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Effect of SSB protein on cleavage of single-stranded DNA by φX gene A protein and A* protein

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ABSTRACT

Gene A protein of bacteriophage ØX174 plays a role as a site-specific endonuclease in the initiation and termination of ØX rolling circle DNA replication. To clarify the sequence requirements of this protein we have studied the cleavage of single-stranded restriction fragments from ØX and G4 viral DNAs using purified gene A protein. The results show that in both viral DNAs cleavage occurs at the origin and at one additional site which shows striking sequence homology with the origin region.

During rolling circle replication the single-stranded viral DNA tail is covered with single-stranded DNA binding (SSB) protein. Therefore, we have also studied the effect of SSB on ØX gene A protein cleavage. In these conditions only single-stranded fragments containing the complete or almost complete origin region of 30 bases are cleaved, whereas cleavage at the additional sites of ØX or G4 viral DNAs does not occur. A model for termination of rolling circle replication which is based on these findings is presented.

Finally, we present evidence that the second product of gene A, the A* protein, cleaves ØX viral DNA at the additional cleavage site in the presence of SSB, not only *in vitro* but also *in vivo*. The functional significance of this cleavage *in vivo* is discussed.

INTRODUCTION

The initiation and termination of bacteriophage ØX174 rolling circle DNA replication are accomplished by the product of the viral gene A, gene A protein (1-7). Gene A protein initiates DNA replication by cleaving only one DNA strand, the viral (or +) strand of supercoiled ØX RF DNA at a unique site, the origin (8). Cleavage creates a free 3'-OH primer for DNA synthesis at one end and a covalent protein-DNA complex at the other (5') end of the cleavage site (7,8). The bond between DNA and protein has recently been identified as a phosphodiesterbond between tyrosine-OH and 5' phosphate of an adenylic residue (9-11).

Termination involves cleavage of the rolling circle at the regenerated origin and the subsequent circularization and release of genome length viral DNA. According to the looped rolling circle model (5,12,13) termination is

accomplished by the covalently linked gene A protein which cleaves the single-stranded tail of the rolling circle at the regenerated origin at the end of each complete round of replication.

In order to gain insight into the mechanisms of DNA initiation and termination we have studied the interaction of supercoiled and single-stranded DNAs with the purified ØX gene A protein. The origin of bacteriophage ØX174 DNA is located in a specific sequence of 30 base pairs, the origin region. This sequence is highly conserved among isometric single-stranded DNA phages (14-16). For cleavage of double-stranded DNA, supercoiling and most i.e. the first 27 base pairs of the origin region are required (16-18). In contrast cleavage of single-stranded DNA requires only the presence of the first ten nucleotides of the origin region (19). Earlier we have presented a model for the initiation of ØX RF DNA replication which is based on these and other findings and which will be dealt with in the discussion.

In this paper we are concerned with experiments that are relevant to the mechanism of termination of ØX rolling circle replication. We report experiments with single-stranded restriction fragments of the viral DNAs of bacteriophages ØX and G4 which show that gene A protein can cleave these viral DNAs at two sites. This indicates that gene A protein requires additional information in order to assure that cleavage during termination occurs at the origin only. In this report we show that single-stranded DNA binding protein from *E. coli* (SSB protein) restricts gene A protein cleavage to the origin and thus can help gene A protein to discriminate between the origin and the additional cleavage sites. Using single-stranded DNA fragments containing various parts of the origin region it was shown that the first 27 nucleotides are required for cleavage in the presence of SSB. These results suggest a model for the termination of rolling circle DNA replication which will be presented.

The A* protein is the result of an internal translation start in gene A in the same reading frame as is used for gene A protein (20). A* protein thus lacks about one third of the amino acids from the N-terminal end of the polypeptide chain of gene A protein. A* protein inhibits host DNA replication (21). The function of A* protein in the replication cycle of ØX is unknown. Previously we have shown that a fraction of the A* protein, as isolated from infected cells is covalently linked to DNA fragments as a result of a cleavage reaction *in vivo* (22). Here we present evidence that cleavage *in vivo* occurs at the additional cleavage site in the ØX genome. The functional significance of these observations will be discussed.

MATERIALS AND METHODS

DNA preparations

Single-stranded ØX174 am3 DNA and ØX174 am3 RFI DNA were isolated as described previously (23). G4 RFI DNA was isolated from *E. coli* C122 infected with bacteriophage G4 as described for the isolation of ØX RFI DNA. Polyomavirus strain A2 DNA was a gift of Dr. J.R. Arrand (Imperial Cancer Research Fund Laboratories, London, UK). Plasmid pAF24a, pAF26 and pAF27 DNAs were prepared as described by Fluit et al. (18). The oligodeoxyribonucleotide CAACTTG was synthesized by G.H. Veeneman and J.H. van Boom (Department of Organic Chemistry, State University of Leiden, Leiden, NL) as described by Marugg et al. (24).

Enzymes and proteins

The restriction endonucleases *Hae*III, *Hpa*II and *Fnu*DII were obtained from New England Biolabs, bacterial alkaline phosphatase from Worthington, T4 polynucleotide kinase from New England Nuclear and *E. coli* single-stranded DNA binding protein (SSB protein) from P.L. Biochemicals. Adenovirus type 5 DNA binding protein (Ad5 DBP) was a gift of Dr. P.C. van der Vliet (Laboratory for Physiological Chemistry, State University of Utrecht, Utrecht, NL) and T7 single-stranded DNA binding protein (T7 DBP) was a gift of Dr. C.W. Fuller (Department of Biological Chemistry, Harvard Medical School, USA). Gene A protein and A* protein were purified according to Langeveld et al. (25).

DNA techniques

Double-stranded DNAs were digested with restriction endonucleases according to the instructions of the manufacturers. Single-stranded ØX DNA was digested with *Hae*III according to Blakesley et al. (26). DNA fragments were labelled at the 5'-ends after dephosphorylation with bacterial alkaline phosphatase with $|\gamma\text{-}^{32}P|$-ATP, 3000 Ci/mmol (New England Nuclear, USA) and T4 polynucleotide kinase using the standard methods as described by Maxam and Gilbert (27).

Incubation with gene A protein and A protein*

Two ng 5' labelled, single-stranded DNA fragments were incubated with 2 ng gene A protein or A* protein in 10 mM Tris/HCl, pH 7.6, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT, 150 NaCl, 2% glycerol and 0.01% Nonidet P40 in a volume of 32 µl. DNA binding protein (from *E. coli*, adenovirus or T7) was added as specified in the legends to the figures. Double-stranded DNA fragments were denatured by heating for 3 min at 100°C. The samples were cooled rapidly by placing the tubes in an ethanol dry-ice mixture. The samples were thawed in ice-water, DNA binding protein was added and the samples were incubated for 5 min at room temperature. Gene A protein or A* protein were added and the samples were incubated

for 30 min at 30°C. Then EDTA was added to a final concentration of 10 mM, 2 µl of a solution of proteinase K, 5 mg/ml, which had been preincubated for 30 min at 37°C was added and the incubation was proceeded for 30 min at 37°C. Five µg tRNA and 10 mM Tris/HCl, pH 7.5, 1 mM EDTA were added to a final volume of 100 µl and the nucleic acids were precipitated with ethanol. The pellets were dissolved in 5 µl 10 mM Tris/HCl, pH 7.6, 1 mM EDTA and analysed on a gel. In some cases one third of the samples was applied directly onto a gel without ethanol precipitation.

Gel electrophoresis

Samples were analysed on 6, 8 or 25% polyacrylamide gels (400 x 300 x 0.45 mm; or 1 mm for the 25% gels only), containing 7 M urea. The gels were made up in 100 mM Tris, 100 mM borate, 2 mM EDTA, pH 8.3. The gels were prerun for 1 h at 45 W. Before applying onto the gel, the samples were mixed with an equal volume of formamide, containing 10 mM NaOH, 0.05% xylene cyanol F and 0.05% bromophenol blue and heated for 3 min at 100°C. Electrophoresis was performed at 45 W until the tracking dyes had migrated the desired distances. The radioactive DNA fragments were detected by autoradiography.

Incubation of A protein with CAACTTG*

Three pmoles 5'-labelled heptanucleotide CAACTTG were incubated with 100 ng A* protein in a reaction mixture as described above with a total volume of 400 µl. After 30 min incubation at 30°C, EDTA was added to a final concentration of 10 mM. 25 µl of a solution of proteinase K, 5 mg/ml which had been preincubated for 30 min at 37°C was added and the incubation was continued for 30 min at 37°C. Protein was denatured by extraction with phenol and the sample was desalted by chromatography over a Sephadex G50 column. Fractions containing radioactivity were freeze-dried, the material was dissolved in a small volume and pooled.

Isolation and analysis of transfer products

The pool of radioactive material, which was obtained as described above, was subjected to electrophoresis on a 25% polyacrylamide gel. The radioactive materials migrating slower than the starting heptanucleotide were eluted from the gel using the elution buffer as described by Maxam and Gilbert (27), desalted over a Sephadex G50 column, freeze-dried and dissolved in a small volume. A sample of this material was subjected to a two-dimensional separation, using high voltage electrophoresis on cellulose-acetate at pH 3.5 in the first direction and homochromatography on Machery and Nagel cel 300 DEAE thin-layer in the second direction as has been described essentially by Brownlee and Sanger (28). The radioactive materials were detected by autoradiography.

RESULTS

Sequence specificity of gene A protein

To determine the sequence requirements of gene A protein, gene A protein was incubated with single-stranded DNA fragments. Therefore, single-stranded ØX viral DNA was digested with the restriction endonuclease *Hae*III, and the fragments were labelled at their 5' ends with ^{32}P. Analysis by electrophoresis on 8% polyacrylamide gel and autoradiography showed that this procedure yields 13 fragments which correspond to the 11 fragments which are found after digestion of double-stranded ØX with *Hae*III and 2 additional fragments, the Z5-Z8 partial and the Z9-Z10 partial (Figure 1 lane a). This mixture of 5'-labelled

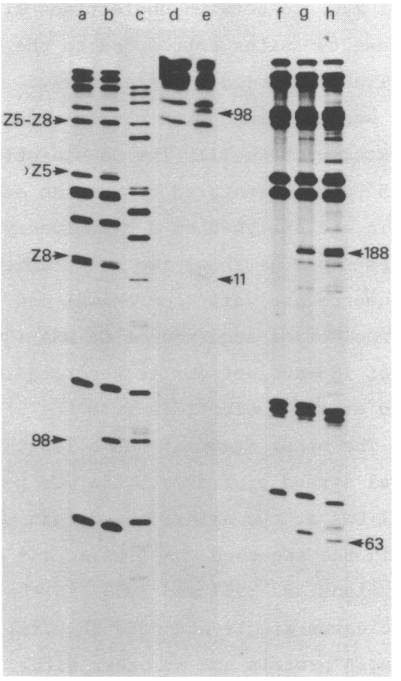

Figure 1. Cleavage of single-stranded DNA by gene A protein and A* protein. Lanes a to e: the 5'-labelled *Hae*III fragments of ØX single-stranded DNA were incubated and the products were analysed by electrophoresis on 8% (lanes a, b and c) or 25% (laned d and e) polyacrylamide gels and autoradiography. Lanes a and d: after incubation without protein; lanes b and e: after incubation with gene A protein; lane c: after incubation with A* protein. Lanes f to h: the 5'-labelled *Hae*III fragments of G4 double-stranded DNA were denatured and incubated and the products were analysed by electrophoresis on 8% polyacrylamide gel and autoradiography. Lane f: after incubation without protein; lane g: after incubation with gene A protein; lane h: after incubation with A* protein.

single-stranded DNA fragments was incubated with gene A protein and analysed (Figure 1 lane b). The disappearance of fragment Z6b and the appearance of a new fragment with a length of 98 nucleotides shows that gene A protein cleaves at the origin, which is located between nucleotides 98 and 99 from the 5' end of Z6b. Also fragment Z8 has disappeared. The labelled product of the cleavage of fragment Z8 was detected on a 25% polyacrylamide gel (Figure 1 lanes d and e). It has a length of 11 nucleotides. The decrease of intensity of the partial Z5-Z8 and the appearance of a fragment slightly longer than fragment Z5 corresponds to cleavage in the Z8 part of the partial Z5-Z8, 11 nucleotides beyond the *Hae*III cleavage site. From these data we conclude that cleavage occurs at two sites in the ØX genome which are located between nucletotides 98 and 99 from the 5' of Z6b and between nucleotides 11 and 12 from the 5' end of Z8, respectively. These two sites correspond to the GA residues in the sequences CAACTTGATA and TTACTTGAGG (29), respectively.

A similar experiment was performed with single-stranded DNA fragments of G4. G4 RFI DNA was digested with *Hae*III. The double-stranded DNA fragments were labelled at their 5' ends, denatured by heating and rapid cooling, incubated with gene A protein and analysed on a 8% polyacrylamide gel. The autoradiogram (Figure 1 lanes f and g) shows two new fragments after incubation with gene A protein. Sequence analysis (not shown) and comparison of the sequences with the known nucleotide sequence of G4 DNA (30) indicated that the fragment with a length of 188 nucleotides is generated by cleavage at the origin, which is located at 188 nucleotides from the 5' end in the viral strand of fragment Z2a. The other fragment has a length of 63 nucleotides and is derived from the viral strand of fragment Z5a. So gene A protein can cleave G4 single-stranded viral DNA at two sites: the origin, between G and A (nucleotides 506 and 507) in the sequence CAACTTGATA, and at one additional site, also between G and A (nucleotides 3957 and 3958) in the sequence ATACTCGAGT.

Purified A* protein cleaves single-stranded ØX viral DNA at the two sites which are cleaved by gene A protein and at other sites (Figure 1 lane c; ref. 31). Single-stranded DNA fragments of G4 are also cleaved at the two gene A protein cleavage sites and other sites by A* protein (Figure 1 lane h).

Cleavage of single-stranded DNA in the presence of SSB protein

During rolling circle replication the single-stranded viral DNA tail is covered with SSB protein. Therefore, the effect of SSB protein on ØX gene A cleavage of single-stranded DNA was also studied. The single-stranded DNA fragments of ØX, obtained by denaturation of the 5'-labelled double-stranded ØX *Hae*III DNA fragments, were incubated with gene A protein in the presence

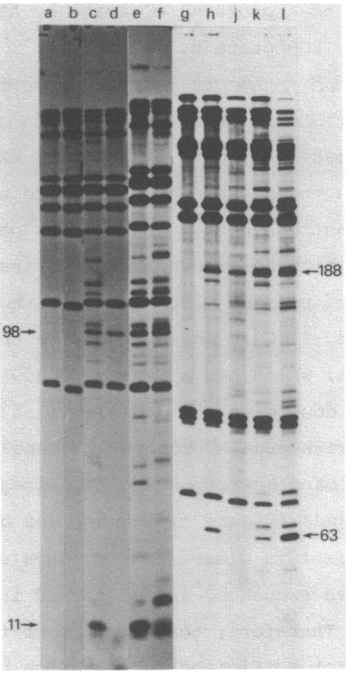

Figure 2. Cleavage of single-stranded DNA by gene A protein and A* protein in the presence of SSB protein. The 5'-labelled fragments were denatured, incubated and analysed by electrophoresis on 6% polyacrylamide gel and autoradiography. Lanes a to f: the *Hae*III ØX DNA fragments after incubation without protein (a), with 2µg SSB protein (b), gene A protein (c), gene A protein and 2 µg SSB protein (d), A* protein (e) and A* protein and SSB protein (f). Lanes g to l: the *Hae*III G4 DNA fragments after incubation without protein (g), gene A protein (h), gene A protein and 2 µg SSB protein (j), A* protein (k) and A* protein and SSB protein (l). The relevant bands are indicated with arrows.

of SSB protein. The autoradiogram (Figures 2 lanes a-d) shows that only the 98 nucleotides fragment is produced under these conditions. So gene A protein cleaves single-stranded ØX DNA only at the origin in the presence of SSB protein. The experiment was also performed with the single-stranded DNA fragments of G4. The autoradiogram (Figure 2, lanes g-j) shows that the 188 nucleotides fragment is formed in the presence of SSB protein. So gene A protein cleaves G4 single-stranded DNA only at the origin in the presence of SSB protein.

The presence of SSB protein during the incubations of single-stranded DNA fragments of ØX and the single-stranded DNA fragments of G4 with A* protein does not suppress cleavage at the origin or the other sites in ØX and G4 viral DNAs which are cleaved by gene A protein (Figure 2, lanes e, f, k and

1). So, in contrast to gene A protein, cleavage by A* protein is not restricted to the origin sequence by SSB protein.

Cleavage of single-stranded fragments containing various parts of the origin region in the presence of SSB protein

Which part of the 30 base pair origin region of ØX is required for cleavage of single-stranded DNA in the presence of SSB protein was investigated using polyoma DNA and recombinant DNA containing various parts of the origin region. The sequence CAACTTGATA which corresponds to the first 10 bases of the origin of ØX occurs in the DNA of polyoma virus (32), in the *Hae*III fragment Z8, between nucleotides 1719 and 1710 in the strand with the same polarity as the late mRNAs. This fragment, which has a length of 209 base pairs, was labelled, isolated, mixed with the double-stranded 5'-labelled ØX *Hae*III DNA fragments, denatured and incubated with gene A protein. The analysis shows that in the absence of SSB protein a band corresponding to a fragment of 49 nucleotides is formed, among other bands (Figure 3, lanes a and b). This fragment, which is expected when gene A protein cleaves the polyoma virus DNA fragment Z8 between G and A in the above sequence, is not formed in the presence of SSB protein (Figure 3, lane c). Therefore, the sequence of the first 10 nucleotides of the origin region is not sufficient for cleavage by gene A protein in the presence of SSB protein. Cleavage at the origin is not inhibited under these conditions as indicated by the presence of a band of 98 nucleotides (Figure 3, lane c).

Plasmid pAF24a contains the sequence of the first 24 nucleotides of the origin region a an insert in the unique *Hin*dIII site of pACYC177 (18). This plasmid was digested with *Hpa*II, the fragments were labelled at their 5' ends and denatured. Analysis of the products obtained after incubation with gene A protein shows a new band which corresponds to a fragment with a length of 173 nucleotides (Figure 3, lanes d and e). This fragment corresponds to cleavage at the origin. SSB protein suppresses the cleavage by gene A protein at this site (Figure 3, lane f).

Similar experiments were carried out with plasmids pAF26 and pAF27. Plasmid pAF26 contains the sequence of the first 26 nucleotides of the origin region of ØX as an insert in the unique *Hin*dIII site in pACYC177 in an orientation opposite to the orientation of the 24 nucleotides insert in pAF24a. Plasmid pAF26 was digested with *Hpa*II, the fragments were labelled, denatured and incubated with gene A protein. The putative gene A protein cleavage site is located in the 210 base pairs fragment, 27 nucleotides away from the 5' end.

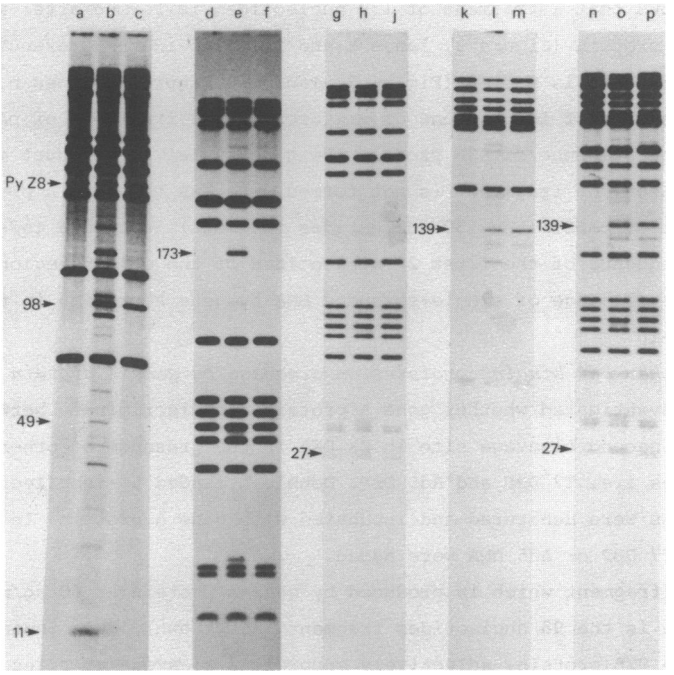

Figure 3. Cleavage of single-stranded DNA fragments containing various parts of the origin in the presence of SSB protein. The 5'-labelled DNA fragments were denatured, incubated: (i) without protein, (ii) with gene A protein, and (iii) with gene A protein and 2 µg SSB protein and analysed in this order by electrophoresis on 6% polyacrylamide gel and autoradiography. Lanes a to c: the products of the incubations of a mixture of the *Hae*III fragments of ØX and fragment Z8 from polyoma virus DNA; lanes d to f: the products of the incubations of the *Hpa*II fragments of plasmid pAF24a; lanes g to j: the products of the incubations of the *Hpa*II fragments of plasmid pAF26; lanes k to m: the products of the incubations of the *Fnu*DII fragments of plasmid pAF27; lanes n to p: the products of the mixtures of the *Hpa*II fragments of plasmid pAF26 and the *Fnu*DII fragments of plasmid pAF27. The relevant bands are indicated by arrows.

The analysis shows that a fragment of 27 nucleotides is formed after incubation with gene A protein (Figure 3, lanes g and h). The presence of SSB protein suppresses cleavage by gene A protein at this site (Figure 3, lane j).

Plasmid pAF27 contains the sequence of the first 27 nucleotides of the origin region of ØX as an insert in the unique *Sma*I site of pACYC177. The plasmid was digested with *Fnu*DII, the fragments were labelled, denatured and incubated with gene A protein. The putative gene A protein cleavage site is located in the 372 base pairs fragment, 139 nucleotides away from the 5' end. The

analysis shows that a fragment of 139 nucleotides is formed after incubation with gene A protein (Figure 3, lanes k and l). Also in the presence of SSB protein this fragment is formed (Figure 3, lane m). Figure 3, lanes n, o and p, shows the results of incubations of mixtures of the fragments of pAF26 and pAF27. In the presence of SSB protein the gene A cleavage product of pAF26 (the 27 nucleotides fragment) is not formed, whereas the gene A protein cleavage product of pAF27 (the 139 nucleotides fragment) is formed indeed. Therefore, the sequence of the first 27 nucleotides of the origin region of ØX is required for cleavage of single-stranded DNA by gene A protein in the presence of SSB protein.

Effect of other DNA binding proteins on cleavage by gene A protein

It was investigated whether gene A protein can discriminate between the origin and the second cleavage site in ØX DNA in the presence of other DNA binding proteins i.e. T7 DBP and Ad5 DBP. Double-stranded 5'-labelled ØX *Hae*III DNA fragments were denatured and incubated with gene A protein. Increasing amounts of T7 DBP or Ad5 DNA were added.

The only fragment which is produced by gene A protein at 60 µg/ml T7 DBP concentrtion is the 98 nucleotides fragment (not shown). This indicates that T7 DBP, like SSB protein, selectively suppresses cleavage at sites other than the origin. Gene A protein can not cleave ØX single-stranded DNA in the presence of Ad5 DBP concentrtion of 50 µg/ml (not shown).

Cleavage site of A protein in vivo*

In a previous study (22) we have shown that the A* protein as isolated from ØX infected cells contains covalently bound oligonucleotides of the types AG, AGG or AGGA. These oligonucleotides probably arise from specific protein A* cleavage *in vivo* followed by degradation of the covalently bound DNA. It is remarkable that the sequence AGGA occurs at the 5' end of the additional gene A protein cleavage site in ØX DNA. This suggests that A* protein cleaves ØX DNA at the additional cleavage site *in vivo*. This possibility was investigated as follows. ^{32}P-labelled CAACTTG, which acts as an acceptor for the covalently bound oligonucleotides, was incubated with A* protein and the products were analysed on a 25% polyacrylamide gel. The autoradiogram (Figure 4a) shows the starting heptamer and a number of transfer products. The major product, 10* corresponds to an oligonucleotide with a length of 10 nucleotides with the sequence CAACTTGAGG (22). The longest product that was detected is 14 nucleotides long. The different transfer products were also analysed in the two-dimensional separation system (Figure 4b and c). In this system each nucleotide has its own characteristic contribution to the mobility of an oligonucleotide (28).

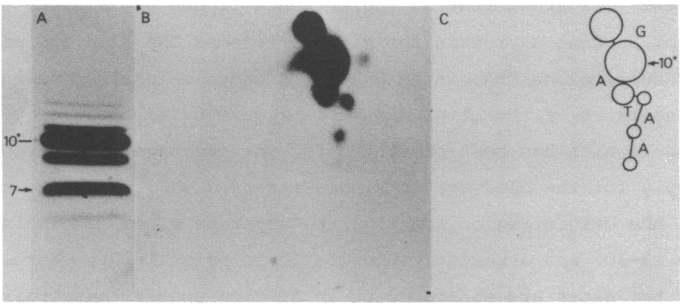

Figure 4. The oligonucleotides which occur covalently bound to A* protein. The products which were obtained after incubation of A* protein with the oligonucleotide |^{32}P|-CAACTTG were analysed on a 25% polyacrylamide gel (a). The transfer products were isolated and subjected to two-dimensional separation (b). Electrophoresis was from the left to the right and homochromatography from the bottom upwards. The major product, 10*, and the nucleotides which cause the shift in mobility of the subsequent radioactive products have been indicated in the scheme (c).

Starting from the major product 10*, the nucleotide sequences of the other transfer products can be deduced from their relative positions. The product which migrated faster than 10* during chromatography contains one G less than 10*. The position of the other products corresponds to, successively, an extra A, AT, ATA and ATAA. The oligonucleotides which have been transferred are thus: AG, AGG, AGGA, AGGAT, AGGATA and AGGATAA. Their sequences show that they have been derived from a single DNA sequence. The sequence AGGATAA occurs once in ØX viral DNA and corresponds to the 5' end of the additional gene A protein cleavage site in ØX viral DNA. Therefore, we conclude that this site is also cleaved by A* protein *in vivo*.

DISCUSSION

We have previously shown that gene A protein cleavage of supercoiled DNA requires the presence of a specific sequence of approximately 30 base pairs the so-called origin region of the single-stranded isometric DNA phages (16-18). In contrast the cleavage of single-stranded DNA requires only the first 10 bases of this origin region (22,33). Since initiation of rolling circle DNA replication involves the interaction of gene A protein with supercoiled DNA, whereas termination involves the interaction of gene A protein with (partially) single-stranded DNA, it has long been thought that the sequence requirements for termination would be less stringent than for initiation.

The present work shows that in the presence of SSB protein the sequence requirements of ØX gene A protein for single-stranded DNA cleavage are as stringent as for supercoiled DNA. In both cases a sequence corresponding to the first 27 base pairs of the origin region is required for cleavage. This suggests that the model which has been presented for the cleavage of supercoiled DNA may also apply for the cleavage of single-stranded DNA. According to this model (16,17) the origin region contains two separate sites, a binding site (base pairs 18-30) and a recognition site (base pairs 1-10), where cleavage occurs. The two sites are separated by an AT-rich spacer (base pairs 11-17). Gene A protein first reacts with the binding site by non-covalent interaction. This binding brings the protein in a proper orientation towards the recognition site and leads to (partial) unwinding of the recognition site which can now be cleaved. Unwinding is driven by the superhelical free energy and is facilitated by the AT-rich sequence. We suggest that the same sequence of events, binding, proper orientation and cleavage, is required for termination of rolling circle replication (Figure 5). This would explain that the additional cleavage sites in ØX and G4 DNA are not cleaved during rolling circle replication since both sites lack the gene A protein binding site which is characteristic of the origin region. This model is in agreement with earlier results (13,34 and Fluit et al. (unpublished)), which show that termination of rolling circle replication requires more than the first 16 bases of the origin region.

The finding that gene A protein cleaves single-stranded ØX DNA in the presence of SSB protein at a unique site shows once more that SSB protein can direct the specificity of protein-DNA interactions in *E. coli*. Other investigators have shown that the specificities of RNA polymerase priming of M13 DNA replication (35), the n' protein initiated primosome assemblage on ØX DNA (36) and the synthesis of the specific primer by dnaG primase on G4 single-stranded DNA (37) depend on the presence of SSB protein. This may be reached by suppression of non-specific binding of these proteins to the DNA and/or promoting secondary structures which are recognized by these proteins.

An alternative explanation for the effect of SSB protein might be that the specific cleavage of single-stranded DNA by gene A protein in the presence of SSB protein is the result of direct protein-protein interaction of SSB protein and gene A protein. However, cleavage at the second site also occurred when small amounts of SSB protein, not enough to cover all single-stranded DNA, but in a molar excess over gene A protein, were added. This makes the latter explanation less plausible.

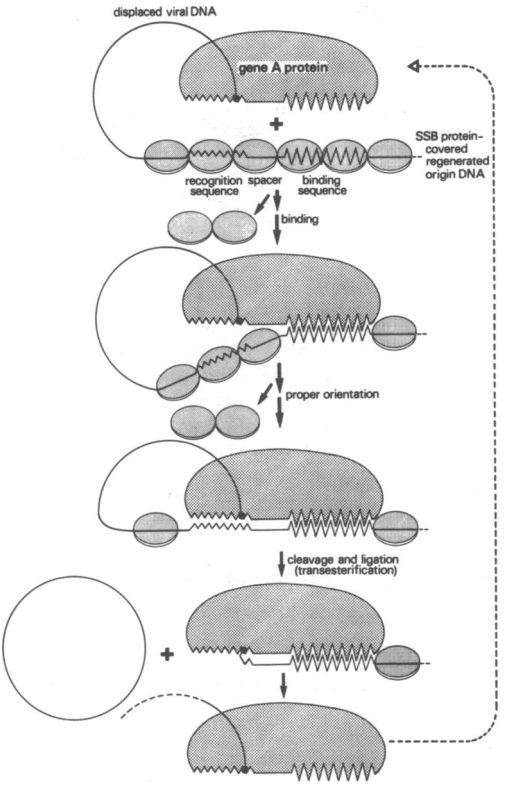

Figure 5. Model for the cleavage of single-stranded DNA by gene A protein in the presence of SSB protein during termination of rolling circle DNA replication. The cleavage and ligation which take place during the termination reaction can be regarded as a transesterification.

The presence of T7 DBP has the same effect on the cleavage of single-stranded DNA by gene A protein as the presence of SSB protein. This and the observation that SSB protein can substitute for T7 DBP during T7 DNA polymerase dependent DNA synthesis (38), suggest that T7 DBP and SSB protein interact with single-stranded DNA in a same way. Ad5 DBP can not substitute for SSB protein. Ad5 DBP prevents all cleavage by gene A protein. Possibly this difference between Ad5 DBP and SSB protein is related to a different strength of binding to single-stranded DNA or to the way both proteins interact with single-stranded DNA: the Ad5 DBP-single-stranded DNA complex shows an extended configuration (39), whereas the SSB protein-single-stranded DNA complex shows a condensed configuration (40).

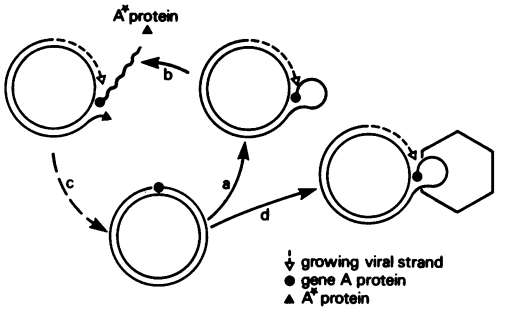

Figure 6. Model for the function of A* protein during stage III ØX DNA replication. Stage III involves coupled rolling circle DNA replication and packaging of single-stranded DNA into phage coats. No RF DNA replication takes place during this stage. The model predicts that RF DNA replication is interrupted by A* protein cleavage at the second cleavage site. This leads to futile replication cycles until sufficient coat proteins are available to package the single-stranded DNA into phage coats which prevents A* protein cleavage.

A* protein cleaves the single-stranded DNA of ØX and G4 at the origin and the additional sites and the presence of SSB protein does not restrict the cleavage of ØX or G4 single-stranded DNA by A* protein to the origin. Obviously, A* protein interacts in a different way with single-stranded DNA than gene A protein. Possibly, SSB protein can be displaced more easily from single-stranded DNA by A* protein than by gene A protein. This may be inferred from the fact that A* protein is eluted from single-stranded DNA cellulose at approximately the same salt concentration as SSB protein, whereas gene A protein is eluted at a lower salt concentration (25,41).

A* protein as isolated from infected cells contains covalently bound oligonucleotides (22). Extensive sequence analysis of these oligonucleotides shows that they are derived from one sequence: AGGATAA. This sequence corresponds to the sequence at the 5' end of the additional gene A protein cleavage site in ØX viral DNA. Therefore, it seems likely that A* protein cleaves ØX viral DNA at this site *in vivo*. The results of Zolothukhin et al. (42) also show that A* protein can cleave ØX DNA *in vivo*. The observations suggest that A* protein may act *in vivo* as outlined in the model shown in figure 6. It is assumed that, when A* protein has accumulated to a certain level, the displaced viral strand of the rolling circle is cleaved by A* protein. This cleavage can occur only if the displaced strand is not packaged (Figure 6a and b). DNA synthesis proceeds (Figure 6c) and after completion of the replication round, the RF II molecule can start a new round of replication which may then be coupled to packaging (Figure 6d). In this way no new, free circular viral DNA

molecules are formed and the *de novo* synthesis of RF DNA is shut off. The replication machinery is no longer involved in synthesis of complementary DNA and will be available for the synthesis of viral DNA. The involvement of A* protein in the switch from RF DNA synthesis to single-stranded DNA synthesis has been suggested before but no mechanism has been suggested (43,44). A second function of A* protein is to stop the host DNA replication (21). This may be reached by the capacity of A* protein to bind to double-stranded DNA (45,46). Next to ØX also other isometric phages encode an A* protein (47,48). A similar protein, gene X protein is essential for the synthesis of the viral DNA strand of the more distantly related single-stranded, filamentous DNA phage f1 (49). These observations may indicate that A* protein is profitable or even required for the replication cycles of the single-stranded, isometric DNA phages.

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