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An endonuclease isolated from Epstein–Barr virus-producing human lymphoblastoid cells

(single-stranded DNA/eukaryotic DNA replication/site-specific nuclease)

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ABSTRACT **An endonuclease has been isolated from human B lymphoblastoid cells that copurifies with an exonucleolytic activity and has been shown to produce double-strand breaks and a high proportion of single-strandedness in phage λ DNA *in vitro*. The data are consistent with a model in which single-strand cuts are made by the endonucleolytic activity, possibly in A+T-rich regions of the DNA, followed by creation of single-stranded regions (gaps) precessing from the site of a cut. Generation of overlapping gaps on opposite strands or of a gap opposite a nick would lead to the creation of the banding patterns that we have seen on electrophoretic gels. This endonucleolytic activity copurifies with other enzymes induced by Epstein–Barr virus that relate to the process of viral DNA replication in productively infected cells. However, a more general role is proposed for this class of eukaryotic endonuclease activities. A marked degree of single-strandedness has been found in the replicating DNAs of many eukaryotes, and these gaps could be generated by endonucleases with associated exonucleolytic activity such as that reported here. This Epstein–Barr virus-induced nuclease activity has been shown to resemble the *recBC* nuclease isolated from the prokaryote *Escherichia coli* and also the endonuclease isolated from the eukaryote *Chlamydomonas*.**

Our laboratory has recently reported that human B lymphoblastoid cell lines that are productively infected with Epstein–Barr virus (EBV) contain EBV-induced DNA polymerase (1) and DNase (2) activities. We have shown that the DNase is separable from the polymerase after several stages of purification and that it digests both native and denatured DNA, used as substrate, to mononucleotides (2). We report here that this EBV-induced exonuclease is inseparable through multiple purification steps from an endonucleolytic activity that also is EBV-induced and acts in a selective manner on phage λ DNA used as substrate. This EBV-induced endonuclease reduces λ DNA to a discontinuous series of large fragments that are then very sensitive to the action of S1 endonuclease. However, DNA fragments of similar length, generated from the large λ genome, result from this endonucleolytic reaction only in the presence of concentrations of salt sufficient to inhibit the active exonucleolytic activity (160–180 mM KCl).

The EBV-induced endonuclease reported here resembles in many important respects an enzymatic activity isolated from the eukaryote *Chlamydomonas* by Burton *et al.* (3). The *Chlamydomonas* endonuclease has been postulated to cut at specific sites on one strand and to then digest one of the two strands of the substrate DNA. The gel electrophoretic pattern of fragments generated by treatment of DNA with various concentrations of the *Chlamydomonas* enzyme resembles the pattern of digests shown in the present report that result from the action of the EBV-induced endonuclease on phage λ DNA.

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We also suggest that this class of eukaryotic nuclease resembles the prokaryotic nuclease *recBC* isolated from *Escherichia coli*. A summary of the similarities and differences of these eukaryotic and prokaryotic nuclease systems is presented in *Discussion*.

MATERIALS AND METHODS

Materials. Phage λ DNA and all prokaryotic restriction enzymes (*Hin*dIII, *Bam*HI, and *Hpa* II) were from Bethesda Research Laboratories (Rockville, MD). Bovine serum albumin ("albumin") was from Miles. Agarose and acrylamide were purchased from Bio-Rad.

Cell Culture. The growth of the human B lymphoblastoid cell line HR-1 was as described by Clough (2) and Goodman *et al.* (1).

Purification and Assay of EBV-Induced Nuclease. The purification of the exonucleolytic activity through DEAE-cellulose, phosphocellulose, and native DNA-cellulose chromatography and the assay system used have been described in detail by Clough (2). The assays used as substrate either 1 μg of native lymphocyte [^{3}H]DNA (2–3 × 10^4 cpm/μg) or 2 μg of [^{3}H]poly[d(A-T)] (2.5 × 10^4 cpm/μg) per 200-μl assay volume, and were incubated for 60 min (DNA) or 15 min (poly[d(A-T)]) at 37°C.

Concentration of EBV-Induced Nuclease Activity. DNA cellulose-purified nuclease activity was made 200 μg/ml in albumin and concentrated by dialysis either against 50% (wt/vol) polyethylene glycol 6000 (Union Carbide, New York) or against crystalline sucrose with subsequent dialysis against column buffer C (2) to remove sucrose from the dialysis bag. All albumin used in these experiments had previously been heat treated in concentrated solution at 45°C for 12 hr to eliminate any traces of contaminating enzymatic activity (see Figs. 2, 4, and 5).

S1 Nuclease. S1 nuclease from *Aspergillis oryzae* was a gift of R. Baker and had been prepared by the method of Sutton (4). This preparation of S1 nuclease has been shown to not break phage λ or φ80 duplex DNA under conditions sufficient for complete hydrolysis of equivalent amounts of single-stranded DNA (5).

Gel Electrophoresis. Both acrylamide tube gel and agarose horizontal slab gel electrophoresis were performed using the Tris/borate/EDTA (TBE) buffer of Peacock and Dingman (6). Acrylamide gel formulas and dye markers (bromophenol blue and xylene cyanol FF) were as described by Maniatis *et al.* (7).

Synthesis of ^{3}H-Labeled Poly[d(A-T)]. This material was prepared by the method of Schachman *et al.* (8) and had a specific activity of 2.5 × 10^4 cpm per μg.

Abbreviation: EBV, Epstein–Barr virus.

RESULTS

Copurification of Exonucleolytic and Endonucleolytic Activity. Goodman *et al.* (1) and Clough (2) recently described the purification of EBV-induced enzymatic activities, using crude extracts from EBV-producer lymphocyte cell lines as starting material. The HR-1 cell line, originally isolated from a Burkitt tumor, was used for the enzyme preparations shown here. Two DEAE-cellulose column chromatography steps were followed by phosphocellulose chromatography (1, 2) and, in the case of the exonuclease, by native DNA-cellulose chromatography (2). Fig. 1*A* depicts such a native DNA-cellulose column, from which each fraction was assayed for exonucleolytic activity, using either native lymphocyte DNA labeled *in vitro* with [^{3}H]thymidine or [^{3}H]poly[d(A-T)] as substrate; under these assay conditions a single peak of exonuclease activity eluted at 375 mM KCl. When a sample of each fraction was diluted into an equal volume of phage λ DNA and buffer (see legend to Fig. 1) and KCl was added to each diluted fraction to a final concentration (180 mM) sufficient to inhibit the exonuclease activity in Fig. 1*A* (2), two species of salt-resistant nuclease activities were apparent upon gel electrophoresis of the digested λ DNA (Fig. 1*B*). One species bound poorly or not at all to native DNA-cellulose and digested λ DNA into a continuous distribution of sizes ranging from almost full sized to very small. A second salt-resistant nuclease eluted in the identical region of the gradient as the salt-sensitive exonuclease depicted in Fig. 1*A*. This latter activity digested λ DNA into what proved to be a discontinuous pattern of predominately large fragments. We also assayed DNA cellulose column fractions with circular DNA molecules such as that of simian virus 40 and certain prokaryotic plasmids (data not shown). The nuclease eluting at 375 mM KCl was capable of converting supercoiled DNA to linear forms, although there was no evidence of any specificity in the digestion. We chose to refer to this enzyme as an endonucleolytic activity to distinguish it from the salt-sensitive exonucleolytic activity shown in Fig. 1*A* with which it copurified. The nuclease activities that were eluted from the DNA-cellulose by 375 mM KCl were too unstable at this advanced stage of purification for us to undertake further attempts at separation. However, it was shown in separate experiments that phosphocellulose-purified exonuclease, when sedimented in glycerol gradients under high-salt conditions, also contained marked endonuclease activity, indicating that glycerol sedimentation under nonaggregating conditions was also not sufficient to separate these two nuclease activities (ref. 2 and unpublished data).

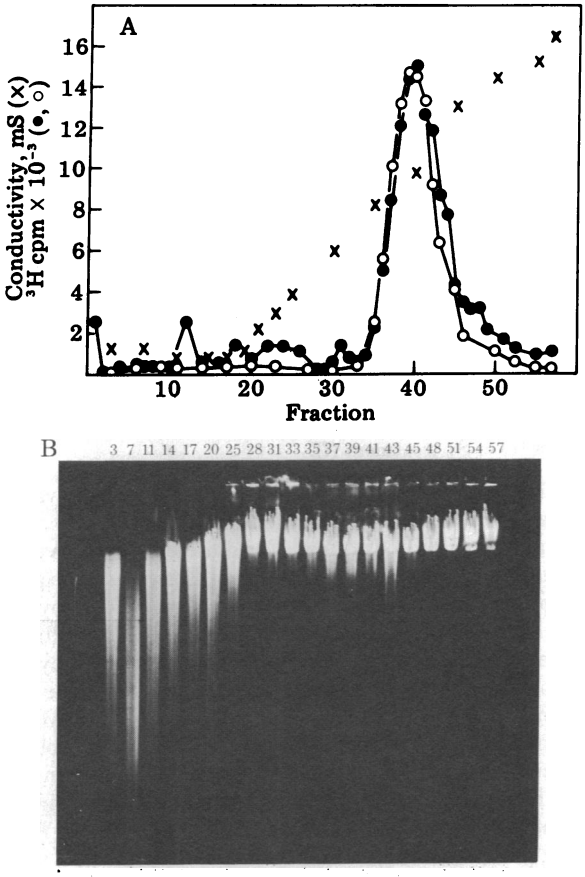

FIG. 1. Coelution of endonucleolytic and exonucleolytic activities from native DNA-cellulose. DNA-cellulose chromatography of phosphocellulose-purified DNase was performed as described in ref. 2, using a 0–0.6 M KCl gradient for elution. (*A*) Aliquots (25 μl) of column fractions were assayed for DNase activity by measuring release of acid-soluble radioactive material from native lymphocyte [^{3}H]DNA (●) or from [^{3}H]poly[d(A-T)] (○) (2). (*B*) Aliquots (25 μl) of selected column fractions were mixed with 25 μl of reaction mix containing 5 μg of λ DNA and Tris/Mg/mercaptoethanol buffer (20 mM Tris·HCl, pH 7.8/10 mM $MgCl_2$/10 mM mercaptoethanol). Final KCl concentration was adjusted to 180 mM, and the reaction was carried out at 37°C for 4.5 hr. Bromophenol blue was added as dye marker and the samples were electrophoresed in a 0.4% agarose gel in a horizontal slab gel electrophoresis apparatus, then stained with ethidium bromide.

Interaction of Concentrated and Unconcentrated Endonuclease with λ DNA. DNA-cellulose-purified endonuclease, concentrated 5-fold or used directly as the column eluant, was incubated with λ DNA in the presence of 160–180 mM KCl. As a control, λ DNA was incubated with buffer, albumin, and KCl without added enzyme activity. Fig. 2 shows the effects of the EBV-induced endonuclease on the substrate DNA.

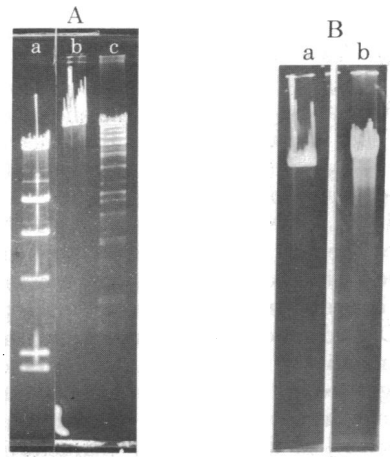

FIG. 2. Effects of concentrated compared to unconcentrated EBV-induced endonuclease on λ DNA. Agarose (0.4%) horizontal slab gel electrophoresis was performed on λ DNA treated with the EBV-induced endonuclease. Reaction mixtures consisted of 50-μl total volume containing 25 μl of reaction mix (5 μg of λ DNA in Tris/Mg/mercaptoethanol buffer) to which 25 μl of 5-fold concentrated enzyme preparation (*A*) or unconcentrated active DNA-cellulose column fractions (*B*) had been added. Final salt concentration was adjusted to 180 mM KCl, and incubation proceeded at 37°C for 60 min (*A*) or 4.5 hr (*B*). Samples were precipitated with ethanol and resuspended in electrophoresis buffer before addition to the gel. (*A*) Concentrated endonuclease treatment of λ DNA: lane a, *Hin*dIII-treated λ DNA as marker; lane b, λ DNA incubated with column buffer and albumin as control; lane c, λ DNA incubated with concentrated endonuclease. (*B*) Unconcentrated endonuclease treatment of λ DNA: lane a, λ DNA incubated with column buffer and albumin as control; lane b, λ DNA incubated with unconcentrated endonuclease-active fractions from a DNA-cellulose column such as that depicted in Fig. 1.

Concentrated enzyme preparations generated a group of high molecular weight fragments of λ DNA (principally between the size of undigested λ and the largest *Hin*dIII λ fragment used as marker) (Fig. 2*A*). This pattern of large fragments was reproducible, although there was variability among batches of enzyme and with degree of concentration. The unconcentrated enzyme generated a similar but fainter banding pattern of less distinct fragments (Fig. 2*B*). However, when a sample of λ DNA was subjected to repeated digestion by fresh batches of enzyme with ethanol precipitation of the DNA performed between incubations, no evidence of further digestion was seen beyond the pattern shown in Fig. 2*A*. Likewise, decreasing the concentration of λ DNA in the reaction mixture did not produce evidence of full double-strand digestion of the DNA. In fact, the endonuclease was most active on λ DNA at concentrations of 50 μg of DNA per ml or higher. It is not known whether the high concentration of DNA actually activates the enzyme.

It was essential to selectively inhibit the active exonuclease activity as depicted in Fig. 1*A* or in ref. 2; otherwise any products of endonucleolytic digestion were rapidly reduced to acid solubility *in vitro*. KCl at 160–180 mM was chosen as the selective exonucleolytic inhibitor for two reasons. First, it was already present in the column buffer so that a dialysis step with the accompanying activity loss could be avoided. Second, other substrates reported to inhibit nucleases were tried unsuccessfully for selective inhibition, including *N*-ethylmaleimide, *para*-hydroxymercuribenzoate, spermine, phosphonoacetic acid, deoxynucleoside monophosphates, and substitution of zinc or calcium for magnesium. Substitution of manganese for magnesium at concentrations of greater than 1 mM also achieved a selective inhibition of the exonuclease in the absence of KCl. However, the use of magnesium and KCl was preferred over the use of manganese alone for selective exonucleolytic inhibition because both of the former compounds were already present in the nuclease-active column fractions and a dialysis step costly of enzymatic activity could be eliminated. The studies using manganese indicate that the endonuclease does not require KCl for its action. However, the concentrations of KCl used here are physiologic in that they accurately reflect intracellular potassium ion concentrations (9).

S1 Sensitivity of λ DNA Treated with the EBV-Induced Endonuclease. The data in Figs. 1 and 2 show that the endonuclease has the ability to cause double-strand breaks in λ DNA. However, the blurred banding pattern seen upon gel electrophoresis of λ DNA digested with unconcentrated enzyme and the apparent inability to achieve full double-strand digestion of the DNA suggested that the enzyme might have other actions on λ DNA even in the presence of a salt concentration sufficient to inhibit over 95% of the previously characterized exonucleolytic activity (2).

The above assays had determined only the ability of this enzyme to generate double-strand breaks in substrate DNA. However, if the EBV-induced endonuclease was acting by digestion on a single strand of substrate DNA in the manner of the *Chlamydomonas* endonuclease (3), then a substantial portion of the λ DNA should become S1 sensitive after treatment with the EBV-induced endonuclease. The gel electrophoretic banding pattern in Fig. 3 shows that after 4.5-hr incubation of λ DNA with the endonuclease and subsequent digestion of the λ DNA with S1 endonuclease, most of the DNA has been sufficiently digested to have run off the 0.4% agarose gel under electrophoretic conditions that normally retain most of the substrate DNA. Therefore, the ability of this endonuclease to generate single-strandedness is far greater than its ability to generate double-strand cuts over a 4.5-hr incubation period. Further evidence of endonucleolytic digestion was not apparent after overnight incubation (data not shown).

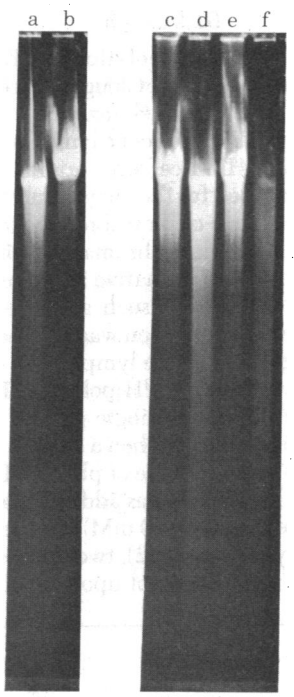

FIG. 3. S1 nuclease digestion of λ DNA pretreated with EBV-induced endonuclease. λ DNA was digested with unconcentrated EBV-induced endonuclease as described for Fig. 2*B* for the incubation times indicated. Each sample from the time course was divided into two portions. One portion was treated as control and the other was treated with S1 nuclease under conditions described in ref. 10. All samples were precipitated with ethanol, resuspended in electrophoresis buffer, and electrophoresed in a 0.4% horizontal agarose slab gel. Lanes a and b, control of λ DNA incubated for 4.5 hr at 37°C in the presence of column buffer, albumin, and KCl. Lane a, subsequent S1 treatment; lane b, no S1 treatment. Lanes c and d, λ DNA incubated with EBV-induced endonuclease and KCl for 3 hr followed by no treatment (c) or S1 treatment (d). Lanes e and f, λ DNA incubated with EBV-induced endonuclease and KCl for 4.5 hr followed by no treatment (e) or S1 treatment (f).

Interaction of EBV-Induced Endonuclease with λ DNA Pretreated with Prokaryotic Restriction Enzymes. The above data showed that this endonuclease attacked λ DNA by generation of some double-stranded breakage and by large amounts of single-strand digestion; however, it was not shown by such experiments whether specific regions of the λ genome were preferentially attacked by this enzyme. In order to answer this question the λ DNA was first digested by the prokaryotic restriction enzyme *Hin*dIII into a set of fragments of known order within the λ genome (11). These fragments were then digested with unconcentrated EBV-induced nuclease for 1 hr. As the banding pattern in the gel electrophoresis study in Fig. 4 shows, two fragments (2.25 and 2.0 kilobases) were selectively eliminated from the banding pattern by the action of the EBV-induced endonuclease. However, new smaller bands of equivalent molecular weight were not seen in the 1% agarose gel. In order to demonstrate the generation of new specific bands by this double enzyme treatment, very large amounts of substrate (25 μg of DNA per 50 μl instead of the usual 5 μg per 50 μl) were required to visualize small amounts of new low molecular weight DNA fragments. The patterns on the 3.5% acrylamide gels shown in Fig. 5 show four new DNA fragments in the size range of 0.4–0.18 kilobases from the *Hin*dIII-digested λ DNA after further digestion of the EBV-induced endonuclease. When other prokaryotic restriction enzymes were used

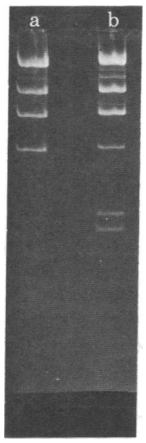

FIG. 4. Effect of EBV-induced endonuclease on λ DNA predigested with the prokaryotic restriction enzyme *Hin*dIII. λ DNA (5 μg) was incubated with Tris/Mg/mercaptoethanol buffer, 50 mM NaCl, and 3–5 units of *Hin*dIII in a 50-μl volume for 2 hr at 37°C. Then 50 μl of unconcentrated EBV-induced nuclease (lane a) or 50 μl of column buffer with albumin (lane b) was added and the final salt concentration was adjusted to 180 mM KCl. Subsequent incubation was carried out at 37°C for 70 min, after which the samples were precipitated with ethanol, resuspended in electrophoresis buffer, and electrophoresed in a 1.0% horizontal agarose slab gel.

to predigest the λ DNA, new small DNA fragments were also generated by the subsequent treatment with the EBV-induced endonuclease; however, they were different in size in the case of each prokaryotic enzyme used.

DISCUSSION

The data reported here and previously by Goodman *et al.* (1) and Clough (2) show that a complex of EBV-induced enzymatic activities is present in cells undergoing active EBV DNA replication, and that one of these is an endonucleolytic activity. Our data indicate that this endonuclease activity, under conditions of high enzyme concentration and short incubation period, can generate double-strand breaks in λ DNA to produce families of relatively high molecular weight fragments. However, long periods of DNA incubation using lower concentrations of enzyme produce increasing S1 endonuclease sensitivity of the substrate. After several hours of incubation large portions of the DNA are caused to become S1 sensitive, with only limited generation of specific length fragments.

Herpes simplex virus induces exonuclease activity similar to that induced by EBV as well as an endonucleolytic activity (12, 13); however, it is not known whether the herpes simplex virus-induced endonuclease has the ability to generate single-stranded regions such as reported here. The EBV-induced endonucleolytic activity reported here resembles in many respects an endonuclease isolated from the eukaryote *Chlamydomonas* by Burton *et al.* (3). They have proposed a model for the *Chlamydomonas* endonuclease activity that fits our data as well. They suggested that this endonuclease cuts at certain sites on one strand. An associated exonuclease activity then digests single strands of DNA starting at that site, leaving long gaps in the DNA. Specific fragments result from generation of overlapping gaps on opposite strands.

Certain data from both the EBV and the *Chlamydomonas* systems suggest that this class of eukaryotic nucleases may act preferentially at A+T-rich sites on substrate DNA. For example, Burton *et al.* (3) determined the nucleotide sequence of terminal regions of adenovirus DNA fragments that had been digested by the *Chlamydomonas* endonuclease and showed that these termini were composed largely of T and A. Our data

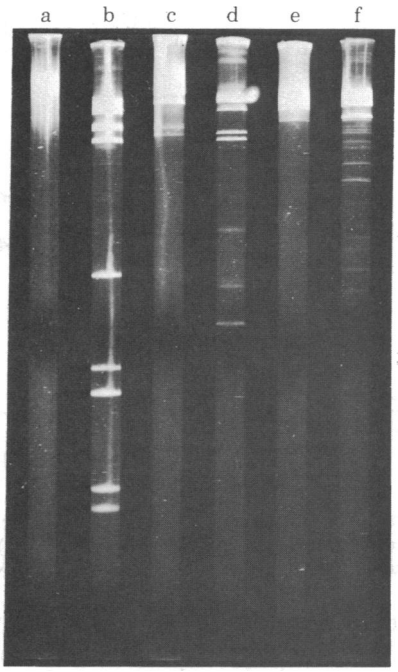

FIG. 5. Low molecular weight DNA fragments produced by EBV-induced nuclease digestion of λ DNA pretreated with prokaryotic restriction enzymes. λ DNA was treated with prokaryotic restriction enzymes as described in the legend for Fig. 4, except that the amount of DNA was increased to 20 μg and the restriction enzyme concentrations were increased proportionately. EBV-induced nuclease or column buffer and albumin was added and incubation was performed as described in the legend for Fig. 4. After ethanol precipitation, the DNA samples were electrophoresed in 3.5% acrylamide tube gels in the presence of bromophenol blue and xylene cyanol FF as markers (7). Lane a, *Hin*dIII-treated λ DNA incubated with column buffer; lane b, *Hin*dIII-treated λ DNA incubated with EBV-induced endonuclease; lane c, *Hpa* II treated λ DNA incubated with column buffer; lane d, *Hpa* II-treated λ DNA incubated with EBV-induced endonuclease; lane e, *Bam*HI-treated λ DNA incubated with column buffer; lane f, *Bam*HI-treated λ DNA incubated with EBV-induced endonuclease.

in Fig. 4 indicate that the EBV-induced nuclease preferentially attacks a certain middle region of the λ genome; portions of this central region have been shown to be very rich in T+A-containing sequences (14–16). Furthermore the exonucleolytic function that is inseparable from the EBV-induced endonuclease not only has a marked preference for single-stranded rather than native DNA (2) but also utilizes poly[d(A-T)], having a mixture of single- and double-stranded regions, with even greater facility. The K_ms of the exonuclease for native DNA, single-stranded DNA, and poly[d(A-T)] are 12.8, 2.2, and 1.1 μg/ml, respectively, as shown in Fig. 6.

The combination of endo- and exonuclease activities reported here and in ref. 2 bears a strong resemblance to the *recBC* gene products isolated from *E. coli*. This prokaryotic nuclease has been shown to contain endonucleolytic and single- and double-stranded exonucleolytic activities that are not separable after extensive purification (17). The *recBC* nuclease may also have some degree of site specificity, possibly cutting preferentially at the χ sites in λ DNA substrate (18, 19). The action of *recBC* nuclease on DNA can result in the formation of oligonucleotides of several hundred bases (see our data in Fig. 5). While *recBC* has been studied as an important component in the process of DNA recombination, it in fact may also be involved in the process of DNA replication (20), a role postulated for the EBV-induced nuclease activities as well (ref. 2 and see below). The *recBC* nuclease can be isolated from the cell

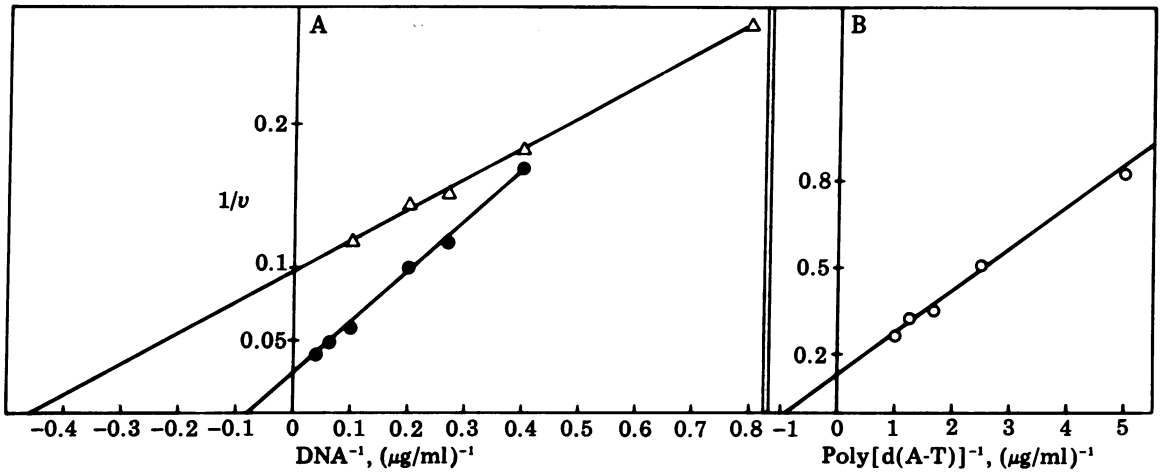

FIG. 6. K_m studies of the EBV-induced exonuclease, using native or single-stranded DNA or poly[d(A-T)] as substrate. Exonuclease assays were performed as described in ref. 2, using native (●) or denatured (△) lymphocyte DNA labeled *in vivo* with ^3H (*A*) or [^3H]poly[d(A-T)] (○) (*B*) as substrate (see *Materials and Methods* and ref. 2) at the indicated concentrations. Assays were performed over time periods in which acid solubilization of substrate was linear (45 min for DNA, 8 min for poly[d(A-T)]). $1/v$ represents (acid-solubilized ^3H cpm)$^{-1} \times 10^3$.

complexed with DNA polymerase (20), as are the EBV-induced nucleases (2). An important difference between the prokaryotic and the eukaryotic nucleases is that *recBC* also contains an active DNA-dependent ATPase and the nuclease has a strong ATP requirement (18). We have not yet demonstrated any ATP requirement for the EBV-induced nucleases.

Finally, it is important to consider the role of such eukaryotic endonucleolytic activity within the cell. As in the case of the EBV-induced exonuclease and DNA polymerase with which it copurifies, the endonuclease is probably associated with productive EBV DNA replication (1, 2). Specifically, many laboratories have shown that the DNA of eukaryotic cells during S phase contains far more single-strandedness than can be accounted for by replicative forks; in fact, 10–20% of such DNA has been shown to be single-stranded by S1 treatment or electron microscopy (21–26). Furthermore, the DNAs of adenovirus, simian virus 40, and certain herpes viruses have been shown to contain large single-stranded regions during replication (27–30). It is not clear at this time whether these single-stranded regions are specifically associated with the process of DNA replication; a role for them in transcription has also been proposed (26, 27). Nevertheless, rapidly accumulating data from these and other sources indicate that these large gaps are universally present in replicating eukaryotic DNA and therefore are involved in some essential function associated with that process. We propose that one mechanism by which these large gapped regions could be generated is by enzymatic activities such as described in this report.

It remains to be determined whether enzymes such as this EBV-induced endonuclease play any role *in vivo* in the processes of recombination or transposition of specific DNA elements.

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