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Cleavage and circularization of single-stranded DNA: a novel enzymatic activity of ϕX174 A* protein

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ABSTRACT

Purified ØX gene A^* protein cleaves ØX single stranded DNA. The cleavage appears to be stoichiometric, whereby a gene A^* protein molecule cleaves a phosphodiester bond and binds to the DNA fragment. The size of the cleavage product was inversely proportional to the ratio of A^* protein to DNA in the reaction mixture.

The cleavage of the DNA resulted in the formation of an A^* protein - ssDNA complex identified on SDS-polyacrylamide gels and by banding in CsCl.

An A^* protein-ssDNA complex was isolated by gel filtration and shown to be active in a ligating reaction in which the two ends of the DNA fragment were joined to form a covalently closed circle. The joining reaction required Mg^{++} ions and was accompanied by the release of the protein from the DNA.

INTRODUCTION

Two polypeptides (A protein, 59 Kdal and A^* protein, 32.5 Kdal), read in the same coding frame, are coded for by the A gene ØX 174 bacteriophage (1). The A protein required in vivo for initiation of duplex RF replication and single stranded DNA synthesis (2-4) displays in vitro a multifunctional role in the replication of RF DNA (5): I, it cleaves the viral (+) strand of supertwisted RFI DNA at the origin of replication (5-8); II, it participates in the formation and movement of replication fork in a complex with rep protein (5); III, it terminates a round of replication by cutting out a unit length viral strand with simultaneous circularization of the DNA to form a covalently closed viral circle (5).

In addition to supertwisted RFI DNA, A protein cleaves ØX ssDNA and forms a tight, presumably covalent complex with the cleaved DNA (9, 10).

Although, a great deal is known today about the role of the A protein in DNA replication, the function of the A^* protein in the life cycle of ØX 174 is not clear. Recently, it was reported that purified A^* protein has double stranded DNA binding and

single stranded DNA specific nuclease activity (9, 11).

We were interested in analyzing the properties of A^* protein in vitro hoping to learn about the mechanism of action of the A protein in the replication of RF DNA.

Here we report some of our findings regarding a new enzymatic activity of purified A^* protein.

MATERIALS AND METHODS

Enzymes: DNase I was from Worthington; proteinase K from Boehringer; ØX 174 A^* protein was purified from ØX infected E. coli cells to better than 90% purity as judged by coomassie blue staining. It migrated as a single band on SDS-polyacrylamide gel of about 32500 daltons. Details of purification scheme will be published elsewhere.

Preparation of ØX 174 single stranded DNA: Radioactively labeled viral (+) ssDNA was prepared in vitro in a reaction containing: ØX supertwisted RF I DNA, rep protein, gene A protein, ssDNA binding protein and DNA polymerase III holoenzyme essentially as previously described (5). ssDNA from phage particles was prepared by published procedures (12). All DNA preparations were purified through an alkaline sucrose gradient and phenol extracted.

Preparation of A^* protein-ssDNA complex

^3H or ^{32}P-labelled ssDNA (in vitro synthesized) was mixed with at least a ten-fold molar excess of ssDNA extracted from purified phage particles, and cleaved by A^* protein as described in legend to Fig. 1. The reaction was stopped by adding EDTA and NaCl to 50 mM and 1 M final concentration respectively. The A^* protein-ssDNA complexes were purified on a Bio gel P-100 column (10 ml) equilibrated with a buffer containing: 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 M NaCl and 2% sucrose. A peak of radioactively labelled material (void volume) was pooled and dialyzed against a buffer of 50 mM Tris·HCl, pH 8.0, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M NaCl and 2% sucrose for 90 minutes. All operations, except where indicated, were carried out at 0-4°C.

DNase I treatment of A^* protein-ssDNA complex: The reaction mixture (50 μl) contained: 100 mM Tris·HCl buffer, pH 8.0, 5 μg carrier salmon sperm DNA, 100 μg/ml BSA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, A^* protein-ssDNA complex and 2 μg of DNase I. The incubation was at 37° for 60-90 minutes.

Proteinase K treatment of A^* protein-ssDNA complex: The reaction mixture (50 μl) contained: 100 mM Tris·HCl buffer, pH 8.0, 0.5% SDS, 4 mM EDTA, 10 μg BSA, A^* protein-

ssDNA complex and 2-5 μg of proteinase K. Incubation was for 2-4 hours at 37°.

RESULTS

A* protein cleaves single-stranded DNA

^{32}P-labelled ssDNA, prepared as described in Materials and Methods, was incubated with purified A* protein and the products were analyzed by sedimentation on alkaline sucrose gradients. The incubation resulted in production of DNA fragments, heterogenous in size (Fig. 1a, b) with an average sedimentation coefficient of 8-9 S. The size of the fragments was influenced by the ratio of enzyme to substrate present in the reaction mixture. With an increased level of substrate, at a constant amount of enzyme, an increase in the size of the fragment was observed (Fig. 2). Longer incubation did not result in additional cleavage of the DNA.

These results suggested that A* protein acts stoichiometrically, whereby it cleaves a phosphodiester bond, binds to the cleaved DNA and does not turn over.

Formation of an A* protein-ssDNA complex

Two different experiments have indicated that an A* protein-ssDNA complex is formed. In the experiment described in Fig. 4a, A* protein-^{3}H-labelled ssDNA complexes (produced as described in Materials and Methods) were mixed with ^{32}P-labelled DNA (extracted from phage particles) and banded to equilibrium in CsCl. A substantial shift of the bulk of ^{3}H-labelled DNA to a lighter density position was observed. That this shift in density is due to the association of a protein to DNA was verified by treating these fragments with proteinase K. The ^{3}H-labelled DNA fragments, after proteinase K treatment, cobanded with protein free ^{32}P-labelled DNA marker (Fig. 4c).

In another experiment, ^{32}P-labelled DNA was cleaved with A* protein followed by digestion with DNase I (Fig. 3). The TCA precipitable ^{32}P-labelled material was electrophoresed on SDS-polyacrylamide gels. A ^{32}P-labelled band comigrated with an A* protein marker after DNase I treatment (Fig. 3). The ^{32}P-labelled band was slightly diffused (Fig. 3), presumably because a various number of nucleotides remained linked to the protein. The band dissappeared upon proteinase K treatment (data not shown), confirming that the ^{32}P-label is attached to a protein.

The DNA ligase activity of A* protein

Purified A* protein-ssDNA complexes (prepared as described in Materials and Methods) were incubated at 30°C in the presence of either EDTA or Mg^{++} followed by banding to equilibrium in CsCl. The bulk of the ^{3}H-labelled complexes banded at a

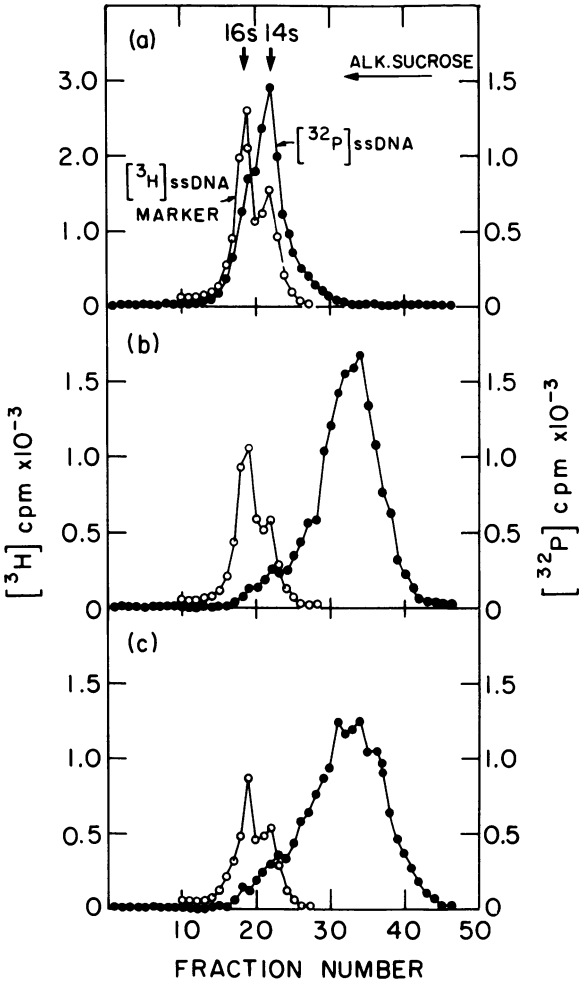

Fig. 1. Sedimentation analysis of ssDNA cleaved by A* protein

^{32}P-labelled ssDNA (25-50 pmol nucleotides) mixed with unlabelled DNA (0.48 μg) was incubated with A* protein in a reaction mixture (100 μl) containing: 0.15 M Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 μg BSA, 10 mM 2-mercaptoethanol and 1.5 μg of A* protein. The reaction was incubated at 30° for 10 minutes, and A* protein-ssDNA complexes purified as described in Materials and Methods. Samples of A* protein-ssDNA complexes were incubated in the presence of either 40 mM EDTA or 5 mM $MgCl_2$ for 30 min at 30°C, then mixed with EDTA, NaOH, sarcosyl, and ^{3}H-labelled ssDNA marker, and sedimented through an alkaline sucrose gradient, essentially as previously described (10). (a) Sedimentation profile of ssDNA incubated without A* protein; (b) sedimentation profile of A* protein-ssDNA complex incubated in the presence of EDTA; (c) sedimentation profile of A* protein-ssDNA complexes incubated with $MgCl_2$.

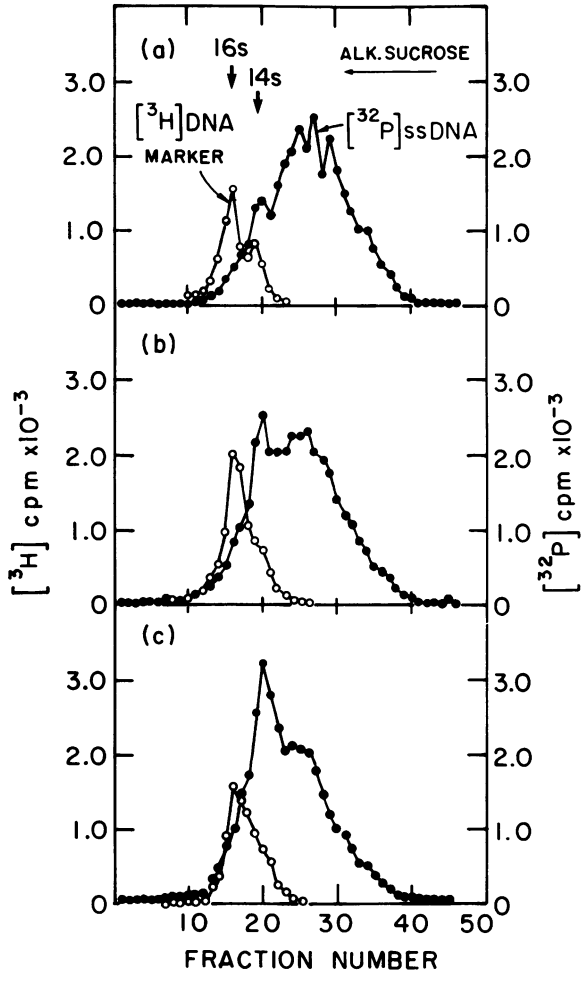

Fig. 2. Size of cleaved DNA influenced by enzyme to substrate ratio

ØX ssDNA was cleaved in a reaction mixture (25 µl) containing: 0.15 M Tris·HCl, pH 7.5, 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol, 100 µg/ml BSA; 0.42 µg A* protein; 20 pmols nucleotides of in vitro synthesized ^{32}P-labelled ssDNA and unlabelled ss circular DNA, panel (a) 0.48 µg ssDNA; panel (b) 0.96 µg ssDNA; panel (c) 1.92 µg ssDNA. The reactions were incubated for 10 min at 30°C and stopped by adding EDTA, NaOH and sarcosyl to a final concentration of 50 mM, 0.2 M and 1% respectively. ^{3}H-labelled ssDNA marker (extracted from phage particles) was added and samples centrifuged as in Fig. 1.

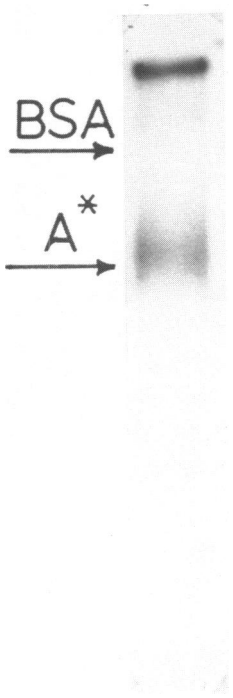

Fig. 3. Electrophoresis of A* protein-ssDNA complex on polyacrylamide gels:

A* protein-ssDNA complexes were prepared by cleaving ^{32}P-labelled ssDNA and purified as described in legend to Fig. 1. The complexes were treated with DNase I as described in Materials and Methods and the reaction stopped by adding an equal volume of a 10% TCA solution. The protein precipitate was collected by centrifugation, washed three times with a 10% TCA solution followed by a wash with ether. The pellet was dissolved in buffer containing 50 mM Tris·HCl, pH 8.0; 20% glycerol; 50 mM EDTA; 50 mM 2-mercaptoethanol and 1% SDS; boiled for 2 minutes and electrophoresed on SDS-polyacrylamide gels as described elsewhere (10). A separate sample, which contained free A* protein (7 μg), BSA (5 μg) was treated identically. The protein bands of marker proteins were identified by coomassie blue staining and the position of the center of the corresponding bands is indicated by the arrows. The ^{32}P labelled band, after DNase I digestion was identified by autoradiography as previously described (10).

lighter density than naked DNA (Fig. 4a). However, as a result of the incubation with Mg^{++} a fraction of the complexes appear to have lost the protein and cobanded with a DNA marker (Fig. 4b). Since the A* protein binds to the 5' end of the cleaved DNA fragment (5' end blocked for polynucleotide kinase action) the release of the protein could have been accomplished by an intramolecular covalent joining of 3' and 5' ends. During such a reaction new covalently closed circles are expected to form, which should result in an increased sedimentation rate of the DNA on alkaline sucrose gradients. As expected, the incubation of the A* protein-ssDNA complexes with Mg^{++} resulted in a more disperse ^{32}P-labelled peak when sedimented through an alkaline gradient with a shoulder of relatively faster sedimenting DNA fragments (Fig. 1b, c).

Electron microscope analysis of the products of the joining reaction

ØX ssDNA (more than 80% circular) was cleaved by A* protein and the A* protein-ssDNA complexes purified as described in Materials and Methods. The complexes were

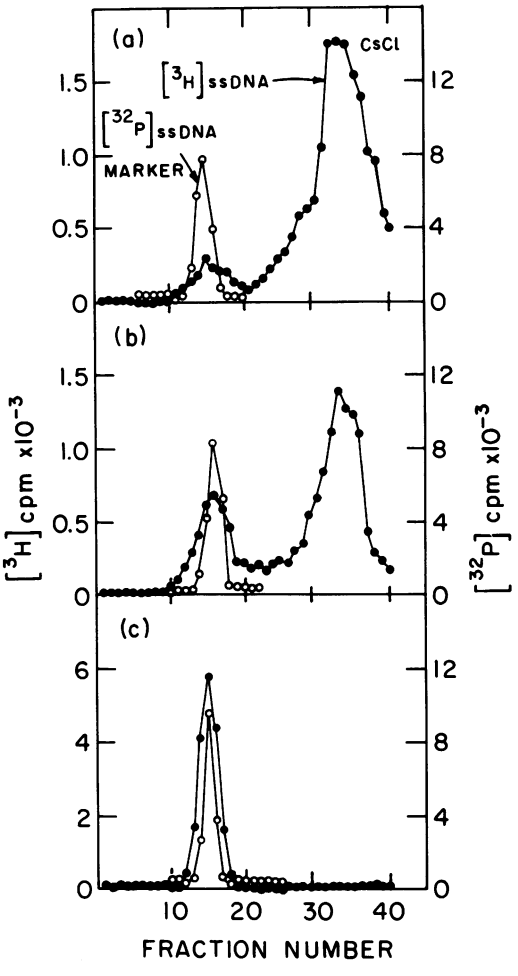

Fig. 4. Analysis on CsCl gradients of the ligating activity of A* protein-ssDNA complex

A* protein-ssDNA complexes were prepared as described in Materials and Methods except that ^{3}H-labelled ssDNA was cleaved by A* protein. Aliquots were incubated at 30° C for 30 minutes in the presence of either 40 mM EDTA (panel a) or 5 mM $MgCl_2$ (panel b) and one aliquot treated with proteinase K (panel c) as described in Materials and Methods. To all reactions 2.9 ml of a buffer containing: 50 mM Tris·HCl, pH 8.0 μg/ml BSA, 0.1% sarcosyl, 40 mM EDTA and ^{32}P-labelled DNA marker was added. Each sample was mixed with 3.6 gr CsCl and centrifuged in the SW 50.1 rotor at 40 K rpm, 20°C for 46 hours. 0.1 ml fractions were collected from the bottom of the tube on GF/C glass fiber filter. Each filter was washed in a 10% TCA solution followed by a wash in ethanol and radioactivity measured in a Toluene based scintillation fluid.

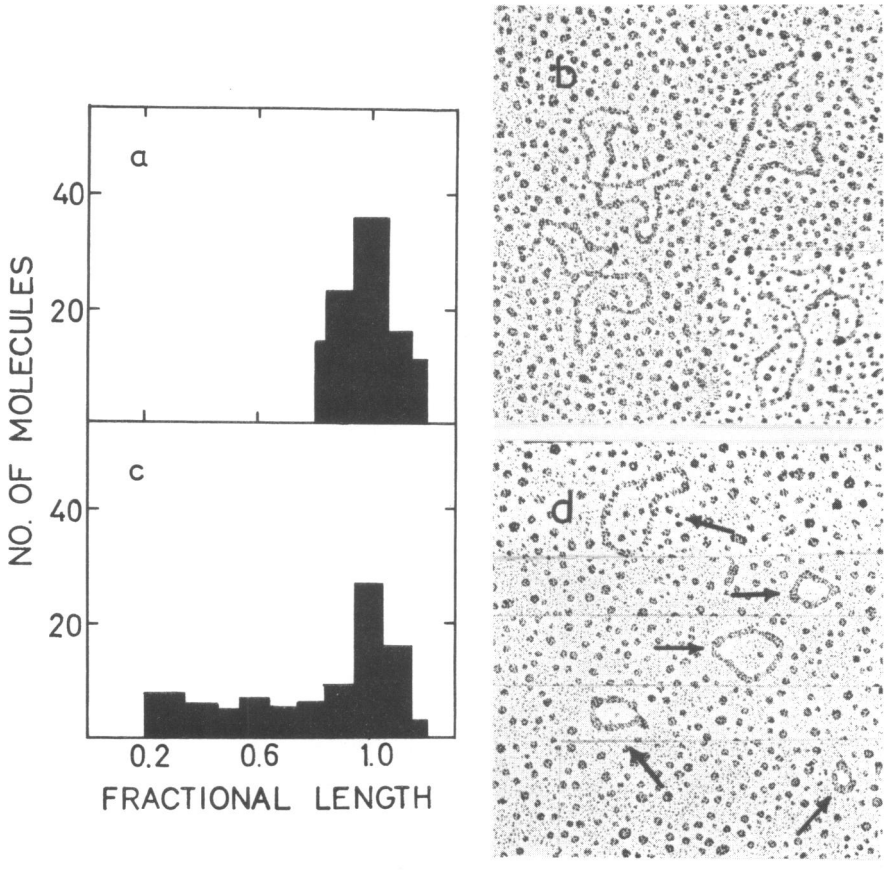

Fig. 5: Electron microscope analysis of the circularization reaction

ØX ssDNA was cleaved by A* protein as in Fig. 2c and A* protein-ssDNA complexes were purified as described in Materials and Methods. The circularization reaction contained: 50 mM Tris, pH 8.0, 10 mM mercaptoethanol, 5 mM $MgCl_2$, 2% sucrose, 0.2 to 0.4 M NaCl and 5-10 μg/ml A* protein-ssDNA complexes. The incubation was at 30° for 30 minutes followed by the addition of EDTA to 40 mM final concentration. In a separate reaction A* protein-ssDNA complexes were incubated in the presence of 40 mM EDTA. All reactions were diluted 10-20 fold into a spreading solution containing: 40% formamide, 0.1 M Tris, pH 8.5, 10 mM EDTA and 0.1 mg/ml cytochrome c. The samples were mounted on grids for EM analysis by the previously published technique (15). A Philips 300 electron microscope was used and DNA molecules were photographed at a magnification of 10,000. The negative plates were enlarged 5-fold, DNA molecules redrawn on paper and contour length of circular DNA was measured. (a) and (b) show a histogram of size distribution of circular ssDNA (100 molecules) and a picture of selected ssDNA circles present in the reaction prior to cutting by A* protein. (c) and (d) show a histogram of size

distribution of circular ssDNA (100 molecules) and a picture of selected smaller than unit-length ssDNA circles seen in the EM after incubating A* protein-ssDNA complexes with Mg^{++}.

In order to estimate the extent of circularization in the different reactions no less than 200 DNA molecules were scored in the electron microscope. Prior to cleaving more than 80% of the DNA was circular. In the cleaved sample (incubated in the presence of EDTA) 29%, the majority of which was of ØX unit length DNA, and in the recircularization reactions, in the presence of 0.1 M and 0.4 NaCl, the amount of circles increased to 42% and 55% respectively. The EM analysis was carried out for at least three different experiments with similar results.

incubated at 30°C in the presence of Mg^{++} to form circular DNA. Samples were mixed with a solution containing 40% (final concentration) formamide and mounted on grids for electron microscope analysis. In the cleaved DNA sample, (cleaved under conditions which do not allow extensive cutting of the DNA, Fig. 2C), 29% of all DNA molecules seen were circular. More than 80% of these circles were of unit length ØX DNA, presumably not cleaved by the A* protein. In two separate circularization reactions the frequency of circles increased to 42% and 54%, indicating that new circles were formed. The newly formed circles were heterogenous in size ranging from 1/5 to full length ØX DNA (Fig.5). A small amount (10-15%) of shorter than unit-length circles were also seen in the ssDNA cleaved sample which was not incubated with Mg^{++}, suggesting that some circularization occurred during the cleavage reaction.

DISCUSSION

We have demonstrated a novel enzymatic activity of the ØX 174 A* protein; the cleavage and ligation of single-stranded DNA. The reaction appears to proceed in two stages; in the first stage, A* protein cleaves single stranded DNA to form linear DNA fragments, and binds tightly to the cleaved DNA. We presume that the protein binds to the 5' phosphorylated end of the DNA fragment since the 3' end is free for terminal transferase activity and the 5' end is blocked for polynucleotide kinase action (9, unpublished observations). In the second stage joining of the 3' and 5' ends occurred with the concomitant release of the bound protein (Fig. 4). The joining reaction required Mg^{++} ions only and no further addition of A* protein. The products of the joining reaction were covalently closed single-stranded circles, heterogenous in size and smaller than unit length ØX DNA. From these results we infer that the joining reaction was carried out by the bound A* protein.

The stability of the A* protein-ssDNA complex (Fig. 3 and 4) and the ability of the

bound A^* protein to restore a phosphodiester bond without any exogenously added source of energy suggests that the binding of protein to DNA is via a high energy covalent bond. A requirement for a high energy protein-DNA covalent bond was previously suggested for the topoisomerases as an intermediate in their nicking closing action (13, 14).

A^* protein does not cleave superhelical RFI DNA and cannot substitute for the A protein in the replication of duplex DNA (unpublished observations). Thus, the N-terminal part of the A protein is essential for the replication activity of the enzyme, probably playing a role in the specific recognition of the origin replication in RFI DNA and the interaction with rep protein to generate a replication fork during the unwinding of duplex DNA.

However, we believe that the single-stranded cleaving and ligating activity of the A^* protein illuminates the action of A protein in termination of a round of replication (5), where it was proposed that A protein, bound to the 5' end of the viral(+)strand, in a complex with rep protein will displace the regenerated origin from it's complementary strand, cut out a unit-length viral DNA strand, and circularize it by joining 3' and 5' ends using the energy of the protein-DNA covalent bond.

Although, an in vivo function for A^* protein cannot be directly inferred from our results, such an ssDNA cleaving-ligating activity might be in volved in vivo in generating deletions or insertions by removing or inserting single strand DNA fragments into replicating DNA molecules. This hypothesis is currently being investigated. Also, we believe that this work will be instrumental in dissecting the various activities of A protein in the replication of duplex DNA.

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ABBREVIATIONS

A and A^* proteins - products of the gene A of ØX 174 bacteriophage
BSA - bovine serum albumin; EM - electron microscope; RF - replicative form
ssDNA - single stranded DNA; SDS - sodium dodecyl sulfate; TCA - trichloroacetic acid

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