 3 U. (B) B77 RSV reverse transcriptase was added as follows: lanes 1, 5, and 9, 0 U; lane 10, $6.2 \times 10^{-5}$ U; lanes 2, 6, and 11, $1.8 \times 10^{-4}$ U; lanes 3, 7, and 12, $6.2 \times 10^{-4}$ U; lanes 4 and 8, $1.8 \times 10^{-3}$ U.

tions, less than 10% of the DNA was found to be nicked (data not shown). Similar results were obtained with the ββ form of Prague C RSV reverse transcriptase and $MgCl_2$. These results indicate that double-stranded DNA is relatively resistant to nicking by the endonuclease associated with reverse transcriptase and are consistent with the data already presented in Table 2. It further suggests that the enzyme nicks the RF I DNA in the single-stranded regions that are induced by DNA supercoiling.

**Activity of the DNA endonuclease on single-stranded DNA.** The above results suggested that the endonuclease associated with reverse transcriptase might act preferentially on single-stranded DNA. To explore this possibility, the single-stranded circular DNA forms of the plasmid M13 DNAs described above were incubated with the αβ or ββ forms of Prague C RSV reverse transcriptase, and the DNA products were separated by agarose gel electrophoresis. A single-stranded circular M13 mp9 DNA was mixed with M13 mp9 1-LTR or 2-LTR DNA (containing either plus or minus strands) for comparison. Upon the addition of either form of reverse transcriptase, the circular DNAs were converted to full-length linear DNA and smaller fragments (data not shown). These small DNA fragments were due to the action of the endonuclease and not to an exonuclease activity contaminating our enzyme preparations. Linear $^3H$-labeled plasmid p13 DNA (18 cpm/pmol, 142 pmol of nucleotide) was heat denatured and incubated with 3.3 U of the RSV or AMV reverse transcriptase for 30 min at 37°C. Under these conditions, less than 2% of the labeled DNA was converted to acid-soluble form, but all of the p13 DNA was degraded to small DNA

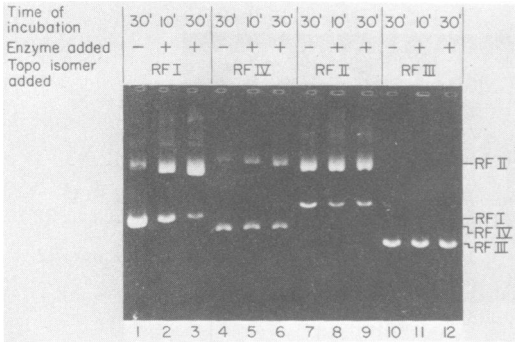


FIG. 5. Gel electrophoresis of different topoisomeric forms of plasmid pBR322 DNA incubated with the AMV reverse transcriptase (αβ). The AMV reverse transcriptase (3 U) was incubated in a 10-μl volume with pBR322 DNA (0.62 nmol of RF I DNA, 0.64 nmol of RF II DNA, 0.33 nmol of RF III DNA, or 0.3 nmol of RF IV DNA) for various lengths of time with $MnCl_2$ as described in the text. After 10 or 30 min of incubation at 37°C, a 3-μl sample of reaction mixture was withdrawn and analyzed on a 0.7% agarose gel containing Tris-phosphate (pH 7.7)–ethidium bromide buffer as described in the text. RF I pBR322 DNA: lane 1, incubated 30 min without enzyme; lane 2, incubated 10 min with reverse transcriptase; lane 3, incubated 30 min with reverse transcriptase. RF IV pBR322 DNA: lane 4, incubated 30 min without enzyme; lane 5, incubated 10 min with reverse transcriptase; lane 6, incubated 30 min with reverse transcriptase. RF II pBR322 DNA: lane 7, incubated 30 min without enzyme; lane 8, incubated 10 min with reverse transcriptase; lane 9, incubated 30 min with reverse transcriptase. RF III pBR322 DNA: lane 10, incubated 30 min without enzyme; lane 11, incubated 10 min with reverse transcriptase; lane 12, incubated 30 min with reverse transcriptase.

fragments. The requirements for the endonuclease to act on single-stranded DNA and on RF I DNA (described above) were found to be similar, with one exception. Nicking of RF I DNA was not affected by the presence of 0.1 M NaCl, but the activity on single-stranded DNA was inhibited about 80% in the presence of 50 mM NaCl.

As was the case with its action on double-stranded DNAs, the endonuclease associated with reverse transcriptase does not require the presence of virus-specific sequences for activity. This is illustrated in the experiment shown in Fig. 6, where the rates of degradation of equimolar amounts of linear single-stranded DNAs of different lengths with and without the viral DNA sequence were compared. These DNA fragments were prepared by digesting the RF I form of M13 mp9 2-LTR DNA with *Cla*I or *Sal*I and *Bam*HI was described above. The first digestion produced two DNA fragments 6,537 and 2,695 base pairs long. The LTR sequence was contained in the larger fragment. The second digest produced two DNA fragments 7,038 and 2,200 base pairs long. In this case, the LTR sequence was in the smaller fragment. Both sets of DNA were heat denatured and then were incubated with the αβ form of the AMV polymerase with 2 mM $MnCl_2$ for various lengths of time (Fig. 6). The presence of the LTR sequence did not influence the rate of reaction since the larger DNA fragment in both cases was degraded faster than the smaller DNA fragment. These results indicate that at the level of single-stranded DNA, the size of the substrate can influence its rate of degradation by the endonuclease associated with reverse transcriptase. A similar result was obtained with heat denatured *Hin*dIII-digested plasmid pGd 111 DNA, which is a pBR322 derivative containing a 3.3-kilobase insert of chicken cellular DNA which includes the site of integration of an endogenous (*ev*1) provirus (7). The AMV reverse transcriptase was found to selectively degrade the larger plasmid pBR322 DNA in preference to the cell DNA. This size preference with the target single-stranded DNA was not observed with double-stranded DNAs (see Fig. 4).

## DISCUSSION

We have characterized the mechanism of action of the DNA endonuclease activity associated with the αβ and the ββ forms of three different avian reverse transcriptases. There is no difference that we can detect in the mode of action or specificity of the three enzymes. The ββ form of enzyme utilizes both $Mg^{2+}$ and $Mn^{2+}$ as a divalent cation, whereas the αβ form of enzyme prefers $Mn^{2+}$ as a divalent cation. Both forms of enzyme introduce nicks containing 3′ OH and 5′ P termini into RF I DNA. A second break can be introduced into the other DNA strand, probably in the vicinity of the first break while the enzyme is still bound to the DNA. This leads to the formation of RF III DNA. The ββ form of enzyme appears to be more efficient than the αβ form in introducing double-strand breaks into DNA. This may be related to the fact that the ββ form of the enzyme contains two active endonuclease sites per enzyme molecule, whereas the αβ form of enzyme has one. Both forms of enzyme exhibit slow rates of reaction on relaxed duplex plasmid pBR322 DNAs, indicating that the RF I pBR322 DNA is being nicked in the single-stranded regions induced by its supercoiling. This has been confirmed by demonstrating that both forms of enzyme introduce multiple breaks into linear and circular single-stranded plasmid M13 DNAs. After long incubation times, the DNA will be degraded to small fragments.

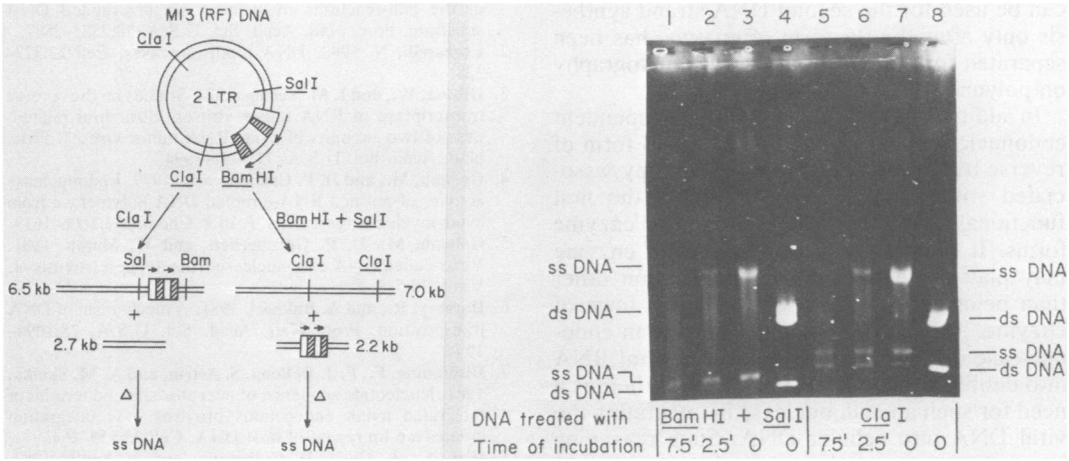


FIG. 6. Gel electrophoresis of single-stranded DNA fragments of M13 mp9 2-LTR DNA treated with the αβ form of AMV reverse transcriptase. The M13 mp9 2-LTR DNAs (0.3 nmol) digested with *Cla*I or with both *Bam*HI and *Sal*I as described in the text were heat denatured at 100°C for 4.5 min and then were cooled quickly in ice. The single-stranded DNAs were incubated with 1.1 U of the αβ form of AMV reverse transcriptase in a 10-μl volume containing 20 mM Tris-hydrochloride (pH 9)–6 mM 2-mercaptoethanol for 5 min at 38°C. Then, 0.02 μmol of $MnCl_2$ was added, and the mixture was incubated for 2.5 or 7 min at 37°C. A 4.5-μl portion was removed and analyzed on a 0.7% agarose gel with Tris-borate buffer as described in the text. M13 mp9 2-LTR DNA treated with *Bam*HI and *Sal*I was incubated as follows: lane 1, 7.5 min with AMV reverse transcriptase; lane 2, 2.5 min with AMV reverse transcriptase; lane 3, 7.5 min without AMV reverse transcriptase; lane 4, unheated, without AMV reverse transcriptase. M13 mp9 2-LTR DNA treated with *Cla*I was incubated as follows: lane 5, 7.5 min with AMV polymerase; lane 6, 2.5 min with AMV reverse transcriptase; lane 7, 7.5 min without AMV reverse transcriptase; lane 8, unheated, without AMV reverse transcriptase. The single- and double-strand DNA markers on the left and the right sides of the figure refer to lanes 1 through 4 and 5 through 8, respectively.

The enzyme activity associated with the αβ and the ββ forms of reverse transcriptase is an endonuclease. Despite multiple tests (Table 3; Fig. 2 and 3), we have been unable to confirm the report of Hizi et al. (8) that there is a topoisomerase activity associated with the reverse transcriptase. These results and the fact that Hizi et al. did not detect the activity in their ββ enzyme preparations suggest that it may be a contaminant of their αβ enzyme preparation. We have also been unable to confirm the report of Meyer-Nisson et al. (18) that the DNA endonuclease activity associated with the αβ form of AMV polymerase contains a $Mg^{2+}$-ATP dependent activity. The addition of ATP to any of our three enzyme preparations had no effect upon the reaction with single- or double-stranded DNAs.

It is interesting to note that the endonucleases associated with reverse transcriptase act in a processive rather than in a nonprocessive fashion. This is shown by the fact that upon incubation of circular single-stranded DNA with reverse transcriptase, we find circular and linear DNAs late in the reaction at a time when there is extensive degradation of some of the DNA. This mode of action is also suggested by the finding that multiple 5′ P termini were detected in plasmid pBR322 DNA treated with the αβ form of reverse transcriptase (Table 2), whereas only 25% of the initial RF I pBR322 DNA was relaxed during the course of the reaction as detected by agarose gel electrophoresis. We have already shown that both the αβ and the ββ forms of B77 RSV reverse transcriptase catalyze the polymerization of deoxynucleotides in a processive fashion (10). Also, the RNase H activity acts processively (15). These catalytic sites must keep the enzyme associated with the DNA template, allowing the endonuclease enough time to introduce multiple breaks into a strand of DNA before the enzyme is released into solution to find another DNA molecule.

The demonstration that there is a $Mg^{2+}$-dependent single-stranded DNA endonuclease associated with reverse transcriptase can explain some of the difficulty in obtaining full-length cDNA clones from mRNAs. Most preparations of the AMV reverse transcriptase (αβ) will contain some of the ββ form of enzyme, which elutes from the phosphocellulose column as a shoulder behind the main peak of enzyme. When these enzyme preparations are used for the synthesis of the second DNA strand, the DNA products will be susceptible to endonuclease cleavage (27). The AMV reverse transcriptase

can be used for the second DNA strand synthesis only after the ββ form of enzyme has been separated from the αβ form by chromatography on polyuridylic acid-cellulose.

In addition, the finding that a $Mg^{2+}$-dependent endonuclease is associated with the ββ form of reverse transcriptase but that little, if any, associated with the αβ form represents the first functional difference between these two enzyme forms. It suggests that the ββ form of enzyme may play some role in virus replication other than being just a precursor to the αβ form of enzyme. There is no obvious need for an endonuclease during the transcription of viral RNA into double-stranded DNA. However, there is a need for such an endonuclease in integrating the viral DNA into cellular DNA. Such reasoning has led us to speculate that the ββ form of reverse transcriptase might function as part of an integration system in which the two subunits of the enzyme could be used to bring the viral DNA into close proximity to a cellular DNA integration site, allowing the endonuclease to introduce single- and double-strand breaks containing 3′ OH termini into each as postulated by Harshey and Bukhari (6). The difficulties encountered in observing preferential cleavage of LTR-containing DNA may be related to the fact that the cloned substrates tested do not resemble the viral DNA as it exists in vivo. Also, our studies were designed to assess alterations in the rate of cleavage and would not necessarily demonstrate preferred sites of cleavage. The latter question is currently under investigation. The endonuclease associated with reverse transcriptase is capable of selecting its substrate and limiting its activity based on the availability of single-stranded regions within double-stranded DNA. This suggests that secondary structure, not primary sequence, may be a crucial element for recognizing LTR sequences in double-stranded DNA. The substrate acted upon by the integration mechanism in vivo may be a viral minichromosome in which the association of viral and host proteins with DNA plays a significant role in directing the course of the integration reaction.

## ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant CA 27414 from the National Institutes of Health (J.L.) and a grant from the Cuyahoga County unit of the American Cancer Society, Ohio Division (J.L.). G.M.D. is supported in part by the National Institutes of Health Training Grant T32-GM-07250 from the National Institute of General Medical Science and is a recipient of a Joseph S. Silber Fellowship from the American Cancer Society, Cuyahoga County unit.

We thank Kathleen Rubino for excellent technical assistance.

## LITERATURE CITED

1. **Been, M. D., and J. J. Champoux.** 1981. DNA breakage and closure by rat liver type 1 topoisomerase: separation of the half-reactions by using a single-stranded DNA substrate. Proc. Natl. Acad. Sci. U.S.A. **78**:2883–2887.
2. **Cozzarelli, N.** 1980. DNA topoisomerases. Cell **22**:327–328.
3. **Gibson, W., and I. M. Verma.** 1974. Studies on the reverse transcriptase of RNA tumor viruses. Structural relatedness of two subunits of avian RNA tumor viruses. Proc. Natl. Acad. Sci. U.S.A. **71**:4991–4994.
4. **Golomb, M., and D. P. Grandegenett.** 1979. Endonuclease activity of purified RNA-directed DNA polymerase from avian myeloblastosis virus. J. Biol. Chem. **254**:1606–1613.
5. **Golomb, M., D. P. Grandgenett, and W. Mason.** 1981. Virus-coded DNA endonuclease from avian retrovirus. J. Virol. **38**:548–555.
6. **Harshey, R., and A. Bukhari.** 1981. A mechanism of DNA transposition. Proc. Natl. Acad. Sci. U.S.A. **78**:1090–1094.
7. **Hishinuma, F., P. J. DeBona, S. Astrin, and A. M. Skalka.** 1981. Nucleotide sequence of acceptor site and termini of integrated avian endogenous provirus *ev*1: integration creates a 6 bp repeat of host DNA. Cell **23**:155–164.
8. **Hizi, A., A. Gazit, D. Guthmann, and A. Yaniv.** 1982. DNA-processing activities associated with the purified α, $\beta_2$, and αβ molecular forms of avian sarcoma virus RNA-dependent DNA polymerase. J. Virol. **41**:974–981.
9. **Hizi, A., and W. K. Joklik.** 1977. RNA-dependent DNA polymerase of avian sarcoma virus B77. I. Isolation and partial characterization of the α, $\beta_2$ and αβ forms of the enzyme. J. Biol. Chem. **252**:2281–2289.
10. **Hizi, A., J. P. Leis, and W. K. Joklik.** 1977. RNA-dependent DNA polymerase of avian sarcoma virus B77. II. Comparison of the catalytic properties of the α, $\beta_2$, and αβ enzyme forms. J. Biol. Chem. **252**:2290–2295.
11. **Jovin, T., P. England, and L. Bertsch.** 1969. Enzymatic synthesis of DNA. XXVI. Physical and chemical studies of a homogeneous DNA polymerase. J. Biol. Chem. **244**:2996–3008.
12. **Ju, G., L. Boone, and A. M. Skalka.** 1980. Isolation and characterization of recombinant DNA clones of avian retroviruses: size heterogeneity and instability of the direct repeat. J. Virol. **33**:1026–1033.
13. **Leis, J., R. Smith, P. Dierks, J. Parsons, M. Collett, and A. Faras.** 1978. *In vitro* transcription of reconstituted 35S RNA-$tRNA_{trp}$ template-primer complexes by the avian oncornavirus DNA polymerase. Effect of temperature on the size of the DNA transcripts. Virology **85**:28–42.
14. **Leis, J. P.** 1976. RNA-dependent DNA polymerase activity of RNA tumor virus. VI. Processive mode of action of avian myeloblastosis virus polymerase. J. Virol. **19**:932–939.
15. **Leis, J. P., I. Berkower, and J. Hurwitz.** 1973. Mechanism of action of ribonuclease H isolated from avian myeloblastosis virus and *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **70**:466–470.
16. **Leis, J. P., P. Scheible, and R. E. Smith.** 1980. Correlation of RNA binding affinity of avian oncornavirus p19 proteins with the extent of processing of virus genome RNA in cells. J. Virol. **35**:722–731.
17. **Linial, M., and W. S. Mason.** 1973. Characterization of two conditional early mutants of Rous sarcoma virus. Virology **53**:258–273.
18. **Meyer-Nisson, J., A. Raae, and I. Nes.** 1981. Effect of ATP on the Friend murine leukemia virus associated endonuclease activity and the endonuclease activity of the avian myeloblastosis virus RNA-directed DNA polymerase. J. Biol. Chem. **256**:7985–7989.
19. **Muster, J. C., Y. S. Lee, J. E. Newbold, and J. Leis.** 1980. Physical mapping of adeno-associated virus serotype 4 DNA. J. Virol. **35**:653–661.
20. **Rho, H. M., D. P. Grandgenett, and M. Green.** 1975. Sequence relatedness between the subunits of avian myeloblastosis virus reverse transcriptase. J. Biol. Chem. **250**:5278–5280.
21. **Schiff, R. D., and D. P. Grandgenett.** 1978. Virus-coded origin of a 32,000-dalton protein from avian retrovirus

cores: structural relatedness of p32 and the β polypeptide of the avian retrovirus DNA polymerase. J. Virol. **28**:279–291.

22. **Shimotohno, K., S. Mizotani, and H. Temin.** 1980. Sequence of retrovirus provirus resembles that of bacterial transposable elements. Nature (London) **285**:550–554.
23. **Smith, R., S. Nebes, and J. Leis.** 1977. Production of large amounts of 35S RNA and complementary DNA from avian RNA tumor viruses. Anal. Biochem. **77**:226–234.
24. **Swanstrom, R., W. Delorbe, J. Bishop, and H. Varmus.** 1981. Nucleotide sequence of cloned unintegrated ASV DNA: Viral DNA contains direct and inverted repeats similar to those in transposable elements. Proc. Natl. Acad. Sci. U.S.A. **78**:124–128.
25. **Verma, I. M.** 1977. The reverse transcriptase. Biochim. Biophys. Acta **473**:1–38.
26. **Verma, I. M., W. S. Mason, S. D. Drost, and D. Balitmore.** 1974. DNA polymerase activity from two temperature-sensitive mutants of Rous sarcoma virus is thermolabile. Nature (London) **251**:1075–1082.
27. **Yoo-Warren, H., M. Cimbala, K. Felz, J. Monahan, J. Leis, and R. Hanson.** 1981. Identification of a DNA clone to phosphoenolpyruvate carboxykinase (GTP) from rat cytosol. J. Biol. Chem. **256**:10224–10227.