ϕ X174 *cis*tron A protein is a multifunctional enzyme in DNA replication*

(*cis*A–RF II complex/origin/strand separation/*rep* protein)

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ABSTRACT The *cis*tron A protein induced by phage ϕ X174 nicks (produces a single-strand break in) the viral strand of the superhelical ϕ X duplex DNA, thereby forming a complex with the DNA. The protein, seen bound to the DNA in the electron microscope, was located in the restriction endonuclease fragment between nucleotides 4290 and 4330 on the ϕ X map [Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchison, C. A., III, Slocumb, P. M. Y. & Smith, M. (1977) *Nature* 265, 687–695]. Replication also was initiated at this point, thus identifying the site of *cis*tron A protein nicking and binding as the origin of replication.

The *cis*A–DNA complex (separated from free *cis*tron A protein), upon the addition of *Escherichia coli* *rep* protein, ATP, and DNA binding protein, is unwound to generate a single-stranded linear [presumably the nicked (+) strand] and a circular [presumably the (–) strand] molecule. The *cis*A–DNA complex, upon the further addition of DNA polymerase III holoenzyme and deoxynucleoside triphosphates, supports replication to generate viral, single-stranded circles, as many as 15 circles per *cis*A–DNA complex.

The replicating intermediates seen in the electron microscope are a novel form of “rolling circle” [Gilbert, W. & Dressler, D. H. (1969) *Cold Spring Harbor Symp. Quant. Biol.* 33, 473–485]. The 5' end (presumably with the *cis*tron A protein bound to it) is locked in the replication fork and loops back to accompany the strand-separation and replication fork around the template [(–) strand] circle. Thus, the multiple functions of *cis*tron A protein include: (i) nicking the viral strand at the origin of replication to initiate a round of replication, (ii) participating in a complex which supports fork movement in strand separation and replication, (iii) nicking again at the regenerated origin to produce a unit-length DNA, and (iv) ligating the newly generated 3'-OH end to the 5'-phosphate-complexed end to form a circular viral molecule.

The life cycle of ϕ X174 bacteriophage includes three stages of DNA replication: I, conversion of the single-stranded (SS), viral (+) circle to a circular duplex, replicative form (RF); II, multiplication of the RF; and III, synthesis of viral strands using the complementary (–) strand of RF as template (3).

Crude enzyme systems sustain RF replication *in vitro*, using exogenously added ϕ X174 superhelical RF I DNA (4, 5). These enzyme systems provided assays for the purification of *cis*tron A protein of ϕ X174 and *Escherichia coli* *rep* protein (4, 6), known from genetic studies to be required for RF replication and SS DNA synthesis *in vivo* (7–10).

The attempt to reconstitute RF replication with purified proteins led to the discovery that four proteins, namely, *cis*tron A (*cis* A) protein, *rep* protein, *E. coli* DNA binding protein (DBP; ref. 11), and DNA polymerase III holoenzyme can use ϕ X174 superhelical RF I DNA to synthesize viral (+) circles in quantities that far exceed the input template (12, 13). In the

absence of holoenzyme, the other three proteins catalyze the separation of the two strands of superhelical RF I DNA (13).

We describe here a multiplicity of functions for the *cis*A protein in the replication of ϕ X DNA, including nicking (production of an SS break), ligation, and participation in a novel replicating structure.

MATERIALS AND METHODS

Source of Enzymes. ϕ X174 *cis*A protein, *rep* protein, holoenzyme, and DBP were prepared as described (13). Restriction endonucleases *Pst* I and *Hae* III were purchased from New England Biolabs; *Hind*III was a gift of Stephen Goff of this department.

Isolation of *cis*A–RF II Complex. Superhelical ϕ X RF I DNA was nicked by *cis*A protein to yield RF II DNA in a reaction mixture (100- μ l volume) containing 50 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol, 10 mM MgCl₂, 1.85 μ g of ³H-labeled ϕ X RF I DNA (14), and 1.8 μ g (7900 units) of *cis*A protein. Incubation at 30° for 20 min was stopped by addition of EDTA to 50 mM and NaCl to 1 M. The mixture was sedimented through a neutral sucrose gradient (14) at 50,000 rpm at 0° for 3 hr. About 80% of input RF I in the reaction was converted to RF II (16S) DNA.

Formation and Replication of *cis*A–RF III Complex. ϕ X174 RF I DNA treated with *cis*A protein (above) was cleaved by *Pst* I restriction endonuclease (6 units for 20 min at 30°). The RF III DNA (linear ϕ X unit-length duplex DNA) was purified through a neutral sucrose gradient (14) and reacted with DBP, *rep* protein, and holoenzyme in a 250- μ l reaction mixture containing 50 mM Tris-HCl (pH 8.1), 10 mM MgCl₂, 4 mM dithiothreitol, 800 μ M ATP, 50 μ M each of dATP, dTTP, dGTP, and [α -³²P]dCTP (3000 cpm/pmol), 0.2 mg of bovine serum albumin per ml, 6 μ g of DBP, 10,000 units of *rep* protein, 200 units of holoenzyme (G4 replication assay), and 4 μ g of RF III DNA per ml. The reaction at 30° for 2.5 min was stopped by addition of EDTA to 50 mM.

³²P-Labeled RF III DNA was purified in a neutral sucrose gradient in the SW 56 rotor at 15° for 3 hr, and concentrated with 2-propanol (14).

Electron Microscopy. DNA–protein complexes were fixed in the reaction mixture with 1% formaldehyde (15 min at 0°), followed by 0.6% glutaraldehyde (15 min at 0°), and examined by electron microscopy as described (15).

Abbreviations: *cis*A protein, *cis*tron A protein; DBP, *E. coli* DNA binding protein; holoenzyme, DNA polymerase III holoenzyme; RF, replicative form DNA; RF I, covalently closed, circular superhelical ϕ X DNA; RF II, a circular DNA molecule with at least one discontinuity in one of the strands; RF III, a linear duplex DNA molecule of ϕ X unit length; SS, single strand.

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FIG. 1. Specific location of the *cisA* protein-RF II complex. The complex was prepared (see *Materials and Methods*) by incubating ϕ X174 superhelical RF I with *cisA* protein; it was fixed with formaldehyde and glutaraldehyde, and purified by sedimentation through a neutral sucrose velocity gradient. (A) *cisA*-RF II complexes. Samples from pooled peak fractions containing RF II DNA were mounted directly on grids for electron microscopic analysis (15). (The distribution of protein-DNA complexes was similar when fixation by formaldehyde and glutaraldehyde was omitted.) Although the background of particulate material was variable, the frequency of unreacted DNA molecules with an attached knob was less than 1%. (B) A complex cleaved by *Pst* 1 nuclease. To locate the knob on the RF molecule, the *cisA*-RF II complex was diluted to 10% of its original concentration with 50 mM Tris-HCl (pH 8.1) and cleaved with *Pst* 1 restriction endonuclease in a mixture of 50 mM Tris-HCl (pH 8.1), 10 mM MgCl₂, 0.1 M NaCl, 0.1 μ g of DNA, and 6 units of *Pst* 1 nuclease. The reaction, carried out at 30° for 20 min, was stopped by addition of EDTA and NaCl to 50 mM and 1 M, respectively. The DNA was mounted directly on grids for electron microscopic analysis. (C) Distance of the "knob" from the near end of the *Pst* 1 cut (B) was determined for 50 molecules (15). The DNA length from the knob-center to the nearest end is expressed as a percent of the total length of the molecule. The location, relative to the nearest end, is 19.82 ± 0.38 (SEM) (1065 ± 20 nucleotides), based on the value of 5375 nucleotides for ϕ X DNA (1).

RESULTS

***cisA* Protein Nicks RF I to Form a Complex at the Replication Origin.** Purified, phage-induced *cisA* protein (13) was shown, as observed earlier (6, 16), to nick ϕ X174 RF I DNA. Inasmuch as only viral (+) strands are synthesized *in vitro*, we inferred (12), as had others (16, 17), that the nick is in the viral strand. Ikeda *et al.* (6) also demonstrated with crude enzyme fractions that a *cisA*-RF complex was formed that could support incorporation of dNTPs.

FIG. 2. Location of origin of replication by restriction endonuclease analysis. ³²P-labeled RF III DNA (see *Materials and Methods*) (150 pmol) was treated with the *Hind*II or the *Hae* III restriction enzymes in a 25- μ l reaction mixture containing: 10 mM Tris-HCl (pH 8.1), 10 mM MgCl₂, 10 mM dithiothreitol, 25 mM NaCl, 40 μ g of bovine serum albumin per ml, 0.6 μ g of unlabeled ϕ X174 RF I DNA marker, and 6 units of either nuclease. The reaction was carried out at 37° for 3 hr; the resulting fragments were electrophoresed on Agarose/acrylamide gels (14); slab gels of 10 $\times$ 10 cm were used. The products of each cleavage were identified by ethidium bromide staining and autoradiography of the ³²P-labeled DNA. The radioactivity in the R3, R8, and R5 fragments of the *Hind*II digest, and the Z6b + Z6a, Z9, Z10, and Z3 fragments of the *Hae* III digest was determined by slicing the gel into 3-mm slices and measuring radioactivity directly in a Nuclear-Chicago gas-flow counter. The number of labeled deoxycytidylate residues in the R3 band was determined using as reference the known number of dC residues [from the sequence map (1)] and recovered radioactivity in the R5 fragment; similarly, Z3 served as a reference standard for Z6b + Z6a. The smaller fragments were not reliable as standards because of variability in recovering them from the gel. In the diagram, the *Hind*II and *Hae* III cleavage sites near the right end of the *Pst* 1 cleavage maps of ϕ X RF I are those of Sanger *et al.* (1). The heavy line in the duplex DNA represents the stretch of newly synthesized, ³²P-labeled viral (+) DNA.

A *cisA*-RF II complex prepared by incubating pure *cisA* protein with superhelical RF I and Mg²⁺ was purified by sedimentation and examined by electron microscopy (Fig. 1). Of 100 DNA molecules observed, 69 had a single globular "knob" attached, 26 had none, 4 had 2, and 1 had 3 (Fig. 1A). Treatment with restriction endonuclease *Pst* 1 converts an RF circle to a linear duplex DNA of ϕ X unit-length (1) (RF III). The knob on the *cisA*-RF II complex was invariably near one end of the DNA (Fig. 1B). By measuring the distance from its center to each end of the molecule, the knob was located precisely at $19.82\% (\pm 0.38\%)$ from the nearest end or 1065 ± 20 nucleotides on the Sanger map (1) from residue 5375 at one end of the restriction cut (Fig. 1C). After treatment of the *cisA*-RF II complex with the *Hae* III restriction nuclease (see below), the protein knob was associated with DNA lengths of about 300 residues, corresponding to the size of the Z6b fragment (data not shown).

The linear *cisA*-RF III complex served as a substrate for replication upon addition of DBP, *rep* protein, holoenzyme, ATP, and dNTPs (including [³²P]dCTP). The strand containing the newly synthesized DNA was 14 S on alkaline sucrose (data not shown), corresponding to the size of ϕ X unit-length linear

FIG. 3. Intermediates in the RF II strand separation and replication reactions. (A, B, and C) Strand separation. *cisA*-RF II complexes were obtained (see *Materials and Methods*) and dialyzed against 50 mM Tris-HCl (pH 8.1), 50 mM NaCl, 4 mM dithiothreitol, and 0.1 mM EDTA for 60–90 min in collodion bags. Dialyzed complexes were mixed at 0° with *rep* protein, DBP, and ATP as in legend of Fig. 4, except that holoenzyme and dNTPs were omitted. After either 30 sec or 15 min at 30°, the mixture was chilled in ice, treated with EDTA to 50 mM, and fixed with formaldehyde and glutaraldehyde (see *Materials and Methods*). The protein-DNA complexes were purified through a neutral sucrose gradient (sedimenting faster than ϕ X RF II) and mounted directly for electron microscopic analysis (15). (A and B) Examples (at 30 sec) of progressive levels of strand separation of the RF II DNA. (C) Final product after 15 min. (D, E, and F) Replication. Replicating intermediates were obtained as in legend of Fig. 4. DNA in a pool of fractions 12–15 was mounted on grids directly for electron microscopic analysis. The molecules in D and E are from the pool of fractions 12–15. Intermediates with free tails were not observed. F is the final product of the replicating reaction, the viral SS circles obtained as in legend of Table 1. The thickness of the SS DNA is due to coating with DBP; the contour length of the DBP-coated SS DNA is contracted by a factor of 2.56 (21); the percentages shown in A–F describe the extent of strand separation and replication as determined by measuring the contour length of the displaced SS loop (corrected for contraction by DBP).

DNA. Thus the initiation of viral (+) strand synthesis was by extension of the 3'-OH end of the parental viral (+) strand generated by the nicking action of the *cisA* protein.

Cleavage by the *Hae* III restriction endonucleases showed (Fig. 2) that the only region replicated was that from which Z6b, Z6a, Z9, Z10, and Z3 fragments of the *Hae* III digest and R3, R8, and R5 fragments of the *Hind*II digest were derived. This result could have been obtained only if the synthesis had been initiated in the region of the Z6b and Z3 fragments and had then proceeded clockwise up to the *Pst* 1 restriction site (1).

The amount of 32 P recovered in the unresolved Z6b + Z6a fragments was compared with that in the Z3 fragment. From the number of deoxycytidylate (dC) and total residues known to be in each of these fragments (1), there is a deficiency of 27 [32 P]dC residues in the Z6b + Z6a fraction. Thus, synthesis was initiated at 19–20% of a ϕ X length from the *Pst* 1 restriction cut. By a similar calculation, there is a deficiency of 26 dC

FIG. 4. Purification of replicating intermediates by sedimentation through a neutral sucrose gradient. Replicating intermediates were synthesized in a 30-sec reaction as in replication of the *cisA*-RF III complex. The reaction was quenched and fixed (see legend of Fig. 3), layered on a 5–20% neutral sucrose gradient, and centrifuged at 50,000 rpm at 5° for 2 hr (see Table 1). Sedimentation is from right to left.

residues in the R3 fragment using the R5 DNA fragment as a reference. Both results agree with the electron microscopic determination of the site where the knob, presumably representing the *cisA* protein, is bound.

Thus, the site at which *cisA* protein nicks and binds is the origin of replication inferred from analyses *in vivo* (14, 18). Because the polarity of the complementary (–) strand is 3' → 5' when the direction of replication is represented as clockwise (5' → 3'), the strand synthesized must be the viral (+) DNA, thus confirming that the nick by *cisA* protein is in the viral (+) strand.

***cisA*-RF II Complex in Conjunction with DBP, *rep* Protein, and ATP Catalyzes Complete Unwinding of the Duplex.** Catalytic strand separation of ϕ X RF I DNA, uncoupled from replication, depends on the *cisA* protein, DBP, and the ATP-utilizing *rep* protein (13). Here we show that *cisA*-RF II complex, in the absence of free *cisA* protein, sustains separation of the two strands of RF II DNA upon the addition of DBP, *rep* protein, and ATP (Fig. 3 A–C).

The products of this reaction seen by electron microscopy were the linear SS [presumably viral (+) DNA], and the circular SS [presumably complementary (–)] in near equal numbers (186 and 213, respectively) (Fig. 3C).

Intermediate structures were seen when the incubation was interrupted after 30 sec at 30° and the proteins interacting with the DNA were fixed with formaldehyde and glutaraldehyde (Fig. 3 A and B). *Single-stranded DNA is identified as the thick fiber coated with DBP.* All intermediates had an SS loop, presumably the viral (+) strand, coming off a duplex circle, having exposed in the process an SS stretch, presumably on the complementary (–) DNA. Intermediates with free ends were not found. The ratio of the contour length of the displaced (+) strand loop to that of the SS portion of the (–) strand was 1.0 ± 0.12 for 24 molecules measured.

Replicating Intermediate Is a “Looped” Rolling Circle. Replicating intermediates were prepared by incubating a *cisA*-RF II complex with DBP, *rep* protein, holoenzyme, [32 P]dCTP, dATP, dTTP, dGTP, and ATP for 30 sec at 30° (see Fig. 4). After fixation, the labeled DNA was purified by sedimentation (Fig. 4). Most of the labeled replicating intermediates sedimented in a broad peak ahead of the RF II DNA. The complexes in a pool of fractions 12–15 (Fig. 4) were analyzed by electron microscopy (Fig. 3 D and E).

All replicating intermediates had an SS, DBP-coated loop coming off a duplex circle (Fig. 3 D and E). Contour lengths

FIG. 5. A scheme for *cisA* protein action illustrating its multiple functions. The *cisA* protein initiates a round of replication by nicking specifically the viral (+) strand at the origin of replication. In the strand separation reaction, uncoupled from replication, the *cisA* protein, bound to the 5' end, participates in the unwinding of the two strands. In replication, this complex with the bound 5' end travels around the complementary (-) template, separating the two strands in advance of replication. After completing a full round, the free active site or subunit of the *cisA* protein (solid circle, see Discussion) nicks the regenerated origin, and the occupied active site (open circle) ligates the newly formed 3'-OH and the bound 5'-phosphoryl groups. Thus an SS viral circle is released and the *cisA*-RF II complex, which can now enter another round of replication, is regenerated. We propose that, in its catalytic action, two active sites or subunits of the *cisA* protein alternate in their covalent attachment to the 5' end of the (+) strand.

of the SS loops in Fig. 3 D and E show that 30 and 60%, respectively, of the duplex circles had been replicated. (A factor of 2.56 was applied to correct for condensation of the SS by DBP.) No intermediates with free ends were found. Rolling circles with free SS tails were found when the purified DNA was examined by electron microscopy in 40% formamide without prior crosslinking of protein to DNA (data not shown).

Thus, the 5' end of the displaced strand, presumably with *cisA* protein attached, is at the replicating fork, moving around the circular template, probably through an interaction with the *rep* protein.

***cisA*-RF II Complex Supports Synthesis of Many Viral (+) Circles without Addition of Free *cisA* Protein.** The *cisA*-RF

Table 1. DNA synthesis starting with a *cisA*-RF II complex as template

Additions and omissions	DNA synthesis, pmol
Complete	680
-DBP	17
- <i>rep</i> protein	0
-Holoenzyme	0

cisA-RF II complex supports net synthesis of viral (+) circular DNA. Complete reaction mixture in a 25- μ l volume contained: 50 mM Tris-HCl (pH 8.1), 10 mM MgCl₂, 4 mM dithiothreitol, 120 μ g of bovine serum albumin per ml, 800 μ M ATP, 50 μ M each of dATP, dGTP, dCTP, and [α -³²P]dTTP (124 cpm/pmol), 2000 units of *rep* protein, 1.6 μ g of DBP, 20 units of holoenzyme (G4 replication assay), and 96 pmol of *cisA*-RF II complex (containing 48 pmol of template nucleotide residues). The reaction at 30° for 15 min was stopped by addition of 0.2 ml of 0.1 M sodium pyrophosphate and 1 ml of 10% trichloroacetic acid. The precipitate was collected on glass fiber filters (Whatman GF/C), washed three times with 1 M HCl/0.2 M sodium pyrophosphate, and dried. Radioactivity was measured in 5 ml of a toluene-based scintillation fluid in a liquid scintillation counter. DNA synthesis is expressed as total nucleotide incorporation.

II complex, when incubated with DBP, *rep* protein, and holoenzyme, supported a 5- to 10-fold higher rate of replication than that of a standard reaction (data not shown). Repeated rounds of replication yielded an average of about 15 viral (+) circles per *cisA*-RF II complex (Table 1; Fig. 3F). This synthesis was dependent upon DBP, *rep* protein, and holoenzyme (Table 1), with no free *cisA* added.

In another series of experiments, to be published elsewhere, ϕ X SS circular DNA, coated with DBP, was cleaved by *cisA* protein. Upon subsequent removal of DBP and free *cisA* protein (by filtration through Bio-Gel A-1.5 m) and incubation of the complex at 30° in the presence of Mg²⁺, but without any further addition of *cisA* protein, the frequency of circular molecules seen by electron microscopy increased from 13 to 45%.

We infer from these results that bound *cisA* protein acts catalytically in a continuous process of excision of ϕ X unit-length DNA and ligation of the two ends to form covalently closed viral (+) circles.

DISCUSSION

The *cisA* protein fulfills multiple functions in the replication of ϕ X174 DNA (Fig. 5): (i) nicking of the viral (+) strand at a specific sequence, the origin, to initiate a round of replication; (ii) participating in the unwinding of the two strands to create the replication fork, presumably by interacting with the *rep* protein; (iii) excising a ϕ X unit-length DNA after completion of a full round of replication; and (iv) ligating the excised DNA to form a covalently closed circular molecule.

We interpret our results as outlined schematically in Fig. 5. The unique nucleotide sequence in an SS region, exposed in superhelical RF I DNA[†] and nicked by the *cisA* protein, is located in the viral (+) strand between nucleotides 4290 and 4330 on the ϕ X sequence map (1). The nicking results in what we

[†] Covalently closed, relaxed ϕ X duplex DNA is not nicked by *cisA* protein (6, 16).

presume to be a covalent attachment of the *ctsA* protein to the 5' end of the viral strand. It seems plausible that this linkage, as in the case of the untwisting enzymes [which carry out repeated nicking and ligating steps without an energy requirement (19, 20)], conserves the energy of the cleaved phosphodiester bond. To explain the nicking and ligating activity of the bound *ctsA* protein, we propose the existence of two active sites or subunits in the bound *ctsA* protein, only one of which (open circle in Fig. 5) is bound covalently to the 5' end in a given complex.

We suggest that the *rep* protein recognizes the origin by its interaction with the bound *ctsA* protein. Unwinding of the two strands, sustained by ATP hydrolysis and DBP, then ensues. Movement of the 5' end into the duplex would displace the viral (+) strand from its complementary (−) circular template. The 3'-OH terminus, at the origin, serves as a primer and is extended by holoenzyme to regenerate the origin. When the *ctsA*-*rep* complex traverses the full length of the template circle, a unit-length ϕ X DNA would be excised by the unoccupied active site of the *ctsA* protein (solid circle in Fig. 5) by displacing and attacking the regenerated origin in the viral (+) strand. Concurrent with the nucleolytic scission carried out by the second *ctsA* subunit (solid circle in Fig. 5), the first subunit of the *ctsA* protein (open circle) ligates the newly created 3' OH with the bound 5' end, using the conserved energy of the covalent protein-DNA bond. In this mechanism, using two active sites or subunits of the *ctsA* protein alternately, the *ctsA*-RF II complex is restored with the completion and release of each viral circle and the catalytic role of the complex is assured. This mechanism ensures efficient use of *ctsA* protein and its proper orientation for the continuous synthesis of viral (+) DNA on a single template.

Initiation of complementary (−) strand synthesis by host proteins (including *dnaB*, *dnaC*, and *dnaG* proteins, proteins *i* and *n*, holoenzyme, and other proteins) takes place either on the displaced viral loop of the replicating intermediate or on the released viral (+) circle. Further studies in which the purified (+) strand synthesizing system is coupled with that for the (−) strand are now required to answer this question.

A mechanism analogous to that for ϕ X RF replication may also operate during the replication of other chromosomes. Initiation of synthesis of one of the strands by extension of a nick introduced by *ctsA*-like protein represents an origin of replication. (In chromosomes that replicate bidirectionally, nicking at the origin would be introduced in both strands.) This "leading" strand, synthesized continuously, is analogous to the ϕ X viral (+) DNA. The other "lagging" strand, analogous to

the ϕ X complementary (−) DNA, would be initiated repeatedly on the exposed parental (+) template.

Further insights into the mechanism of DNA replication may emerge from future efforts to reconstitute the enzyme system for the concerted, rapid, and efficient replication of RF, as well as the replication stages that precede and follow it.

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