The bond in the bacteriophage ϕX174 gene A protein–DNA complex is a tyrosyl-5′-phosphate ester

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The bacteriophage ϕX174 gene A protein cleaves the viral strand of the double-stranded replicative form (RF) DNA of the phage at a specific site, the origin. It leaves a free 3′-OH at nucleotide 4305 (G) of the ϕX DNA sequence and binds covalently to the DNA. The nature and position of the covalent bond have been determined using the octadecadesoxyribonucleotide CAACTTG[^{32}P]ATATTAATAAC. This octadecamer, which corresponds to nucleotides 4299–4316 of ϕX viral DNA, is cleaved by gene A protein. Gene A protein is bound to the labelled phosphate via a tyrosyl residue, indicating that binding occurs to the nucleotide corresponding to 4306 (A) of the ϕX viral DNA strand.

Bacteriophage ϕX174 *Gene A protein* *DNA replication* *Protein–DNA complex* *Synthetic oligonucleotide* *Tyrosyl-5′-phosphate ester*

1. INTRODUCTION

Gene A protein of bacteriophage ϕX174 (ϕX) is a multifunctional enzyme in DNA replication of the phage [1]. It creates a starting point for replication of the DNA by cleaving the viral strand of the supercoiled double-stranded replicative form DNA (RFI). The 3′-nucleotide, the G at position 4305 [2] in the complete nucleotide sequence of ϕX DNA [3], has a free 3′-OH, which serves as a primer for the subsequent rolling circle DNA replication. During the cleavage reaction gene A protein binds to the 5′-end of the nick [1,2,4], but the nature and position of this bond are unknown. At the end of one replication round, the genome length tail of the rolling circle is cut off and circularized. This reaction is supposed to take place by the cleavage-ligation-transfer of the covalently bound gene A protein with the regenerated origin in the single-stranded DNA tail of the rolling circle [5,6]. To clarify the mechanism by which these complex reactions proceed it is important to know the nature of the protein–DNA bond in the gene A protein–DNA complex. Here, we report the analysis of the nature and position of this bond. For this purpose we made use of the observation that gene A protein can cleave oligonucleotides which have at least the first 10 nucleotides in common with the highly conserved sequence of 30 nucleotides which represents the origin region of ϕX and related phages [7–9]. We constructed an oligonucleotide with a ^{32}P-labelled phosphate at the position of the phosphodiester which is broken by gene A protein.

A* protein is the product of an internal translation start in gene A [10]. It has retained a number of enzymatic activities of gene A protein [11,12]. A portion of the A* protein molecules in preparations of purified A* protein carry a covalently linked specific oligonucleotide, suggesting that A* protein performs a cleavage reaction during phage development [13]. The function of this cleavage reaction is not yet clear. The experiments with gene A protein were also performed with A* protein.

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2. MATERIALS AND METHODS

2.1. *Materials*

Gene A protein and A* protein were purified as in [14]. T4 polynucleotide kinase was purchased from Boehringer (Mannheim, FRG), [γ-^{32}P]ATP (3000 Ci/mmol) was from NEN (Boston, USA). Oligodesoxyribonucleotides and phosphotyrosine were synthesized as in [15]. Phosphoserine and phosphothreonine were purchased from Sigma (St. Louis, USA). T4 DNA ligase was a gift from P.J. Weisbeek (Dept. Molecular Cell Biology, University of Utrecht, The Netherlands).

2.2. *Construction of the internally labelled octadecamer CAACTTG[^{32}P]-ATATTAATAAC*

The undecamer $_{HO}$ATATTAATAAC$_{OH}$ (60 pmol) was labelled at the 5'-end by incubation for 1 h at 37°C with [γ-^{32}P]ATP and T4 polynucleotide kinase as in [7] in a volume of 75 μl. Then the mixture was incubated for 3 min at 90°C to inactivate T4 polynucleotide kinase. After cooling to room temperature the heptamer $_{HO}$CAACTTG$_{OH}$ (60 pmol), the hexadecamer $_{HO}$TATTAATATCAAGTTG$_{OH}$ (60 pmol), 1.5 μl of 0.75 mM dithiothreitol (DTT) and 3.75 μl of 20 mM ATP were added and the mixture (total volume 85 μl) was incubated for 1 h at 4°C to anneal the heptamer and the undecamer to the hexadecamer. Then 2 μl T4 DNA ligase were added and incubation was continued for 1 h at 4°C. The reaction was stopped by the addition of 10 μl of 0.1 M EDTA (pH 8.0). The mixture was extracted with phenol followed by extraction with ether, mixed with 70 μl of 98% deionized formamide containing 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanole F, heated for 3 min at 100°C, applied onto a 25% polyacrylamide gel (24 cm high, 16 cm wide, 0.1 cm thick) in 0.1 M Tris, 0.1 M borate, 0.002 M EDTA (pH 8.3) (2 × TBE buffer) and 7 M urea and subjected to electrophoresis for 1 h at 750 V. The radioactive products were detected by autoradiography and the band containing the internally labelled ligation product, the octadecamer CAACTTG[^{32}P]ATATTAATAAC, was excised and eluted [16]. The sample was desalted by chromatography on a Sephadex G-50 column, using 0.1 mM Tris–HCl (pH 7.5), 0.01 mM EDTA as elution buffer. Peak fractions were lyophilized, dissolved in H_2O and pooled. The yield of this procedure was about 20 pmol of labelled octadecamer.

2.3. *Incubation of gene A protein and A* protein with the ^{32}P-labelled octadecamer*

Labelled octadecamer (0.3 pmol) was incubated with 50 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 mM NaCl, 2% glycerol and 0.01% Nonidet P40 (NP40) in a volume of 320 μl for 30 min at 30°C. Then 40 μl of 0.1 M EDTA (pH 8.0) and 100 μg lysozyme (20 μl of a solution of 5 mg/ml) were added (at this stage samples of 5% were taken for analysis on SDS-polyacrylamide gels). Lysozyme serves as a carrier at the protein precipitation which was performed by adding 360 μl ice-cold 20% trichloroacetic acid. After standing for 20 min at 0°C protein was spun down, pellets were washed with 1 ml ice-cold 10% trichloroacetic acid, dissolved in 100 μl of 50 mM NH_4HCO_3 and extracted with ether to remove residual trichloroacetic acid.

2.4. *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was carried out on a 12.5% polyacrylamide gel (24 cm high, 16 cm wide, 0.1 cm thick), using the gel system of [17]. Samples were mixed with 20 μl buffer containing 0.125 M Tris–HCl (pH 6.0), 2% SDS, 12.5% glycerol, 12.5% β-mercaptoethanol and 0.005% bromophenol blue. The samples were heated for 3 min at 100°C, applied to the gel and electrophoresis was performed for 19 h at 50 V. Proteins were stained with silver as in [18]. Radioactive bands were detected by autoradiography.

2.5. *Acid hydrolysis*

Ten μl of the protein samples obtained by trichloroacetic acid precipitation were lyophilized and dissolved in 20 μl H_2O containing 0.2% NP40. Twenty μl of 12 N HCl were added and the samples incubated in sealed capillary glass tubes for 3 h at 110°C. Then the samples were lyophilized and dissolved in 20 μl H_2O. Ten μg of each of the marker phosphoamino acids, phosphoserine, phosphothreonine and phosphotyrosine, were added, the samples lyophilized, dissolved in 20 μl H_2O, lyophilized and finally dissolved in 10 μl H_2O and applied on Whatman 3M paper.

2.6. *High-voltage paper electrophoresis*

Paper electrophoresis was performed in 5% acetic acid, adjusted to pH 3.5 with pyrimidine for 1¾ h at 1500 V. The paper was dried and the marker phosphoamino acids were visualized by spraying with a solution of ninhydrin (0.2% ninhydrin, 5% acetic acid in ethanol) and heating for 30 min at 60°C. The radioactive products were detected by autoradiography.

3. RESULTS AND DISCUSSION

The octadecamer carrying an internal radioactive phosphate, $_{HO}$CAACTTG[^{32}P]ATATTAATAAC$_{OH}$, was obtained by coupling $_{HO}$CAACTTG$_{OH}$ to 5′-labelled [^{32}P]ATATTAATAAC$_{OH}$ with T4 DNA ligase using a complementary oligonucleotide as shown in fig.1. This octadecamer corresponds to nucleotides 4299–4316 of the ϕX DNA sequence [3] and is specifically cleaved after the guanylic residue by the gene A and A* proteins [7]. Gene A protein or A* protein was incubated with the ^{32}P-labelled octadecamer and analyzed on an SDS-polyacrylamide gel. The autoradiogram (fig.2) shows that both proteins become radioactively labelled by the reaction with the octadecamer. They form tight complexes which are resistant to heating in the presence of SDS (which is done before the samples are applied to the gel). A gene A protein–DNA complex which is resistant to heating in SDS has been reported in [5]. These authors also showed that the complex is not dissociated by treatment

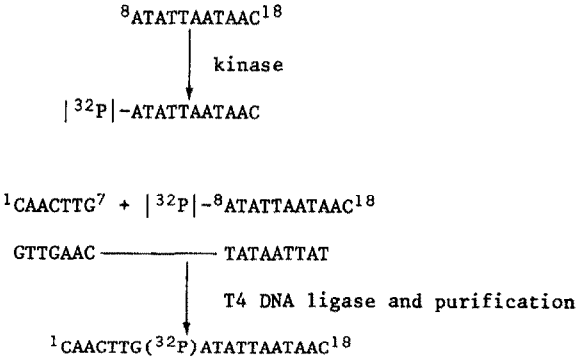

Fig.1. Construction of the ^{32}P-labelled octadecadesoxyribonucleotide. The sequence 1–18 corresponds to the sequence 4299–4316 of the ϕX DNA sequence [3].

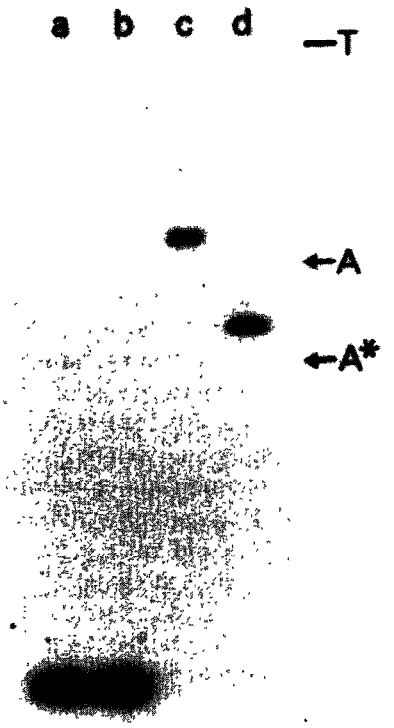

Fig.2. SDS-polyacrylamide gel analysis of gene A protein and A* protein after reaction with the ^{32}P-labelled oligonucleotide. Autoradiogram of the polyacrylamide gel. Lanes a,b: control incubations of gene A protein (a) and A* protein (b) with the oligonucleotide in the presence of 0.01 M EDTA. Lanes c,d: incubations of the oligonucleotide with gene A protein (c) and A* protein (d). The arrows show the positions of gene A protein and A* protein when stained with silver. T, top of the running gel.

with 0.2 M NaOH. The positions of the radioactive bands show that the mobilities of the protein–oligonucleotide complexes are reduced compared to the unlabelled proteins (fig.2). This is probably due to the presence of the 11-nucleotide fragment, which remains bound after cleavage of the octadecamer. These complexes are not formed if the incubations are performed in the presence of 0.01 M EDTA (fig.2). Under these conditions the oligonucleotide is not cleaved by gene A protein or A* protein.

To determine the nature of the bond between gene A protein or A* protein and the

oligonucleotide, the ^{32}P-labelled protein–DNA complexes were subjected to partial hydrolysis in 6 N HCl. The hydrolysates were analyzed by high-voltage paper electrophoresis. Radioactive products were detected by autoradiography. Phosphoserine, phosphothreonine and phosphotyrosine were coelectrophoresed as references and visualized with ninhydrin (fig.3). The major radioactive product of the hydrolysis of the gene A protein–oligonucleotide complex is present at the position of P_i. The second radioactive product coincides with the position of phosphotyrosine. The radioactive products at the other positions did not coincide with one of the references. They were eluted from the paper and yielded P_i and a radioactive product at the position of phosphotyrosine when treated with HCl and subjected to paper electrophoresis for a second time. Therefore, these products were probably incompletely hydrolyzed complexes (peptide–oligonucleotide or peptide–phosphate complexes). Analysis of the radioactive A* protein–oligonucleotide complex gave the same results (fig.3).

The results indicate that gene A protein and A* protein are linked via a tyrosyl residue to the 5′-phosphate of the adenylic residue at position 8 of the octadecamer (fig.1). It was shown in [7] that cleavage of the hexadecamer CAACTTGATATTAATA by the gene A protein or A* protein produced the heptamer $CAACTTG_{OH}$. Together these results indicate that the reaction of the gene A and A* proteins with these oligonucleotides involves the lysis of the phosphodiester bond between position 7 (G) and 8 (A), creating a 3′-OH at one end and a tyrosyl-5′-phosphate ester bond at the other end. This type of phosphodiester bond cleavage may therefore be called tyrosinolysis.

The results obtained are also pertinent to the cleavage reactions which are carried out by the ϕX gene A protein during ϕX RF DNA replication. It has been shown [2] that during initiation ϕX gene A protein cleaves the viral strand of ϕX RFI DNA, creating a 3′-OH group at position 4305 (G) in the viral strand and that the gene A protein becomes covalently linked to the DNA [1,2,4]. Our results clarify the nature of this linkage and show that the protein is covalently bound to the 5′-phosphate of the adjacent adenylic residue in the DNA chain at position 4306. Therefore, no gap is created in the cleavage reaction.

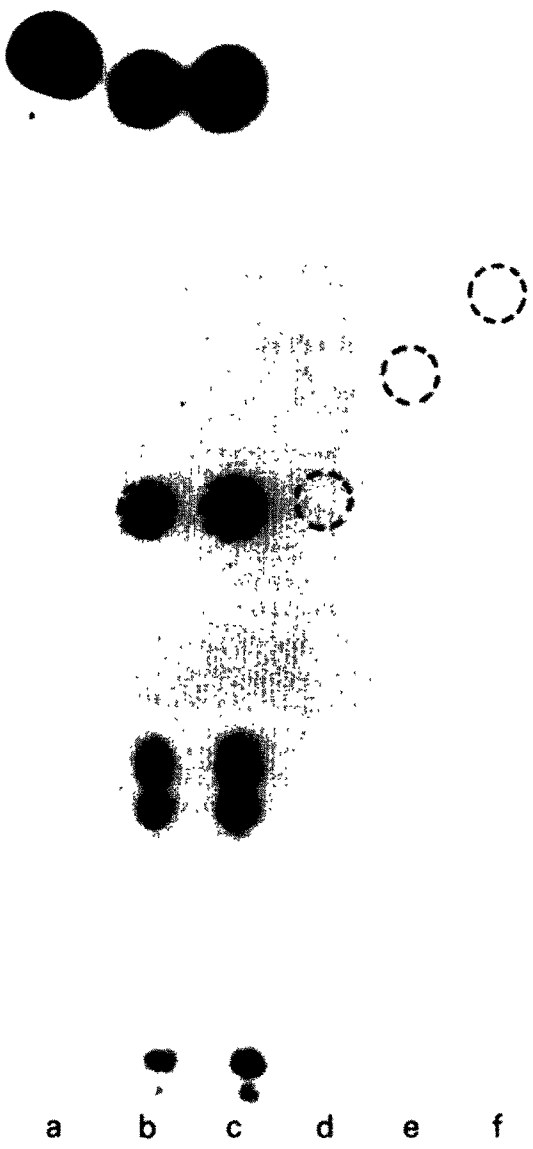

Fig.3. Analysis of the gene A protein–oligonucleotide and A* protein–oligonucleotide complexes after acid hydrolysis. Autoradiogram after high-voltage paper electrophoresis. Lane a: control, the oligonucleotide after acid hydrolysis. Lanes b,c: the gene A protein–oligonucleotide (b) and A* protein–oligonucleotide (c) complexes after hydrolysis. The hydrolysates were mixed with phosphoamino acids. The dotted circles show the positions of the phosphoamino acids, detected with ninhydrin. Lanes d–f: the dotted circles indicate the positions of phosphotyrosine (d), phosphothreonine (e) and phosphoserine (f) after detection with ninhydrin.

The results show that A* protein is bound to the DNA in the same way. A* protein lacks the N-terminal part (1/3 of the polypeptide chain) of the A protein; it starts probably at nucleotide 4497 in the ϕX DNA sequence [3]. A* protein has retained some of the enzymatic activities of gene A protein; it can cleave and ligate single-stranded DNA [11–13,19]. This suggests that the first 5 tyrosyl residues in the polypeptide chain of gene A protein, which occur in the part that lacks A* protein, are not involved in the covalent binding of gene A protein to DNA.

Amino acid analysis or sequencing of radioactive peptides which can be obtained after cleavage of the A protein–oligonucleotide complex with proteolytic enzymes could reveal which of the tyrosine residues in gene A protein are involved in cleavage of and binding to DNA. However, these analyses require more gene A protein than is available and need a better production method of gene A protein, for example, by cloning gene A in a plasmid such that its expression is greatly increased.

Topoisomerase I of *E. coli* and *M. luteus* and subunit A of *M. luteus* gyrase form, like ϕX gene A protein, covalent protein–DNA complexes with DNA in which the protein is linked to the 5′-phosphate of the DNA via a tyrosyl residue [20]. These enzymes cleave and ligate DNA without the need for additional energy in the form of, for example, ATP. Obviously, the energy which is released at the cleavage of the phosphodiester bond is conserved in the phosphotyrosyl bond and can be used to form a new phosphodiester bond. The phosphotyrosyl bonds in these protein–DNA complexes may be regarded as energy-rich in analogy with tyrosyl–phosphate bonds in other proteins such as glutamine synthetase–tyrosyl-*O*-adenylate [21] and *src* gene kinase phosphorylated immunoglobin [22]. ϕX gene A protein can thus be considered to be a sequence-specific breakage and reunion enzyme which has, in contrast to the topoisomerases, a protracted coupling period [23]. Gene A protein shows a relaxing activity like the topoisomerases I have when it is incubated with ϕX RFI DNA under rather unphysiological conditions, in the presence of Mn^{2+} instead of Mg^{2+} [14].

The bond between the genome-linked protein VPg and poliovirus RNA is also a tyrosyl-phosphate ester [24,25]. VPg presumably acts as a primer at the replication of the viral RNA [26]. Thus, it differs from the above proteins although it has not been excluded completely that gene A protein itself could act as a primer at the DNA synthesis.

Recently it has also been shown, using a different approach, that gene A protein is linked to DNA via a tyrosyl-phosphate diester (Roth, M.J., Brown, D.R. and Hurwitz, J., personal communication).

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