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Purification and properties of DNA endonuclease associated with Friend leukemia virus

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ABSTRACT

An endonuclease associated with the core of Friend leukemia virus (FLV) has been purified more than 10^3-fold by ion exchange chromatography and gel filtration. Its molecular weight was determined by gel filtration to be about 40,000. Divalent cations were required for the endonuclease to function and KCl concentrations above 50 mM inhibited the enzyme activity. In the presence of Mg^{++} the purified enzyme nicked preferentially supercoiled circular DNA duplexes and in most of these molecules only one single-stranded nick was introduced per strand. The regions into which the nick could be introduced appeared to be randomly distributed on the circular molecule. When Mn^{++} was substituted for Mg^{++} the number of nicks introduced into DNA by the purified enzyme was greatly increased, and both relaxed circular and linear DNA duplexes were nicked as well as supercoiled circular DNA duplexes. Prior to its purification, however, in the presence of Mn^{++} the endonuclease activity in the virus extract was able to differentiate between circular and linear DNA duplexes, since both supercoiled and relaxed circular duplexes were nicked much more readily than linear duplexes. Single-stranded DNA functioned poorly as a substrate for the purified enzyme.

INTRODUCTION

The importance of a virus endonuclease in the replication of retroviruses is suggested by the fact that such an enzyme is present in the viral core of the avian myeloblastosis virus (AMV) (1). It appears that this activity resides in both the β-subunit of the viral RNA-directed DNA polymerase (2,3) and in the p32 protein, which is derived by proteolytic cleavage of the polymerase β-subunit (1). In the presence of divalent cations the endonuclease activity associated with the AMV p32 protein converts supercoiled circular DNA duplexes (RFI) to the relaxed form (RFII) by introducing single-stranded nicks into the DNA (1). When Mg^{++} is present, the enzyme introduces in most of the supercoiled duplexes only one nick per strand, whereas a large number of nicks are introduced into both strands of the supercoiled DNA in the presence of Mn^{++} (1). In contrast to circular duplex-

es, linear duplexes (RFIII) and single-stranded DNA function relatively poorly as substrate for the enzyme (1).

We recently reported the presence of an endonuclease activity in crude extracts of Friend leukemia virus (FLV) (4). The activity in the FLV extract was shown to have all of the above properties which are characteristic of the endonuclease activity of the AMV p32 protein. The fact that two retroviruses from different species appear to contain very similar endonucleases suggests that they are bonafide virus enzymes which are important in the biology of these viruses. This notion is also supported by the fact that the AMV p32 protein is coded for by the AMV genome. In the present work we report the purification of the FLV associated endonuclease and the characterization of the purified enzyme.

MATERIALS AND METHODS

Cell line, culture conditions and virus purification. FLV was propagated in Eveline cells (5). The cells were maintained in suspension culture in Eagle's minimum essential medium with Earle's balanced salt solution and 10% foetal calf serum (Gibco Biocult). The medium was supplemented with 100 units/ml of penicillin and 100 µg/ml of streptomycin. The cell density was kept at 2 to 4 x 10^5 cells/ml by adding an equal volume of fresh medium every 24 h. The FLV was purified from the cell culture as earlier described (4).

Purification of virus associated endonuclease. Purified viruses at a concentration of 1 mg protein/ml in buffer A (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA and 10 mM β-mercaptoethanol) were disrupted by adding Triton X-100 to a final concentration of 0.2%. Two volumes of buffer A were added to the virus extract after incubating the extract for 30 min at 0^oC. The extract was then applied to a DEAE-cellulose column (about 1 mg of protein per ml bed volume) which was subsequently washed with two volumes of buffer A. The proteins were eluted from the column with a linear gradient (10 column volumes) of 0-600 mM NaCl in buffer A, and fractions equivalent to 4% of the gradient volume were collected and assayed for endonuclease activity. The endonuclease which eluted at about 50 mM NaCl was then applied to a phosphocellulose column (about 1 mg of protein per ml bed volume). The phosphocellulose column was washed with two volumes of buffer A and the proteins were then eluted from the column using a linear gradient (10 column volumes) of 0-600 mM NaCl in buffer A. Fractions equivalent to

4% of the gradient volume were collected and assayed for endonuclease activity. The endonuclease activity which eluted at about 300 mM NaCl was concentrated by dialysis against a 50% (v/v) glycerol buffer which contained 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.1 mM EDTA and 10 mM β-mercaptoethanol. After dialysis, the endonuclease was applied to a Sephadex G-75 column (1.68 x 68 cm) which had previously been equilibrated with buffer B (20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.1 mM EDTA and 10 mM β-mercaptoethanol. The column was calibrated with ovalbumin, molecular weight (m.w.) 45,000; chymotrypsinogen, m.w. 25,000; lysozyme, m.w. 14.000 and RNase, m.w. 13,5000.

Preparation of øX174 DNAs. The isolation of [^{3}H]thymidine øX174 RFI and RFII DNA was as earlier described (6). The viral form of øX174 DNA was purified by the method of Pagano and Hutchison (7). Unit length linear øX174 DNA duplexes (RFIII) were generated by EndoR.Ava I (New England Biolab) cleavage of the RFI form.

Endonuclease assay. The endonuclease assay was carried out in a reaction mixture of 100-500 µl which contained 20 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, either 10 mM Mg^{++} or 10 mM Mn^{++} unless otherwise indicated, and radioactive øX174 DNA and enzyme in the amounts indicated in the legends to figures. The reaction mixture was incubated at 37^{o} for 15 min unless otherwise stated. The endonucleolytic activity was monitored either by the nitrocellulose filter technique as earlier described (6), or by alkaline sucrose gradient centrifugation or gel electrophoresis of the DNA.

Alkaline sucrose gradient centrifugation and gel electrophoresis. The procedure used for alkaline sucrose gradient centrifugation of DNA was as earlier described (4). Alkaline gel electrophoresis of DNA was performed essentially as described by McDonell et al. (8) using horizontal 1% agarose gels.

Protein determination. Protein concentrations were determined using the Bio-Rad Protein Assay Kit.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate gel electrophoresis was carried out according to the slab gel technique described by Laemmli and Favre (9).

RESULTS

Endonuclease activity associated with the FLV core. In order to show that the endonuclease which is activated upon detergent disruption of FLV (4) actually is located in the interior of the virus particle, a preparation of

FLV was treated with proteinase K such that the viral envelope protein (gp 71) and all nonviral proteins were digested (Fig. 1).

The viral cores obtained after the proteinase K digestion were then sedimented through a sucrose cushion in order to remove the proteinase K, and subsequently disrupted by Triton x-100 and assayed for endonuclease activity (Fig. 2). The endonuclease activity proved to be completely resistant to the proteinase K treatment of the virus particles (Fig. 2). These results indicate that the endonuclease is primarily associated with the virus core. Analogous results to those shown in Figure 2 were also obtained when Mg^{++} was substituted for Mn^{++} in the reaction mixture.

Purification of virus associated endonuclease. The FLV associated endonuclease was purified by DEAE- and phosphocellulose ion exchange chromatography, followed by gel filtration on a Sephadex G-75 column (see Materials and Methods). The enzyme was eluted from the DEAE- and phosphocellulose columns at concentrations of about 50 and 300 mM NaCl respectively (Fig. 3A and B). Only one major peak of endonuclease activity was detected on each column. When this activity was subsequently applied on a Sephadex G-75 column in the presence of 300 mM NaCl, one peak of endonuclease activity was eluted at a molecular weight of approximately 40,000 (Fig. 3 C).

It is apparent from these results that the endonuclease activity does not reside in the FLV RNA-directed DNA polymerase whose molecular weight is

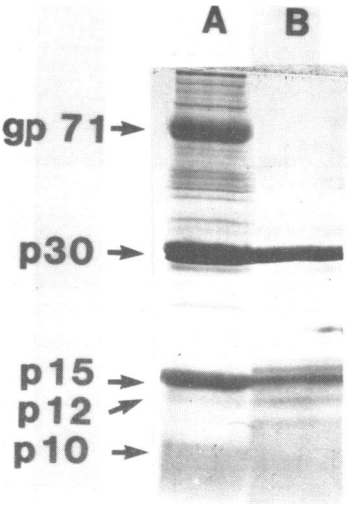

Fig. 1. The effect of proteinase K on FLV. FLV pelleted from 1000 ml cell culture was resuspended in 1 ml 20 mM Tris-HCl, pH 7.5 (about 1 mg protein/ml) and divided into two equal aliquots. One of the aliquots was then incubated with 12 µg of proteinase K for 20 min. at 37°C. The virus particles in both aliquots were then pelleted through a 20% sucrose cushion in 20 mM Tris-HCl (70,000 x g for 1 h) and resuspended in 0.5 ml 20 mM Tris-HCl (pH 7.5). The proteins in each of the two preparations were then analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. (A) Proteins from the FLV preparation which had not been treated with proteinase K and (B) proteins from the FLV preparation which had been treated with proteinase K.

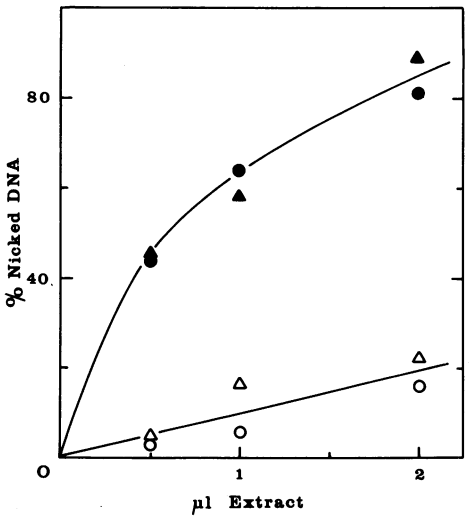

Fig. 2. The effect on the FLV endonuclease activity of treating the virus with proteinase K. The two virus samples (with and without proteinase K treatment) prepared as described in the legend to Figure 1 were both divided into two equal aliquotes (250 µl each). Triton X-100 was added to a final concentration of 0.2% to one of the aliquotes which had been preincubated with proteinase K and to one of the aliquotes which had not been treated with proteinase K. The endonuclease activity in each of the aliquotes was assayed in the presence of 10 mM Mn^{++} and øX174 RFI [^{3}H]DNA (0.02 µg/ assay), using the nitrocellulose filtration technique as described in Materials and Methods. Endonuclease activity in FLV preparation which had been incubated with both proteinase K and Triton X-100 (–●–), incubated without proteinase K and with Triton X-100 (–▲–), incubated with proteinase K and without Triton X-100 (–0–), and incubated with neither proteinase K nor Triton X-100 (–Δ–).

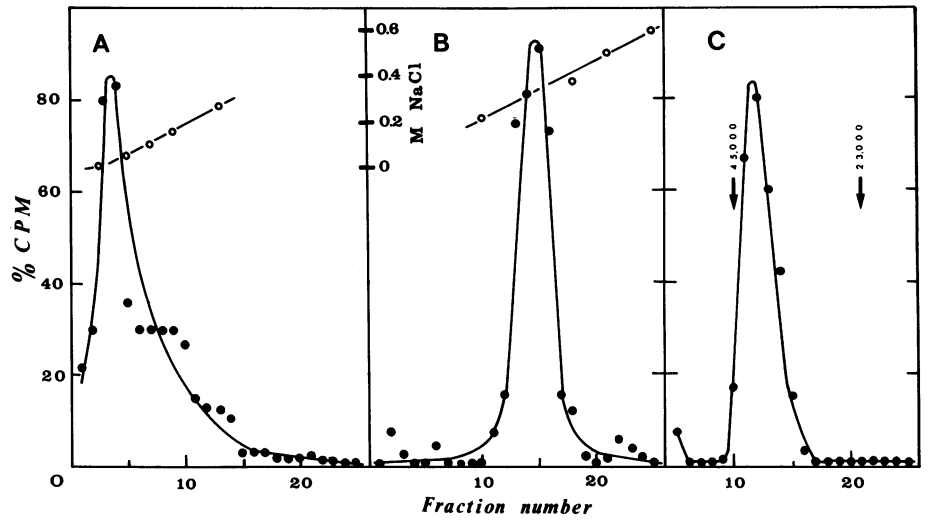

Fig. 3. Purification of the FLV endonuclease by chromatography on (A) DEAE-cellulose, (B) phosphocellulose and (C) Sephadex G-75 columns. The purification procedure was as described in Materials and Methods (starting with about 5 mg of virus proteins). The endonuclease activity in 10 µl from each column fraction was assayed by the nitrocellulose filter technique using supercoiled øX174 RFI [^{3}H]DNA (0.02 µg/assay) as a substrate.

about 84,000 (10).

When starting with a virus extract containing 5 mg of protein less than 1 µg protein was obtained in the purified endonuclease fraction after gel filtration. The purification of the enzyme resulted consequently in at least a 10^3-fold increase in the specific activity. An exact determination of the specific activity, however, was not obtained, since the low protein content (< 0.1 µg/ml) in the purified enzyme preparation made it difficult to measure the protein concentration. The added amount of purified enzyme in the following experiments will be quantitated in µl rather than µg. The number of µl refers in each case to the volume applied from a 10 ml preparation of FLV endonuclease which had been purified from 5 mg virus protein.

Properties of virus associated endonuclease. The endonuclease was characterized with respect to the effect various salt and divalent cation concentrations had on the enzymatic activity. The presence of divalent cations was required for the endonuclease to function, as 10 mM EDTA completely inhibited the activity.

The enzyme was active in the presence of either Mg^{++} or Mn^{++} and the optimal concentration was the same for both cations (Fig. 4 A). However, Mn^{++} greatly enhanced the endonuclease activity (Fig. 4 A). Salt concentrations above 50 mM inhibited the enzyme activity in the presence of either Mn^{++} or Mg^{++} (Fig. 4 B).

The FLV associated endonuclease converts øX174 RFI DNA to the open RFII form rather than to linear molecules as judged by gel electrophoresis of nicked DNA (4). Hence, the endonuclease introduces single rather than double-stranded breaks into DNA. Information about how extensively the purified endonuclease nicked RFI DNA in the presence of either Mn^{++} or Mg^{++} was obtained by analysing the sedimentation characteristics of the nicked DNA under denaturing conditions (Fig. 5).

The sedimentation profiles in Figure 5A show that RFI DNA was nicked extensively by the enzyme in the presence of Mn^{++} and the extent of nicking correlated to the amount of enzyme which was added. In contrast to these results, in the presence of Mg^{++} only a very limited number of nicks were introduced into the supercoiled DNA by the purified enzyme and the number of nicks did not increase significantly upon a 10-fold increase in the enzyme to DNA ratio (Fig. 5B). Unit length linear single-stranded DNA was the predominant DNA species in the alkaline gradient (Fig. 5B), indicating that in the presence of Mg^{++} the main reaction product was a relaxed circular duplex with one nick in either one or both strands. The data suggests that

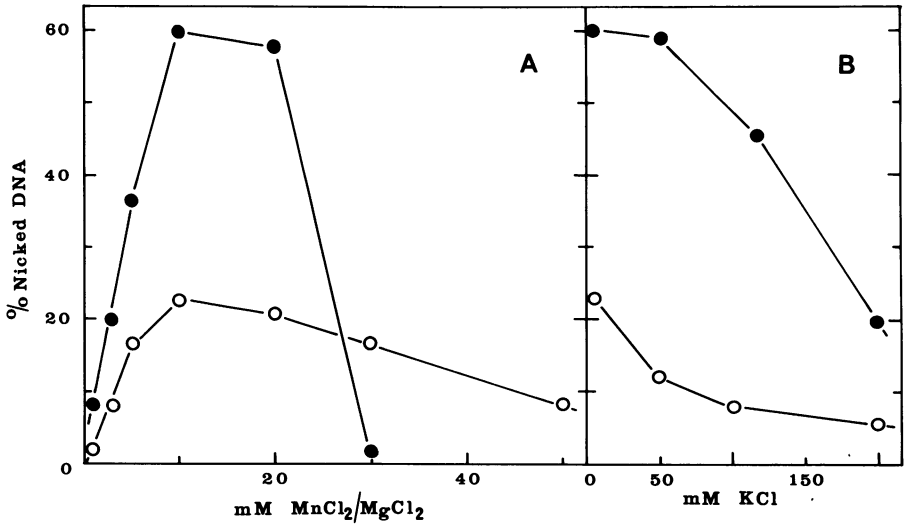

Fig. 4. The effect of divalent cations and salt on the FLV associated endonuclease activity. (A) Endonuclease activity as a function of the concentration of Mn^{++} (–●–) or Mg^{++} (–0–) in the reaction mixture. (B) Endonuclease activity as a function of the KCl concentration in the presence of 10 mM Mn^{++} (–●–) or 10 mM Mg^{++} (–0–). Each assay mix contained 0.02 µg øX174 RFI [^{3}H]DNA and 20 µl or 80 µl of the purified FLV endonuclease when assayed in the presence of Mn^{++} or Mg^{++} respectively. The endonuclease activity was assayed using the nitrocellulose filter technique as described in Materials and Methods.

the endonuclease in the presence of Mg^{++} acts more readily on supercoiled than relaxed DNA.

In order to determine the distribution on the øX174 RFI DNA of the sites which could be nicked by the purified FLV endonuclease in the presence of Mg^{++} the following experiment was performed. An excess of øX174 RFI DNA was treated with the purified FLV endonuclease and the nicked RFII molecules were then separated from the RFI DNA by centrifugation in a neutral sucrose gradient. The majority of the RFII DNA molecules contained only one single-stranded nick as revealed by the fact that an equal number of circular and unit length linear single-stranded molecules were obtained upon alkaline sucrose gradient centrifugation of an aliquot of the RFII DNA (data not shown). The remaining RFII DNA was then divided into two equal aliquots, one of which was treated with the EndoR. AvaI endonuclease which introduces one double-stranded break at a specific site on the øX174 DNA duplex. The DNA in both aliquots were then analyzed by gel electrophoresis

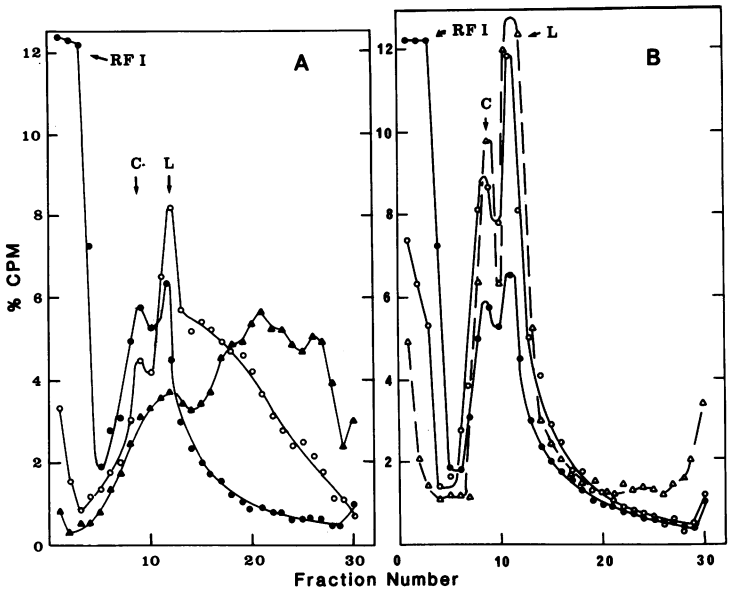

Fig. 5. Alkaline sucrose gradient centrifugation analysis of the FLV endonuclease activity on øX174 RFI [^{3}H] DNA. (A) The sedimentation profile of DNA after incubating 0.2 µg RFI DNA with 0 µl (–●–), 100 µl (–○–) and 150 µl (–▲–) of the purified FLV endonuclease in the presence of 10 mM Mn^{++}. (B) The sedimentation profiles of DNA after incubating 0.2 µg RFI DNA with 0 µl (–●–), 200 µl (–○–) and 2500 µl (–△–) of purified FLV endonuclease in the presence of 10 mM Mg^{++}. The reaction was carried out at 37°C for 15 min. The alkaline sucrose gradient centrifugation was as described in Materials and Methods. The C and L denote circular and unit length linear single-stranded øX174 DNA respectively.

under denaturing conditions (Fig. 6).

The DNA which had been treated only with the FLV endonuclease migrated as single-stranded circular and unit length linear molecules. About 50% of the DNA which had first been treated with the FLV endonuclease and subsequently with the EndoR. AvaI endonuclease migrated as unit length single-stranded DNA. This was expected in view of the fact that prior to cleavage by EndoR. AvaI the majority of the RFII molecules contained one strand which was not nicked by the FLV endonuclease. The remaining DNA migrated as expected if the regions in the DNA which were recognized by the FLV associated endonuclease in the presence of Mg^{++} were evenly distributed on the circular øX174 DNA molecule since no dominant peak other than the unit length linear DNA was detected (Fig. 6). Both strands of the DNA

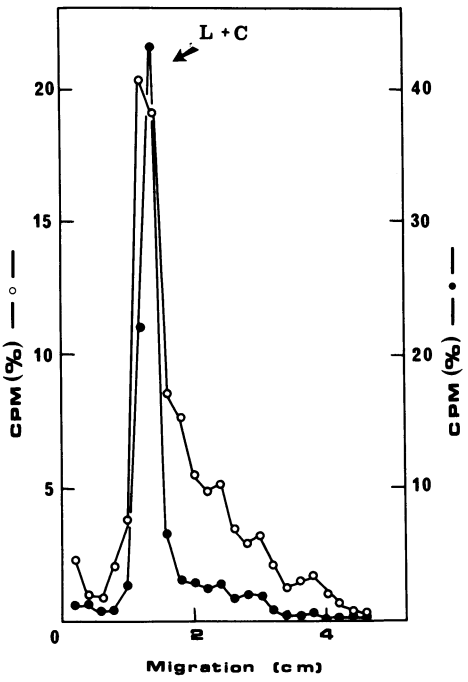

Fig. 6. Alkaline gel electrophoresis analysis of the distribution on øX174 RFI [^{3}H]DNA of the sites which may be nicked by the purified FLV endonuclease in the presence of Mg^{++}. øX174 RFI ^{3}H-DNA (1 µg) was incubated 15 min with 700 µl of the FLV endonuclease in the presence of 10 mM Mg^{++}. The nicked RFII DNA was then separated from the RFI DNA by sedimentation in a neutral 5-20% sucrose gradient (in 20 mM sodiumphosphate pH 7.0, 1 mM EDTA) using an International SB-283 rotor and centrifuging at 38,000 rpm for 8 h at 15°C. After centrifugation the RFII DNA was collected and concentrated by precipitation in ethanol. One aliquot of this DNA was then analyzed by alkaline gel electrophoresis using horizontal 1% agarose gels (—●—), whereas another aliquote of the DNA was treated with EndoR. AvaI endonuclease prior to analysis by alkaline gel electrophoresis (—○—). The C and L denote circular and unit length linear single-stranded øX174 DNA respectively.

molecule could be nicked by the FLV endonuclease, since higher enzyme to DNA ratios than used in this experiment resulted in the nicking of both strands of the same DNA duplex (Fig. 5B).

Numerous of single-stranded nicks are introduced randomly into supercoiled $ColE_1$ DNA by the endonuclease associated with the β-subunit of the AMV polymerase in the presence of Mn^{++}, whereas under similar conditions the enzyme nicks linear DNA duplexes poorly (2). It thus seems that this enzyme may have the property that it nicks circular duplexes more readily than linear duplexes. A somewhat similar observation has been made with the S1 endonuclease of Aspergillus oryzae, which cuts both supercoiled and relaxed circular avian sarcoma virus DNA intermediates, but not the linear viral DNA duplex (11). These observations suggest that some of the endonuclease sensitive sites which are present in supercoiled DNA due to the special structural configuration of this DNA may also to some extent be present in open circular DNA duplexes, whereas they are not frequently present in linear duplexes. With this in mind the ability of the FLV associated endonuclease to nick linear as compared to circular DNA duplexes was

studied. Prior to purification the FLV associated endonuclease activity behaved similarly to the AMV associated activity, since linear øX174 duplexes were not readily nicked when incubated with FLV lysates under conditions which introduced many nicks into supercoiled circular øX174 duplexes (Fig. 7 A and B).

Moreover, even though approximately 0.5-fold fewer nicks were introduced into the DNA when using open circular rather than supercoiled circular DNA as the initial substrate (Fig. 7 A and C), the open circular DNA functioned also much better as a substrate for the endonuclease in the FLV lysates than the linear duplexes (Fig. 7 B and C). However, though the endonucleolytic activity in the FLV lysates invariably nicked circular duplexes more readily than linear DNA and thus appears to resemble the endonucleolytic activity associated with purified AMV polymerase, after purification the FLV asso-

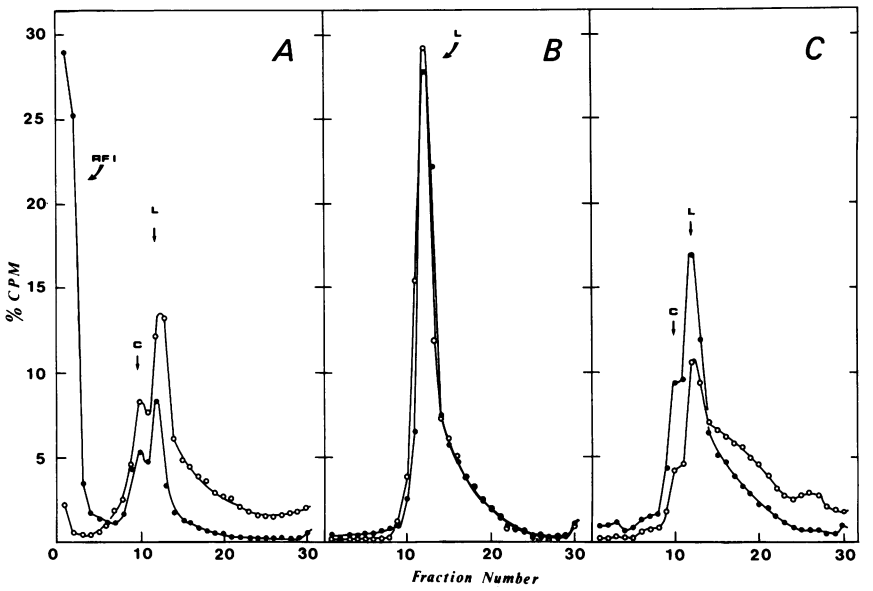

Fig. 7. Alkaline sucrose gradient centrifugation analysis of the endonuclease activity in FLV extracts in the presence of Mn^{++}. The DNA sedimentation profiles after incubating (A) supercoiled circular, (B) linear and (C) open relaxed circular DNA duplexes with (–0–) and without (–●–) 25 µg of protein from Triton X-100 (0.2%) disrupted FLV. The reaction mixture contained 0.2 µg of øX174 [^{3}H]DNA and 10 mM Mn^{++}. The assay conditions and alkaline sucrose gradient centrifugation were as described in Materials and Methods. The C and L denote circular and unit length linear single-stranded øX174 DNA respectively.

ciated endonucleolytic activity appeared to have lost this specificity since linear and circular DNA duplexes were nicked about equally well when treated with the purified enzyme (data not shown). At the present time it is not clear whether this loss of specificity is due to a partial destruction of the endonuclease during the purification or whether it is due to the loss of a factor which influences the specificity of the enzymatic activity. Prior to purification the endonuclease nicked single-stranded circular ϕX174 DNA poorly, and this characteristic was, however, retained by the enzyme after its purification (data not shown).

DISCUSSION

An endonuclease has been shown to be associated with FLV and the enzyme has been purified more than 10^3-fold. Its molecular weight was determined by gel filtration to be about 40,000. The endonuclease activity apparantly does not reside in the FLV RNA-directed DNA polymerase whose molecular weight is about 84,000 (10).

An endonuclease activity resides in the β-subunit of the AMV RNA-directed DNA polymerase (2,3). Some of the β-subunits undergo a cleavage processing which results in the formation of the polymerase α-subunit and the p32 protein. The p32 protein appears to have retained the β-subunit endonuclease activity (1). The FLV associated endonuclease activity has characteristics in common with the endonuclease activity associated with the AMV p32 protein. Both of these enzymes introduce in most supercoiled DNA duplexes only one single-stranded nick per strand when Mg^{++} is present, whereas a great number of nicks are introduced into both strands in the presence of Mn^{++}. Neither enzyme acts readily on single-stranded DNA.

The enzyme described in this study was the only major endonuclease activity observed to be associated with FLV, which suggests that in contrast to the AMV polymerase, the FLV polymerase does not contain an endonuclease activity. This would be in agreement with the fact that in the presence of Mn^{++} the purified FLV polymerase is unable to convert supercoiled $ColE_1$ DNA into relaxed circular duplexes (10). However, it is possible that the FLV endonuclease originates from the viral polymerase by a process similar to the cleavage processing of the AMV p32 protein, but that the cleavage occurs very early in FLV maturation. This would be in keeping with the fact that the pol gene of murine leukemia virus has a much greater coding capacity than what is required for the coding of the viral polymerase (12).

In the presence of Mg^{++} the purified FLV endonuclease appears to introduce largely one nick per strand in supercoiled DNA duplexes. As determined by analysis of the nicked DNA by alkaline gel electrophoresis, the regions in the DNA where this nick was introduced were fairly evenly distributed on the circular DNA duplex. This is consistent with earlier results obtained with an unpurified enzyme preparation (4). Our data are compatible with the idea that the nick is introduced either completely aspecifically or at one of a number of possible enzyme recognition sites which are fairly evenly distributed on the supercoiled circular duplex. In contrast to the FLV endonuclease, the AMV p32 protein has been reported to nick supercoiled DNA at a very few preferred sites (1). This difference may be due to a difference in the two viral enzymes, or it may be due to the fact that the supercoiled DNA duplexes used as substrates in the two studies were different.

The fact that covalently closed circular virus DNA may be synthesized in vitro using detergent disrupted retroviruses (13) suggests that these viruses contain all the enzymes necessary for the synthesis of the mature viral DNA. A function for the virus associated endonuclease in this DNA synthesis is not yet obvious. The most intriguing aspect of this endonuclease is that in virus lysates in the presence of Mn^{++} it seems to differentiate between circular (RFI and II) and linear duplexes since the former molecule is nicked more readily than the latter. A somewhat similar observation has been made with endonuclease activity associated with AMV (2) and with the S1 endonuclease (11), and it suggests that under certain conditions open circular DNA duplexes may differ structurally from its linear counterpart in a manner which enables some endonucleases to differentiate between them. The nature of such a recognition site in the circular duplex is unknown. It could not, however, purely be a base sequence which is being recognized, since otherwise the endonuclease should also recognize the site in the linear DNA molecule. The biological significance of this apparent specificity in case of the FLV associated endonuclease, however, remains uncertain, since the specificity was not retained upon purification of the endonuclease activity. Nevertheless, the fact that the purified enzyme in the presence of Mg^{++} appears to preferentially nick supercoiled DNA suggests that the circular supercoiled proviral DNA may be the biological substrate for the endonuclease and that it somehow participates in the replication of this DNA.

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