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A Vaccinia Virus DNase Preparation Which Cross-Links Superhelical DNA

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Multiple DNA-dependent enzyme activities have been detected in highly purified preparations of a single-strand-specific nuclease from vaccinia virus. These enzyme preparations were extensively purified and characterized by using superhelical DNAs as substrates. In particular, the nuclease activity was monitored by the extent of conversion of supercoiled closed duplex DNA (DNA I) to nicked circular DNA (DNA II), which could subsequently be converted to duplex linear DNA (DNA III) by prolonged incubation with the enzyme. DNA species which were not substrates for the enzyme included relaxed closed duplex DNA, DNA II which had been prepared by nuclease S1 treatment or by photochemical nicking of DNA I, and DNA III. With plasmid pSM1 DNA as substrate, the extent of cleavage of DNA I to DNA II was found to increase with superhelix density above a threshold value of about −0.06. The linear reaction products were examined by gel electrophoresis after restriction enzyme digestion of the DNAs from plasmids pSM1 and pBR322 and of the viral DNAs from bacteriophage φX174 (replicative form) and simian virus 40, and the map coordinate locations of the scissions were determined. These products were further examined by electron microscopy and by gel electrophoresis under denaturing conditions. Electron micrographs taken under partially denaturing conditions revealed molecules with terminal loops or hairpins such as would result from the introduction of cross-links at the cutting sites. These species exhibited snapback renaturation. The denaturing gel electrophoresis experiments revealed the appearance of new bands at locations consistent with terminal cross-linking. With pSM1 and pBR322 DNAs, this band was shown to contain DNA that was approximately twice the length of a linear single strand. The terminal regions of the cross-linked linear duplex reaction products were sensitive to nuclease S1 but insensitive to proteinase K, suggesting that the structure is a hairpin loop not maintained by a protein linker. A similar structure is found in mature vaccinia virus DNA.

Vaccinia virus contains numerous virus-specific enzymes, including all those required to support the early transcription of its own genome. Among the enzymes previously detected in preparations from virus cores are two DNase activities: one an exonuclease with a pH optimum of 4.5, and the other an endonuclease with a pH optimum of 7.8, both requiring single-stranded DNA (26, 27). The low-pH enzyme has been reported to possess both exo- and endonucleolytic activities (32, 33). Preparations of these enzymes are known to remove the terminal cross-links from mature vaccinia virus DNA (12, 26), but the in vivo role of the enzymes has not yet been demonstrated. In common with other single-strand-specific endonucleases, these enzyme preparations have been shown to cleave superhelical DNA (32). Starting with vaccinia virus cores, we have purified and characterized nuclease preparations having a pH optimum near 6.5 and have found evidence for a much more complex pattern of activity than reported heretofore.

In the present study we report the results of experiments in which several supercoiled plasmid and viral DNAs are cleaved by a purified vaccinia virus nuclease under nearly neutral pH conditions. We present evidence that this activity is multifaceted, capable of catalyzing nicking, linearization, and interstrand cross-linking reactions. Nicked circular DNA produced by other means, such as photochemically in the presence of ethidium bromide (EtBr) or with nuclease S1, is not linearized or cross-linked by the enzyme, nor is relaxed closed circular DNA. The nick introduced by the enzyme is highly specific, and its location has been mapped on several plasmid and viral DNAs by restriction enzyme analysis. The cross-link produced by the enzyme appears to form a terminal hairpin under nondenaturing conditions. The enzyme thus possesses the appropriate activities to play a key role in the initiation of DNA replication in vaccinia virus and, in addition, in the processing of replicative intermediates into progeny genome-length DNA molecules, each cross-linked at both termini.

MATERIALS AND METHODS

Assay for endonuclease activity. Standard reaction mixtures (25 μl) contained 0.1 μg of DNA, 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid, pH 6.5], 1 mM EDTA, 100 μg of bovine serum albumin (heat denatured) per ml, and the appropriate fraction of the vaccinia virus DNase (see below). After incubation at 37°C for 30 min, the reaction was stopped by chilling on ice or by addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.1%. The product distributions were not affected by the method of terminating the reaction. One unit of activity was defined as that amount of enzyme needed to convert 50% of a 0.1-μg sample of pSM1 DNA I to open DNA in 60 min at 37°C in the standard assay

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TABLE 1. Purification of vaccinia virus nuclease

Fraction no.	Prepn	Protein (mg)	Activity (10^3 U)	Sp act (10^3 U/mg)	Purification (fold)
I	Purified virus	26.6			
II	Cores	18.7	100	5.4	1.0
III	Deoxycholate-treated cores	16.6	200	12	2.3
IV	Core polypeptides	6.2	100	16	3.0
V	DEAE-cellulose flowthrough	5.4	92	17	3.2
VI	Hydroxyapatite peak fractions	0.51	32	63	11
VII	Glycerol gradient	0.032	8.2	270	50

buffer. The 50% conversion point was determined by a serial dilution of the enzyme stock.

Purification. Vaccinia virus strain WR was purified from infected HeLa or mouse L cells through two sucrose density gradients as described previously (9). The procedure for purifying the nuclease was identical to that described for the vaccinia virus type I topoisomerase through fraction IV (3). Fraction IV was applied to a column (0.8 by 4.0 cm) of DEAE-cellulose (Whatman DE-52) previously equilibrated with buffer A (0.25 M Tris-hydrochloride, pH 8.5, 0.2 M NaCl, 1 mM EDTA, 2 mM dithiothreitol [DTT], 0.1% Triton X-100, 10% [vol/vol] glycerol). The flowthrough (fraction V) was applied to a column (0.9 by 6.0 cm) of hydroxyapatite (DNA grade; Bio-Rad Laboratories) equilibrated with buffer A containing no EDTA. The column was washed first with buffer A and then with buffer B (0.01 M sodium phosphate, pH 6.8, 0.2 M NaCl, 2 mM DTT, 0.1% Triton X-100, 10% [vol/vol] glycerol). The column was eluted with a 0.01 to 0.40 M sodium phosphate gradient containing 2 mM DTT and 10% glycerol. The peak of nuclease activity eluted at 0.15 to 0.18 M sodium phosphate, pH 6.8, yielding fraction VI. Equal portions of fraction VI (1.5 ml each) were layered onto 10 to 30% linear glycerol gradients (10 ml) containing 0.18 M sodium phosphate, pH 6.8, 2 mM DTT, and 1 mM EDTA. Sedimentation was conducted in an SW40.1 rotor at 38,000 rpm for 24 h at 4°C. The gradient was collected from the bottom into 28 fractions, and fractions 6 through 10 were combined to obtain fraction VII.

Protein determinations were done by the method of Bradford (5) with reagents obtained from Bio-Rad. The amount of protein in fraction VII was below the limit of detection by this technique; hence, the amount was approximated from silver-stained SDS gels (18) by comparison with known amounts of low-molecular-weight standards (10,000 to 100,000 daltons; Bio-Rad). Fractions 6 through 9 of the glycerol gradient were examined by this technique, and the predominant band was a 50,000-dalton species (manuscript in preparation). The only other band observed after silver staining in these fractions was a small amount (<10%) of a 110,000-dalton species in fraction 7 only. The results of the purification are presented in Table 1.

Measurement of sedimentation coefficient. The peak fraction of enzyme from hydroxyapatite chromatography, fraction VI was concentrated about ninefold by negative pressure dialysis (Micro-ProDiCon apparatus from Bio-Molecular Dynamics). This solution was then applied to a 15 to 35% glycerol gradient containing 40 mM Tris-hydrochloride, pH 7.5, 0.2 M NaCl, 1 mM EDTA, 2 mM DTT, and 0.1% Triton X-100. Catalase, aldolase, and cytochrome *c* were used as markers in a parallel tube. Sedimentation was performed in an SW50.1 rotor at 48,000 rpm for 22 h at 4°C. Vaccinia virus DNase activity sedimented at 5.6S.

Agarose gel electrophoresis. For agarose gel electrophoresis at neutral pH, samples were adjusted to contain 0.5 μg of EtBr per ml, 17% sucrose, and 0.017% bromphenol blue. The samples were subjected to electrophoresis on 1% agarose gels (14 by 10 cm) at 70 V for 3.5 h. The gel and chamber buffers contained 40 mM Tris-acetate, pH 8.3, 20 mM sodium acetate, 2 mM EDTA, and 0.5 μg of EtBr per ml for vertical gels. For submarine horizontal gels, the gel and chamber buffers contained 89 mM Tris-hydrochloride, pH 8.3, 89 mM boric acid, and 2.5 mM EDTA. The DNA bands were visualized with short-wavelength UV light and photographed with Polaroid 55 P/N film through a Tiffar 23A filter. The extent of cleavage was determined by scanning the negative with a Joyce-Loebl recording microdensitometer and measuring the peak areas with the aid of a Numonics digital planimeter. For electrophoresis under denaturing conditions, samples were adjusted to 0.1 M NaOH, 5.0 mM EDTA, 17% glycerol, and 0.003% bromcresol green. Electrophoresis was performed as described above except that it was done at 40 V for 12 h and the gel and chamber buffers contained 30 mM NaOH and 2.0 mM EDTA. The denaturing gels were equilibrated with 0.1 M Tris-hydrochloride, pH 7.5, and 0.5 μg of EtBr per ml before visualization.

Preparation of DNAs with various superhelix densities. Completely relaxed pSM1 closed duplex DNA was prepared by incubation with fraction V vaccinia virus type I topoisomerase as described previously (9). A series of DNAs of various superhelix densities was prepared by incubating 25 μg of pSM1 DNA I with vaccinia virus topoisomerase in the presence of various concentrations of EtBr (0 to 1.5 mM) (2). After removal of protein and EtBr by phenol and chloroform extraction and extensive dialysis, the DNAs were supercoiled to a degree determined by the concentration of EtBr in the topoisomerase reaction medium. The superhelix densities of these DNAs were determined by EtBr-CsCl buoyant density centrifugation (29) with pSM1 DNA I and DNA II as markers.

Electron microscopy. Linear simian virus 40 (SV40) DNAs which had been prepared by treatment with either the vaccinia virus DNase or with *Eco*RI were partially denatured as described previously (16) by incubation for either 15 or 45 min at 30°C in 10% formaldehyde–20 mM Na_2CO_3–3 mM EDTA after the pH was adjusted to 10.9 with 3 N NaOH (21). Denatured specimens were diluted to a concentration of 0.1 to 0.5 μg/ml in a hyperphase containing 60% formamide, 20 μg of cytochrome *c* per ml, and 0.1 μg of pBR322 DNA I per ml as a size marker. The hyperphase was spread on a hypophase consisting of 20% formamide in double-distilled water. Specimens were collected on Parlodion-coated grids, stained with uranyl acetate, rotary shadowed with platinum-palladium (7), and examined with a Joel 100cx electron microscope.

RESULTS

Catalytic activity. The reactions catalyzed by the vaccinia virus DNase preparation described here included the formation of nicked circular DNA from supercoiled DNA (reaction R1), the formation of linear duplex DNA from the nicked circular DNA produced by reaction R1 (reaction R2), and the formation of terminal cross-links between the two strands of the linear duplex DNA molecules produced by

reaction R2 (reaction R3). We present evidence partially characterizing each reaction as indicated.

Supercoiled DNAs from SV40, φX174, and plasmids pSM1 and pBR322 were all substrates for the nicking reaction (R1). The DNA II species so generated were also substrates for the linearization reaction (R2) and the cross-linking reaction (R3) under appropriate conditions, as described below. DNA II molecules generated by photochemical nicking of pSM1 DNA I in the presence of EtBr, as described previously (19), or by nicking with nuclease S1 were not substrates for reactions R2 or R3 at 37°C. These species could, however, be cleaved (reaction R2) at 57°C after a 50-fold excess of the amount of DNase required to cleave DNA I under the same conditions was added. Linear DNA III molecules produced by nuclease S1 or *Eco*RI digestion of pSM1 DNA I were not cleaved by the vaccinia virus enzyme under the conditions employed.

Superhelix density. Completely relaxed closed duplex pSM1 DNA was not cleaved by the vaccinia virus DNase under standard assay conditions in which supercoiled DNA was readily cleaved. A minimum superhelix density of the substrate DNA is therefore required for enzyme activity in reaction R1. To characterize this requirement, several pSM1 DNAs of various superhelix densities were prepared as described above and incubated with the vaccinia virus DNase under standard conditions (Fig. 1). No cleavage of DNA was observed at negative superhelix densities of magnitude less than 0.06, confirming the requirement for a minimal negative superhelix density for nuclease activity with closed duplex DNA substrates. At negative superhelix densities of greater than 0.06, the nuclease cleaved pSM1 DNA more effectively with increasing superhelix density. Supercoiling in DNA I molecules might stabilize secondary structures which are recognized by the vaccinia virus nuclease or, alternatively, might induce regions of partial denaturation. These possibilities are discussed in greater detail below.

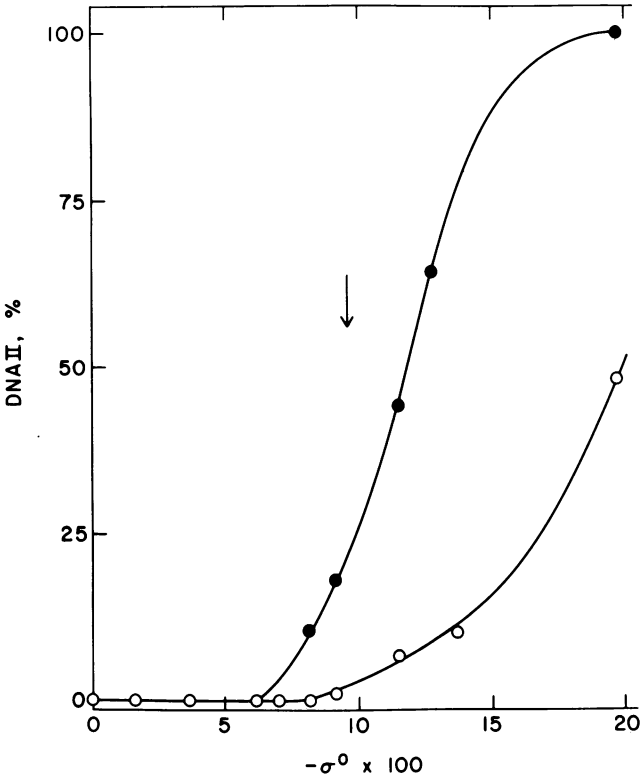

FIG. 1. Effect of superhelix density on vaccinia virus DNase activity. Plasmid pSM1 DNA I preparations of different superhelix densities were prepared by incubating pSM1 DNA I with vaccinia virus type I DNA topoisomerase (3) with various amounts of EtBr in a total volume of 2.5 ml. The pSM1 DNAs of various superhelix densities were incubated with hydroxyapatite-purified vaccinia virus nuclease (fraction VI) under conditions in which cleavage to DNA II* predominated (40 mM Tris-acetate, pH 8.3, 20 mM sodium acetate, 2 mM EDTA at 25°C for 2 h). Samples were analyzed by gel electrophoresis as described in the text. The data shown are from two separate experiments in which either 3 (○) or 15 (●) U of fraction VI nuclease were assayed with 0.1 to 0.2 μg of pSM1 DNA I. The quantity σ^0 denotes the number of superhelical turns per duplex turn and assumes an EtBr unwinding angle of 26°. Arrow, Superhelix density of native pSM1 DNA I (19).

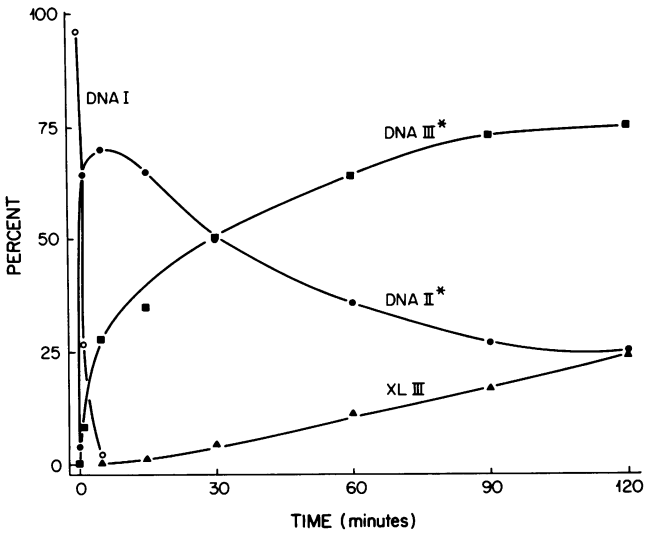

FIG. 2. Time course of pBR322 DNA I cleavage by vaccinia virus DNase. Cleavage reaction mixtures containing 3 μg of supercoiled DNA and 44 U of glycerol-gradient-purified nuclease (fraction VII) in a total volume of 120 μl were incubated at 57°C in the standard assay buffer. At the indicated times, samples were added to an SDS stop solution and examined by electrophoresis as described in the text. The percentages of DNAs I, II*, and III* were determined by removing 4-μl portions (0.10 μg of DNA) and separation by gel electrophoresis at neutral pH. The amount of cross-linked product was determined by removing 8-μl portions (0.20 μg of DNA) and separation by gel electrophoresis at alkaline pH. The data for the native DNAs represent the intensity of each band as a percentage of the total. The ordinate for the cross-linked (XL) DNA indicates the percentage of linear DNA that was cross-linked.

Time course. The kinetics data (Fig. 2) were consistent with a sequence of reactions in the order indicated above; i.e., nicking followed by linearization followed, in turn, by cross-linking. Under substrate-limiting conditions, supercoiled pBR322 DNA I was rapidly and quantitatively converted to nicked circular DNA in reaction R1 (Fig. 2). The intermediate product nicked DNA was more slowly and less efficiently converted to duplex linear DNA in reaction R2. Since the DNA II generated by nuclease S1 or photochemical nicking was not cleaved by the vaccinia virus nuclease under these conditions (data not shown), the nicked and linear DNAs generated by enzyme activity may either contain a specific cleavage or be DNA-protein complexes. We use the designations DNA II* and DNA III*, respectively, to indicate this. The addition of proteinase K to the reaction

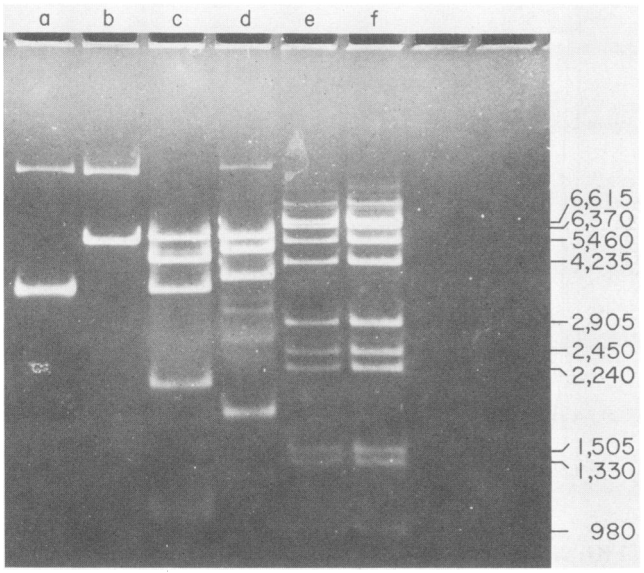

FIG. 3. Mapping vaccinia virus DNase cleavage sites by restriction enzyme analysis. Samples were loaded onto a 10-cm-long 1% agarose gel, and electrophoresis was done at 70 V for 3.5 h at room temperature. (a) Control pSM1 DNA I (0.5 μg). (b) Products formed by incubation of pSM1 DNA (0.5 μg) with 18 U of fraction VII vaccinia virus nuclease for 4 h at 37°C under standard assay conditions. (c) Products from incubation b were adjusted to *Eco*RI reaction conditions and digested with 5 U of *Eco*RI at 37°C for 1 h. (d) Products from incubation b were adjusted to *Bgl*II reaction conditions and digested with 5 U of *Bgl*II at 37°C for 1 h. (e and f) *Sma*I digest of adenovirus 2 DNA as size markers; 0.5 and 1.0 μg of DNA were added, respectively. The lengths of the marker pieces (in base pairs) are indicated at the right.

mixtures immediately prevented further cleavage of pBR322 DNA but had no effect on the electrophoretic mobility of the DNA products under either native or denaturing conditions. This result suggests that the interstrand cross-link is probably not maintained by a protein linker. Cross-linked material (reaction R3) was assayed on alkaline gels, as described below, and could not be detected before the appearance of linear duplex DNA.

pH optimum. The pH optimum for complete cleavage of pSM1 DNA I to DNA III* was approximately 6.5 in MES buffer. There was little formation of DNA III* below pH 6.0 or above pH 8.0. Between pH 8.0 and 9.0, the DNA II* product predominated.

Ionic strength. The reaction, as measured by the removal of supercoiled DNA in reaction R1, was inhibited by increased ionic strength. Above 25 mM added NaCl, the conversion of DNA II* to DNA III* was strongly suppressed, and at higher NaCl concentrations, DNA II* was the predominant product. Virtually no cleavage of DNA I occurred at concentrations above 150 mM NaCl. The substitution of KCl, NH_4Cl, or Tris-hydrochloride for NaCl produced inhibitory effects on enzyme activity indistinguishable from those seen with NaCl at comparable ionic strength.

Temperature. Cleavage of supercoiled DNA by the vaccinia virus nuclease (reaction R1) was not observed below 23°C. The extent of DNA cleavage above 23°C increased with increasing temperatures (see Fig. 5). However, above 57°C nonspecific nicking of DNA competed effectively with nuclease activity, resulting in DNA fragments of heterogeneous sizes. The vaccinia virus DNase-mediated conversion of DNA II* to DNA III*, reaction R2, was maximal at 57°C in the standard incubation buffer. The marked enhancement of reaction R2 by elevated temperatures appears to reflect a requirement for partial denaturation of the substrate DNA, since the effect can be mimicked by the addition of denaturing solvents at physiological temperatures (M. Reddy and W. R. Bauer, manuscript in preparation).

Reactivity with single-stranded DNA. Single-stranded circular φX174 DNA was cleaved to heterogeneous lengths by the vaccinia virus DNase. The activity on single-stranded DNA was not quantitatively determined, but extensive degradation of φX174 DNA was observed with less than 10% of the amount of enzyme required to nick pSM1 DNA I. Under the conditions employed, there was no detectable difference in the extent of degradation of φX174 single-stranded DNA at 37 and 57°C. This is consistent with the hypothesis, mentioned above, that the effect of elevated temperatures is to promote the formation of single-stranded regions in DNA I molecules which are recognized by the relatively heat-stable vaccinia virus DNase.

Site specificity. We have determined that the vaccinia virus nuclease cleaves superhelical DNA at specific locations. The linear reaction products formed in reaction R2 by treatment of pSM1 DNA I with the vaccinia virus enzyme were digested with *Eco*RI (which cleaves pSM1 DNA at one site) and analyzed by electrophoresis on agarose gels (Fig. 3). Discrete bands were observed, indicating that the vaccinia virus nuclease cleavage locations are highly specific. The sizes of these DNA fragments, as well as those produced by cleavage with *Bgl*II (which also cleaves pSM1 DNA at one site), were determined. By this restriction enzyme analysis,

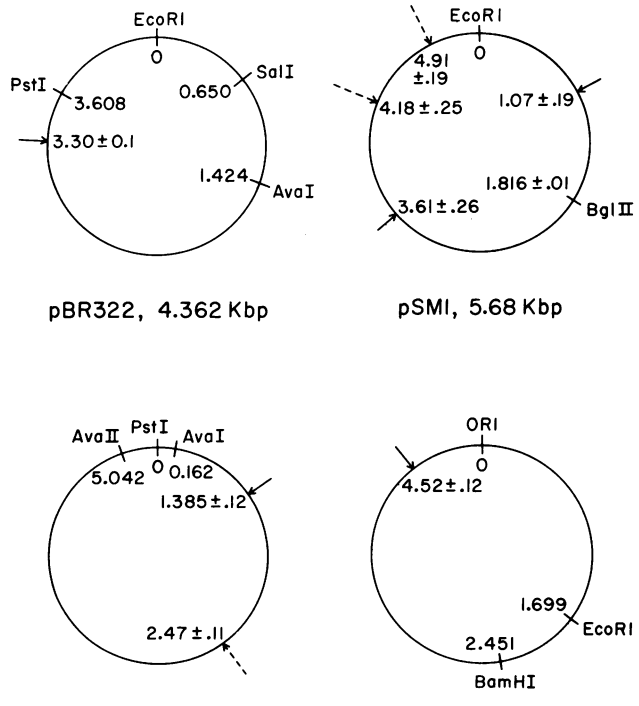

FIG. 4. Vaccinia virus DNase cleavage sites on SV40, φX174 RF, pSM1, and pBR322 DNAs. Major (→) and minor (⇢) cleavage sites (<5% by frequency) on φX174 RF and pSM1 DNAs are indicated. Distances (in kilobase pairs) measured clockwise from the unique *Eco*RI site (pSM1 and pBR322), the unique *Pst*I site (φX174 RF), and the SV40 DNA replication origin are indicated.

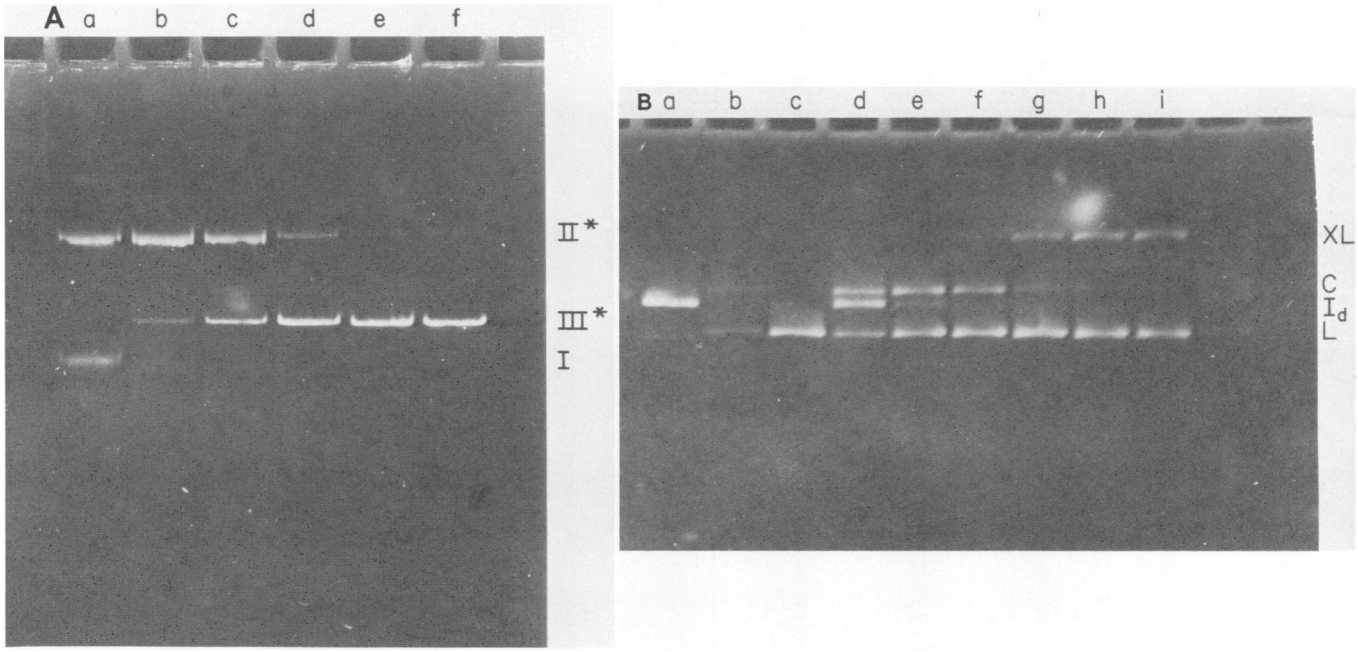

FIG. 5. Temperature dependence of vaccinia virus nuclease activity. (A) Electrophoresis on a 10-cm-long 1% agarose gel at 70 V for 3.5 h at neutral pH. Reaction products were formed by incubating pSM1 DNA I (0.24 μg) with 5 U of fraction VII vaccinia virus nuclease in a volume of 65 μl for 14 h at (a) 23, (b) 30, (c) 37, (d) 43, (e) 50, or (f) 57°C. A 20-μl portion of each reaction mixture was used per lane. (B) Electrophoresis on a 10-cm-long 1% agarose gel at 40 V for 16 h at alkaline pH. (a) pSM1 DNA I control, no added enzyme; (b) products of digestion of pSM1 DNA I with nuclease S1; (c) pSM1 DNA I linearized by digestion with *Eco*RI; (d through i) 40-μl portions (0.16 μg of DNA) of the incubation reaction products shown in lanes a through f of panel A. The bands are identified as cross-linked linear DNA (XL), single-stranded circular DNA (C), denatured DNA I (I_d), and single-stranded linear DNA (L).

the vaccinia virus DNase cleavage sites on pSM1 DNA were mapped. Two preferentially cleaved sites were located, as well as at least two additional sites which represented fewer than 5% of the cleavages. Similar analyses were performed with SV40 DNA I by using *Eco*RI and *Bam*HI. The vaccinia virus nuclease preferentially cleaved at a single site on SV40 DNA and at least three additional sites at lower frequency. φX174 replicative form (RF) DNA I was also analyzed in this way with *Ava*I and *Ava*II. The vaccinia virus nuclease cleaved φX174 RF DNA at two sites, one major and one minor. Similar mapping was performed on pBR322 DNA, with the result that a single preferred site for cleavage was found. The sites of vaccinia virus nuclease cleavage of these DNAs are shown in Fig. 4.

Cross-linking. When the final reaction products of pSM1 DNA I treated with vaccinia virus nuclease were examined by electrophoresis under denaturing conditions, a fraction of the molecules migrated as double-length single-stranded linear forms (Fig. 5B). No such structures were observed in control DNA I samples or in samples in which DNA I was first treated with nuclease S1 or *Eco*RI and then with vaccinia virus nuclease. These results suggest that the vaccinia virus nuclease formed a covalent cross-link between the two DNA strands (reaction R3). Such molecules should rapidly renature after alkaline denaturation; indeed, when vaccinia virus nuclease products were denatured in 0.2 M NaOH, neutralized with HCl, and examined by electrophoresis under native conditions, 10 to 20% of the linear products were renatured. Likewise, when the cross-linked product was electroeluted from alkaline gels, it comigrated precisely with its double-stranded unit-length DNA counterpart under neutral pH electrophoresis conditions. In contrast, all DNA II* products as well as products formed by digestion with nucleases S1 and *Eco*RI migrated as unit-length single-stranded molecules after similar treatment. The cross-linked DNA was sensitive to cleavage by nuclease S1 but resistant to proteinase K treatment.

Electron microscopy. Treatment of SV40 DNA with vaccinia virus DNase resulted in cleavage of the superhelical molecule and the formation predominantly of linear products (80% DNA III*). These DNAs were partially denatured and spread for electron microscopy as described above. Examination of 62 molecules revealed three distinct morphologies. The most common form had free single strands at both ends. Seven molecules (11%) had a closed single-stranded loop at one end (Fig. 6A, C, and D), and one molecule appeared to have closed single-stranded loops at both ends (Fig. 6B). Examination of 63 SV40 DNA molecules treated with *Eco*RI revealed only one molecule that appeared to have a closed loop at one end.

Length of the cross-linked product. The results of electron microscopy and renaturation experiments suggested that a hairpin-like structure was formed at the termini of nuclease-linearized DNA. To examine this possibility further, products from the linearization reaction (reaction R2) were examined by gel electrophoresis under alkaline conditions. The results are shown in Fig. 7 for plasmids pBR322 and pSM1. Restriction fragments from bacteriophage λ DNA cut to completion with *Hin*dIII were used as size markers, and the lengths of the products were determined from a plot of the logarithm of the distance migrated versus the quantity $(M_r)^{1/2}$ (39). The ratio of the length of the cross-linked products to that of the noncross-linked products was 1.94 and 2.07 for pBR322 and pSM1 DNAs, respectively.

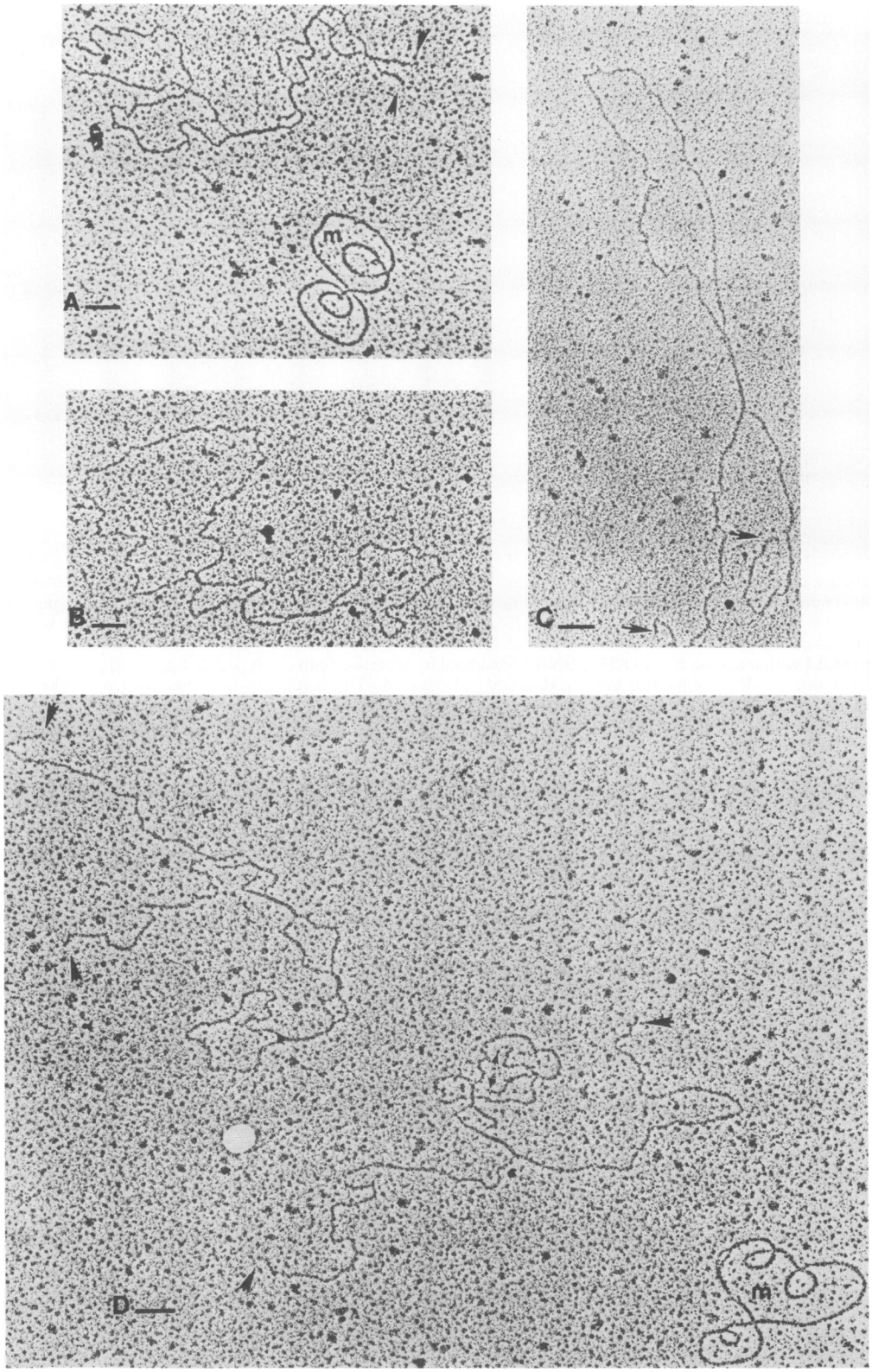

FIG. 6. Electron micrograph of SV40 DNA treated with vaccinia virus DNase and partially denatured at pH 10.9. (A and C) DNA molecules with a closed loop at one end and free single strands at the other. (B) DNA molecule in a circular configuration with single- and double-stranded regions. (D) One molecule has a closed loop at one end, the other has free single strands at both ends (arrows). pBR322 DNA I used as a size marker. Bar, 100 nm.

DISCUSSION

We have isolated from vaccinia virus cores and partially characterized a nuclease possessing a nearly neutral pH optimum and have found evidence for a much more complex pattern of activity than has been reported previously. The molecular characterization of this enzyme is not yet complete, and we have not ruled out the possibility that this is a novel nuclease, distinct from the known vaccinia virus DNases. However, our present evidence is consistent with the interpretation that the enzyme is derived from the previously reported nucleases (26, 27). In particular, our enzyme preparation had a molecular weight of 50,000 by SDS-polyacrylamide gel electrophoresis (Reddy and Bauer, manuscript in preparation), the same value as that of both the neutral and acid nucleases. In addition, our preparation of vaccinia virus nuclease eluted from hydroxyapatite columns at the same position (0.15 to 0.17 M phosphate) as that reported for the vaccinia virus acid exonuclease (33). This identical chromatographic behavior might reflect a physical association between two distinct enzymes, a possibility which has been suggested by others based on studies which detected the presence of both DNase activities at two different isoelectric points (27).

The possibility remains, however, that the acid and neutral nuclease activities are manifested by a single polypeptide which self-associates to form a dimer. Sedimentation of fraction VI of the nuclease preparation through glycerol gradients revealed activity with a sedimentation coefficient of 5.6S, a value which is in close agreement with that reported (5.7S) for acid exonuclease preparations (33). The apparent molecular weight of 105,000 determined by combined sedimentation and gel exclusion chromatography (33) might, therefore, result either from 1:1 heterologous association between two polypeptide chains of the same M_r or from homologous dimerization. The pH optimum of 6.5 is significantly different from that reported for the neutral DNase (pH 7.8), but the assay procedures were quite different. In the present communication, pH 6.5 represents the optimum for linearization of covalently closed double-stranded DNA. Previous assays involved digestion of thermally denatured single-stranded DNA to oligonucleotides. For comparison, the Bal 31 nuclease has an optimum pH for cutting single-stranded DNA of 8.8, but the optimum with supercoiled DNA is pH 8.0 (15).

In these studies we measured activity by monitoring the conversion of supercoiled DNA to nicked and linear forms. This assay method detects the hydrolysis of a single phosphodiester bond per molecule for the conversion of DNA I to DNA II*. Thus, the sensitivity of this assay (5 to 10 fmol of phosphodiester bond hydrolysis) is much greater than that of previous assays for vaccinia virus nucleases, which measured the solubilization of nucleotides from labeled DNAs after precipitation with trichloroacetic acid (27, 32). Using supercoiled DNA as the substrate, we detected nuclease activity in fractionated extracts of vaccinia virions under physiological conditions (pH 7.5, 25 mM NaCl, 37°C). The nuclease could be readily separated from the vaccinia virus type I topoisomerase by chromatography on hydroxyapatite; the nuclease elutes at 0.17 M potassium phosphate, whereas the topoisomerase elutes at 0.33 M potassium phosphate (9).

The lack of activity of the vaccinia virus nuclease with relaxed closed DNA suggests a minimum superhelix density requirement. We determined this requirement by assaying the nuclease with a series of pSM1 DNAs of various

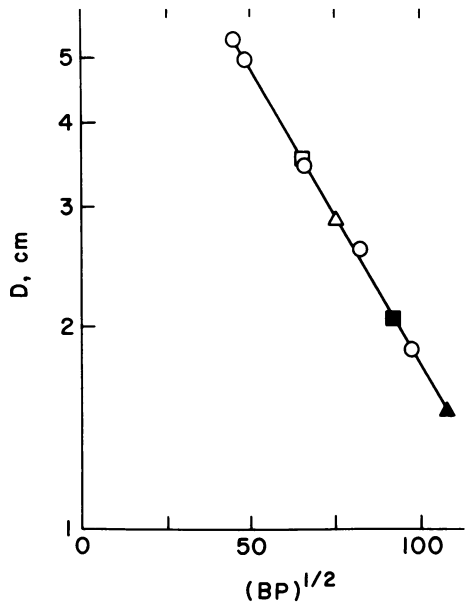

FIG. 7. Length determination of cross-linked reaction products by alkaline gel electrophoresis. The logarithm of the distance migrated (D) for the denatured DNA fragments is plotted as a function of $(M_r)^{1/2}$ (expressed as [base pairs]$^{1/2}$). ○, λ DNA cut to completion by digestion with *Hin*dIII and used as length markers; unit-length (□, △) and double-length (■, ▲) single-stranded linear DNAs formed by incubating 0.25 μg of pBR322 (□, ■) or pSM1 (△, ▲) DNA I with 10 U of fraction VI of the vaccinia virus nuclease for 12 h at 57°C. Electrophoresis was conducted on a 15-cm-long 1% agarose gel at room temperature.

superhelix densities. Nuclease activity was detected with DNAs with a negative superhelix density greater than 0.06, and greater activity was observed with increasing negative superhelix density (Fig. 1). Therefore, supercoiling must stabilize a DNA structure which can be recognized by the enzyme. Nuclease S1 cleavage of SV40 DNA I also requires a minimum superhelix density (37). These results, along with our observation that single-stranded circular φX174 DNA is readily degraded by the vaccinia virus nuclease to heterogeneous oligonucleotides, confirm that the enzyme requires single-stranded DNA. The favorable effect of elevated temperatures (57°C) on the vaccinia virus nuclease activity may be due to partial denaturation of the substrate DNA to structures of sufficient single-strandedness for enzyme recognition, coupled with the pronounced thermostability of the enzyme. This conclusion is supported by the ability of denaturing solvents, such as formamide, to act as the equivalent of a temperature increase (Reddy and Bauer, manuscript in preparation).

The vaccinia virus nuclease recognition or cleavage reactions or both are characterized by both site specificity and a requirement for single-strand character. We used small circular DNAs of known nucleotide sequence to examine the bases for these characteristics. With plasmid pSM1 DNA, the two major cleavage sites were mapped near possible stem-loop structures occurring both at the end of insertion element IS*1* (22) and at the origin of DNA replication (34). The vaccinia virus nuclease might recognize such a structure, which is expected to be stabilized by supercoiling (38). With pBR322 DNA, the vaccinia virus nuclease cleavage site did not coincide with the major S1 (17) or T7 (24) nuclease cleavage sites, both of which occur at palindromes. It did, however, map near the nuclease S1-sensitive minor

palindromic site at 3220 base pairs (bp). The vaccinia virus nuclease cleavage sites did not map near palindromic sequences in either SV40 or φX174 DNA.

It is also possible that the nuclease specificity is related to a preference for A+T-rich regions of the DNA, which are expected to denature most readily in supercoiled DNA. Indeed, the vaccinia virus nuclease cleavage sites map very close to A+T-rich regions in pSM1 (34), pBR322 (40), φX174 (36), and SV40 (30) DNAs. However, such A+T-rich regions are clearly not sufficient for specificity, because several other A+T-rich sequences occur in these DNAs which are not cleavage sites for the enzyme. Denaturation mapping experiments with SV40 DNA (11) revealed two early melting regions at 4120 and 4532 bp coinciding with two of the three locally A+T-rich clusters at 2757, 4120, and 4532 bp. The vaccinia virus nuclease cleavage site lies at only one of these early melting regions (also cut by nuclease S1 at elevated temperatures, near 4532 bp). Similar experiments with φX174 DNA show one principal early melting region (at 1300 bp), where the vaccinia nuclease cutting site is located. A+T-rich clusters occur, however, throughout the genome. Considering all the available evidence, the vaccinia virus nuclease cutting sites are invariably located in the immediate vicinity of the DNA region that is most readily denatured. These early melting regions are often, but not invariably, associated with A+T-rich clusters and with palindromes. The 104-bp termini of vaccinia virus DNA are 92% A+T and can assume two distinct hairpin structures (1). Nuclease preparations from vaccinia virus have previously been shown to cleave the terminal cross-links of vaccinia virus DNA at pH 7.9 (12).

A fraction of the linear DNA products of vaccinia virus DNase treatment migrated on denaturating gels as double-length single-stranded linear molecules, suggesting the formation of a cross-link at one end of the molecule. Similarly, a fraction of these molecules rapidly renatured after denaturation with 0.2 M NaOH and neutralization. Nuclease S1 (but not proteinase K) destroyed the cross-links formed by the vaccinia virus nuclease. Finally, electron microscopy under partial denaturing conditions and gel sizing under alkaline conditions confirmed the formation of terminal covalent cross-links in the product linear molecules. The DNA cross-linking activity of the vaccinia virus nuclease does not exhibit a requirement for Mg^{2+}, ATP, or deoxynucleotide triphosphates, all of which are required for in vitro cross-link formation in previously described systems (25, 35, 44) which require DNA polymerase and DNA ligase.

The vaccinia virus nuclease provides an excellent model for understanding certain aspects of DNA processing in eucaryotic systems. In the first place, the vaccinia virus enzyme is remarkably similar to several procaryotic multifunctional proteins, including the φX174 A protein (31), the φX174 A* protein (43), and the fd gene II protein (13). The φX174 A protein, for example, cleaves a specific A+T-rich sequence in supercoiled φX174 DNA, forms a covalent bond with the 5′ terminus of the hydrolyzed DNA strand, and subsequently rejoins the cleaved strand, resulting in formation of a single-stranded circle (6). The vaccinia virus enzyme is also multifunctional and has a repertoire of activities very similar to those of φX174 A* protein in the presence of Mn^{2+}: (i) single-strand specificity and acceptability of supercoiled DNA as substrate, (ii) site specificity, (iii) formation of nicked, linear, or relaxed circular DNAs as products, and (iv) ability to cross-link the ends of linear product DNA molecules. In the second place, the vaccinia virus replication system shows promise of serving as a model for understanding the mechanism of telomere resolution in eucaryotes. Inverted repeats constitute the replication intermediates of cross-linked telomeres; in addition to vaccinia virus (1, 20), these structures have been found in *Paramecium* mitochondrial DNA (28), in *Saccharomyces cerevisiae* telomeres (41), and in *Tetrahymena* extrachromosomal rDNA elements (4). The cloned vaccinia virus telomere is not recognized by *S. cerevisiae* replicative enzymes as a palindrome (8), indicating that the yeast and virus telomere resolution mechanisms are distinct.

The in vivo function of the vaccinia virus nuclease has not been rigorously established, but these studies, and those reported previously suggest that it may be required for the initiation and termination of viral DNA replication (1, 20). The existence of cross-linked DNA termini in other eucaryotic systems (10, 14, 23, 42) suggests that the breaking and rejoining of these DNA strands may represent a generalized mechanism for the initiation and termination of DNA replication in eucaryotes.

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