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The A* protein of φX174 is an inhibitor of DNA replication

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ABSTRACT

Extracts prepared from ØX174 infected E. coli cells inhibited in vitro RF replication The inhibition was dependent upon the presence of A* protein in the reaction and served as an assay to highly purify the A* protein. Purified A* protein bound tightly to duplex DNA as well as single-stranded DNA. The binding of the A* protein to duplex DNA inhibited (I) its single-stranded DNA specific endonucleolytic activity; (II) in vitro synthesis of viral (+) single stranded DNA on an A-RFII DNA complex template; (III) ATP hydrolysis by rep protein and unwinding of the strands of RF DNA.

We propose that this inhibitory activity is responsible in vivo for the shut off of E. coli chromosome replication during ØX174 infection, and has a role in the transition from semiconservative RF DNA replication to single-stranded DNA synthesis in the life cycle of ØX174.

INTRODUCTION

Two polypeptides are coded for by the A gene of ØX174, the A protein (59 Kdaltons) and the A* protein (32.5-35 Kdaltons) (1). The A protein is required in vivo for the semiconservative replication of RF DNA and asymmetric synthesis of viral (+) ssDNA (2-4). Using in vitro systems for RF replication the role of A protein in DNA replication has been now extensively described (5, 6).

The function of the A* protein in the life cycle of ØX174 is not clear. Recently, it has been shown in vitro that purified A* protein, like the A protein, cleaves single-stranded DNA and forms a covalent complex with the cleaved DNA fragment (7-9).

In vivo, at 10-15 minutes after infection, the replication of the E. coli chromosome and the semiconservative replication of ØXRF DNA is shut off (10). The shut off of host DNA synthesis has been correlated with the presence of A* protein in the cell, implicating the A* protein in this process (11, 12). The in vivo effect of A* protein on ØXRF DNA replication could not be elucidated, since a mutation in the A* protein inactivates the A protein - a key function in the replication of RF DNA.

Here we report new properties of the A^* protein, identified in vitro, which we propose are related to the in vivo role of the A^* protein in the life cycle of ØX174.

MATERIALS AND METHODS

Organism: E. coli 4720 has been described (13). ØX174 amN14 and ØX174 amS29, mutants in the A gene were kindly provided by Dr. E. Tessman.

Enzymes: ØX A protein, E. coli rep protein and DNA polymerase III holoenzyme were purified as previously described (14-16). E. coli SSB protein was a gift of Dr. C. McHenry. ØX A^* protein was purified from ØX-infected E. coli cells. Purified A^* protein (7.5 μg) migrated on SDS-polyacrylamide gel as a single band of 32.5-35 Kdaltons. Details of the purification will be published elsewhere. Pst I and Hae III restriction endonucleases were from Biolab.

DNA preparations: ^{3}H-labeled ØXRFI and RFII DNA were prepared as previously described (17). ^{32}P-labeled ØX ssDNA was prepared in vitro with purified proteins in a (+) strand synthesis reaction (18). SV40 Form I DNA and plasmid PBR313 DNA were a gift of Dr. E. Winocour and D. Salomon respectively.

Preparation of A-RFII DNA complex: Superhelical (^{3}H)-ØXRFI DNA was cleaved in vitro by A protein, and the A-RFII DNA complex purified through neutral sucrose gradients, essentially as previously described (18). The purified complexes were dialyzed at 4°C against 50 mM Tris-HCl buffer, pH 8 containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 2% sucrose. When stored on ice, the complex remained active in replication for several weeks.

Analysis on GF/C glass fiber filters for formation of a protein-DNA complex: A^* protein was mixed with duplex DNA, on ice, in a 25 μl reaction mixture containing 50 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA and NaCl at the appropriate concentration. The mixture was filtered through a GF/C glass fiber filter (0.6 cm in diameter) supported by a GF/A filter (2.4 cm in diameter), essentially as described by Thomas et al. (1979). After the application of the sample the filters were washed five times (0.1 ml each) with the buffer of the reaction mixture.

RESULTS

Extracts from ØX174-infected E. coli cells inhibited RF DNA replication

Unlike the amS29 mutation (located at the C-terminal part of the A gene) which blocks the synthesis of the A and A^* proteins, the amN14 mutation (located at the N-

terminal part of the A gene) permits the synthesis of the A^* protein from an internal initiation codon within the A gene (1).

Extracts, prepared from amN14-infected E. coli 4720 cells, completely inhibited in vitro synthesis of viral (+) DNA in a reaction including DNA polymerase III holoenzyme, SSB protein and A-RFII DNA complex as template (table 1). Extracts from amS29-infected E. coli 4720 cells were by an order of magnitude less inhibitory, whereas extracts prepared from uninfected cells had no effect on the synthesis of viral (+) DNA (table 1). These findings suggested that A^* protein has a role in the in vitro inhibition of RF DNA replication. Using this inhibition reaction as an assay, the A^* protein was highly purified (manuscript in preparation).

Purified A^* protein binds to duplex DNA

Since DNA could be retained on a GF/C glass fiber-filter only through it's binding to a protein, the GF/C filters provide a rather simple tool to examine the formation of a protein-DNA complex (19).

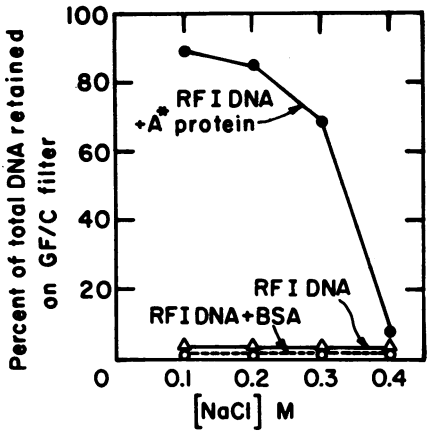
Using the GF/C filter binding assay, the A^* protein was shown to bind tightly to duplex DNA. The binding of A^* protein to ØX RFI DNA was salt sensitive and a complex was formed at a salt concentration as high as 0.3 M NaCl (Fig. 1).

Relaxed ØX RFII DNA, ØX RFI DNA treated with PstI or HaeIII restriction endonucleases, and SV40 DNA were also retained on the GF/C filters when mixed with the A^* protein (table 2). Thus, it appears that the binding of A^* protein is not affected by topo-

Extracts added	Protein (μg)	DNA synthesis (pmol)
-	-	350
Uninfected E. coli 4720	5.0	360
" "	25.0	320
ØX amS29/E. coli 4720	1.3	210
" "	6.3	130
ØX amN14/E. coli 4720	1.3	80
" "	6.3	5

Table 1. Extracts from ØX-infected E. coli cells inhibit RF DNA replication

Viral (+) DNA synthesis was performed as described in Legend to Fig. 3. The replication reactions were supplemented with extracts prepared from I, uninfected E. coli 4720; II, ØX amN14-infected E. coli 4720; III, ØX amS29-infected E. coli 4720 cells. The growth of cells and preparation of cell extracts (ammonium sulfate precipitate of gently lysed cells) was by a previously published procedure (14). DNA synthesis was measured as in Legend to Fig. 3.



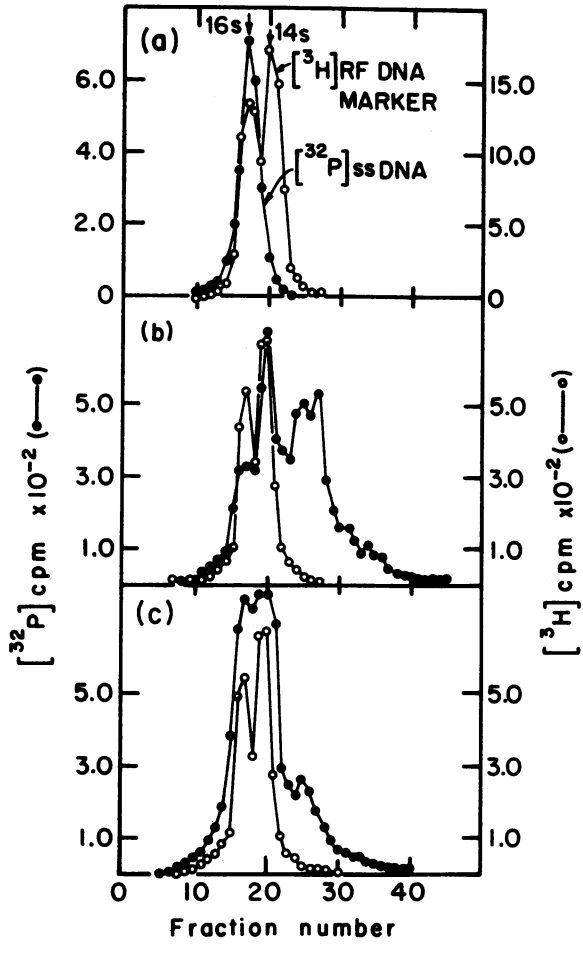
Legend to Figure 1:
A* protein binds to ØX RFI DNA
0.25 μg of A* protein were mixed with 0.15 μg of ØX RFI DNA and filtered through a GF/C glass fiber filter as described in Materials and Methods. The different reactions contained increasing levels of NaCl (0.1 M to 0.4 M), and the washings of the filters were performed with Tris·HCl buffer, pH 7.5 containing the appropriate amounts of NaCl (0.1 M to 0.4 M) ●–● RFI DNA retained on GF/C filter after incubation with A* protein; Δ–Δ RFI DNA retained on GF/C filter without prior incubation with A* protein; o–o RFI DNA retained after incubation with 2 μg of BSA

logical conformation of the duplex DNA nor is it specific for ØX DNA template.

The apparent binding of A* protein to duplex DNA could also be inferred indirectly from the results of the experiment described in Fig. 2. The extent of cleavage of ØX ssDNA was markedly reduced when a 25-fold molar excess of RFI DNA was added to the reaction (Fig. 2)' Thus, duplex DNA inhibits the ssDNA specific endonucleolytic activity of A* protein, presumably by competing with the ssDNA for the A* protein molecules.

DNA source	% of DNA retained on GF/C filter
ØX RFI DNA	92
ØX RFII DNA	70
PstI treat. ØX DNA	81
HaeIII treat. ØX DNA	81
SV40 FormI DNA	79

Table 2: A* protein binds to duplex DNA
250 pmol (as nucleotides) of duplex DNA was mixed with 0.5 μg of A* protein in a reaction mixture containing: 50 mM Tris-HCl buffer, pH 7.5; 1 mM EDTA; and 0.1 NaCl. The reactions were analyzed for the formation of an A* protein-DNA complex by the GF/C glass fiber filter binding assay as described in Materials and Methods. PstI and HaeIII treated DNA were obtained by cleaving ØX RFI DNA with the restriction endonucleases as previously described (18). All DNA preparations were tritium labeled. In the absence of A* protein, less than 1% of any of the DNA preparations tested was retained on the GF/C filter.



Legend to Figure 2: Duplex DNA represses the ssDNA specific endonucleolytic activity of the A* protein

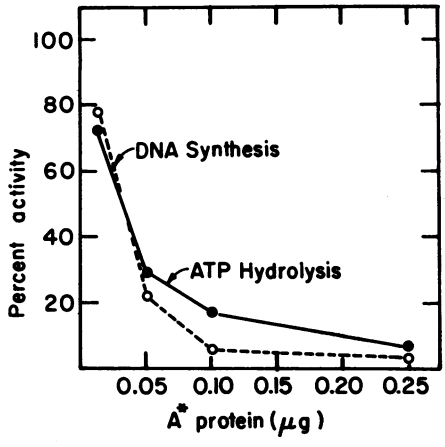
32P-labeled circular ØX ssDNA (prepared in vitro) was cleaved by A* protein in a reaction mixture (25 µl) containing: 10 mm $MgCl_2$; 10 mM 2-mercaptoethanol; 0.1 mg/ml BSA; 0.2 M Tris·HCl buffer, pH 7.5; 150 pmol (as nucleotides) of ssDNA and 0.1 µg of A* protein. (a), ssDNA incubated without A* protein; (b) ssDNA incubated with A* protein; (c) ssDNA incubated with A* protein in the presence of 5 µg of unlabeled ØX RFI DNA. The incubation was at 30° for 5 minutes and stopped by the addition of EDTA, NaOH and sarcosyl to 70 mM, 0.15 M and 2% final concentration respectively. 20 µg of salmon sperm carrier DNA and 2 µg of 3H-labeled ØX RFII DNA were also added and the reactions were analyzed by sedimentation through an alkaline sucrose gradient as previously described (9).

Purified A* protein inhibits in vitro RF DNA replication

The A protein initiates a round of replication on RFI DNA by cleaving the viral (+) parental DNA strand at the origin of replication (5, 6, 21, 22). The cleavage results in the covalent attachment of the protein to the 5' end to form an A-RFII DNA complex (5, 6, 22). The A-RFII DNA complex serves as a template for DNA polymerase III holoenzyme, rep protein and SSB to synthesize viral (+) ss circular DNA (18). It was proposed that this reaction represents the mechanism for the continuous synthesis of the leading strand during the semiconservative RF DNA replication (6).

Addition of increasing amounts of A* protein to the viral (+) strand DNA synthesis reaction caused a proportional decrease in the amount of DNA synthesized (Fig. 3). At a ratio of 100-150 molecules of A* protein for each A-RFII DNA complex present in the reaction, the synthesis of the DNA was completely inhibited. Furthermore, the A* protein inhibited ATP hydrolysis by the rep protein (Fig. 3) suggesting that the protein influences the formation and movement of the replication fork during RF DNA replication.

In support of this notion is the fact that the A* protein appeared to inhibit ongoing



Legend to Figure 3:
Inhibition of ATP hydrolysis and viral (+) DNA synthesis by A* protein
Viral (+) DNA synthesis was performed essentially as previously described (18). The reaction mixtures (25 μl) contained: 10 mM $MgCl_2$; 10 mM 2-mercapto-ethanol; 0.12 mg/ml BSA; 40 μM of dATP, dGTP, dCTP; 20 μM of (^{3}H)-TTP at a specific activity of 200 cpm/pmol; 200 pmol (as nucleotides) of A-RFII DNA complex, DNA polymerase III holoenzyme, rep protein and SSB (as before 18). Incubation was at 30° for 10 minutes, followed by the addition of EDTA to 50 mM final concentration and chilling the tubes on ice. To determine the amount of ATP hydrolyzed 2 μl aliquots were withdrawn and chromatographed on PEI plates by a previously published procedure (23). To the remaining reaction mixture, 0.5 ml of 10% TCA - 0.2 M sodium pyrophosphate solution were added and the amount of DNA synthesized was determined as previously described (18). A* protein was added to the DNA replication reactions at amounts indicated in the graph. In the absence of A* protein, 450 pmol nucleotides of viral (+) DNA and 1200 pmol ATP were synthesized and hydrolyzed respectively. The values were calculated for a 25 μl reaction mixture.

DNA replication (table 3). When added at intervals, after initiating the reaction, an immediate stop of any further incorporation of deoxyribonucleoside triphosphates was observed (table 3).

The A* protein inhibits DNA strand unwinding

To ascertain the possibility that the inhibition of RF DNA replication is a result of a block in the movement of the replication fork, we have examined the effect of A* protein on the unwinding of duplex DNA. Two proteins, rep protein and SSB, unwind the two strands of the A-RFII DNA complex completely separating the two strands (18). This reaction is characterized by the hydrolysis of ATP by the rep protein, the source of energy for the unwinding (23). ATP hydrolysis dependent upon the rep and A-RFII DNA complex was inhibited upon treatment with A* protein (table 4). Also, as expected, the formation of ssDNA was blocked (Fig. 4). The A-RFII DNA complex has not changed its sedimentation coefficient upon treatment with A* protein (Fig. 4c), indicating that the DNA in the complex remained intact.

An excess of duplex DNA overcomes the inhibitory activity of A* protein

The high stoichiometry of A* protein molecules needed to completely inhibit the replication of RF DNA (Fig. 3) suggested that inhibition occurs by a direct interaction of A* protein with the DNA.

When the RF replication reactions, inhibited by A* protein, were supplemented with increasing amounts of ØX RFI DNA, the synthesis of viral (+) DNA was restored proportionally (table 5). The synthesis was A-RFII DNA dependent, since in the absence of the

Time of A* protein addition (minutes)	DNA synthesis (pmol)
0	4
2	90
4	190
6	280
-	390

Table 3: A* protein inhibits ongoing DNA replication

ØX174 viral (+) DNA synthesis was performed as described in Legend to Fig. 3. The incubation was at 30°. Four separate reactions were supplemented, at 0, 2, 4 and 6 min, with 0.25 μg of A* protein, and the incubation was stopped at 10 minutes by chilling the tubes on ice and adding 0.5 ml of a 10% TCA - 0.2 M sodium pyrophosphate solution. One reaction was incubated for 10 minutes in the absence of A* protein. Amount of DNA synthesis was determined as in Legend to Fig. 3.

Additions and omissions	ATP hydrolysis (pmol)
Complete	160
-rep	1
-A-RFII DNA	0
+A^* protein (0.1 μg)	90
+ " " (0.25 μg)	10

Table 4: A^* protein inhibits ATP hydrolysis by rep protein

A complete reaction mixture contained: 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 200 μM (α ^{32}P)-ATP (60 cpm/pmol); 0.12 μg BSA; 400 pmol (as nucleotides) of A-RFII DNA complex; 0.92 μg of SSB and 0.075 μg of rep protein. Incubation was for 2 minutes at 30°. The reaction was stopped by adding EDTA to 50 mM and placing the tubes on ice. 2 μl aliquots were withdrawn and chromatographed on PEI plates to determine the amounts of ATP hydrolyzed as previoysly described (23). The values are for a 25 μl reaction.

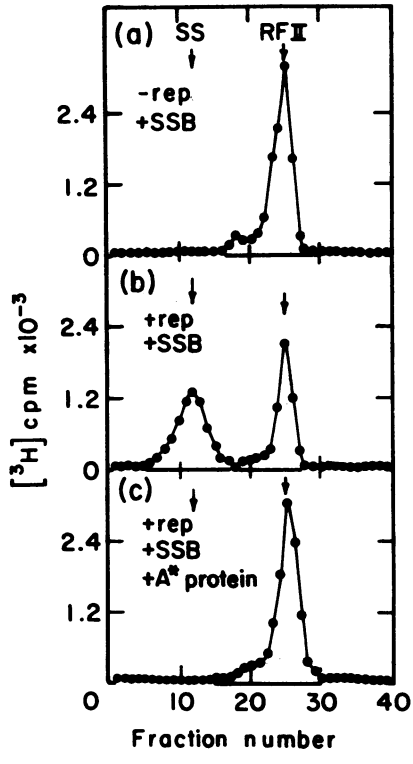
complex, DNA was not formed (data not shown). Addition of SV40 FormI DNA and plasmid PBR313 DNA were also effective in overcoming the inhibitory activity of A^* protein (data not shown), indicating that the competitive effect of duplex DNA is not specific for ØX DNA.

DISCUSSION

In vitro enzymatic activities of A^* protein

Purified A^* protein displays in vitro multiple activities; (a) it cleaves single-stranded DNA and forms a covalent complex with the 5' end of the cleaved DNA fragment (7, 8). Some topoisomerases have been recently shown capable of breaking single-stranded DNA and binding covalently to the cleaved DNA (25, 16). (b) Covalently bound A^* protein ligates the 5' and 3' ends of the DNA fragment to form a covalently closed, single-stranded, circular DNA molecule (8); (c) it binds to duplex and single-stranded DNA (24) (Fig. 1 and table 2). We believe that the cleaving-ligating activity of the A^* protein reflects the action of the A protein in terminating a round of replication.

Here, we have demonstrated that the A^* protein inhibits in vitro ØX RF DNA replication. The high stoichiometry of A^* protein molecules to A-RFII DNA complex (100-150:1) required to completely repress DNA replication (Fig. 3); the fact that upon treatment of the unwinding reaction with A^* protein, the DNA in the A-RFII DNA complex remains intact (Fig. 4); and the competitive effect of duplex DNA added to the replication reaction, would all argue that the inhibition of DNA replication is by



Legend to Figure 4: A* protein inhibits strand unwinding of duplex DNA
1000 pmol (as nucleotides) of A-(^{3}H) RFII DNA complex (prepared as described in Materials and Methods) were incubated at 30° for 10 minutes in a reaction mixture (30 µl) containing: 6.7 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 1.5 mM ATP, 1.8 µg of SSB. (a) Incubated without rep protein, (b) incubated with 0.75 µg rep protein; (c) incubated with 0.75 µg rep protein and 1 µg of A* protein. The incubation was stopped by chilling the tubes on ice and adding EDTA, sarcosyl, and NaCl to 50 mM, 2% and 1 M respectively. ^{32}P-labeled ssDNA marker was also added and the samples were sedimented through a neutral sucrose gradient as previously described (14). The position of the ssDNA marker is indicated by the arrow.

a direct interaction of the A* protein with the duplex DNA. The inhibition of the ATPase activity of the rep protein and DNA strands unwinding, in absence of DNA synthesis (Fig. 4, table 4), suggests that the replication is impaired at the level of formation and movement of the replication fork.

Two alternative mechanisms are being considered to explain the inhibitory activity of A* protein: 1, the interaction of the A* protein with the DNA template intro-

ØX RFI DNA added (μg)	DNA synthesis (pmol)
-	3
0.14	30
0.28	80
0.56	110

Table 5: Addition of ØX RFI DNA overcomes inhibition by A^* protein

The DNA replication reaction mixture, inhibited by A^* protein was like in Legend to Fig. 3, except that in all reactions 0.25 μg of A^* protein was used. Three reactions were supplemented with unlabeled ØX RFI DNA at amounts indicated in the table. The incubation was for 5 minutes at 30°. The amount of DNA synthesized was determined as in legend to Fig. 3. When A^* protein was absent, 150 pmols (as nucleotides) of DNA was synthesized.

duces a conformational change in the DNA, which either prevents or disrupts the interaction of the rep protein with the A-RFII DNA complex; II, the bound A^* protein forms a physical barrier for the movement of the rep-A protein complex preventing the unwinding of the DNA strands.

To discriminate between these possibilities, the stoichiometry of A^* protein molecules bound at equilibrium to duplex DNA, and the nature of the A^* protein-DNA interaction has still to be determined.

A model for the in vivo role of A^* protein in the life cycle of ØX174

We propose that the duplex DNA binding property of the A^* protein has at least two functions in the cell: I, it is responsible for the turning off of the E. coli chromosome replication. When accumulated in the cell, the A^* protein would bind to chromosomal DNA, ahead of the replication fork, hindering the unwinding of duplex DNA. Such an inactivation of host DNA replication by a viral function would be most advantageous to the virus, enabling it to mobilize the host enzymes and DNA precursors for its own reproduction. II, it has a role in the transition from semiconservative RF DNA replication to asymmetric viral (+) ssDNA synthesis, the last stage (stage III) in the life cycle of ØX174.

We suggest that this transition proceeds in two steps: 1, complete inhibition of semiconservative DNA replication by the binding of the A^* protein to duplex RF DNA; 2, Relief of this inhibition by the involvement of additional proteins coded for by the virus (products of gene B, C, D, F and G). This relief of inhibition would be specific for ØX template because of a specific interaction of some of these proteins with A and rep proteins at the origin of replication. The ØX gene F product may have a key role in

this process since it has been suggested to interact, in vivo, with A protein and rep (27).

The involvement of ØX proteins in ssDNA synthesis, overcoming the inhibition by A^* protein, may represent a novel mechanism for the formation and movement of a replication fork, different from that operating during semiconservative RF DNA replication.

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ABBREVIATIONS

A-RFII DNA, A protein-RFII DNA complex. BSA, bovine serume albumin; RFI, circular covalently closed, superhelical, duplex DNA; RFII, circular duplex DNA with at least one discontinuity in one of the strands; ssDNA, single stranded DNA; SSB, E. coli single-stranded binding protein; TCA, trichloroacetic acid.

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