P1 plasmid replication requires methylated DNA

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Plasmids driven by the plasmid replication origin of bacteriophage P1 cannot be established in *Escherichia coli* strains that are defective for the DNA adenine methylase (*dam*). Using a composite plasmid that has two origins, we show that the P1 origin cannot function even in a plasmid that is already established in a *dam* strain. An *in vitro* replication system for the P1 origin was developed that uses as a substrate M13 replicative-form DNA containing the minimal P1 origin. The reaction mixture contains a crude extract of *E. coli* and purified P1 RepA protein. In addition to being RepA dependent, synthesis was shown to be dependent on methylation of the *dam* methylase-sensitive sites of the substrate DNA. As the P1 origin contains five such sites in a small region known to be critical for origin function, it can be concluded that methylation of these sites is a requirement for initiation. This suggests that the postreplicational methylation of the origin may control reinitiation and contribute to the accuracy of the highly stringent copy-number control of the origin *in vivo*. Key words: *Escherichia coli*/plasmid/DNA/replication/methylation

Introduction

The prophage of bacteriophage P1 is maintained in its *Escherichia coli* host as a plasmid whose copy number is approximately one per host chromosome (Prentki *et al.*, 1977). Despite this low copy number, generation of cured cells during growth is extremely rare (Rosner, 1972; Austin *et al.*, 1981) implying that highly accurate replication controls exist that ensure a single round of replication per cell cycle. The minimal P1 plasmid replicon consists of some 2 kb of DNA. It contains an origin of replication in a region of ~250 bp, an open reading frame for the essential replication protein RepA, and a copy-control element *incA* (Abeles *et al.*, 1984). Both the origin region and the copy-control locus contain multiple copies of a 19-bp consensus sequence that has been shown to be a specific binding site for the RepA protein (Abeles, 1986). RepA binding to the origin appears to be essential for initiation (Austin *et al.*, 1985) and also to modulate the activity of the *repA* gene (Chattoraj *et al.*, 1985a,b). The binding of RepA to the repeats in the control region appears to limit the free RepA concentration, thus limiting the availability of RepA for initiation (Chattoraj *et al.*, 1984). The origin contains two notable features in addition to the 19-bp repeated sequences (Figure 1). There are two copies of a sequence thought to be the binding site for the host DnaA protein. P1 replication has been shown to be DnaA dependent and may require these sequences for DnaA activity during replication (Hansen and Yarmolinsky, 1986). Placed between the DnaA binding sites and the 19-bp repeats is a region that contains five closely spaced GATC

sequences. This sequence constitutes the substrate for the DNA adenine methylase of *E. coli* that adds methyl groups to the symmetrically placed adenine residues after DNA replication (Geier and Modrich, 1979; Lacks and Greenberg, 1977; Marinus and Morris, 1973). The origin region containing these sequences is important for origin function as a single base change in one of these GATC sites (the *rep-30* mutation) blocks plasmid maintenance (Austin *et al.*, 1985). Moreover, attempts to introduce P1 miniplasmids into a *dam* strain were unsuccessful, suggesting that origin function might be impaired when the origin is unmethylated (Austin *et al.*, 1985).

Results

Comparison of P1 and F miniplasmid establishment in a dam strain

Both P1 and F miniplasmids have been constructed by cloning fragments of the parental plasmids into the λ vector λ c1857 D15 b538 *nin5 sr* λ 3. λ -P1:5R (Sternberg and Austin, 1983) and λ -F:583 (Austin and Abeles, 1983) have the complete plasmid-maintenance regions of P1 and F respectively, introduced at the unique *EcoRI* site of the vector. The λ vector used in these constructs lacks the λ att site and cannot integrate into the host chromosome. Thus, both the P1 and F constructs form lysogens by replicating as low-copy-number plasmids under P1 or F control. On measuring the lysogenization frequency of these plasmid prophages in a *dam*⁺ and a *dam* host strain, we found that although lysogeny was *dam*-independent with the F construct, lysogens of the P1 construct were only formed in the *dam*⁺ cells (Table I). As λ -P1:5R could infect and grow lytically on the *dam* cells (data not shown), it was concluded that the DNA was injected normally into cells. Introduction of plasmid pMQ3 that contains a wild-type *dam*⁺ gene into the *dam* strain fully restored λ -P1:5R lysogeny. Thus, some step in the establishment or maintenance of the plasmid prophage is *dam* dependent.

The P1 origin cannot support the replication of a plasmid already established in a dam strain

Plasmid pALA1005 contains the complete plasmid-maintenance region of F and the essential plasmid replication region of P1. Its construction and configuration are illustrated in Figure 2A. The plasmid transformed and was stably maintained in the *dam* strain. In order to determine which of the two replication origins were functional under these conditions, the strain was subsequently transformed with a second plasmid, pFAMK, that carries the F replication region and selectively switches off the F origin. The F replicon region includes the *F incB* and *incC* elements that contain groups of repeat sequences which act by titration of the essential F replication-initiation protein.

Table II shows that pALA1005 was displaced from the population of *dam* cells by the F plasmid (pFAMK). The competing F plasmid did not have any effect on the maintenance of pALA1005 in a *dam*⁺ strain. Thus, both the F and P1 origins are capable of function in a *dam*⁺ strain so that if one is switched off, the other can support replication. However, in the *dam*

Fig. 1. The DNA sequence of the minimal P1 origin cloned into the M13mp10 and 11. The numbers above the sequence correspond to the numbering scheme adopted previously (Abeles *et al.*, 1984). The large box near bp 390 encloses the proposed DnaA recognition site. The small boxes mark short repeats which surround each of the underlined GATC (*dam*) sites. The long arrows, numbered 14–10, mark the repeats which share the same consensus sequence with the *incA* region of the replicon (Abeles *et al.*, 1984). The location of the *rep-30* mutation is marked.

Table I. Lysogenization frequency

Miniplasmid	Host		pMQ3 ^a (<i>dam</i> ⁺)
	<i>dam</i> ⁺	<i>dam</i> [−]	
λ-F:583	2.8%	2.8%	n.t.
λ-P1:5R	3.0%	<0.1%	4.5%

^apMQ3 is a high-copy plasmid carrying the *E. coli dam*⁺ gene that was introduced into GM 2150 (*dam*) before lysogenization with the phage. Infection was carried out at a multiplicity of infection of 5. Lysogenization frequency is expressed as % viable cells lysogenized.

Table II. Replication of P1 in *dam*⁺ and *dam*[−] hosts – stability of plasmids in *dam*⁺ or *dam*[−] hosts after transformation with a plasmid^a which blocks replication from the F origin

Plasmid tested	<i>dam</i> ⁺	<i>dam</i> [−]
P1ori (pALA318)	100/100	(0) ^b
P1oriFori (pALA1005)	100/100	0/100

^apFAMK *amp*^R which carried the complete *Frep* region was used to transform the *dam*⁺ and *dam*[−] strains containing pALA318 or pALA1005. Transformants were selected for pFAMK on ampicillin and then tested for the presence of pALA318 or pALA1005 by stabbing into agar containing chloramphenicol.

^bpALA318 will not transform the *dam*[−] host.

strain, only the F origin is capable of function so that the plasmid becomes sensitive to the switching off of the F origin. We conclude that the P1 origin cannot function properly in a *dam* strain even if the plasmid is already established in the strain.

Replication from the P1 origin in an *in vitro* replication system requires RepA

We have developed an *in vitro* replication system for the P1 origin based on the technique developed by Fuller, Kaguni, and Kornberg for the replication of *oriC* plasmids (Fuller *et al.*, 1981). The substrate for this reaction consisted of M13 replicative form (RF) DNA containing 245 bp of P1 sequence that encompass the P1 origin (Figure 2B). A crude extract of *E. coli* cells was used to supply the necessary enzymes and was supplemented with highly purified RepA made as previously described (Abeles, 1986). This system incorporates radiolabelled nucleotides into acid-precipitable material dependent on the addition of the exogenous DNA and RepA protein (Table III). Incorporation is approximately proportional to added RepA except at high RepA

concentrations, at which a saturation of the response was observed (Figure 3). Without added RepA, the incorporation was low and indistinguishable from that given by controls without any exogenous DNA template or with M13 DNA that does not contain the P1 origin. The plasmid containing the *rep-30* mutation which blocks origin function *in vivo* (Austin *et al.*, 1985) also showed no RepA-stimulated incorporation.

Adenine methylation of the template is essential for *in vitro* replication

The replication-effective M13 P1ori RF DNA was isolated from *dam*⁺ cells. When DNA isolated from *dam* cells was used, only background levels of incorporation were observed in the *in vitro* system and added RepA had no effect (Table IV). Moreover, when this DNA was methylated *in vitro* using purified *dam* methylase prior to its addition to the *in vitro* system, its template activity was fully restored (Table IV). The same effect was observed when the DNA was preincubated with methylase and S-adenosyl methionine in the replication system prior to addition of the *E. coli* extract. We conclude that replication from the P1 origin is absolutely dependent on the state of adenine methylation of the DNA.

Discussion

Although the P1 origin appears to be unique so far in its absolute functional requirement for efficient DNA adenine methylation, the effects of methylation on origin function appear to be common to a number of replicons. The presence of multiple GATC sequences within the chromosomal origins of several Gram-negative species, including *E. coli*, suggests an important role for methylation in these systems (Zyskind *et al.*, 1983; Meijer *et al.*, 1979; Sugimoto *et al.*, 1979). Moreover, plasmids containing the *E. coli* origin, *oriC*, transform *dam* strains poorly or not at all (Messer *et al.*, 1985; Smith *et al.*, 1985). The requirement for *oriC* methylation appears to be a partial one. Although the methylation state of *oriC* plasmids affects the efficiency of *in vitro* replication, the effects are modest, with the incorporation rates for unmethylated templates being about half that of the normal template (Hughes *et al.*, 1984; Messer *et al.*, 1985; Smith *et al.*, 1985). In the absence of *dam*-directed methylation, replication appears to proceed from the normal *oriC* site, but the frequency of initiation is reduced (Hughes *et al.*, 1984).

What is the function of these methylation sites in the

Fig. 2. Construction of clones used in this work. (A) pALA1005. The *EcoRI* site of pALA206 was converted to a *BamHI* site. The plasmid then was digested with *BamHI* and the F region isolated and cloned into pALA318 which had been digested with *BamHI*. (B) The structure of the M13-P1 origin clones. A 327-bp fragment encompassing bp 3609 to 3736 of pBR322 and the P1 origin was isolated from pALA159 (Chatteraj *et al.*, 1985) and cloned into the *BamHI* to *PstI* sites of M13mp10 and 11 to form M13-P1ori-#49 and M13-P1ori-#38, respectively. The orientation of the inserted region is shown to the side of the plasmid. An enlargement of this region is shown below the plasmid. The numbers are the base pair coordinates of the pBR322 and P1 sequences. The restriction sites used for cloning are indicated.

Table III. *In vitro* replication

DNA	pmol dTTP incorporated	
	RepA	
	-	+
M13mp10	7	10
M13mp11	7	9
M13-Pori-#38	7	68
M13-Pori-#49	8	58
M13-Pori-#88 ^a	12	11
<i>oriC</i> ^b	214	216

^aThis plasmid contains the *rep-30* mutation (Figure 1).

^bThe *E. coli oriC* origin was carried on pAL3, a pACYC184 derivative. Incorporation of dTTP was based on the amount of [³H]dTTP incorporated into a trichloroacetic acid precipitate under the assay conditions described in Materials and methods. Each reaction contains about 0.04 pmol of DNA and 7 pmol of RepA.

chromosomal and P1 origins? We do not think that the tight clustering of methylation sites within the origin, so well illustrated by the P1 case, is fortuitous. It seems more likely that methylation plays some dynamic role in origin control. The hemimethylated products of replication appear to persist for some time before methylation is complete (Marinus, 1976). Thus, this change in the methylation state of the DNA might be involved in the timing of reinitiation (Messer *et al.*, 1985). In the case of *oriC*, Smith *et al.* (1985) have suggested that methylation may regulate initiation by altering the potentially complex secondary structure of the origin region. At least in the P1 case, this type

Fig. 3. Dependence of *in vitro* P1 replication on RepA protein. *In vitro*, P1-dependent incorporation of [³H]dTTP into acid-precipitable nucleotides depends upon the presence of RepA protein. The assay was performed as described in Materials and methods. Each reaction contained about 0.1 μ g of M13 DNA (20 fmol).

of mechanism is unlikely as there are no significant secondary structure possibilities involving the methylation sites in the functional minimum origin sequence (Austin *et al.*, 1985). Rather, it seems more likely that regulation of the P1 origin occurs by modulation of the binding of *trans*-acting factors to the GATC-rich region that are required to form an active initiation complex as suggested for the *oriC* system by Messer *et al.* (1985). The absolute requirement of the P1 origin for methylation relative to that of *oriC* might reflect a more stringent dependence for binding of such factors or that a greater proportion of the GATC sequences need to be methylated.

Table IV. Dependence of P1 replication on methylation — incorporation of [³H]-dTTP by the *in vitro* replication system using M13-P1ori-#49 grown in *dam*⁺ or *dam*⁻ hosts

Host	<i>In vitro</i> methylation	RepA		Methylation in replication system	RepA	
		-	+		-	+
<i>dam</i> ⁺	-	9	42	-	8	40
	+	9	33	+	6	59
<i>dam</i> ⁻	-	12	11	-	8	8
	+	10	47	+	5	60

Values are expressed as pmol dTTP incorporated into TCA precipitable material per assay in 1 h.

Materials and methods

Strains, bacteriophage and plasmids

The strains used for testing P1 and F lysogeny were GM30 (F⁻ *supE44 thr-1 leuB6 his-4 thi-1 tonA31 galK2 galT22 ara-14 lacY1 xyl-5 mth-1 tsx-78 rpsL136*) and the *dam-13::Trn9* derivative, GM2150 from M. Marinus (1973; see also Marinus *et al.*, 1983). The strains used to test plasmid replication under *dam* conditions were BU40 [Δ (*pro-lac*) *trp-8 rpsL*] (Bukhari and Metlay, 1973) and its derivative BU1416 [*dam-3*, Δ (*pro-lac*) *trp-8, rpsL*] (constructed and supplied by Tom Patterson). The strain used for the *E. coli* fraction II extract was C600 (Appleyard, 1954). The strains used for preparing the M13 RF DNA were JM103 (Messing *et al.*, 1981) and GM119Nal^R/F'Km^R (*dam, supE*) from Bauer *et al.* (1985). The λ phage used were the mini-P1 derivatives λ -P1:5R-3 (Sternberg and Austin, 1983) and λ -P1:5Rrep-30 (Austin *et al.*, 1985), and the mini-F derivative, λ -F:583 (Austin and Abeles, 1983). The M13 phage used were M13mp10 and mp11 (Messing and Vieira, 1982). The P1 origin fragments cloned into the M13 vectors were isolated from pALA159 (Chattoraj *et al.*, 1985) and from the λ -P1:5Rrep-30 origin mutant (Austin *et al.*, 1985). The plasmids used were pMQ3 which carries the *E. coli dam* gene (Arraj and Marinus, 1983), pFAMK which carries the F replication region (Koop *et al.*, 1987) and pALA318, a derivative of pALA317 (Chattoraj *et al.*, 1985) with *Bam*HI linkers inserted at the *Xma*I site in the pBR322 sequence. The *oriC* plasmid was pAL3, a pBR322 derivative (Leonard *et al.*, 1980).

Media

Cells for most experiments were routinely grown in L broth (Gottesman and Yarmolinsky, 1968) supplemented with 50 μ g/ml ampicillin, 25 μ g/ml chloramphenicol or 25 μ g/ml tetracycline when required. JM103 and GM119 were grown in YT medium (Miller, 1972). C600 for the fraction II extract was grown in L broth supplemented with 0.4% glucose.

Enzymes, special chemicals and buffers

Restriction enzymes, linkers and adapters, ligase, and phosphatase were obtained from Bethesda Research Labs, New England Biolabs, Boehringer Mannheim, International Biotechnologies, Inc., or Pharmacia and used according to their directions. Creatine kinase was from Sigma, DNA gyrase from Bethesda Research Labs and *E. coli dam* methylase was from New England Biolabs. The other chemicals used for the *in vitro* replication reactions were from Pharmacia or Sigma. The [³H]-methyl dTTP was the 50–80 Ci/mmol dTTP from New England Nuclear. The buffer for suspending DNA was TE (Maniatis *et al.*, 1982) and the buffers for electrophoresis were TBE (Maniatis *et al.*, 1982) and TASE (Sternberg, 1979).

Gel electrophoresis

Gel electrophoresis was performed in 0.8–1.2% agarose with TBE or TASE and in 5% polyacrylamide in TBE buffer.

DNA fragment isolation, cloning, and transformation

DNA fragments for cloning were isolated from acrylamide by the 'crush and soak' method (Maxam and Gilbert, 1980) and from agarose by the 'phenol-freeze' method (Silhavy *et al.*, 1984). Preparation of plasmid DNA, general cloning techniques and transformations were described earlier (Abeles *et al.*, 1984).

Phage techniques

Procedures for preparing phage and lysogens were described previously (Austin *et al.*, 1982).

Construction of plasmids for this work

The construction of plasmids for this work are shown in Figure 2. The plasmid pALA206 (Austin and Wierzbicki, 1983) was digested with *Eco*RI, and ligated to an *Eco*RI–*Bam*HI adapter that destroys the *Eco*RI site and adds a *Bam*HI site. Following ligation the plasmid was digested with *Bam*HI. The DNA en-

compassing the complete F plasmid-maintenance region was isolated and ligated to pALA318 which had been digested with *Bam*HI and treated with calf alkaline phosphatase. The new plasmid is pALA1005 and carries the complete F plasmid-maintenance region, the P1 replicon deleted for most of the *incA* repeats, and the chloramphenicol acetyl transferase gene. The M13-P1ori clones #38 and #49 (Figure 2B), were constructed by digesting pALA159 with *Bam*HI and *Pst*I and ligating the purified P1ori fragment into M13mp10 and mp11, digested with *Bam*HI and *Pst*I, to form M13-P1ori-#49 and M13-P1ori-#38, respectively. The M13-P1ori clone M13-P1ori-#88 that contains the *rep-30* mutation was constructed by digesting λ -P1:5Rrep-30 with *Asp*718 and *Eco*RI, purifying the 1.8-kb fragment encompassing the P1 origin, digesting the fragment with *Dra*I and *Hin*dIII, isolating the 520-bp fragment of P1 (bp 87-606; Abeles *et al.*, 1984) and ligating in into M13mp10 which had been digested with *Hin*dIII and *Hinc*III.

Preparation of M13 RF DNA

JM103 and GM119/F' were grown in an air shaker at 37°C in YT to an OD₅₉₀ = 0.3 and inoculated with M13 derivatives at an MOI of ~5. The cultures were then grown to OD₅₉₀ = 2. Chloramphenicol was added to 0.1 mg/ml and the cultures were incubated with shaking for 3 h. The cells from 250 ml of culture were pelleted and the DNA extracted by the alkaline lysis method (Birnbom and Doly, 1979). Following the isopropanol precipitation step, the DNA was resuspended in 4 ml TE and treated with 0.05 mg/ml RNase for 30 min at 37°C. The DNA was then phenol extracted and ethanol precipitated. After drying, it was resuspended in TE, banded in CsCl–ethidium bromide and purified as described by Maniatis *et al.* (1982).

E. coli fraction II extract

The fraction II extract for the *in vitro* replication experiments was prepared essentially as described by Fuller *et al.* (1981).

In vitro replication assay

Each reaction contained in 25 μ l: 40 mM Hepes buffer (pH 7.6); 0.5 mM each GTP, CTP, UTP; 2 mM ATP; 300 μ M each dNTP (with 120 μ Ci [³H]-dTTP/ml); 12 mM Mg(OAc)₂; 40 mM creatine phosphate, 100 μ g/ml creatine kinase; 0.05 unit gyrase; 50 μ g/ml bovine serum albumin; 5% polyvinyl alcohol; 10–20 mg/ml fraction II extract and 20–50 fmol supercoiled DNA. Purified P1 RepA protein (Abeles, 1986) was added up to 22 pmol/reaction (~0.6 μ g). Some experiments also included 10–15 units of *Dam* methylase and 80 μ M S-adenosyl methionine. The reactions were assembled at 0°C. If S-adenosyl methionine and *Dam* were added, the reactions were preincubated at 30°C for 30 min before the replication reactions were initiated by the addition of 200–300 μ g of fraction II extract and RepA, and incubated at 30°C for 1 h. The reactions were stopped by the addition of 0.5 ml of 0.2 M NaPP₃ and 1.5 ml 10% trichloroacetic acid followed by incubation on ice for 15 min. The precipitated nucleic acids were collected by filtration through Whatman GF/C glass fiber filters and the counts of incorporated [³H]dTTP determined. The pmol of dTTP incorporated into precipitable nucleotides were calculated based on the specific activity of the [³H]dTTP solution (100–300 c.p.m./pmol dTT).

In vitro methylation

DNA was methylated *in vitro* either as a separate reaction or in the *in vitro* replication buffer. For the separate reaction, 10–15 μ g of DNA was reacted with 100–150 units of *E. coli dam* methylase (New England Biolabs) in 150–200 μ l of 2 mM Tris–Cl, pH 7.5, 10 mM EDTA, 5 mM 2-mercaptoethanol, and 80 μ M S-adenosyl methionine for 30 min at 37°C. The reactions were then heated to 65°C for 10 min, phenol extracted twice with 200 μ l neutral phenol and alcohol precipitated. The DNA was dried, resuspended in TE to 0.1 μ g/ml and tested in the *in vitro* reactions. DNA from the *dam* host showed some nicking by *Dpn*I before *in vitro* methylation and was completely digested by *Dpn*I following methylation (data not shown). When the DNA was methylated in the replication buffer, the reactions were assembled at 0°C as described above but without *E. coli* extract or RepA. In addition, each reaction contained 80 μ M S-adenosyl methionine and 10–15 units of methylase. The reactions were incubated for 30 min at 30°C before the replication reactions were started by the addition of the *E. coli* extract and RepA (where used).

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