Characterization of the origins of replication of bacteriophage ϕ 29 DNA

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ABSTRACT

The origins of replication of ϕ 29 DNA have been studied by analyzing the activity as templates in the ϕ 29 in vitro replication system of *E. coli* recombinant plasmids and M13 derivatives containing ϕ 29 DNA terminal sequences. Plasmid pTR, containing the 6 bp long inverted terminal repeat of ϕ 29 DNA, was shown to be essentially inactive. The analysis of a series of deletion derivatives of plasmid pD13, that contains the 73 and 269 bp from the left and right ϕ 29 DNA ends, respectively, indicated that the minimal origins of replication are comprised within the terminal 12 bp at each DNA end. Point site-directed and random mutagenesis at these sequences was carried out. Changes of the second or third A into a C completely abolished the template activity. In the case of changes at position from 4 to 12, only 3 out of 14 mutations reduced the template activity; these 3 mutations were double changes and 2 of them affected the inverted terminal repeat. The results suggest that the sequence requirement at the end-proximal region of the origin of replication is more strict than that at the distal region.

INTRODUCTION

The *Bacillus subtilis* phage ϕ 29 has a linear, double-stranded DNA genome, 19,285 bp long (1), with an inverted terminal repeat (ITR) 6 bp long and a viral-coded protein covalently linked to each 5' end (2). ϕ 29 DNA replication starts at either DNA end and proceeds by a mechanism of strand displacement (3,4). Two copies of the terminal protein p3 are involved in the initiation step of ϕ 29 DNA replication at two different levels: a) A newly synthesized molecule of protein p3 acts as a primer of replication: the hydroxyl group of a specific serine residue (5), is used by the viral-encoded DNA polymerase, protein p2, to incorporate the first nucleotide of the nascent chain, dAMP (6). b) The p3 molecule linked to ϕ 29 DNA, named parental terminal protein, plays an important role as a structural part of the template (7,8), probably by setting a direct interaction with the

priming protein p3 (9). However, the specificity of the template is determined not only by the parental terminal protein. We have recently shown that protein-free terminal fragments of ϕ 29 DNA are active templates for the formation of the initiation complex p3-dAMP in an in vitro system with purified proteins p2 and p3 (10), indicating that specific base sequences may function as origins of replication. Furthermore, the DNA of pID13, an E. coli recombinant plasmid containing the terminal 73 bp from the left ϕ 29 DNA end and the terminal 269 bp from the right end, is also an active template in the in vitro initiation reaction, provided it has been previously digested with the restriction endonuclease DraI to release the cloned ϕ 29 terminal sequences at DNA ends (11). The template activity is lower than that of ϕ 29 DNA with the parental terminal protein, but the initiation site is the correct one, at the very end of the DNA, and full replication of the ϕ 29-specific restriction fragments can take place.

The availability of plasmid pID13 enabled us to study in more detail the template requirements of the ϕ 29 protein-priming reaction. Here we report the production of deleted and point-mutated derivatives of cloned terminal sequences of ϕ 29 DNA and their activity as origins of replication in the ϕ 29 in vitro system.

MATERIALS AND METHODS

Bacterial strains and cloning vectors. The recombinant plasmid pID13, containing the ends of ϕ 29 DNA, has been described elsewhere (11). The E. coli strain W3110 lac I^q (12), used as host for the plasmid vector pKK223-3 (13) and its derivatives, was obtained from J. Alonso. The E. coli strains JM103 (14) and JM109 (15) were used as hosts for derivatives from phages M13 mp8 (16) and M13 mp19 (17), respectively. Competent cells of each E. coli strain were prepared as described by Hanahan (18).

Nucleoside triphosphates and enzymes. (γ -³²P)ATP (~3000 Ci/mmol), (α -³⁵S)dATP (~600 Ci/mmol) and (α -³²P)dATP (~410 Ci/mmol) were from the Radiochemical Centre (Amersham). ATP and deoxynucleoside triphosphates were from P-L Biochemicals.

Restriction nucleases were from New England Biolabs, Boehringer Mannheim and Amersham, and were used according to the three-buffers system of Maniatis et al. (19). Klenow fragment of E. coli DNA polymerase I and exonuclease Bal 31 were from New

England Biolabs; mung bean nuclease from P-L Biochemicals and exonuclease III, from Bethesda Research Laboratories, were used according to the directions of Stratagene, cloning systems. All other enzymes were from Boehringer, and were used according to the supplier.

DNA manipulations. Plasmid DNAs and the replicative form of phage M13 DNA and its derivatives were isolated as described by Clewell and Helinski (20). The single-stranded form of M13 DNA and its derivatives was prepared as described by Sanger et al. (21). For analysis, plasmid DNA was prepared as described by Birnboim and Doly (22).

Deletion derivatives of plasmid pID13 (see Fig. 2) were first prepared by linearizing 10 μ g of plasmid pID13 with SalI or HindIII, next to the cloned left or right ϕ 29 DNA ends, respectively. The DNA was precipitated with ethanol and resuspended in 50 μ l of the Bal 31 buffer recommended by the supplier. Two units of this enzyme were added and the samples were incubated at 30°C for 14 min. Samples of 5 μ l were taken each minute, beginning at the 5th minute of incubation, and stopped with EGTA. One half of each sample was treated with a second restriction enzyme and analyzed in 1% agarose gels. The remainder of those samples containing DNAs appropriately degraded was ethanol-precipitated, treated with Klenow fragment for 30 min at room temperature and with T4 DNA ligase overnight at 15°C. The DNA was then used to transform W3110 lac I^q competent cells and transformants were screened by restriction and sequence analysis.

Exonuclease III and mung bean nuclease were also used to generate deletions in p Δ 59, one of the Bal 31 derivatives of pID13. About 6 μ g of plasmid p Δ 59 were linearized with StuI, the DNA was ethanol-precipitated and resuspended in 40 μ l of 10 mM Tris-HCl, pH 8, and 1 mM EDTA (TE buffer); 50 μ l of the 2x exonuclease III buffer and 5 μ l of 0.4 M β -mercaptoethanol were added and the mixture was warmed at 25°C for 1 min. Approximately 50 units of exonuclease III were added and samples of 33 μ l were taken after 45, 75 and 105 sec of incubation. The three samples were then joined, adjusted to the mung bean nuclease buffer, and further incubated with this enzyme for 1 h at 30°C. The mixture was phenol-extracted and the DNA was digested with SalI, releasing the target ϕ 29 DNA sequence in fragments ranging in size from about 300 to 400 bp. These fragments were isolated by

polyacrylamide gel electrophoresis in a buffer containing 0.1 M Tris- borate, pH 8.3, 2 mM EDTA (TBE), extracted from the gel by electroelution and coprecipitated with the SalI/SmaI-cut replicative form of the DNA of phage M13 mp19, and ligated overnight. The ligation mixture was used to transform *E. coli* JM109 competent cells and those transformants giving a white plaque were screened by sequence analysis.

The 17-mers used to generate a synthetic linker with the ϕ 29 ITR sequences and the 24-mers used to change the 2nd or the 3rd position at the cloned ϕ 29 DNA ends were a gift of Antibióticos, S.A. The 32-mers mixture containing point mutations in the ϕ 29 origins of replication was prepared in a 380A Synthesizer of Applied Biosystems. All synthetic oligonucleotides were purified by polyacrylamide gel electrophoresis as recommended by Applied Biosystems.

Point mutants at the 2nd or 3rd positions of ϕ 29 DNA terminal sequences subcloned in phage M13 mp8 DNA were prepared by the directed mutagenesis procedure described by Zoller and Smith (23). Two synthetic 24-mers were used as mutagens for the single-stranded DNA of clone G7, which is an M13 mp8 derivative containing the wild-type sequences of the 73 and the 125 terminal bp from the left and right ϕ 29 DNA ends, respectively. The mutagenic reaction was carried out with both oligonucleotides mixed, and the DNAs of the resultant mutants were separately screened on nitrocellulose filters with a radioactive probe of each one.

The saturation mutagenesis method described by Derbyshire et al. (24) was used to prepare DNA molecules containing point mutations randomly distributed along the terminal 12 bp of each ϕ 29 DNA end. A mixture of synthetic oligonucleotides was prepared using mixed stocks of monomers, each one containing 1/48 parts of each of the other three. The canonical sequence, showed in Fig. 6A, consisted of both ϕ 29 origins of replication joined head to head and two tails cohesive with XmaI and PstI sites, respectively. The expected frequency of wild-type sequences would be about 21%; the frequency of single, double and triple point mutations, randomly distributed between the restriction tails, was expected to be about 73%; quadruple and higher mutations would reach a 6%. The mixture of oligonucleotides was inserted in phage M13 mp19 DNA, the DNA was used to transform competent JM109 cells and mutants were selected by sequence analysis of the transformants.

The DNA fragments to be tested in the replication assays were isolated by electrophoresis in gels containing 3.5% polyacrylamide in TBE, and extracted from the gel and purified as described (25).

DNA sequencing was carried out by the chemical method (26) on pKK223-3 derivatives and by the enzymatic method (27) on M13 derivatives.

Assays for the formation of protein p3-dAMP initiation complex and for protein p3-primed DNA replication. The incubation mixture for the initiation reaction contained, in 25 μ l, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM spermidine, 20 mM $(NH_4)_2SO_4$, 0.25 μ M (α - ^{32}P) dATP (2.5 μ Ci), purified proteins p2 (6) and p3 (28) as indicated and the DNA fragments indicated in each case as templates (3-12 fmol). After incubation for 10 min at 30°C, the reaction was stopped by addition of EDTA to 10 mM and heating for 10 min at 68°C and the samples were treated with micrococcal nuclease. The unreacted (α - ^{32}P)dATP was removed by filtration through a Sephadex G-50 spun column in the presence of 0.1% SDS and the samples were subjected to SDS-polyacrylamide gel electrophoresis as described (29). The ^{32}P -labelled p3-dAMP was detected by autoradiography of the dried gel.

Those initiation assays carried out with DNA templates lacking a fully active ϕ 29 origin of replication gave rise to a background amount of p3-dAMP initiation complex. As this complex turned out to elongate very inefficiently, the p3-primed replication assay was systematically carried out with all DNAs to check the results obtained in the initiation assays. For the DNA replication reaction, the incubation mixture was as described above except that it contained 10 μ M (α - ^{32}P)dATP (2.5 μ Ci) and 20 μ M each dGTP, dCTP and dTTP. After incubation at 30°C, the reaction was stopped by addition of EDTA to 10 mM and SDS to 0.1% and heating at 68°C for 10 min. The unincorporated (α - ^{32}P)dATP was removed from the samples as described for the initiation assay and the labelled DNA was subjected to electrophoresis in 1% agarose gels after digestion with proteinase K; the gels were dried and autoradiographed.

RESULTS

Cloning and template activity of the ϕ 29 ITR sequences. To test the possibility that the 6 bp long ITR of ϕ 29 DNA was the minimal origin of replication, two complementary oligonucleotides 17

Figure 1. Cloning and template activity of the Ø29 ITR sequences.

A) 10 µg of each oligonucleotide, in 10 µl, were phosphorylated with ATP and polynucleotide kinase. EDTA to 10 mM was added and the samples were mixed and heated at 90°C for 2 min; NaCl to 50 mM was added and the mixture was placed at 55°C and allowed to cool down slowly to 4°C, to facilitate hybridization. Plasmid pKK223-3 was cut with EcoRI and HindIII and the large DNA fragment was isolated by agarose gel electrophoresis and ligated in different ratios to the hybridized oligonucleotides, to produce the recombinant plasmid pITR. **B)** 19 ng of each restriction mixture, pID13/DraI (a), pITR/DraI (b), or pKK223-3/DraI (c), were used as templates in an initiation assay with 6 ng of protein p2 and 86 ng of protein p3, in the conditions described in Materials and Methods, except that the incubation time was 20 min. The position of the initiation complex p3-dAMP is indicated. **C)** 19 ng of the restriction mixtures pID13/DraI (a,b) or pITR/DraI (c,d) were used as templates in a replication assay with 6 ng of protein p2 in the presence (b,d) or in the absence (a,c) of 86 ng of protein p3, as described in Materials and Methods. The positions of the input DNA fragments are indicated.

The sizes of the pID13/DraI A, B and C fragments are 2,956, 993 and 692 bp, respectively, and those of the pITR/DraI fragments are 3,114, 729 and 692 bp, respectively. The label along lane (b) not coinciding with input fragments is considered to be caused by single-stranded intermediates of ϕ 29-specific replication, as judged by nuclease S1 sensitivity.

bases long were prepared containing the sequences of two copies of the ϕ 29 ITRs, joined head to head, and tails cohesive with EcoRI and HindIII restriction sites, respectively (Fig. 1A). The 17-mers were hybridized to one another and ligated to the EcoRI/Hind III linearized DNA of plasmid pKK223-3. The ligation mixture was used to transform competent W3110 lac I^q cells. Recombinant plasmid pITR was identified among transformants by restriction and sequence analysis.

Treatment of pITR with the restriction nuclease DraI releases 4 DNA fragments. Each one of the largest fragments, A and B, 3,114 and 729 bp long, respectively, contains one copy of the ϕ 29 ITR. Besides, the sequence flanking the ITR in fragment A is identical to that at the right ϕ 29 DNA end up to position 7, and the sequence flanking the ITR in fragment B is identical to that at the left ϕ 29 DNA end up to position 9 (see Fig. 4). Fig. 1B shows that the use of this plasmid after treatment with DraI as template in the in vitro initiation assay, gave rise to the formation of a very small amount of p3-dAMP initiation complex (lane b) as compared to the DraI-treated plasmid pID13, containing 73 and 269 bp from the left and right ϕ 29 DNA ends, respectively (lane a). The template activity of the DraI-cut pITR was only slightly higher than the background given by the DraI-cut vector pKK223-3, which lacks ϕ 29 DNA sequences (lane c).

Similar results were obtained in the replication assay (Fig. 1C). DraI fragments A and B of plasmid pITR, each containing the 6 bp long ITR of ϕ 29 DNA, showed little protein p3-dependent incorporation of label (lanes c and d), whereas the pID13/Dra I fragments A and B, containing the left and right ϕ 29 DNA ends, respectively, specifically incorporated (α -³²P)dATP label in the presence (lane b), but not in the absence (lane a) of the priming protein p3. The 692 bp long DraI fragment, without ϕ 29 DNA sequences (fragment C), from the vector regions of both plasmids, pID13 and pITR, did not replicate, as expected.

These results indicated that the ϕ 29 ITRs do not contain all the sequences needed to function as ϕ 29 origins of replication.

Figure 2. Production and template activity of Bal 31 deletions of plasmid pID13.

A) The SalI- or the HindIII- linearized plasmid pID13 was digested with exonuclease Bal 31 to progressively shorten the left or the right cloned ends of $\phi 29$ DNA. $\phi 29$ DNA inserts in the figure are indicated with double line and the products of the action of Bal 31 with discontinuous line. The 6 bp long ITR sequences are represented by striped double-line. $\uparrow$, DraI sites. B) 34 ng of each DraI fragment containing the left $\phi 29$ DNA end, isolated from plasmids pID13 (a), pI Δ 46 (b), or pI Δ 12 (c), were used as templates in an initiation assay as described in Fig. 1B. Eight ng of the 692 bp long DraI fragment of pID13, without $\phi 29$ DNA sequences, were also used as a negative control (d).

Taking into account the sequences present in pITR, these origins seemed to extend beyond position 9 at the left ϕ 29 DNA end and beyond position 7 at the right end.

Production and template activity of pID13 deletion derivatives.

Exonuclease Bal 31 was used on linearized plasmid pID13 to obtain two series of pID13 derivatives with partially deleted left or right terminal sequences of ϕ 29 DNA (Fig. 2A). Two clones were selected, pID46 and pID12, that contained only 46 and 12 bp from the left ϕ 29 DNA end, respectively, as indicated in their name. Another two, pDA59 and pDA34, contained only 59 and 34 bp from the right end, respectively. An additional plasmid, named pDA10, containing the 10 terminal bp from the right end, was obtained by direct subcloning of a restriction fragment of plasmid pID13 in plasmid pKK223-3 (not shown).

The DraI fragments containing ϕ 29 deleted sequences were isolated from each clone and used as templates for the in vitro initiation reaction. Fig. 2B shows that the DNA fragments 2,254 bp long from plasmids pID46 and pID12, respectively, were still active (lanes b and c), at the same level as the 2,956 bp long pID13 fragment containing the 73 terminal bp from the left ϕ 29 DNA end (lane a). A DNA fragment without ϕ 29 DNA sequences produced only a background level of p3-dAMP initiation complex (lane d). The results were less clear for the right-end derivatives pDA59, pDA34 and pDA10, as the initiation activity of the fragments, 560, 500 and 755 bp long, respectively, was rather low and it progressively decreased to the background level (results not shown). The right-end series was completed by treatment of the StuI-linearized plasmid pDA59 with exonuclease III and mung bean nuclease (Fig. 3A). Six clones, mDA40, mDA25, mDA22, mDA12, mDA10 and mDA9, were selected, each one containing the number of terminal bp from the right ϕ 29 DNA end indicated in its name. An effect similar to that mentioned for the pDA series was observed in the initiation reaction with the DraI fragments 560-530 bp long from the mDA series, as shown in Fig. 3B, but, in this case, the activity of clones mDA10 and mDA9 (lanes d and e) did not seem to be significant relative to the background (lane f).

The in vitro replication assay was then carried out with all DNA fragments containing the deletions described before to check the results obtained in the initiation assay. The results on the

Figure 3. Production and template activity of exonuclease III deletions of plasmid pDΔ59.

A) The StuI-linearized plasmid pDΔ59 was digested with exonuclease III and mung bean nuclease. The DNA was further digested with SalI and the subfragment containing the partially deleted sequence of the right end was isolated and inserted in phage M13 mp19 DNA. Ø29 inserts are indicated with double line and the products of the action of exonuclease III and mung bean nuclease with discontinuous line. †, DraI sites. B) 3 ng of each DraI fragment, about 530 bp long, containing deletions of the right Ø29 DNA end, isolated from the M13 derivatives mDΔ25 (a), mDΔ22 (b), mDΔ12 (c), mDΔ10 (d) or mDΔ9 (e), were used as templates in an initiation assay with 25 ng of protein p2 and 42 ng of protein p3. Four ng of the unspecific 692 bp long pID13/DraI fragment

were used as control (f). C) 3 ng of each *Dra*I fragment containing deletions of the right ϕ 29 DNA end, isolated from the M13 derivatives mD Δ 22 (a,b), mD Δ 12 (c,d) or mD Δ 10 (e,f) were used as templates in a replication assay with 25 ng of protein p2, in the presence (a,c,e) or in the absence (b,d,f) of 86 ng of protein p3.

initiation activity of the deletion mutants at the left ϕ 29 DNA end were confirmed (not shown). For the deletion mutants at the right end (Fig. 3C), a low, but significant p3-dependent replication was observed with the DNA of clones mD Δ 22 (lanes a and b) and mD Δ 12 (lanes c and d), but not with that of mD Δ 10 (lanes e and f).

These results indicated that the minimal sequences active as ϕ 29 origins of replication were comprised within the terminal 12

RIGHT END		ACTIVITY
pID13	AAAGTA--//-----AATAAGCTT ²⁷⁴ _____	+
pD Δ 59	AAAGTA--//-----TTCGACAAG ⁵⁹ _____	+
pD Δ 34	AAAGTA--//-----ACCATTTCC ³⁴ _____	+
mD Δ 25	AAAGTA--//-----ACAACATAC ²⁵ _____	+
mD Δ 22	AAAGTA--//-----ACAACA ²² _____	+
mD Δ 12	AAAGTAGGGTAC ¹² _____	+
pD Δ 10	AAAGTAGGGT ¹⁰ _____	-
pITR-A	AAAGTAG ⁷ _____	-
pITR-B	AAAGTA ⁶ _____	-
LEFT END		
pID13	AAAGTA-----//----ACCATGATC ⁷⁷ _____	+
pI Δ 46	AAAGTA-----//----AATATCGAC ⁴⁶ _____	+
pI Δ 12	AAAGTAAGCCCC ¹² _____	+
pITR-B	AAAGTAAGC ⁹ _____	-
pITR-A	AAAGTA ⁶ _____	-

Figure 4. Deletion map of the origins of replication of ϕ 29 DNA.

Only the ITR and the end-distal ϕ 29 DNA sequences are presented for those clones containing more than 12 bp from ϕ 29 DNA. The sequences of both fragments of pITR (A and B) are compared to each ϕ 29 DNA end. The template activity was tested in the initiation and replication assays as described in Materials and Methods, and is indicated as positive (+) for initiation and replication values from 50–100% or negative (-) for initiation values <20% and replication values <5%.

Figure 5. Site-directed mutagenesis at positions 2 or 3 of the Ø29 DNA ends. Template activity of the mutants.

A) The procedure described by Zoller and Smith (23) was followed. Transformants were screened on nitrocellulose filters by separate hybridizations with a radioactive probe of each oligonucleotide, MS1 and MS2, at room temperature; the filters were

washed at different temperatures up to 45°C and 55°C, respectively, to detect the mutants from which clones R161 and H7 were finally segregated. The autoradiography of one of the filters showing two positive clones, in duplicate, is presented. Double-line represents the cloned ϕ 29 sequences. The 6 bp long ITR sequences, represented by striped double-line, are shown. The target bases and the changes introduced are underlined. The mutated left or right ITR sequences are indicated with an asterisk. $\uparrow$, DraI sites. B) The initiation assay was carried out using as templates: 4 ng of the left origin-containing fragments isolated from the RsaI/DraI digestion of clones G7, with the wild-type sequence (a), or R161, with the A $\rightarrow$ C transversion at position 2 (b), 8 ng of the analogous fragments from the DraI digestion of clone G7, with the wild-type sequence (c), or from the HpaI/DraI digestion of clone H7, with the A $\rightarrow$ C transversion at position 3 (d), 17 ng of a fragment without ϕ 29 sequences (e), and 3 ng of the right origin-containing fragments isolated from the DraI digestion of clone G7, with the wild-type sequence (f), or from the HpaI/DraI digestion of clone H7, with the A $\rightarrow$ C transversion at position 3 (g). The conditions of the reactions were those indicated in Fig. 3B. The label at the bottom of the lanes corresponds to residual (α - 32 P)dATP.

bp at each ϕ 29 DNA end. A compilation of the mapping analysis is shown in Fig. 4.

Point mutagenesis at the ϕ 29 DNA origins of replication. Two different strategies, site-directed and random mutagenesis, were followed to obtain point mutations at the origins of replication, depending on the bases selected as target.

The terminal 3 bp of each cloned ϕ 29 origin of replication produced the DraI site used to release the ϕ 29 sequences at the ends of linear DNA fragments. Thus, mutations at any of the 3 terminal positions of one of the cloned origins must be concomitant with another palindromic change at the other origin, and both changes should generate a new restriction site replacing the one lost. Attending to these requirements, there were only two possible mutations at that region in both ϕ 29 DNA ends, an A $\rightarrow$ C transversion at the 2nd or at the 3rd position, generating RsaI or HpaI sites, respectively. The strategy depicted in Fig. 5A was followed and mutants R161 and H7 were obtained, containing the change A $\rightarrow$ C at the 2nd or the 3rd position, respectively. Figure 5B shows the result of an initiation reaction using as templates the DNA fragments isolated from clone R161, mutated at the 2nd position in the left ϕ 29 DNA end (lane b), and from clone H7, mutated at the 3rd position in the left (lane d) or right (lane g) ϕ 29 DNA ends. The initiation activity of these mutants, when detectable, was very low, similar to the background of a DNA

Figure 6. Random point mutagenesis along the Ø29 DNA origins of replication. Template activity of the mutants.

A) The procedure described by Derbyshire et al. (24) was followed. The 3'-end of the oligonucleotides was first ligated to the PstI-linearized vector. The DNA was then converted to full double-strand, cut with SmaI to produce blunt ends, and incubated with ligase after 30-fold dilution to favour circularization.

Double-line represents double-stranded DNA. The synthetic DNA is represented with wavy line and the repaired strand with discontinuous line. $\uparrow$, DraI sites. The XmaI site at the 5'-end of the oligonucleotides was relevant for a different cloning strategy not shown here. B) 31 ng of the DraI-digested DNAs of clones PM3 (a), PM5 (b), PM9 (c), PM12 (d), PM28 (e), PM31 (f), or PM46 (g), were used as templates in a replication reaction as described in Fig. 3C. The absence (-) or presence (+) of priming protein p3 in each reaction is indicated. The position of the input DNA fragments 1,626 and 522 bp long, containing the ϕ 29 left and right origins of replication, respectively, is also indicated. Additional bands not coinciding with the input fragments are considered to be caused by single-stranded intermediates of ϕ 29-specific replication as judged by nuclease S1 sensitivity. The mutations affecting the left origin in each clone are presented in Figure 7. Clone PM46 contains a wild-type right origin at the fragment 522 bp long.

fragment without ϕ 29 DNA sequences (lane e) and much lower than that of the control fragments carrying the wild-type sequences (lanes a, c and f). Although the mutation at the 3rd position of the left ϕ 29 DNA end did not seem to inactivate completely its template activity in the initiation assay, the lack of activity of the three mutated DNAs was further confirmed in replication assays (results not shown). The right ϕ 29 DNA end with an A→C mutation at the 2nd position, contained in R161 DNA, could not be assayed. As it has been indicated, the release of the mutated ϕ 29 DNA ends from R161 DNA required treatment with RsaI, and there is a target for this enzyme at position 10 in the right ϕ 29 DNA end, inside the origin of replication.

Fig. 6A shows the saturation mutagenesis strategy followed to obtain point mutants along the terminal 12 bp of both ϕ 29 DNA ends. The DNA sequence of 55 transformants was determined and 14 of them, carrying point substitutions beyond position 3 at each origin of replication of ϕ 29 DNA, were selected. Fig. 6B shows the template activity of 7 out of these 14 selected clones. The mutated DNAs were cut with DraI and directly assayed in the replication reaction, without further isolation of the origin-containing DNA fragments. The left origin was released in a DNA fragment 1,626 bp long and the right one in a fragment 522 bp long. As it can be seen, only the fragment 1,626 bp long became labelled in the presence of priming protein p3, and it did so in almost all mutants as efficiently as in a wild-type fragment (not shown). Only in one clone of those shown in the Figure (PM31, lane f) was the labelling markedly reduced, to about 20% of that

	1	2	3	4	5	6	7	8	9	10	11	12	ACTIVITY
WT	A	A	A	G	T	A	A	G	C	C	C	C	+
R161		C											-
H7			C										-
PM31				T								G	±
PM4						C						G	±
PM22										A		G	±
PM3												G	+
PM5						G							+
PM9									G				+
PM12										A			+
PM21									T	A			+
PM27						T						G	+
PM36									A			G	+
PM37									T				+
PM42									G			A	+
PM46											T		+

Figure 7. Point mutations at the left origin of replication of ϕ 29 DNA.

Only the bases different from those at the same position in the wild-type sequence are shown. The template activity was tested in the initiation and replication assays for clones R161 and H7, and only in the replication assay for all the others. Symbols (+), (+/-) and (-) represent normal (replication values from 80-100%), reduced (replication values from 20-50%) and undetectable (initiation values <20% and replication values <5%) activities, respectively.

of the other mutant or wild-type clones, as determined by densitometry of the autoradiographs. Two more mutants out of the 14 assayed also showed a significant decrease of template activity (see PM4 and PM22 in Fig. 7). The template activity of the mutated right origin of replication could not be determined, since the DNA fragments 522 bp long hardly incorporated any label in the presence of protein p3, even if containing a wild-type origin (lane g). This lack of template activity was shown to be an effect of the small size of the right origin-containing fragment. When a particular mp18 derivative was used instead of the mp19 ones, the wild-type right origin was released in the *Dra*I fragment 1,626 bp long and the wild-type left origin in the fragment 522 bp long; again, only the larger fragment was active as template (results not shown). We have previously reported a size effect on the activity of ϕ 29-specific templates in the *in vitro* initiation reaction (8,11).

Fig. 7 shows a summary of the sequences of the different point mutations introduced in the left origin of replication of ϕ 29 DNA and their effect on the in vitro template activity.

DISCUSSION

One particular feature common to all DNAs known to have a terminal protein covalently linked is the presence of inverted repeats at the ends of the molecule (30). Most of these DNAs, which appear in a wide variety of prokaryotic and eukaryotic organisms, have ~100-600 bp long ITRs, so called "long" ITRs, as compared to the 6-8 bp long highly homologous "short" ITRs found in the DNAs of the ϕ 29-related bacteriophages of B. subtilis. For the adenovirus system, the best studied one with a "long" ITR, it has been already demonstrated that, although the repetition seems to be somehow involved in the adenovirus multiplication cycle (31), the sequences acting as DNA origins of replication are much smaller than the whole ITR (32).

It had been thought that the 6 bp repeated at the ends of ϕ 29 DNA, as well as the short ITRs of the other phage DNAs, could by themselves be the origins of replication. Our results with plasmid pITR, containing not only the ϕ 29 ITRs, but also 3 and 1 additional bp from the left and right ϕ 29 DNA ends, respectively, showed that this is not the case. In the ϕ 29 in vitro replication system in the absence of the parental protein p3, although pITR DNA showed a detectable template activity above the background, it was not significant as compared with that of pID13 DNA, with longer terminal sequences of ϕ 29 DNA. Therefore, it seems that some bases beyond the ITRs are needed. With the aid of enzymatically produced deletion derivatives of plasmid pID13 we have mapped their extent. Although a great number of different deletions were not obtained for the left end of ϕ 29 DNA, the results clearly indicate that the terminal 12 bp from the left end present in pID12 DNA has as much template activity as the 73 bp present in pID13 DNA. The vector sequences flanking those 12 bp do not seem to affect significantly this activity (results not shown).

The template activity of the DNA fragments bearing a deleted right end was always lower than that of the left end, most likely due to their smaller size. The template activity of the deletions at the right origin of replication contained in DraI fragments

~530 bp long from the mDA series could be determined in the replication assay only when the isolated fragments were tested (Fig. 3C). When DraI restriction mixtures were used as templates in the replication assay, it became difficult to detect the specific labelling of origin-containing fragments about 500 bp long (see Fig. 6B). It seems as if the DNA fragments present in the restriction mixture compete for proteins p2 or p3, with a decrease of the labelling of the smaller origin-containing fragments. The size effect could also be related to the observed gradual loss of template activity of the right Ø29 DNA end in fragments from clone pDA59 through clone mDA12. It is possible that additional signals located beyond position 12 at the right Ø29 DNA end can enhance the initiation of replication promoted by the minimal origin. The lack of these signals would imply a special sensitivity of the origin of replication to the size of the DNA fragment, in the *in vitro* system.

The fact that the sequences at the left and right Ø29 DNA ends are quite different beyond position 6 (Fig. 4), suggests that the degree of sequence requirement at that distal region is less strict than that at the end-proximal region. The results obtained with the point mutants support this idea. Only two mutations completely abolish the template activity of Ø29 DNA ends, and both of them changed terminal bases, the 2nd or the 3rd ones. For the left end, only 3 out of 14 mutations reduced the template activity; all 3 were double changes, and 2 of them affected the ITR, at positions 4 and 6, respectively, in addition to the C at position 12. Most of the mutations affecting only the distal half of the origin of replication did not have a significant effect on its function. Although required as a part of the origin, this region may not be recognized nucleotide by nucleotide, but rather as a whole structure, disturbed only by some double mutations such as the one in clone PM22, affecting positions 10 and 12 at the left origin. Clone mDA10, a deletion derivative that can be considered as a double mutant at positions 11 and 12 at the right origin, was also inactive.

The Ø29 ITR sequence may represent, according to this data, the "core" of each origin of replication, identical at both DNA ends because most of the changes inside it, and specially any one close to the first positions, would have been selected against. As has been mentioned, the length of the ITR sequences at the DNA

ends of ϕ 29-related phages is not the same, and in one of the phages even bases inside the ITR are not homologous to that of the others. However, in agreement with our results, the sequence AAA is conserved at the very ends in all cases.

The results presented here indicate a direct involvement of the "short" ITRs in the function of the origins of DNA replication, presumably as binding sites for the priming protein or for a complex between the priming protein and the ϕ 29 DNA polymerase. The "long" ITRs have been proposed to be involved in the replication of completely displaced single-strands of parental DNA by facilitating the formation of panhandle structures with a double-stranded DNA end (33). Such structures have not been observed in the ϕ 29 group and they are not likely to be formed because of the short length of their ITRs (34).

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