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Cleavage of single-stranded DNA by the A and A* proteins of bacteriophage φX174

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ABSTRACT

The purified A protein and A* protein of bacteriophage φX174 have been tested for endonuclease activity on single stranded viral φX174 DNA. The A protein (55.000 daltons) nicks single-stranded DNA in the same way and at the same place as it does superhelical RFI DNA, at the origin of DNA replication. The A* protein (37.000 daltons) can cleave the single-stranded viral DNA at many different sites. It has however a strong preference for the origin of replication. Both proteins generate 3'OH ends and blocked 5' termini at the nick site.

INTRODUCTION

The replication of the double-stranded DNA of bacteriophage φX174 is initiated by the endonucleolytic action of the viral gene A protein by introduction of a nick in the viral(+)strand of superhelical DNA (RFI DNA)(1). The 3'OH end produced functions as a primer for the subsequent synthesis of new viral strand DNA in a looped rolling circle type of DNA replication (2, 3). The cleavage site is therefore the origin of RF DNA replication. The position of this discontinuity has been determined in vitro (3, 4) and lies between nucleotides 4305 (G) and 4306 (A) of the complete φX-DNA sequence (5). The main feature of the nucleotide sequence around the origin is a large region of high A-T base content. In the related phage G4 an identical (30 nucleotides) A-T rich region is found near the origin (6, 7). Only superhelical RF DNA is a substrate for the A protein; relaxed covalently-closed RF DNA and RFII DNA are not nicked (8). Together with the A-T rich region near the origin this fact suggests the need for (partially) single-stranded DNA in the A protein action. Results of in vivo experiments (9) showed gene A to be involved also in single-stranded DNA synthesis. In vitro experiments (3, 8, 10) showed the A protein to nick single-stranded DNA of φX174 at least once. In the work presented here the action of the A protein on single-stranded DNA in vitro is

investigated in more detail. Also the purified A^* protein was tested for its activity as a nuclease. This protein is coded for by the same gene as the A protein, by means of an internal restart in the same reading frame as used for the A protein (11). The A^* protein functions late in the phage life cycle but the exact nature of its activity is yet unknown. It has no endonuclease-activity on double-stranded ϕX DNA (3, 8, own results). Results will be presented here that show the A^* protein to be a single-stranded DNA-dependent endonuclease. It cleaves ϕX single-stranded DNA in approx. 30 discrete fragments. The endonuclease-activity however has a very strong preference for the origin-sequence.

EXPERIMENTAL PROCEDURE

Materials. $|^{32}P|$ α-r UTP and $|^{32}P|$ γ-ATP (2000-3000 Ci/mmole) were purchased from New England Nuclear.

Enzymes. The A and A^* proteins are purified by affinity chromatography using single-stranded DNA-cellulose and Sepharose-heparin columns. A more detailed description of this procedure will be published elsewhere. Enzymatic activity on RFI DNA was measured by using vertical agarose slab gel electrophoresis (12). The A protein preparation showed no detectable contaminating protein bands when run on SDS-polyacrylamide gels. The A protein preparation converted 90% of RFI DNA into RFII DNA during a 30 min incubation at 37°C, in a molar ratio of protein to DNA of 5:1. The A^* protein preparation was also free of other proteins as judged from SDS-polyacrylamide gels. No nicking of RFI DNA by the A^* protein was observed not even with a molar ratio of protein to DNA of 50:1. T4 polynucleotide kinase and terminal deoxynucleotidyl transferase from calf thymus were from Boehringer Mannheim, alkaline phosphatase (BAPF) was from Worthington and proteinase K from Merck.

Single-stranded DNA fragments. Viral single-stranded DNA was isolated from viral particles by phenol extraction. 30 µg of viral ss DNA was digested with HaeIII restriction enzyme in a 0.5 ml reaction mixture containing 50 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol and 1 mM EDTA. The amount of enzyme units needed for complete digestion of single-stranded DNA was 100 times the amount needed for the digestion of double-stranded DNA. Incubation proceeded for 16 hr at 37°C. The reaction was terminated by the addition of EDTA to 50 mM and 500 µg proteinase K. Incubation was continued for 30 min at 37°C. The mixture was treated with phenol followed by precipitation of the DNA frag-

ments with ethanol. Finally the DNA fragments were dissolved in 100 µl 50 mM Tris-HCl at pH 7.5, treated with alkaline phosphatase (BAPF) and labelled at their 5' termini with $|^{32}P|$ γ-ATP and T4 polynucleotide kinase as described by Maxam and Gilbert (13).

The overall recovery of single-stranded fragments after 5' terminus labelling was estimated to be 50%. ^{32}P-labelled fragments were run on a neutral preparative 5% polyacrylamide slab gel in 40 mM Tris-acetate at pH 7.7, 20 mM Na-acetate and 2 mM EDTA. The gel was autoradiographed and the fragments were eluted from the gel as described by Maxam and Gilbert (13).

METHODS

Assay for nuclease activity on ss DNA fragments. 10 µl reaction mixtures containing ss DNA fragment, A or A^* protein, 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA were incubated during 90 min at 37°C. Reactions were terminated by addition of EDTA to 50 mM and 10 µg of proteinase K. The incubation was continued for 30 min at 37°C. Finally 20 µl formamide containing 20% sucrose, 0.25% xylene cyanol, 0.25% bromophenol blue and 0.2% sarkosyl was added. The mixtures were heated 3 min at 100°C and layered on a denaturing slab gel.

Denaturing gel electrophoresis. Denaturing gels were all 6% polyacrylamide gels made up in 98% formamide as described by Maniatis et al. (14).

Terminal deoxynucleotidyl transferase reaction. The reaction mixture contained DNA fragment (1-3 pmoles 3'OH termini), 250 mM Na-cacodylate at pH 7.0, 50 µM dithiothreitol, 50 units of terminal transferase, 2.5 mM $CoCl_2$, 100 µM Zn-acetate and 50 pmoles $|^{32}P|$ α-r UTP. After 1 hr incubation at 30°C 1 mMol unlabelled rUTP was added and the incubation was continued for 30 min at 30°C. The reaction was stopped by addition of EDTA to 25 mM and sarkosyl to 0.1%. After phenol extraction the labelled DNA fragments were separated from the non-incorporated rUTP by gel filtration on a Sephadex G75 column, concentrated by alcohol precipitation and run on formamide gels.

EXPERIMENTAL

Preliminary experiments with circular φX174 single-stranded DNA indicated that both the A and A^* proteins introduced one or more nicks in this DNA, but these experiments also made it clear that a very sensitive assay was needed to

analyse properly the activities on single-stranded DNA of the A and A^* proteins. Therefore the following experimental approach was used to detect fragmentation of single-stranded ϕX174 DNA: (i) viral (+) strand DNA was digested with the restriction enzyme HaeIII, (ii) the combined fragments were labelled at their 5' termini by T4 polynucleotide kinase and $|^{32}P|$ γ-ATP and separated on polyacrylamide gels, (iii) each 5' labelled DNA fragment was incubated with the A protein or with the A^* protein, and (iv) the reaction mixtures were run on polyacrylamide gels and after autoradiography each reaction mixture was screened for fragmentation of the HaeIII DNA fragment tested.

Digestion of ϕX174 single-stranded DNA with HaeIII gives a fragmentation pattern that is identical to that of ϕX174 double-stranded DNA with the exception of one additional DNA fragment (15) (Figure 1). This additional fragment which is repeatedly found turned out to be a partial digestion product which contains the fragments Z5 and Z8.

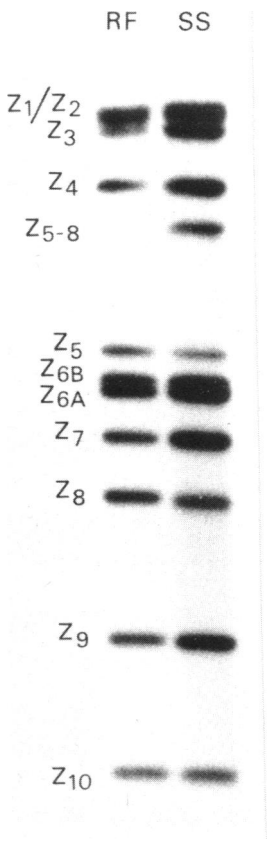

Figure 1. Analytical formamide polyacrylamide gel with the HaeIII digestion patterns of ϕX am3 double-stranded DNA (RF) and viral single-stranded DNA (ss) after labelling of the 5' termini with $|^{32}P|$ γ-ATP and T4 polynucleotide kinase.

RESULTS

Nuclease activity of the A protein

Incubation of all HaeIII single-stranded DNA fragments with the A protein, followed by electrophoresis on formamide-polyacrylamide gels and autoradiography, showed that of all DNA fragments tested, only fragment $Z6_B$ was cleaved. This fragment contains the origin of DNA replication (4, 16). Fragment $Z6_B$ gives one extra labelled band of approx. 98 nucleotides in length (Figure 2). The nucleotide sequence of this subfragment was determined with the M-G-procedure (13). The sequence autoradiograph is shown in Figure 3 and the complete sequence, together with the origin sequence of ϕX174 is given in Figure 4. It shows that the A protein cleaves the single-stranded $Z6_B$ fragment between nucleotides 4305 and 4306. The conclusion therefore is that the A protein nicks both single-stranded and superhelical double-stranded ϕX174 DNA exactly at the same place, at the origin of DNA replication. By nicking RFI DNA the A

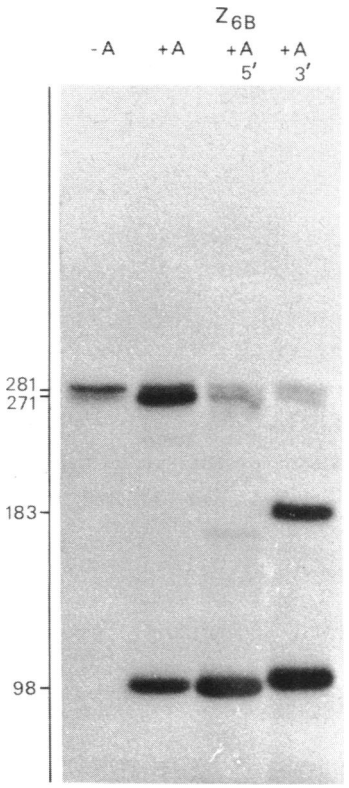

Figure 2. Cleavage pattern of the restriction fragment $Z6_B$ after incubation with and without A protein on a formamide-polyacrylamide gel. 0.4 µg of $Z6_B$ fragment was incubated with 3 µg A protein in a 35 µl reaction mixture as described in materials and methods. (- A) $Z6_B$ fragment incubated without A protein. (+ A) $Z6_B$ fragment analysed after digestion with A protein. (+ A, 5') $Z6_B$ fragment analysed after A protein digestion followed by alkaline phosphatase treatment and relabelling of the 5' termini, (+ A, 3') after relabelling of the 3'OH termini.

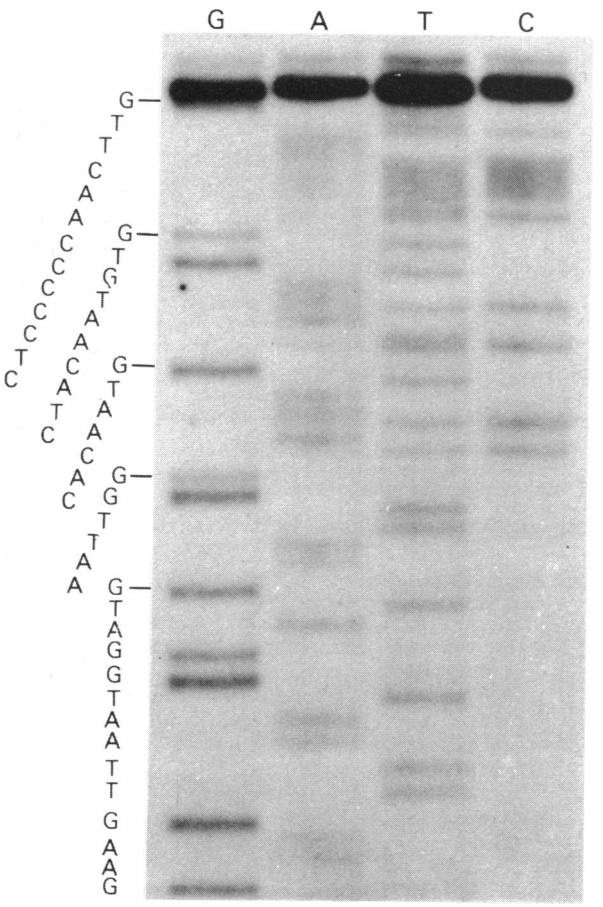

Figure 3. Sequence gel of the 98 nucleotide subfragment generated from $Z6_B$.

protein generates RFII DNA with a free 3'OH end and a blocked 5' end. This 5' end is blocked for ^{32}P-labelling with T4 polynucleotide kinase and $|^{32}P|$ γ-ATP, even after alkaline phosphatase treatment. The reason for this is most likely that the A protein becomes covalently attached to this 5' end (3). The

Figure 4

(a) 5' ..... GAAGTTAATGGATGAATTGGCACAATGCTACAATGTGCTCCCCCAACTTG 3'

(b) 5' ..... GAAGTTAATGGATGAATTGGCACAATGCTACAATGTGCTCCCCCAACTTGATATTAATAACA 3'

4256 ↑ 4317

(a) Sequence deduced from figure 3, (b) origin sequence of φX174 (5). The arrow marks the position of the A protein nick in RFI DNA.

possible presence of a blocked 5' end at the nick-site in the single-stranded $Z6_B$ DNA fragment was checked in the following way. The reaction mixture of the $Z6_B$ fragment incubated with the A protein (containing intact labelled $Z6_B$, a labelled 98 nucleotides long subfragment and an unlabelled 183 nucleotides long subfragment) was treated with alkaline phosphatase to remove all 5' terminal phosphates, including the 5' ^{32}P-phosphates. Part of this DNA mixture was then incubated with T4 polynucleotide kinase and $|^{32}P|$ γ-ATP to label the free 5' ends; the other part was labelled with terminal transferase and $|^{32}P|$ α-r UTP to label the 3'OH ends. Figure 2 shows the result of this experiment. Both subfragments can be labelled at their 3' end with terminal transferase. The small decrease in mobility is due to the addition of one or two UMP's to the 3' end. When T4 polynucleotide kinase is used only the 5' end of the 98 nucleotides fragment is labelled, showing that the 5' end of the 183 nucleotides fragment which is the 5' end of the nick-site is blocked. This shows that the nuclease activity of the A protein has similar characteristics on single-stranded DNA as on RFI DNA. In Figure 2 (lane +A, 5') after repeated labelling of the 5' end, a very weak extra band of approx. 170 nucleotides can be seen. It is not clear to us what this band represents but it contains less than 5% of the ^{32}P-label found in the 98 nucleotides fragment.

Nuclease activity of the A^* protein

A similar set of experiments was performed with the A^* protein. Each HaeIII fragment was incubated with the A^* protein and autoradiographed after gel electrophoresis. This showed the presence of at least 30 discrete subfragments indicating the same number of specific cleavage sites. An example is seen in Figure 5 for the fragments Z1, Z2 and Z3 which yield at least 20 different subfragments. Due to the very partial nature of the reaction, most or all nick-sites can be deduced from the 5' labelled subfragments. There is a striking difference in affinity for the different cleavage sites. This is shown more clearly in Figure 6. Here, fragment $Z6_B$ was mixed with Z5 and $Z6_A$ and incubated with increasing amounts of A^* protein. The first subfragment found is a 98 nucleotides subfragment of $Z6_B$, next appear two subfragments, one of 130 nucleotides of Z5 and/or $Z6_A$ and another of 180 nucleotides of Z5. The latter two become only visible when the digestion of the $Z6_B$ fragment is almost complete. The A^* protein has therefore a strong preference for the origin region.

The subsequent appearance of different subfragments suggests a stoichiometrical reaction mechanism. We therefore asked whether also the A^* protein produced a

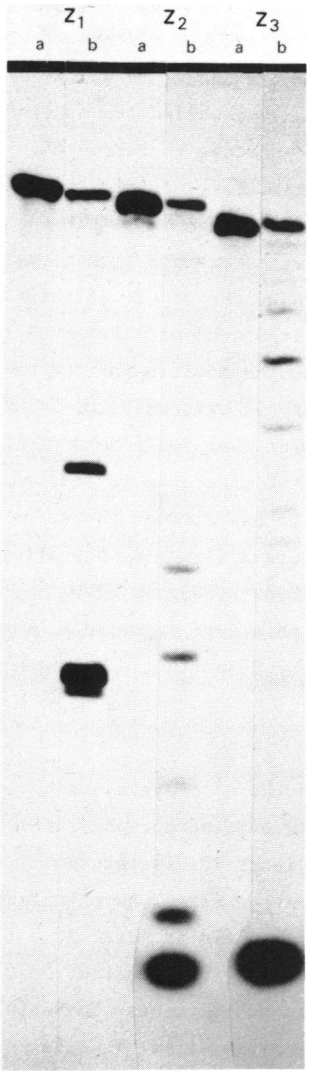

Figure 5. Formamide gel electrophoresis of the restriction fragments Z1, Z2 and Z3 after incubation with (b) and without (a) A^* protein. 0.05 µg of restriction fragment was incubated with 0.035 µg of A^* protein.

blocked 5' end. Figure 7 shows that treatment of fragment Z4 with T4 polynucleotide kinase or terminal transferase in the same way as described for the A protein, demonstrates again the presence of free 3' ends and blocked 5' ends at the cleavage sites. To make sure that the cleavage of the ss DNA at the origin site cannot be attributed to small amounts (less than 5%) of A protein in the A^* protein preparation, we isolated the A^* protein from cells that do not produce the A protein. When suppressor-less cells are infected

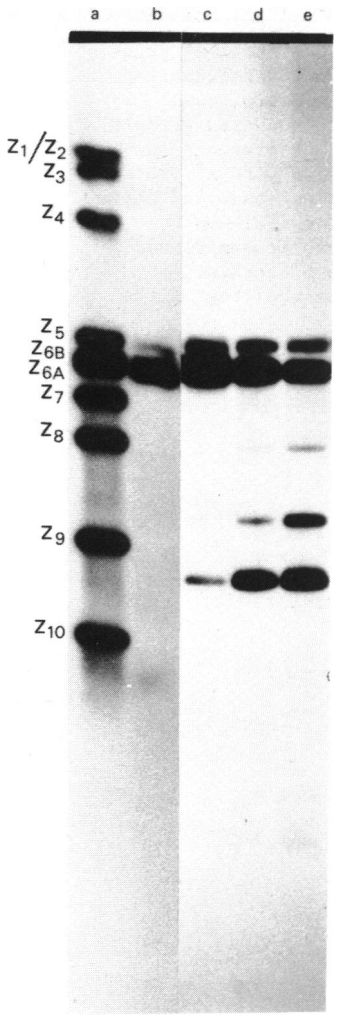

Figure 6. Formamide gel showing a mixture of restriction fragments Z5 (0.004 µg), $Z6_A$ (0.009 µg) and $Z6_B$ (0.009 µg) incubated with 0.001 µg A^* protein (c), 0.006 µg A^* protein (d), 0.012 µg A^* protein (e) and without A^* protein (b). Markers: single-stranded restriction fragments Z1-Z10 (a).

with ϕX-amber mutant am86 (A), there is no synthesis of the A protein but near normal amounts of the A^* protein are present. The A^* protein purified from these infected cells gave the same results as described above.

DISCUSSION

The results presented here show that both the A and A^* protein possess a strong sequence-specific endonuclease-activity on single-stranded DNA as can be concluded from the high efficiency of nicking of the origin containing

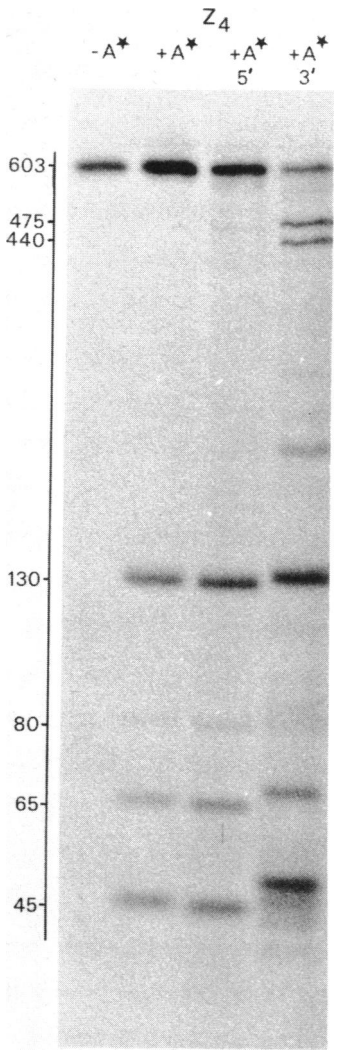

Figure 7. Formamide gel electrophoresis of the restriction fragment Z4 after incubation with and without A* protein. 1.0 µg of Z4 fragment was incubated with 0.6 µg of A* protein. (- A*) Z4 fragment incubated without A* protein. (+ A*) Z4 fragment analysed after digestion with A* protein. (+ A*, 5') Z4 fragment analysed after A* protein digestion followed by alkaline phosphatase treatment and relabelling of the 5' termini, (+ A*, 3') after relabelling of the 3'OH termini.

fragment. It is also evident from the large number of additional cleavage sites that the specificity of the nuclease-activity of the A* protein is much more relaxed than that of the A protein.

Both proteins produce free 3'OH ends and blocked 5' termini. In this respect they act in the same way as how the A protein nicks RFI DNA. The specific activity of the A protein on single-stranded DNA stresses again the very likely reason why superhelical ϕX-RF is cleaved and not the RFII DNA or the relaxed RFI DNA; the proper DNA sequence has to be present in a more or less single-

stranded form. This result does not imply persé that in vivo the A protein is active late in the infection in the synthesis of progeny viral DNA. It however supports the evidence for the involvement of the A protein in the processing of the newly synthesized viral strand during DNA synthesis in vitro (3).

The activity of the A^* protein on single-stranded DNA is quite surprising and it gives new ideas about the function of this protein in the phage life cycle. Uptill now it was suggested (17) that this protein functions late in the phage infection, possibly in the synthesis of viral single-strands and/or during the packaging of this DNA into mature phage. The here described properties of the A^* proteins tend to point to comparable functions for both the A and A^* proteins, at least during the synthesis of single-stranded DNA. It could well be that both proteins compete for the same single-stranded DNA substrate and that this competition determines whether or not the DNA synthesis switches from double-stranded to single-stranded DNA. This could also explain why the A^* protein is only found late in the infection cycle.

One might expect that given the large number of cleavage sites for the A^* protein, it would be quite easy to find the recognition sequence for this protein within the known ϕX-sequence. Comparison of the approximate cleavage site did however not yield a nucleotide sequence that is common to all. It appears that the better a sequence resembles the origin, the better it is cut by A^*.

A comparison of the amino acid sequence of the A and A^* proteins of the related phages ϕX174 and G4 (5, 6) shows a rather strong conservation of the A^* proteins (21% amino acid changes) whereas the A protein has an overall change of 37%. This means that the NH_2-part of the A protein that is absent in the A^* protein has 51% amino acid changes.
In both phages the A (and likely also A^*) protein have comparable functions (18). It therefore appears that the A^* part of the gene A can tolerate relatively few changes. The functions that reside in this part of the gene can be deduced from a comparison between A and A^*. Both proteins cleave single-stranded DNA, only the A protein nicks RFI DNA. Both proteins bind to single-stranded DNA whereas only the A^* protein binds to double-stranded DNA (unpublished results). Therefore the A^* part contains the DNA binding information and the single-stranded DNA specific nuclease-activity. The additional amino acid sequence in the A protein seems to determine the ability to cleave RFI DNA and also to determine the loss of the ds DNA binding capacity. These two extra properties apparently do tolerate a substantial change of the amino acid sequence (51%).

Genetically both proteins are very much related. The A^* protein cannot

change its amino acid sequence without a change in the A protein. Biochemically both proteins show a number of similar or comparable properties. This makes it very attractive and likely that in vivo both proteins functionally interact. It could therefore well be that the presence and ratio of these two proteins determine the mode of ϕX DNA replication in the infected cell.

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