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Studies of the recognition sequence of ϕX174 gene A protein. Cleavage site of ϕX gene A protein in St-1 RFI DNA

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ABSTRACT

It is already known that ØX gene A protein converts besides ØX RFI DNA also the RFI DNAs of the single-stranded bacteriophages G4, St-1, α3 and ØK into RFII DNA. We have extended this observation for bacteriophages G14 and U3. Restriction enzyme analysis placed the ØX gene A protein cleavage site in St-1 RF DNA in the *Hinf*I restriction DNA fragment F_{10} and in the overlapping *Hae*III restriction DNA fragment Z_7. The exact position and the nucleotide sequence at the 3'-OH end of the nick were determined by DNA sequence analysis of the single-stranded DNA subfragment of the nicked DNA fragment F_{10} obtained by gelelectrophoresis in denaturing conditions. A stretch of 85 nucleotides of St-1 DNA around the position of the ØX gene A protein cleavage site was established by DNA sequence analysis of the restriction DNA fragment Z_7F_1. Comparison of this nucleotide sequence with the previously determined nucleotide sequence around the cleavage site of ØX gene A protein in ØX174 RF DNA and G4 RF DNA revealed an identical sequence of only 10 nucleotides. The results suggest that the recognition sequence of the ØX174 gene A protein lies within these 10 nucleotides.

INTRODUCTION

Bacteriophage ØX174 gene A protein is the only phage-coded protein required for ØX RF DNA replication, as was first shown by Tessman (1) for the closely related bacteriophage S13. Initiation of ØX RF DNA replication is mediated by the endonucleolytic action of the gene A protein, which introduces a single nick in the viral strand of ØX RFI DNA (2). This nick serves as the origin of ØX RF DNA replication.

The origin of ØX RF DNA replication has been placed by *in vivo* (3) and *in vitro* studies (4,5) in a particular region of the *Hae*III restriction DNA fragment Z_{6B} which is located within the viral gene A of the ØX genome. The exact position of the ØX gene A protein cleavage site in ØX RF DNA has been determined by Langeveld *et al.* (6). Their results indicate that ØX gene A protein cleaves the 3' sugar-phosphate bond of the guanylic acid residue which corresponds with nucleotide 4305 (7) in the viral strand of ØX RFI DNA.

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In studies on the recognition sequence of the ØX gene A protein, van Mansfeld *et al.* (8) have shown that RFI DNA of the ØX related bacteriophage G4 is also nicked at a unique site by incubation with ØX gene A protein. The ØX gene A protein cleaves the 3' sugar-phosphate bond of the guanylic acid residue which corresponds with nucleotide 506 (9) in the viral strand of G4 RFI DNA. Comparison of the viral strand DNA sequences around the ØX gene A protein cleavage sites in ØX RF DNA and G4 RF DNA revealed a stretch of 30 completely conserved nucleotides (8, 10). In order to determine which nucleotides within that region are essential for the origin function we have studied the action of ØX gene A protein on RFI DNAs of other single-stranded DNA containing bacteriophages. The results, presented in this paper, confirm that the RFI DNAs of bacteriophages St-1 and α3 are also nicked by ØX gene A protein (11) and extend this observation for bacteriophages G14 and U3. The DNA sequence around the cleavage site in St-1 RF DNA has been established and compared with the corresponding sequences in ØX and G4.

MATERIALS AND METHODS

Preparation of RF DNA

Escherichia coli was grown in a medium containing 10 g Bacto-Tryptone, 5 g KCl, 10 mM $MgSO_4$ and 2 mM $CaCl_2$ per liter. As a host for St-1 and U3 Escherichia coli W3110 was used and for ØX174, α3 and G14 E. coli BTCC 122. At a cell density of about 3×10^8/ml the culture was infected with phage at a multiplicity of infection of 3-4. Chloramphenicol was added 2 min after infection to a concentration of 40 μg/ml. RF DNA was prepared according to the method of Jansz *et al.* (12). Pure RFI DNA was isolated by CsCl buoyant density centrifugation in the presence of 200 μg/ml ethidium bromide (13).

Enzymes

ØX174 gene A protein was purified by affinity chromatography using single-stranded DNA cellulose and Sepharose-heparin columns and showed no detectable contaminating protein bands as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Details of the purification procedure will be published elsewhere. The restriction endonucleases *Hae*III and *Hinf*I were isolated according to a combination of the methods of Takanami and Kojo (14) and Takanami (15) as described by Vereijken (16). T4 polynucleotide kinase and the restriction endonuclease *Alu*I were purchased from Boehringer (Mannheim); bacterial alkaline phosphatase (BAPF) was from Worthington (Freehold, NJ), proteinase K was from Merck (Darmstadt). Restriction endonuclease digestions

were performed in a buffer containing 6.6 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 6.6 mM β-mercapto ethanol and 50 mM NaCl.

Chemicals

$|\gamma$-$^{32}P|$ATP (specific activity 2000-3000 Ci/mmole) was purchased from The Radiochemical Centre (Amersham, UK); acrylamide (99%) was from Merck-Schuchardt (München), bis-acrylamide (ultra pure) was from Eastman-Kodak Co (Rochester, NY). Urea (99.5%) was purchased from BDH (Poole); dimethylsulfate (98%) was from Merck-Schuchardt (München), hydrazine (95+%) from Eastman-Kodak Co (Rochester, NY) and piperidine (98%) was from Baker Chemicals (Deventer).

ØX gene A protein incubation

RFI DNA (0.5 μg) was incubated with gene A protein (0.08 μg) in a reaction mixture of 25 μl containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA and 120 mM NaCl for 30 min at 37°C. In control experiments, no gene A protein was added to the mixture. The reaction was terminated by adding EDTA to 40 mM and 50 μg proteinase K (preincubated for 30 min at 37°C). The incubation was continued for 30 min at 37°C and then the conversion of RFI DNA into RFII DNA was analyzed by electrophoresis for 2.5 hrs at 150 Volts on horizontal 1% agarose slab gels, containing 1 μg/ml ethidium bromide (17).

Preparation and analysis of restriction endonuclease DNA fragments

For preparative purposes, 30 μg St-1 RFI DNA was incubated with 2.6 μg ØX gene A protein in a reaction volume of 2.0 ml (composition as described above) for 90 min at 37°C. The reaction was terminated by adding EDTA to 40 mM and proteinase K (preincubated for 60 min at 37°C) to 300 μg/ml. The incubation was continued for 60 min at 37°C and followed by extraction with phenol. After ethanol precipitation the DNA was dissolved in 80 μl restriction enzyme buffer and an appropriate amount of restriction enzyme was added. The mixture was incubated for 2 hrs at 37°C and the reaction was terminated by adding EDTA to 10 mM. After phenol extraction, the DNA fragments were precipitated with ethanol and redissolved in 80 μl 50 mM Tris-HCl (pH 8.0). The restriction DNA fragments were treated with alkaline phosphatase and labelled at their 5' ends with $|\gamma$-$^{32}P|$ATP by T_4 polynucleotide kinase as described by Maxam and Gilbert (18) with omittance of the heat-denaturing step prior to the kinase reaction. The DNA fragments were separated by electrophoresis on 0.15 cm thick x 30 cm wide x 40 cm long 5% polyacrylamide slab gels in 40 mM Tris-acetate (pH 7.8), 20 mM Na-acetate and 2 mM EDTA. DNA fragments were

visualized by autoradiography and eluted from the gels as described by Maxam and Gilbert (18). After elution the DNA fragments were precipitated with ethanol and dissolved in 20 µl formamide containing 0.05% xylene cyanol and 0.05% bromophenol blue. The mixtures were heated for 3 min at 100°C and layered on denaturing slab gels of varying polyacrylamide concentrations. For analytical purposes the gels were made up in 98% formamide (19), for preparative purposes they were made up in 7 M urea.
In experiments with non-radioactive fragments, the DNA was stained by immersing the gels in electrophoresis buffer containing 1 µg/ml ethidium bromide.

Sequence analysis
In order to obtain DNA fragments, appropriate for the chemical degradation method (18), two strategies were used; 1) single-stranded DNA fragments labelled at the 5' ends were eluted from urea-polyacrylamide gels and directly subjected to the sequence analysis technique; 2) double-stranded DNA fragments labelled at both 5' ends were subjected to strand separation on polyacrylamide slab gels as described by Maxam and Gilbert (18). The separated strands were eluted from the gel after autoradiography and used in the chemical degradation method (18). Specific cleavage at guanine residues was achieved by methylation with dimethyl sulfate, at adenine residues by ring-opening with alkali, at cytosine and thymine residues by hydrazinolysis and at cytosine residues alone by hydrazinolysis in 2 M NaCl. Strand-scission was achieved by incubation with piperidine in all cases. For resolution of the cleavage products, 20% acrylamide, 7 M urea gels were run in 50 mM Tris-borate (pH 8.3), 1mM EDTA.

RESULTS

Conversion of RFI DNAs of bacteriophages St-1, α3, U3 and G14 into their respective RFII DNAs by ØX gene A protein
ØX gene A protein nicks the viral strand of ØX RFI DNA (6) and G4 RFI DNA (8) at a specific site, the origin of viral strand DNA replication. In order to study the effect of ØX gene A protein on the RFI DNAs of St-1, α3, U3 and G14, these RFI DNAs were incubated with ØX gene A protein. Subsequently, the conversion of RFI DNA into RFII DNA was analyzed by agarose gel electrophoresis (Fig. 1). In all cases the incubation of RFI DNA with ØX gene A protein resulted in the formation of RFII DNA. This indicates that ØX gene A protein introduces at least one single-stranded break into the RFI DNAs of bacteriophages St-1, α3, U3 and G14, respectively.

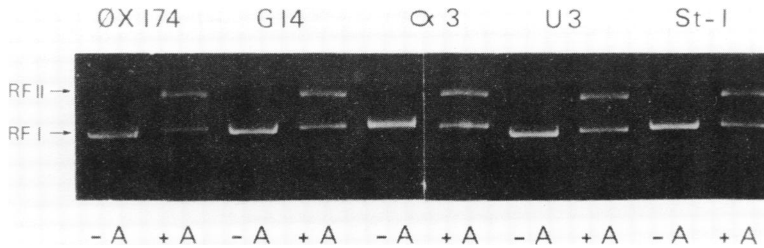

Figure 1:
The conversion of ØX174, G14, α3, U3 and St-1 RFI DNAs into RFII DNAs by ØX gene A protein. After incubation of the different RFI DNAs with ØX gene A protein the reaction products were analyzed on horizontal 1% agarose slab gels as described in Materials and Methods. Migration was downwards from the top. +A refers to the experiment with gene A protein; -A refers to the control experiment in which the gene A protein was omitted.

Localization of the ØX gene A protein cleavage site in St-1 RF DNA
For the localization of the gene A protein nick in St-1 RF DNA the following strategy was used: St-1 RFI DNA was incubated with as well as without ØX gene A protein and subsequently digested with the restriction endonuclease *Hae*III. After dephosphorylation the DNA fragments were labelled at their 5' ends with ^{32}P and separated by electrophoresis on a neutral polyacrylamide slab gel. In both cases after digestion with *Hae*III 12 DNA fragments were obtained {results not shown, (20)}. Each DNA fragment was eluted from the gel and subsequently analyzed under denaturing conditions. In the gelsystem used the two strands of a restriction DNA fragment are not separated. Fragments that contain nicked DNA can yield two extra labelled single-stranded DNA fragments. However for ØX174 (6) and G4 (8) the 5' end of the gene A protein nick cannot be labelled by the combined action of phosphatase and kinase because the gene A protein is covalently bound to the DNA at the 5' end of the nick (4, 6, 8, 21, 22). Therefore only one extra band is observed in denaturing gels representing the DNA at the 3'-OH end of the nick. Also for St-1 only one restriction DNA fragment (Z_7, approx. 325 b.p.) showed one extra labelled band on the denaturing gel {Fig. 2 ; results of the fragments Z_1-Z_6 and Z_9-Z_{12} not shown}. The length of this single-stranded DNA fragment (approx. 200 nucleotides) does not allow an accurate determination of the nucleotide sequence at the 3' end starting from the labelled 5' end. For that reason other restriction sites in the region around the gene A protein cleavage site in St-1 RFI DNA were traced. The restriction DNA fragment Z_7 and the neighbouring DNA fragments Z_2 and Z_6

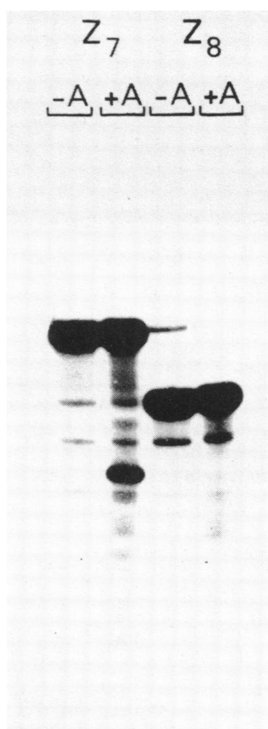

Figure 2:
Autoradiograph of the slabgel, containing the St-1 RF *Hae*III DNA fragments Z_7 and Z_8 after electrophoresis in denaturing conditions. The +A lanes contain the Z-fragments obtained from the experiment in which St-1 RFI DNA was incubated with ØX gene A protein. The -A lanes contain the Z-fragments, obtained from the control experiment in which gene A protein was omitted. The Z_7 and Z_8 samples were electrophoresed on a 5% polyacrylamide gel in 98% formamide.

(20) were redigested with several restriction endonucleases. Analysis of the redigestion products revealed that the restriction endonuclease *Hinf*I could be a useful tool in elucidating the nucleotide sequence at the ØX gene A protein cleavage site in St-1 RF DNA.
Redigestion of 5'-^{32}P-labelled DNA fragment Z_7 with *Hinf*I yielded two labelled subfragments of approximately 100 b.p. and 190 b.p. respectively. When 5'-^{32}P-endlabelling was performed after redigestion of DNA fragment Z_7 with *Hinf*I, 4 fragments were detected on a neutral gel of about 15 b.p. (Z_7F_4), 20 b.p. (Z_7F_3), 100 b.p. (Z_7F_2) and 190 b.p. (Z_7F_1) respectively. Redigestion of 5'-^{32}P-labelled DNA fragment Z_6 with *Hinf*I yielded two labelled subfragments of

approximately 15 b.p. and 50 b.p..

The DNA sequence at the 3' end of the ØX gene A protein nick in St-1 RF DNA

St-1 RFI DNA was incubated with ØX gene A protein and digested with the restriction endonuclease *Hinf*I. After labelling their 5' ends with ^{32}P the DNA fragments were subjected to electrophoresis on a neutral polyacrylamide slab gel. Autoradiography of this gel showed approximately 30 bands, which figure is in good agreement with Godsons results (23), taking into account that at least 12 of the St-1 *Hinf*I restriction DNA fragments are smaller than 100 b.p.. After elution from this neutral gel, the *Hinf*I DNA fragment F_{10} (approx. 205 b.p.) yielded one extra labelled band with a length of about 65 nucleotides (results not shown) when subsequently electrophoresed in denaturing conditions. When the gene A protein incubation was omitted no specific subfragment from DNA fragment F_{10} could be detected.
These results, together with the redigestion data of the DNA fragments Z_7 and Z_6 (see above) yield a cleavage map of the St-1 genome in the region of the ØX gene A protein cleavage site as shown in Figure 3.
The sequence at the 3' OH end of the ØX gene A protein cleavage site was deduced by chemical degradation (18) of the F_{10}-subfragment. From the autoradiograph (shown in Figure 4) a stretch of 32 nucleotides up to the 3'-OH end can be read.

The DNA sequence around the ØX gene A protein nick in St-1 RF DNA
In order to determine the nucleotide sequence around the gene A protein cleavage site in St-1 RF DNA, the *Hae*III restriction DNA fragment Z_7 was redigest-

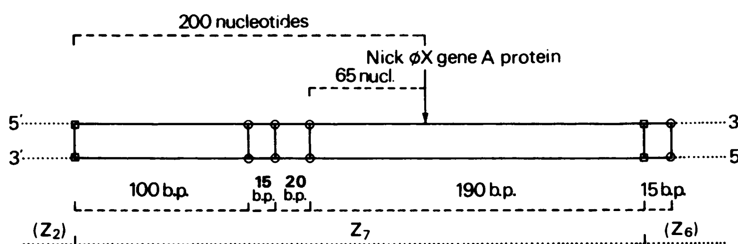

Figure 3:
Cleavage map of part of the St-1 genome containing the region around the ØX gene A protein nick site. The alignment of the Z-fragments is taken from Grindley and Godson (ref. 20). Open squares (□) refer to *Hae*III recognition sequences, open circles (o) refer to recognition sequences cleaved by *Hinf*I. The order of the two small *Hinf*I DNA fragments was not determined.

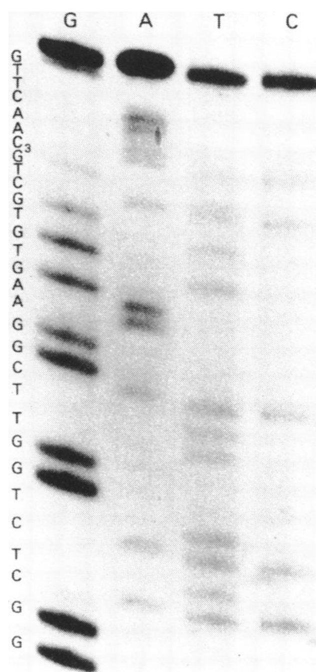

Figure 4:
Autoradiograph of the sequence gel of the single-stranded F_{10}-subfragment with the deduced sequence. Products, shorter than approx. 35 nucleotides have run off the gel. The last 7 nucleotides at the 3'-OH end are completely identical with the DNA sequences at the 3'-OH end of the ØX gene A protein nick in ØX (6) and G4 (8).

ted with *Hinf*I. After labelling their 5' ends with ^{32}P the DNA fragments were separated on a neutral polyacrylamide slab gel. The Z_7F_1 DNA fragment with a length of about 190 b.p., which according to the physical map (Fig. 3) should contain the DNA sequence nicked by ØX gene A protein, was eluted and subjected to strand separation as described in Materials and Methods. The autoradiograph of the sequence gel of the slowest moving band is shown in Figure 5. A sequence of 85 nucleotides containing the 32 nucleotides up to the 3'-OH end of the gene A protein cleavage site (see Fig. 4) can be read.

DISCUSSION

Previous experiments (6,8) have shown that *in vitro* ØX gene A protein nicks ØX174 RFI DNA and G4 RFI DNA in a common region of 30 nucleotides at the same

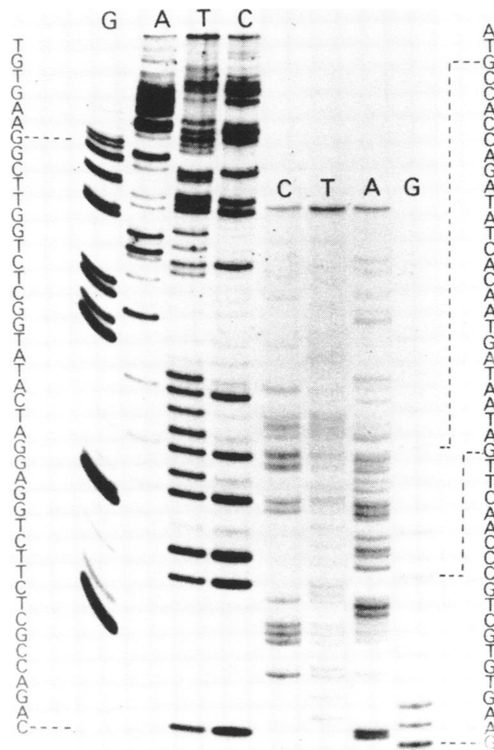

Figure 5:
Autoradiograph of the sequence gel of the slowest migrating band (see text) of Z_7F_1. The chemical degradation products were loaded twice onto the gel. The left four lanes show the short run, the right four lanes show the longer run, both with the deduced sequence.

unique site (Fig. 6).

In order to determine which nucleotides within that region are essential for origin function the action of ØX gene A protein on RFI DNAs of the related bacteriophages St-1, α3, U3 and G14 was studied.

From the results presented in this paper we conclude that:

- ØX gene A protein introduces at least one single-stranded break into the RFI DNAs of bacteriophages α3, U3 and G14 respectively;
- ØX gene A protein nicks St-1 RFI DNA only once thereby creating, as has been observed in the case of ØX RFI DNA and G4 RFI DNA, a 3' OH-G terminus and a 5' terminus, presumably an A residue to which the gene A protein is covalently bound.

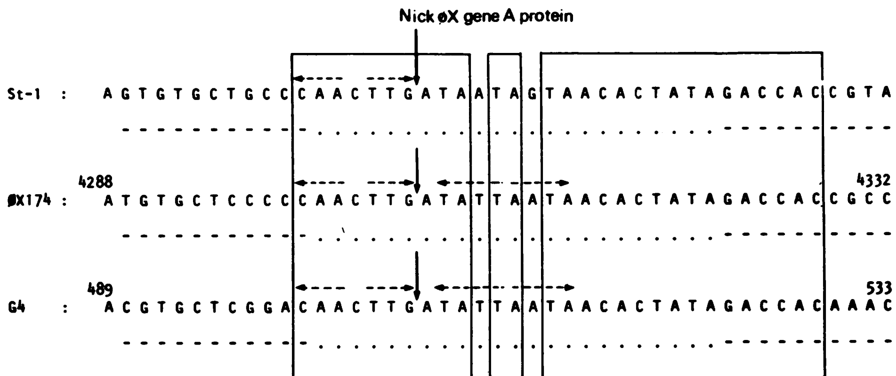

Figure 6:
The nucleotide sequences in St-1, ØX and G4 RF DNA in the region around the ØX gene A protein cleavage site. The nucleotide sequence and the numbering of ØX174 are from Sanger *et al.* (7). The nucleotide sequence and the numbering of G4 are from Godson *et al.* (9). The site of the ØX gene A protein nick is indicated by vertical arrows. Horizontal arrows indicate short, self-complementary sequences. GC-rich tracts are indicated by broken lines, AT-rich tracts are marked with dots. Nucleotide sequences which are identical in all three phages are boxed.

A comparison of the DNA sequence around the ØX gene A protein nick site in St-1 RF DNA with the sequences around the nick in ØX174 RF DNA and G4 RF DNA is made in Fig. 6. The St-1 DNA sequence in the region where the cleavage site has been located shows strong homology with the conserved region of 30 nucleotides surrounding the gene A protein cleavage site in the viral DNA strands of ØX174 and G4. However, within this region the St-1 DNA sequence shows two remarkable changes:

T (nucleotide 4309 in ØX; nucleotide 510 in G4) → A (in St-1) and

A (nucleotide 4312 in ØX; nucleotide 513 in G4) → G (in St-1).

The implications of these nucleotide changes are twofold: first, the self-complementary sequence TATT-AATA found near the 5'end of the nick in ØX and G4 DNA is absent in the analogous part of the St-1 DNA sequence. Therefore we suppose that the self-complementariness of this sequence is not essential for the nickase activity of the ØX gene A protein.
Second, perfect sequence homology around the nick site in the RF DNAs of St-1, ØX174 and G4 is only 10 nucleotides, suggesting that the recognition sequence of the ØX gene A protein lies within this region. The short self-complementary sequence CAA-C-TTG found near the 3'-OH end of the gene A pro-

tein nick is preserved in these 10 nucleotides.

It is striking that, besides the two nucleotide changes, also in St-1 the common region of 30 nucleotides found in ØX and G4 around the gene A protein cleavage site is present. Outside this region many nucleotide changes in the DNA sequences of the three bacteriophages have been detected. This suggests a special role for this region in the process of DNA replication. We envisage the following possibilities. The gene A protein nicks single-stranded ØX DNA (25) and double stranded ØX RFI DNA (24), only if it contains superhelical turns. Both are nicked once at the same specific site, the origin of DNA replication. Therefore partial denaturation of the origin region in a double stranded DNA molecule is required for the nicking activity of ØX gene A protein. The conserved region consists of an AT-rich sequence, flanked on both sides by a GC-rich stretch, which may favour partial denaturation of this region in a superhelical DNA molecule.

Alternatively this region may be needed after nicking of the viral strand in the elongation and the termination step of ØX DNA replication. Eisenberg *et al.* (5) have shown that ØX gene A protein in co-operation with the *rep* and DNA-binding protein separates the strands of the template ahead of the replication fork and that after a round of DNA replication the gene A protein is probably responsible for the formation of mature, circular viral strands by a second nicking and ligation activity.

Finally it is possible that the coding properties of the conserved region are essential for the enzymatic activity of the gene A protein. The isometric phages code for the same series of proteins and these proteins have probably the same function during the infection cycle of these bacteriophages. In ØX and G4 the origin region is located within the viral gene A. By analogy it is

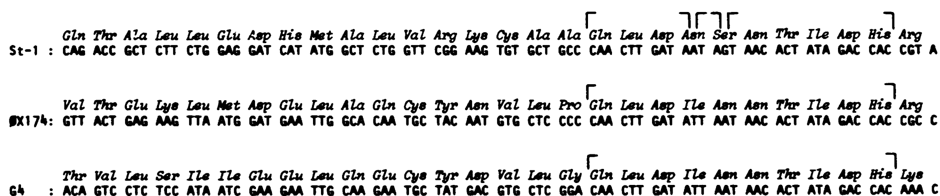

Figure 7:
The nucleotide sequences of St-1, ØX and G4 RF DNA in the region of the ØX gene A protein nick with the known amino acid composition of part of the ØX and G4 gene A protein and the amino acid sequence where the St-1 DNA sequence is presumed to code for.

assumed that this is also the case for St-1. When we further assume that the nicked strand in St-1 RFI DNA is the viral strand and that the complementary strand of St-1 RFI DNA is the template strand for m-RNA synthesis, only one of the three reading frames for St-1 contains no stop codons. This frame is the same one, that is used in ØX (7) and G4 (9). In this reading frame the two nucleotide changes in St-1 DNA are second position changes in the gene A codons which result in the following amino acid changes in the gene A protein of St-1 (see Fig. 7):

Ileu (ØX and G4) → Asn (St-1) and
Asn (ØX and G4) → Ser (St-1).

This at least excludes the possibility that the whole conserved region is required for essential amino acids in the gene A protein. However, further experiments are needed to elucidate the role of the conserved region of 30 nucleotides in the process of DNA replication and the minimum recognition sequence of the gene A protein.

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Abbreviations used:

RF DNA, double-stranded circular replicative form DNA;
RFI DNA, supercoiled replicative form DNA with both strands closed;
RFII DNA, replicative form DNA with one or more discontinuities in either strand; *Alu*I, *Hae*III and *Hinf*I are restriction endonucleases from Arthrobacter luteus, Haemophilus aegyptius and Haemophilus influenza Rf respectively;
A, F and Z are restriction endonuclease fragments produced by *Alu*I, *Hinf*I and *Hae*III respectively;
b.p., base pairs

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