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DNA endonucleases associated with the avian myeloblastosis virus DNA polymerase

(RNA-dependent DNA nucleotidyltransferase/hybrid nuclease/superhelical DNA)

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ABSTRACT **A DNA endonuclease, Endo-I, which cleaves superhelical DNAs, has been isolated from avian myeloblastosis virions stripped of their coats by mild detergent treatment. The enzyme has a broad pH optimum around 7.5–8.0 and requires Mg^{2+} for activity. A second endonuclease, Endo-II, with a requirement for Mn^{2+}, also present in viral cores, copurified with avian myeloblastosis virus $\alpha\beta$ DNA polymerase (reverse transcriptase, RNA-dependent DNA nucleotidyltransferase) and similarly cleaved superhelical DNAs. Heat denaturation and sodium fluoride and *N*-ethylmaleimide inhibition studies were carried out to demonstrate a possible relationship between the two endonucleases and the viral DNA polymerase and RNase H activities. It appears that Endo-II may be an intrinsic activity of the polymerase.**

In cells infected with RNA tumor viruses, a superhelical DNA provirus is expressed in the nuclei and then integrated into the host genome (1–3). Several studies provide evidence that the superhelical form of the proviral DNA exists in the nucleus of the infected host (1–4) and is a prerequistite for the integration of viral information into the cellular genome (1). Enzymes have been implicated in the relaxation process of superhelical DNAs (5, 6) that potentially facilitates recombination and integration events (7–9). The introduction of a single-strand break in superhelical DNAs has been postulated as a requirement in the formation of a swivel required in DNA replication. DNA tumor virus endonucleases with specificity for single-stranded DNA (ssDNA) have also been implicated in such biological functions (6).

In this report we describe two endonuclease activities that are present in viral cores and that utilize superhelical DNAs as substrates. The first activity, Endo-I, converts superhelical DNAs to the corresponding relaxed form. It requires Mg^{2+} for activity, and can be separated from the avian myeloblastosis virus (AMV) $\alpha\beta$ DNA polymerase (reverse transcriptase, RNA-dependent DNA nucleotidyltransferase). The second activity, Endo-II, also converts superhelical DNAs to their relaxed form but remains associated with the AMV DNA polymerase and requires Mn^{2+} for activity.

Several conditions that inhibit AMV $\alpha\beta$ DNA polymerase and RNase H activities also inhibited the Endo-II activity, but showed no effect on the Endo-I activity. A preliminary report of this work has been made.§

MATERIALS AND METHODS

Reagents. Templates for reverse transcriptase and RNase H assays, agarose, and simian virus 40 (SV40) DNA were obtained from Bethesda Research Laboratories (Rockville, MD). [^{3}H]dTTP and [^{3}H]dGTP were purchased from New England Nuclear. CM-cellulose-bound trypsin was purchased from Miles. Calf thymus DNA was from P-L Biochemicals and cellulose (Cellex 410), from Bio-Rad.

Preparation of DNA-Cellulose. Calf thymus DNA-cellulose, single- or double-stranded, was prepared by the procedure of Alberts and Herrick (10).

Isolation and Purification of AMV Virions and Viral Cores. AMV was obtained as frozen pellets from Life Sciences (St. Petersburg, FL), or was isolated from high titer leukemic chicken plasma by the procedure of Bonar *et al.* (11). Prior to use the virus was extensively purified by equilibrium density centrifugation by the method of Verma and Baltimore (12). Virions stripped of their coats by mild detergent (AMV cores) were prepared by modification of the procedure of Bolognesi *et al.* (13) and Stromberg (14).

Digestion of AMV with Bromelain. AMV was incubated at 37°C for 3 hr with bromelain as described by Compans *et al.* (15), followed by isopycnic centrifugation in a 20–55% (wt/vol) sucrose gradient at 36,000 rpm in a Beckman SW 40 rotor for 4 hr at 4°C (16). The banded virus was pooled, diluted to 5 ml in the same buffer, and pelleted by centrifugation at 50,000 rpm in a Beckman SW 50.1 rotor for 1 hr.

Enzyme Assay. DNA polymerase and RNase H activities were determined as described (17). AMV Endo-I activity was determined by incubating superhelical (form I) DNA (0.3 μg) in a reaction mixture containing 6 mM Tris·HCl (pH 7.5) and 5 mM $MgCl_2$. AMV Endo-II activity was determined in the same reaction mixture, substituting 0.5 mM $MnCl_2$ as the divalent cation. Both endonuclease reaction mixtures were in a final volume of 20 μl. Reactions were carried out at 37°C and stopped by the addition of 5 μl of a solution containing 0.02% bromophenol blue, 1% (wt/vol) sodium dodecyl sulfate, and 5% (vol/vol) glycerol. DNA fragments were separated by electrophoresis on a 1.4% agarose gel at 10 V/cm for 2 hr at 23°C, with 40 mM Tris·HCl/20 mM sodium acetate/2 mM sodium EDTA (pH 7.8) as the buffer. Gels were stained with an ethidium bromide solution at 0.5 μg/ml.

Sucrose Gradient Fractionation of AMV Endo-I and Endo-II Activities. Purified AMV (10 mg) was lysed by incubation with Nonidet P-40, 0.2% final concentration, at 37°C for 5 min (12). The viral lysate was then brought up to 200 μl with a 10 mM Tris·HCl (pH 7.5) buffer containing 0.1 mM Na_2EDTA, 2mM dithiothreitol, 0.02% Nonidet P-40, 0.4 M NaCl and layered onto a 10–30% (wt/vol) sucrose gradient in the same buffer and centrifuged at 100,000 × *g* at 4°C for 15 hr.

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Abbreviations: AMV, avian myeloblastosis virus; SV40, simian virus 40; ssDNA, single-stranded DNA; MalNEt, *N*-ethylmaleimide.

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§ Samuel, K. P., Papas, T. S. & Chirikjian, J. G. (1978) *Abstracts of the Annual Meeting, American Society for Microbiology*, Las Vegas, NV, S4, p. 213.

Analysis of AMV Endo-I-Treated Superhelical DNAs. AMV Endo-I-treated SV40 form I [^{3}H]DNA with a specific activity of 72,000 cpm/μg and a relaxed circular (form II) SV40 [^{14}C]DNA marker were layered onto a 10–30% alkaline sucrose gradient. The gradient was centrifuged at 100,000 × *g* at 10°C for 16 hr. Fractions were neutralized by the addition of glacial acetic acid and their radioactivities were determined.

Limited Tryptic Digestion of Purified AMV $\alpha\beta$ DNA Polymerase. The $\alpha\beta$ form of AMV DNA polymerase was digested as reported (18). The resulting tryptic digestion products were assayed for the polymerase, RNase H, and endonuclease activities.

Inhibition Studies. *Sodium fluoride inhibition.* Purified AMV $\alpha\beta$ DNA polymerase (250 μg/ml) was preincubated with 0–40 mM NaF at 4°C for 30 min. The NaF-treated enzyme was then assayed for DNA polymerase and RNase H and endonuclease activities. *Thermal denaturation.* Purified AMV $\alpha\beta$ polymerase (250 μg/ml) was diluted 1:5 in a buffer containing 20 mM Tris·HCl (pH 8.0), 0.1 mM EDTA, 2 mM dithiothreitol, bovine serum albumin at 100 μg/ml, and 5% glycerol. The diluted enzyme was incubated for 15 min at various temperatures, quickly cooled to 4°C, and assayed for DNA polymerase, RNase H, and endonuclease activities. *N-Ethylmaleimide (MalNEt) inhibition.* Purified AMV $\alpha\beta$ DNA polymerase (250 μg/ml) was preincubated with 0–5.0 mM MalNEt at 4°C for 30 min. Endo-I and Endo-II activities were determined in their respective reaction mixtures. *Sodium pyrophosphate.* Endo-I and Endo-II activities were determined in the presence of 0–5.0 mM sodium pyrophosphate.

RESULTS

Presence of DNA Endonuclease Activities within AMV. Protease-treated AMV, viral cores, and untreated virions were examined for the presence of DNA endonuclease activity. In order to determine whether the enzymes were bound nonspecifically to the viral surface or were present in the viral core, bromelain-treated AMV virions were further purified by isopycnic sucrose gradient centrifugation. Parallel experiments were run with untreated AMV virions and assayed for the presence of DNA endonuclease activity.

When lysed untreated AMV was incubated with superhelical DNA of SV40, a linear form, a smear due to nonspecific exonuclease, and the relaxed form were obtained. Bromelain-treated AMV showed a substantial reduction in the generation of linear SV40 DNA and exonuclease activity. By contrast, protein extracts from purified AMV cores converted superhelical SV40 DNA to the corresponding relaxed form exclusively, while unlysed cores were devoid of exonuclease and DNA polymerase activity (Fig. 1). These results suggested that the endonuclease activity is present within the viral structure and not on the outer surface.

Fractionation and Properties of Endonucleases. Protease-treated virus was lysed with 0.2% Nonidet P-40, and the viral lysate was further fractionated. Sedimentation of the viral lysates on sucrose velocity gradients separated two endonuclease activities. As shown (Fig. 2), Endo-II cosediments with the AMV $\alpha\beta$ DNA polymerase, while the Endo-I activity is separated from the polymerase. Fractions of Endo-I and Endo-II from the sucrose gradient were pooled separately and absorbed onto separate DEAE-cellulose columns equilibrated with 20 mM Tris·HCl (pH 7.5)/0.1 mM EDTA, 2 mM dithiothreitol/10% (vol/vol) glycerol buffer. Activities were recovered by elution with DEAE-cellulose buffer in 0.5 M KCl and further fractionated on ssDNA-cellulose equilibrated with 20 mM Tris·HCl

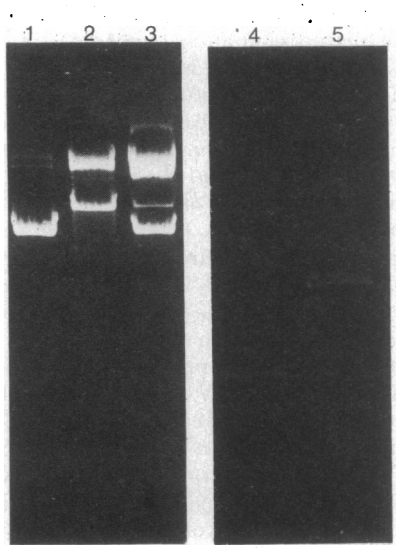

FIG. 1. Presence of DNA endonuclease in purified virions of AMV and virions stripped of their coats by mild detergent. AMV (10 mg) was incubated in the presence (*Left*) or absence (*Right*) of bromelain. After rebanding in a sucrose gradient and pelleting by high-speed centrifugation, the virus pellet was resuspended in 0.5 ml of buffer and further treated. Aliquots (5 μl) were examined for DNA endonuclease activity. Lanes 1 and 4 of the agarose gel pictures represent a SV40 DNA control and lanes 2, 3, and 5 represent SV40 DNA with untreated AMV, repurified bromelain-digested AMV, and AMV lysed cores, respectively.

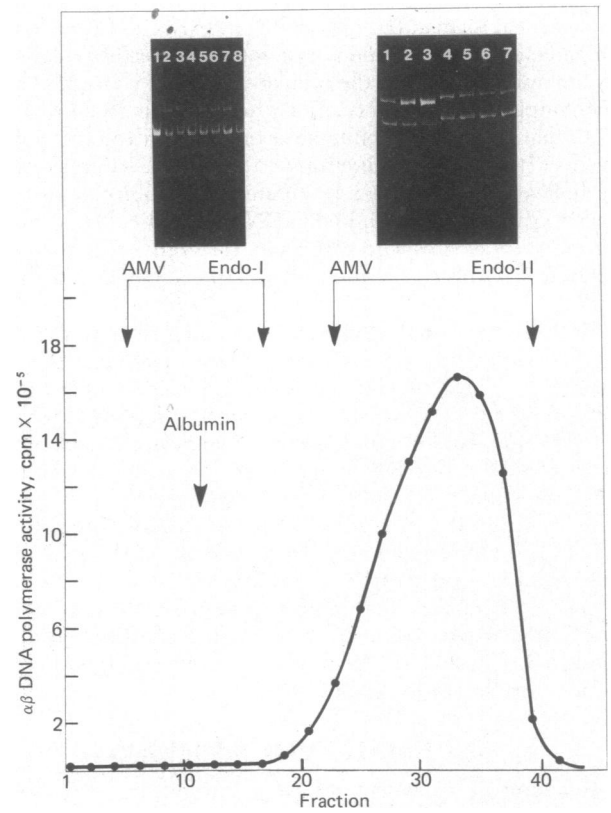

FIG. 2. Sucrose gradient fractionation of AMV-associated Endo-I and Endo-II activities. The detergent-lysed AMV virions were layered onto a 10–30% (wt/vol) sucrose gradient and centrifuged with bovine serum albumin as a standard. AMV Endo-I activity sedimented between fractions 3 and 10. (*Left Inset*) Assay gel as in Fig. 1. Lane 1 of the gel corresponds to a SV40 form-I DNA control; lanes 2–8 are the corresponding gradient fractions. (*Right Inset*) Endo-II assays of fractions 20–37, lanes 1–7, respectively.

Table 1. Summary of elution profiles for AMV DNA polymerase-associated activities from calf thymus ss-DNA-cellulose

Enzyme activity	Enzyme form	KCl concentration for elution from ssDNA-cellulose, M
DNA polymerase	α subunit	0.11
DNA polymerase	$\alpha\beta$ holoenzyme	0.22
Mg^{2+}-requiring	Endo-I	0.05 (broad peak)
Mn^{2+}-requiring	Endo-II	0.22

(pH 8.0)/0.1 mM EDTA/2 mM dithiothreitol/10% (vol/vol) glycerol. Endo-I eluted from ssDNA-cellulose as a broad peak of activity around 0.05 M KCl. Endo-II eluted at 0.22 M KCl, cochromatographing with the $\alpha\beta$ DNA polymerase (Table 1).

Endo-I shows an absolute requirement for Mg^{2+}, with a broad optimum around 5 mM in Tris·HCl, pH 7.5–8.0 (Table 2). On a sucrose velocity gradient, Endo-I sedimented with an apparent molecular weight of 50,000 (Fig. 2). The AMV $\alpha\beta$ DNA polymerase-associated Mn^{2+}-dependent activity Endo-II, which sedimented with the polymerase (molecular weight 160,000 ± 10,000), also has a broad pH optimum between pH 7.5 and 8.0 in Tris·HCl buffer; however, Endo-II has a Mn^{2+} requirement, with an optimum around 0.5 mM. Endo-II copurifies with the AMV $\alpha\beta$ DNA polymerase through both anion and cation exchange chromatography, as well as on sucrose velocity gradient centrifugation.

Effect of Endo-I and Endo-II on Superhelical DNA. The digestion pattern of SV40 form-I [^{3}H]DNA by Endo-I activity was examined both by nondenaturing gel electrophoresis on a 1.4% agarose gel and under denaturing conditions on sucrose gradients, to determine internal breaks in DNA. In alkaline sucrose gradients, Endo-I-treated SV40 DNA sedimented as the 16S linear and 18S circular forms (Fig. 3), indicating that Endo-I acts as a DNA "nickase," converting superhelical SV40 DNA into the relaxed circular form.

Endo-II also converted labeled superhelical SV40 DNA primarily to its corresponding 16S and 18S forms but in addition generated small fragments with low sedimentation values (data not shown).

Effect of Limited Protease Digestion, Inhibitors, and Thermal Denaturation on AMV Endo-I and Endo-II. Our previous studies had shown that storage of AMV $\alpha\beta$ DNA polymerase resulted in the degradation of the β subunit to the α subunit and smaller polypeptides (18). We therefore exposed purified AMV $\alpha\beta$ DNA polymerase that contained both Endo-I and Endo-II activities to protease digestion to determine the effect of such treatment on the two endonuclease activities relative to the RNase H and DNA polymerase activities.

The Endo-II, DNA polymerase, and RNase H activities were not appreciably affected by the limited tryptic treatment. However, complete loss of Endo-I activity was observed under the same conditions (Table 2).

Properties of the two endonucleases were examined by using different inhibitors and thermal denaturation. In all reactions superhelical SV40 DNA was used as a substrate. Thus Endo-I was inhibited by 0.1 mM (MalNEt) (Fig. 4, gel A), was not affected by 30 mM NaF (Fig. 4, gel C), and was resistant to thermal treatment for 15 min at 53°C (Fig. 4, gel E). Endo-II was also inhibited by 0.1 mM MalNEt (Fig. 4, gel B); by contrast, it was inhibited by 30 mM NaF (Fig. 4, gel D) and was labile to thermal treatment (Fig. 4, gel F). Because Endo-II copurifies with DNA polymerase, we examined the effect of these treatments on both the polymerase and RNase H activities in order to determine a relationship between the endonucleases and the intrinsic activities of AMV DNA polymerase. These results can be summarized as follows: Both the polymerase and RNase H activities were inhibited by MalNEt. The polymerase activity was not inhibited by 30 mM NaF, whereas the RNase H lost more than 60% of its activity, when compared to the activity without the inhibitor (Fig. 4 *center*). Under our reaction conditions both the polymerase and RNase H were sensitive to thermal denaturation, although the polymerase was more labile than the RNase H (Fig. 4 *right*).

We examined the effect of sodium pyrophosphate on the AMV DNA polymerase-associated Endo-I and Endo-II activities because this reagent had been reported to enhance cDNA copy synthesis on the viral 35S RNA in a reconstituted system (19). Complete inhibition of Endo-II was observed at 0.3 mM sodium pyrophosphate, whereas the Endo-I and DNA polymerase activities were partially inhibited (Fig. 5). In spite of the partial inhibition of the polymerase, better yields of double-stranded DNA have been reported.

DISCUSSION

Our studies localized two DNA endonucleases within virions of AMV stripped of their coats by mild detergent. Endo-I requires Mg^{2+} for optimal activity and introduces a break into the phosphodiester backbone of superhelical DNA (Fig. 3). The enzyme is stable to thermal treatment for 15 min at 53°C (Fig. 4), has a broad pH optimum, and is activated severalfold by 100 mM NaCl. The second activity, Endo-II, is closely associated with the AMV DNA polymerase and shares similar DNA-binding properties, on the basis of its coelution from native and denatured DNA-cellulose columns (Table 1). The enzyme is more labile to thermal denaturation than Endo-I (Fig. 4), but has similar reaction requirements, except for the Mn^{2+} divalent cation requirement.

Because endonuclease activity was found to be associated with the AMV DNA polymerase, we screened for Endo-I and Endo-II activities in samples of the $\alpha\beta$ DNA polymerase at different stages of purity that had been stored for various periods of time. Little or no Endo-I activity was detected in newly

Table 2. Properties of AMV-associated Endo-I, Endo-II, DNA polymerase, and RNase H activities

Property	Endo-I	Endo-II	DNA polymerase	RNase
Apparent molecular weight	50,000	165,000	165,000	165,000
Divalent cation requirement	Mg^{2+}	Mn^{2+}	Mg^{2+}, Mn^{2+}	Mg^{2+}
Effect of 100 mM NaCl	Activated	Inhibited	Inhibited	Inhibited
Effect of inhibitors				
30 mM NaF	Not affected	Partially inhibited	Not affected	Inhibited
0.1–5 mM MalNEt	Inhibited	Inhibited	Inhibited	Inhibited
0.3 mM $NaPP_i$	Not inhibited	Completely inhibited	Inhibited	Not determined
Limited tryptic digestion	Loss of activity	Not affected	Not affected	Not affected
Thermal denaturation	Not affected	Completely inhibited	Completely inhibited	Inhibited

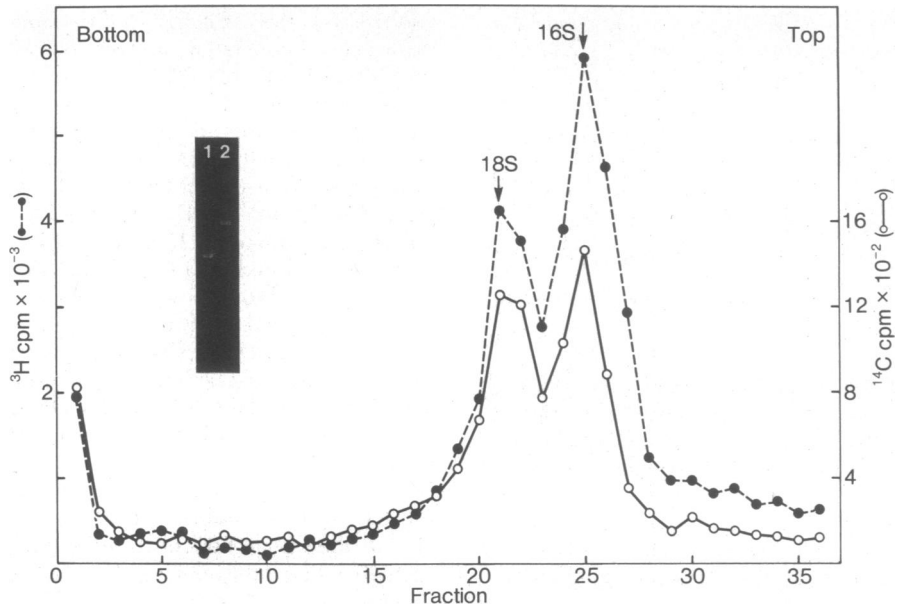

FIG. 3. Alkaline sucrose density gradient analysis of the digestion products of AMV Endo-I activity on SV40 form-I [^{3}H]DNA. A sample of AMV Endo-I (10 μl) from the calf thymus ssDNA-cellulose column pool was incubated with 1.0 μg of SV40 form-I [^{3}H]DNA at 37°C. After incubation, aliquots of the Endo-I-digested DNA and the control DNA were analyzed by electrophoresis on a 1.4% agarose slab gel (lanes 2 and 1, respectively, of *Inset*). The other aliquot was layered onto a 10–30% (wt/vol) alkaline sucrose gradient and centrifuged. ○—○, SV40 [^{14}C]DNA marker; ●---●, enzyme-treated SV40 [^{3}H]DNA. Undigested DNA sediments to the bottom of the tube.

prepared AMV DNA polymerase samples, by contrast to stored or aged enzyme preparations. Moreover, Endo-II activity was present in all samples of AMV αβ DNA polymerase examined, regardless of the age of the enzyme. We extended these observations by exposing AMV αβ DNA polymerase to limited protease digestion (18). The DNA polymerase, RNase H, and Endo-II activities were essentially unaffected, while a loss in Endo-I activity was detected.

To determine the catalytic properties of the two endonucleases and to demonstrate a possible relationship to the DNA polymerase and RNase H activities, inhibition and thermal denaturation studies were carried out. Inhibitor studies showed sodium fluoride, which differentially inhibits RNase H activity (20) had no effect on Endo-I but partially inhibited Endo-II (Fig. 4). MalNEt, which partially inhibits RNase H and fully inhibits the DNA polymerase, also inhibited Endo-I and Endo-II (Fig. 4). Thermal denaturation at 53°C for 15 min showed essentially no loss of Endo-I activity, while the Endo-II activity was completely inhibited and the polymerase and RNase H activities were differentially affected.

Both AMV DNA polymerase and the associated Endo-II activity are inhibited by high salt concentrations (Table 2). By contrast, Endo-I activity increases severalfold in the presence of 100 mM NaCl. The requirement for high salt by Endo-I occassionally generated intermediate DNA banding patterns characteristic of a DNA topoisomerase (21). The importance of the ionic environment for reverse transcription has been documented, and cDNA transcripts of larger than usual size have been obtained when no NaCl was added to the reaction (22), and the Mg^{2+}/Mn^{2+} ratio has been similarly implicated (23).

It is of interest to note that concentrations of sodium pyrophosphate that completely inhibited the Endo-II activity and partially inhibited Endo-I (Fig. 5) optimized the synthesis of

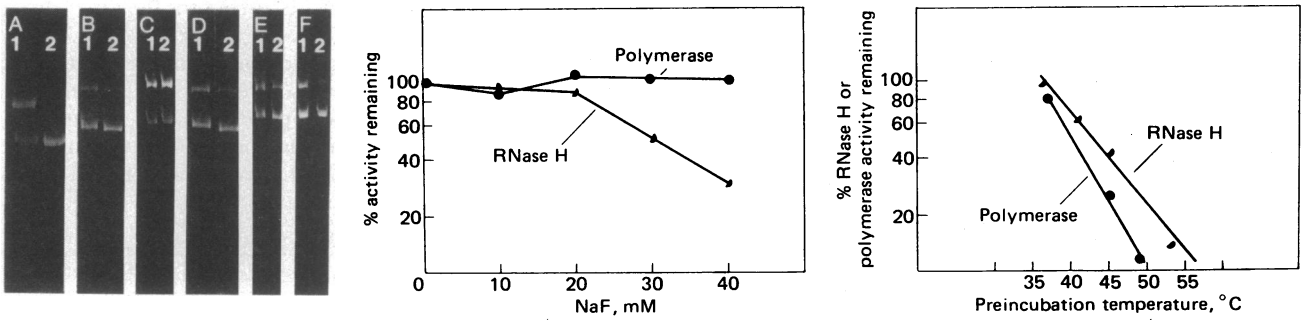

FIG. 4. Effect of inhibitors and thermal denaturation on AMV-associated DNA polymerase, RNase H, and DNA endonuclease activities. (*Left*) Endonuclease assay gels. Lane 1 of each gel represents the control enzymes with no inhibitor present or unheated. *MalNEt*. AMV αβ DNA polymerase (250 μg/ml) was preincubated at 4°C with MalNEt at either 0 or 3 mM. Shown are samples (3 μl) from the 3.0 mM MalNEt that were assayed for Endo-I (gel A) or Endo-II (gel B), at 37°C for 3 hr. *NaF*. Aliquots of AMV αβ DNA polymerase (250 μg/ml) were preincubated at 4°C with NaF at either 0 or 40 mM for 30 min. Shown are samples (3 μl) from the 30 mM NaF that were assayed for Endo-I and Endo-II activities (*Left*, gels C and D), and 2 μl for DNA polymerase and RNase H activities (*Center*). *Thermal denaturation*. DNA polymerase (250 μg/ml) was diluted 1:5 with dilution buffer and processed. The polymerase and RNAse H activities are represented graphically in *Right*, and Endo-I and Endo-II activities in gels E and F (*Left*). In all experiments dealing with the polymerase and RNase H the activity level prior to treatment is taken as 100%.

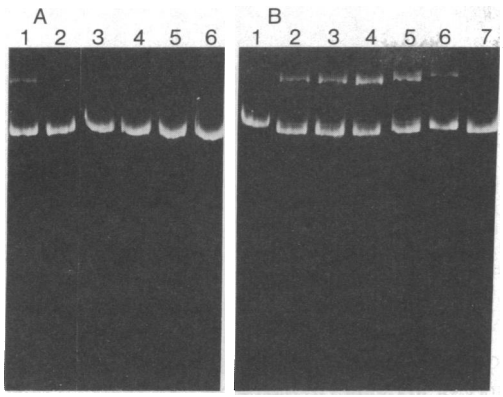

FIG. 5. Effect of sodium pyrophosphate on the AMV-associated Endo-I and Endo-II activities. Endo-I and Endo-II (5 μl) from the ssDNA-cellulose fractionation were incubated in a reaction mixture containing 0.33 μg of SV40 form-I DNA, 6 mM Tris·HCl (pH 7.5), either (*A*) 5 mM $MgCl_2$ (for Endo-I activity) or (*B*) 0.5 mM $MnCl_2$ (for Endo-II activity), and increasing concentrations of sodium pyrophosphate. (*A*) Endo-II activity in 0, 0.1, 0.3, 1.0, 3.0, and 5.0 mM sodium pyrophosphate is represented by lanes 1–6. (*B*) The corresponding Endo-I activity is shown in lanes 2–7. Lane 1 is a DNA control.

large cDNA copies of AMV 35S RNA by AMV DNA polymerase. Myers *et al.* (19) reported that inclusion of 4 mM sodium pyrophosphate in the reconstituted transcription system yielded larger cDNA copies. We have also observed that AMV DNA polymerase samples, free of Endo-I activities, and under conditions in which the Endo-II activity was inhibited, consistently yield large cDNA copies (unpublished data).

It is attractive to speculate on the basis of the results obtained (Table 2) that Endo-II has several properties that are similar to those of AMV DNA polymerase and that it is an intrinsic activity of the polymerase. We cannot at this time completely rule out the possibility, although unlikely, of trace amounts of an endonuclease activity copurifying with AMV DNA polymerase. Possible roles for the two endonucleases in the viral transcriptional process have not yet been clarified.

Note Added in Proof. Since the submission of the original manuscript, we have obtained evidence that RNA also serves as a substrate for both nucleases. We therefore examined the effect of AMV Endo-I and Endo-II on $tRNA^{Trp}$ (bovine) as a potential mechanism for the unfolding of the primer molecule. Limited exposure of $tRNA^{Trp}$ to either enzyme generated as the predominant fragment a 16-nucleotide fragment from the 3′ end. This fragment has been shown to be required for base pairing as a prerequisite to the transcription of the viral genome (24).

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