

Alteration of the ATG start codon of the A* protein of bacteriophage ϕ X174 into an ATT codon yields a viable phage indicating that A* protein is not essential for ϕ X174 reproduction

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Bacteriophage ϕ X174 gene A encodes two proteins: the gene A protein and the smaller A* protein, which is synthesized from a translational start signal within the A gene in the same reading frame as the gene A protein. The gene A protein is involved in initiation, elongation and termination of rolling circle DNA replication. The role of the A* protein in the life cycle of ϕ X174, however, is unknown. Using oligonucleotide-directed mutagenesis a viable ϕ X174 mutant was constructed in which the ATG start codon of the A* protein was changed into an ATT codon. This mutant, ϕ X-4499T, does not synthesize A* protein. The burst size of ϕ X-4499T amounted to 50% of that of wild type ϕ X174. This indicates that A* protein, although advantageous for phage reproduction, is not essential during the life cycle of bacteriophage ϕ X174.

A* protein; Oligonucleotide-directed mutagenesis; ATG start codon; (Bacteriophage ϕ X174)

1. INTRODUCTION

The function of the initiator gene A protein in the life cycle of bacteriophage ϕ X174 has been elucidated by studying conditional lethal gene A mutants and by studying its role in *in vitro* ϕ X174 DNA synthesizing systems (reviews [1-3]). ϕ X174 rolling circle DNA replication is initiated by gene

A protein cleavage of the viral strand of the parental RFI DNA at a unique site, the origin of replication [4]. During this cleavage reaction an ester bond is formed between either one of the tyrosyl residues in position 343 or 347 of the gene A protein and the phosphate of the adenylic acid residue (no. 4306 of the ϕ X174 DNA sequence [5]) at the 5'-end of the cleavage site [6]. The covalently bound gene A protein participates in the replication complex during elongation and termination of DNA replication. At the end of a replication round gene A protein cleaves the regenerated origin and ligates the 3'- and 5'-end of the viral strand to form a circle.

The role of the A* protein, which is synthesized from an internal translation start within gene A [7], during the life cycle of bacteriophage ϕ X174 is less clear. Mutational analysis of the A* protein is

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Abbreviations: RFI, replicative form DNA with both strands closed containing superhelical turns; ss, single-stranded; ds, double-stranded; c, circular

complicated, because any A* mutation also results in the same mutation in the gene A protein.

A* protein has been purified and its properties have been studied extensively in vitro. A* protein binds strongly to ds- and ss-DNA [8]. It has retained nuclease activity on the ss-DNA of gene A protein, although with a more relaxed sequence specificity [9]. It also binds covalently to the 5'-end of the cleaved DNA and is able to transfer the attached DNA to a 3'-OH-terminated acceptor [10,11].

A* protein has lost the ability to cleave the viral strand of ϕ XRFI at the origin. It is not required in the RF \rightarrow ss (c) in vitro ϕ X174 DNA synthesizing system or in the stage III in vitro replication system in which the synthesized viral strand DNA is directly packaged into viral proheads [12]. It inhibits ϕ X174 DNA synthesis in vitro by aspecific binding to ds-DNA [13]. A* protein has been implicated in shutoff of host DNA synthesis [14,15]. Shutoff of host DNA synthesis occurred only in ϕ X174 infections in which the A* protein was present. Recent cloning of the A* protein showed that the A* protein is lethal for the host cell [16]. Upon induction of A* protein synthesis, *Escherichia coli* DNA synthesis was inhibited.

In order to obtain more information about the role of ϕ X174 gene A* protein during the life cycle of ϕ X174 we have mutagenized the ATG start codon of the A* protein using a mixed oligonucleotide primer. We have obtained a viable ϕ X174 mutant, which synthesizes no A* protein upon infection of *E. coli* C. This indicates that the A* protein is not essential for the reproduction of bacteriophage ϕ X174.

2. MATERIALS AND METHODS

2.1. Phage and bacterial strains

E. coli strain C112 (*E. coli* C) was used as the normal host for plating and propagation of wild type ϕ X174 and ϕ X174 mutants. *E. coli* K58 *ung*⁻, *sup*⁺ [17] was used as the host in the spheroplast assay for ϕ X174 DNA. ϕ X174 *am3* is a lysis defective ϕ X174 mutant [18].

2.2. Construction, isolation and characterization of ϕ X174 mutant phage

ϕ X-4499T, a mutant phage with a G \rightarrow T substitution at position 4499 of the ϕ X174 DNA se-

quence, was constructed using two oligonucleotide primers on wild type ϕ X174 DNA as a template essentially as described by Zoller and Smith [19]. The 5'-P mutagenic primer, GATTTXXYAGTG-GAGG, in which X represents 25% of each nucleotide and Y 80% T (wild type) and 20% C, is complementary to nucleotides 4489-4504 of the ϕ X174 DNA sequence. As wild type 5'-OH primers ACCGTCTTCTCGTTCT and CAAGCA-TTGGGGATTG complementary to nucleotides 4360-4375 and 4089-4104 of the ϕ X174 DNA sequence, respectively, were used. Complementary strand ϕ X174 DNA was synthesized at 15°C during 16 h by *E. coli* DNA polymerase I, Klenow fragment (Boehringer, Mannheim, FRG) in the presence of T₄ DNA ligase (Boehringer, Mannheim). After transfection of *E. coli* K58 *ung*⁻, *sup*⁺ spheroplasts with ϕ X174 DNA the resulting plaques were screened for mutants by filter hybridization using the wild type oligonucleotide complementary to nucleotides 4489-4504 of the ϕ X174 sequence as probe. Hybridization and washing were performed at 35°C. The hybridization solution [20] contained 1-2 \times 10⁶ dpm/ml end labelled ³²P-oligonucleotide. After a second screening a mutant plaque was propagated in a 150 ml culture of *E. coli* C at a cell density of about 10⁸/ml in TK broth (10 g Bacto Tryptone/l, 5 g KCl/l, 10 mM MgSO₄, 2 mM CaCl₂). The DNA sequence of the mutant was determined by sequence analysis according to the chain termination method [21,22] using a wild type, ³²P-end labelled oligonucleotide complementary to nucleotides 4532-4547 of the ϕ X174 DNA sequence as a primer.

2.3. Determination of the burst size of bacteriophage ϕ X174

E. coli C was grown in TK broth to a density of 3 \times 10⁸ cells/ml. To 0.85 ml of *E. coli* 0.1 ml of 0.03 M KCN was added to synchronize the subsequent infection. After 10 min *E. coli* C was infected with 1 \times 10⁸ phage suspended in 0.05 ml TK broth. 10 min later the culture was diluted 10⁴ in prewarmed TK broth and incubated under vigorous shaking at 37°C. Samples at different time intervals were taken and the number of infective centers was determined by titration. The burst size of the phage was calculated by dividing the average number of infected centers after lysis by

the average number of infected centers before lysis of the *E. coli* vells.

2.4. Detection of ϕ X174 gene A protein and ϕ X174 A* protein in ϕ X174 infected cells

E. coli C was grown in TK broth to a density of 5×10^8 cells/ml. The cells were infected with ϕ X174 wild type, ϕ X174 *am3* or ϕ X-4499T phages at a multiplicity of infection of 5. Samples of 10 ml were taken from the different cultures just before infection and at 10, 30 and 60 min after infection. The samples were rapidly cooled and centrifuged for 15 min at 10 000 rpm at 0°C in a Sorvall SS34 rotor. The pellets were resuspended in 1 ml of 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, spun down, resuspended in 1 ml of 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM β -mercaptoethanol and stored at -70°C. After thawing the cells were lysed by the addition of 10 μ l solution of lysozyme (10 mg/ml) in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and incubated for 30 min at 4°C and 2 min at 37°C. Then 250 μ l of 5 M NaCl were added and the suspensions were mixed gently for 30 min at 0°C. The samples were centrifuged for 30 min at 16 000 rpm at 0°C in a Sorvall SS34 rotor. The supernatants (lysates) were transferred to Eppendorf tubes and used in the following assay. 4 μ l of the above lysates were incubated with 0.03 pmol of the internally 32 P-labelled oligonucleotide CAACTTG[32 P]ATATTAATAAC (approx. 50 000 cpm) in a volume of 16 μ l as described before [6]. The incubations were stopped by the addition of 2 μ l of 0.1 M EDTA, pH 8.0. Then 14 μ l of 5 mM Na-phosphate, pH 7.0, 1% SDS, 30 mM dithiothreitol, 10% glycerol, 0.05% bromophenol blue were added, the samples were heated for 2 min at 100°C and subjected to electrophoresis on a SDS-polyacrylamide gel (20%) as described by Laemmli [23]. After electrophoresis the radioactive material was visualized by autoradiography. The gels corresponding to the position of the gene A protein-oligonucleotide and A* protein-oligonucleotide complexes were cut out and the Čerenkow radiation was determined in a liquid scintillation counter.

3. RESULTS

Mutation of the ATG start codon of ϕ X174 A* protein in such a way that initiation of A* protein

synthesis is inhibited may provide insight into the function of the A* protein during reproduction of bacteriophage ϕ X174. If it is possible to create a viable ϕ X174 mutant, which does not synthesize A* protein, the conclusion that A* protein is not essential for ϕ X174 reproduction is justified. However, the fact that any mutation in the ATG start codon of the A* protein will also change the methionine residue in position 173 of the gene A protein further complicates the approach. If this part of the gene A protein is important for the function of the gene A protein, substitution of this methionine residue may result in an inactive gene A protein. Therefore failure to obtain a viable ϕ X174 mutant with an altered ATG start codon of the A* protein may be explained in either one or two ways, namely that the A* protein is essential for ϕ X174 reproduction or that the presence of a methionine residue in position 173 of the gene A protein is important for the function of the gene A protein.

A mixed oligonucleotide, GATTTXXYAGTG-GAGG (X represents 25% of each nucleotide and Y 80% T, wild type, and 20% G, respectively) complementary to nucleotides 4489-4504 of the ϕ X174 DNA sequence, was synthesized to induce ϕ X174 mutants with an altered ATG start codon of the A* protein. By using this oligonucleotide as a mutagenic agent the ATG start codon of the A* protein may be changed into any other triplet except a triplet with a G or T in the first position. These nucleotides were deliberately omitted during the synthesis of the oligonucleotide, because it is known that GTG and TTG may be used as initiation codons for protein synthesis [24]. The mutagenesis experiment was performed essentially as described by Zoller and Smith [19] using, besides the phosphorylated mutagenic oligonucleotide, a second wild type 5'-OH oligonucleotide primer. Screening of 1000 plaques, obtained after transfection of *E. coli* K58 *ung*⁻, *sup*⁺ spheroplasts with ds ϕ X174 DNA synthesized in this way, yielded one mutant phage. DNA sequence analysis of this mutant and wild type ϕ X174 as a control, according to the chain termination method, showed that the ATG start codon of the A* protein has been changed into an ATT codon (fig.1). The mutant will be designated as ϕ X-4499T, indicating the nucleotide change in position 4499 of the ϕ X174 DNA sequence. This nucleotide change alters the

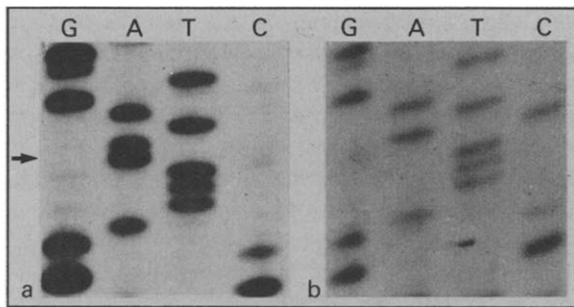


Fig. 1. DNA sequence of ϕ X-4499T (a) and wild type ϕ X174 (b) in the region of the mutation, ϕ X no. 4492-4507. The arrow indicates the position of the mutation.

methionine residue in position 173 of the gene A protein into an isoleucine residue. The DNA sequence analysis also revealed that our wild type ϕ X174 contains at position 4518 an A residue instead of a G residue as published by Sanger et al. [5] for their *am3cs70* ϕ X174 phage. This nucleotide change results in an amino acid substitution of an alanine into a threonine residue in the gene A protein as well as in the A* protein. The ϕ X-4499T mutant forms smaller plaques than ϕ X174 wild type phage on an *E. coli* C lawn. This smaller plaque morphology is correlated to the burst size of the mutant. The burst size of ϕ X-4499T, measured in a one-step growth experiment, is 60 compared to 124 found for wild type ϕ X174 in the same experiment.

To analyze if the ϕ X-4499T mutant produced A* protein during infection, cell lysates obtained at various times after infection were incubated with the internally ^{32}P -labelled oligonucleotide CAACTTG[^{32}P]ATATTAATAAC. This octadecamer corresponds to the first 18 nucleotides of the ϕ X174 replication origin. Upon incubation with gene A protein or A* protein this oligonucleotide is specifically cleaved after the G residue [25]. After cleavage gene A protein and A* protein are radioactive due to covalent linkage of the radioactive oligonucleotide [^{32}P]ATATTAATAAC to a tyrosyl residue of these proteins. The radioactive gene A and A* proteins are separated by polyacrylamide gel electrophoresis. Fig. 2 shows the autoradiogram of the polyacrylamide gel of this analysis. As a control the infection was also carried out with wild type ϕ X174 and the lysis defective

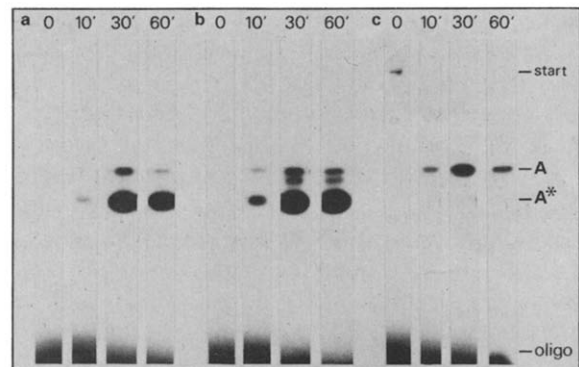


Fig. 2. SDS-polyacrylamide gel electrophoresis of cell lysates, obtained at various times after infection of *E. coli* C with wild type ϕ X174 (a), ϕ X174 *am3* (b) and ϕ X-4499T (c), respectively, after reaction with the ^{32}P -labelled octadecamer, CAACTTG[^{32}P]ATATTAATAAC.

ϕ X174 *am3*. The infection with ϕ X174 *am3* shows increasing amounts of gene A protein and A* protein as the infection proceeds. The drop in the amount of gene A protein (and A* protein) observed 60 min after infection with wild type ϕ X174 and ϕ X-4499T is due to lysis of the cells. In contrast to wild type ϕ X174 and ϕ X174 *am3* no A* protein could be detected during infection with the ϕ X-4499T mutant. Fig. 3 shows a quantitation of

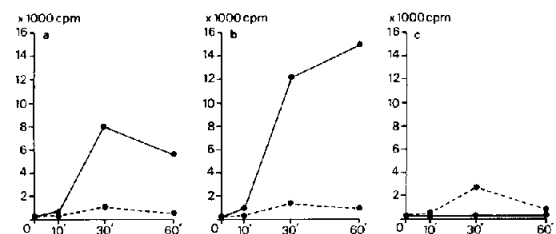


Fig. 3. Amount of gene A protein (---) and A* protein (—) present at different times after infection of *E. coli* C with ϕ X174 wild type (a), ϕ X174 *am3* (b), and ϕ X-4499T (c), respectively. The relative amounts of gene A protein and A* protein were determined by counting the Čerenkov radiation present in the gel at the position of the gene A protein-oligonucleotide complex and the A* protein-oligonucleotide complex as obtained after reaction with the ^{32}P -labelled octadecamer, CAACTTG[^{32}P]ATATTAATAAC and separation on a SDS-polyacrylamide gel (fig. 2).

this result. The level of A* protein in the ϕ X-4499T infected culture is reduced at least 100-fold compared to the wild type infection. Despite the lower burst size of ϕ X-4499T the culture infected with ϕ X-4499T contained even higher levels of the gene A protein than infected wild type cells. It is concluded from these experiments that A* protein, although advantageous for phage reproduction, does not fulfill an essential role in the life cycle of bacteriophage ϕ X174.

4. DISCUSSION

Alteration of the ATG start codon of the A* protein of bacteriophage ϕ X174 into an ATT codon by means of oligonucleotide-directed mutagenesis has resulted in a viable ϕ X174 mutant phage. This mutant ϕ X-4499T, produces functional gene A protein and no A* protein. The 50% lower burst size compared to wild type ϕ X174 is caused either by the changed gene A protein, a methionine \rightarrow isoleucine substitution in position 173, or by the absence of A* protein. We favor the latter possibility. The shutoff of host DNA replication by the A* protein in wild type infection makes the host replication proteins available for ϕ X174 DNA synthesis. This will increase the number of replicating RF molecules late in infection and may enhance in this way the burst size.

Recently we have proposed a model in which the A* protein is implicated in the switch from stage II (RF \rightarrow RF) to stage III (RF \rightarrow ss-DNA-phage) replication [26]. This model is based on the finding that part of the A* molecules carry a covalently bound oligonucleotide, that appeared to arise from cleavage of the viral strand in the intergenic region between the genes J and F [11]. Since free ss viral DNA is not present in the infected cell and since A* protein does not cleave ds ϕ X174 DNA, cleavage of the displaced viral strand of the rolling circle by the A* protein probably takes place. This can only occur if the displaced strand is not packaged. As the A* protein concentration increases during infection, cleavage of the viral strand will block RF \rightarrow RF replication. No completed circular viral DNA is synthesized and consequently the replication machinery is no longer involved in the synthesis of complementary strand DNA and is available for the synthesis of viral DNA during stage III replication. Recent in vitro

studies have shown that the ϕ X174 gene C protein also inhibits viral strand synthesis by binding after a replication round to the replication complex [27]. Addition of proheads results in the starting of stage III replication. So the amount of gene C protein seems to be an important factor in determining the transition of RF replication to ss-DNA synthesis.

Similar results to those here for the A* protein have been obtained for the gene K product of ϕ X174. This gene is also not essential for phage viability, although its absence decreases the burst size 3–6-fold. In this case no clues exist for the profitable function of gene K product during the infection cycle [28].

The initiator protein of the filamentous phages (M13, f₁, f_d), gene II protein, has a similar function to gene A protein during the infection cycle of the isometric phages. The filamentous phage genome also encodes a smaller protein, the gene X protein, which is synthesized from an internal start signal in the same reading frame as the gene II protein. However, site-directed mutagenesis of the ATG start codon of the gene X protein, which was essential for the viability of the filamentous phages [29]. ss-DNA synthesis, but not RF replication was blocked when the gene X protein was absent. Apparently there exists a fundamental difference in the function of the gene X protein of the filamentous phages and the A* protein of the isometric phages. Shutoff of host DNA synthesis, which appeared the most important activity of the A* protein, does not occur in filamentous phage-infected hosts. These cells continue to grow and divide, extruding continuously newly synthesized phage particles.

To our knowledge one example of initiation of prokaryotic protein synthesis using an ATT codon has been described in the literature [30]. The synthesis of the *E. coli* initiation factor IF3 starts effi-

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IF3      : A T T G G A G G A A T A A G G T A T T A A A
φX-4499T: G C T G G A G G C C T C C A C T A T T A A A
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Fig.4. Comparison of the DNA sequence of ϕ X-4499T (ϕ X no. 4481–4502) with the DNA sequence at the initiation site of *E. coli* initiation factor IF3. The ATT codon and the GGAGG Shine-Dalgarno sequence are underlined.

ciently on an ATT codon. Comparison of the DNA sequence of ϕ X-4999T with the DNA sequence around the initiation site of IF3 synthesis shows a remarkable homology (fig.4). Eight nucleotides upstream from the ATT codon the same, strong Shine-Dalgarno sequence, GGAGG, is found. In both cases the sequence shows a T and an A residue on the 5'- and 3'-sides of the ATT codon respectively. These nucleotides have considerable influence on the formation of the initiation complex probably by extra hydrogen bonds with the nucleotides flanking the anticodon in the anticodon loop of fMet-tRNA [31-34]. Despite these similarities in DNA sequence between the ϕ X-4499T mutant and the initiation site of IF3 protein synthesis, no initiation of A* protein synthesis has been observed in ϕ X-4499T infected cells. Therefore subtle differences in the primary structure, the sequence between the Shine-Dalgarno sequence and the ATT codon, and the secondary structure may have important consequences for the initiation of protein synthesis.

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