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Evidence for Two Levels of Control of P1 *oriR* and Host *oriC* Replication Origins by DNA Adenine Methylation

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A mutant mini-P1 plasmid with increased copy number can be established in Dam⁻ strains of *Escherichia coli*, where mini-P1 plasmid replication is normally blocked. Comparison of this plasmid and a plasmid driven by the host *oriC* replication origin showed that both origins are subject to control by methylation at two different levels. First, both origins appear to be subject to negative regulation acting at the level of hemimethylation. This probably involves the sequestration of the hemimethylated DNA produced by replication, as has been previously described for *oriC*. Second, both origins show a positive requirement for adenine methylation for efficient function in vivo. This conclusion is supported by the behavior of the P1 origin in an improved in vitro replication system. In vitro, where sequestration of hemimethylated DNA is not expected to occur, the hemimethylated P1 origin DNA was fully functional as a template. However, the activity of fully unmethylated DNA was severely restricted in comparison with that of either of the methylated forms. This in vitro uncoupling of the two effects of origin methylation suggests that two separate mechanisms are involved.

The prophage of bacteriophage P1 is maintained in its *Escherichia coli* host as an autonomous plasmid whose replication is under stringent control. The plasmid has multiple adenine methylation (GATC) sites clustered within the origin, P1 *oriR* (2) (Fig. 1). This feature is rare among bacterial plasmids but is shared by the *oriC* system that replicates the host bacterial chromosome (38) (Fig. 1). Wild-type P1 miniplasmids cannot be maintained in strains that lack DNA adenine methyltransferase activity (Dam⁻ strains) (2), suggesting a stringent requirement for adenine methylation for the origin to fire. However, origin methylation is not essential for *oriC*, as it is able to function in Dam⁻ strains. Rather, *oriC* methylation is implicated in the timing of initiation within the cell cycle (8, 11). Immediately following replication from the fully methylated *oriC* origin, the GATC sites are hemimethylated. This causes sequestration of the origin by binding to the bacterial membrane (32). Sequestration prevents a second round of replication until methylation is completed, an event that takes a considerable portion of the cell cycle for the origin GATC sites (16).

Although P1 *oriR* and *oriC* seem to respond differently to methylation, the similarity of organization of the origins and their methylation sites suggests some commonality of mechanism. We present studies that examine this possibility.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The *E. coli* strains used for the in vivo studies were the Dam⁺ strains MC1061 (30) and AB1157 (20) and Dam⁻ strains S1540 *dam::kan-16* (15), GM3819 *dam::kan-16* (34), and RS5033 *dam-4* (28). The high-copy-number P1 *oriR* plasmid pSP102 (33) and *oriC* plasmid pOC15 (31) were used. Plasmid pALA109 is a wild-type mini-P1 carrying P1 *oriR* and the complete P1 *par* region. It was constructed by ligating together the following three fragments: the 3.7-kb *Eco*RI-to-*Hin*dIII fragment from pALA33 (4) that encompasses the complete P1 *oriR* replicon including *repA* and *incA*, the 3.3-kb *Hin*dIII fragment from Tn*5* that encompasses the kanamycin resistance gene (23), and the 6.1-kb *Hin*dIII-to-*Eco*RI fragment from pALA17 (7) that encompasses the complete P1 *par* region. The *Hin*dIII fragment is oriented such that the *neo* gene (conferring kanamycin resistance) is located close to the P1 *repA* gene. Plasmids pDS596 (22), pMQ148 (6), and pSSH6 (37) were used to supply DnaA, *E. coli* Dam methylase, and T4 Dam methylase proteins, respectively. For the in vitro replication studies, the FII extract was prepared from strain C600 (5). The phages used were f1h_0 (26) and its derivative, flP1, which was constructed as follows. The P1 *oriR* fragment from M13 P1 *ori* clone 49 (2) was digested with *Pst*I and *Bal* 31, which removed all P1 bases up to bp 373, and then recircularized by ligation to a synthetic blunt-end linker containing an internal *Eco*RI site. The resulting circles were digested with *Eco*RI, and the *Eco*RI piece encompassing the P1 origin (bp 373 to 610) was then cloned into the *Eco*RI site of f1h_0 to form f1P1. The insert is oriented such that the vector *Hin*cII site is proximal to the left of the origin, as illustrated in Fig. 1.

In vitro replication. Template f1P1 replicative-form DNA was isolated from the Dam⁺ host JJ119 (26) or the Dam⁻ host RS5033 (28) as described previously (2). Hemimethylated f1P1 DNA was prepared from double-stranded methylated replicative-form DNA and circular phage DNA. The fully methylated double-stranded DNA, isolated from JJ119, was linearized with *Sna*B1 and annealed to the circular phage DNA isolated from f1P1 that had been propagated by several lytic passages through the Dam⁻ strain RS5033. The hemimethylated DNA was then prepared as described by Lu et al. (28). The FII extract was prepared as described by Fuller et al. (18). The in vitro replication reactions were performed as described elsewhere (3). This method differs from that previously employed (2) in being enriched with additional DnaA protein to increase the signal to background ratio. Each 25-μl reaction mixture contained 0.04 pmol of DNA, 2 pmol of purified DnaA protein (a gift from Jon Kaguni), and, when present, 6 pmol of purified P1 RepA (1). For cases in which the template DNA was methylated in vitro, purified *dam* methylase (10 to 15 U; New England Biolabs) and *S*-adenosylmethionine (to 80 μM) were

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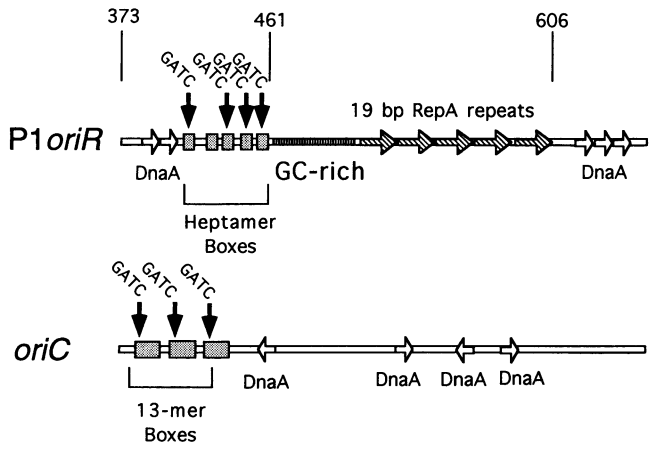

FIG. 1. Physical maps of the P1 *oriR* and *E. coli oriC* replication origins. Open and hatched arrows mark the repeat sequences responsible for binding of the DnaA and P1 RepA proteins, respectively. The heptamer and 13-mer repeat sequences (stippled boxes) contain clustered GATC sequences (solid arrows) that are substrates for DNA adenine methylase. The P1 region present in f1P1 does not include the three rightmost DnaA binding sequences which are not essential for origin function (3). The numbers above the P1 map indicate the conventional coordinates (4).

added and the reaction mixtures were incubated for 30 min at 30°C before the addition of the FII, RepA, and DnaA to start in vitro replication (2).

Transformations. Competent cells were prepared by the $CaCl_2$ method (17). The initial transformations with pOC15 and pSP102 used 0.1 μg of DNA in 100 μl of Dam^+ cells or 1 μg of DNA in 100 μl of Dam^- cells. All incubations were done at 32°C. The initial transformations used DNA purified by banding through cesium chloride-ethidium bromide gradients (36). DNA concentration was determined by spectrophotometry. All other DNA concentrations were estimated from band intensities of suitable restriction digests subjected to gel electrophoresis in comparison with a suitable DNA standard. Concentration of the unmethylated pSP102 DNA recovered from Dam^- cells by the minipreparation technique was too low to estimate precisely. The concentration of 0.01 μg/μl assumed here should be taken as a maximum estimate. For testing the effect of increased levels of DnaA protein, competent cells with increased DnaA production were prepared by growing MC1061/pDS596 and S1540/pDS596 in L broth supplemented with 20 μg of ampicillin per ml and either 0, 0.1, or 0.7% arabinose.

Stability tests. Transformants were purified by being streaked onto selective agar, L agar with 10 μg of chloramphenicol per ml for pSP102 or with 15 μg of ampicillin per ml for pOC15. Eight colonies were picked from each transformation and restreaked for single colonies on the same medium. The colonies were then restreaked without selection onto L agar (first-generation colonies) and then restreaked again without selection. Approximately 10 of the resulting second-generation colonies were tested for antibiotic resistance retention by being stabbed into L agar with or without antibiotic. The proportion of colonies retaining antibiotic resistance was taken as a measure of plasmid retention during the approximately 25 generations required to generate the first-generation colonies. Despite the fact that they were derived from colonies grown under selection, some of the first-generation colonies originated from single cells that had already lost the plasmid prior to plating. The colonies contained no antibiotic-resistant cells at all. These uninformative clones were identified by being stabbed into plates with antibiotic and eliminated from the analysis. For the transformations of strains carrying pDS596, all media contained 20 μg of ampicillin per ml to ensure retention of pDS596, and, when relevant, induction of the pDS596 *dnaA* gene was maintained by including 0.1 or 0.7% arabinose in all media.

Southern blotting of plasmid DNA. Total cell DNA was prepared essentially by the method of Berman et al. (9) by using 5 ml of overnight cultures grown in L broth with either 15 μg of ampicillin or 10 μg of chloramphenicol per ml as appropriate. Cell pellets were resuspended in 250 μl of buffer. Proteinase K treatment was carried out for 3 h. DNA was precipitated with ethanol and collected by centrifugation rather than spooling. The final samples were dried for 5 to 10 min under a vacuum and resuspended in 100 μl of 50 mM Tris-Cl–1 mM EDTA. After being held overnight at 4°C, the DNA was digested with the relevant restriction endonuclease and electrophoresed in a 0.8% agarose gel in 1× Tris-borate-EDTA buffer. The gel was soaked in 0.25 M HCl for 15 to 20 min and washed twice for 20 min in denaturing solution (Digene Diagnostics) and twice for 20 min in neutralizing solution (Digene Diagnostics). The gels were blotted onto nylon transfer membranes (Magna; Micron Separations, Inc.) by capillary transfer overnight (36). The membranes were treated in a Stratagene UV cross-linker, dried for 30 min under a vacuum, prehybridized with Digene Fastpair reagent for 1 h at 42°C, and then hybridized overnight at 42°C with 10^6 cpm of ^{32}P-labeled plasmid probe per ml. The membranes were then washed twice for 10 min at 42°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and once for 20 min at 65°C in 0.2× SSC–0.1% SDS, and then they were autoradiographed. Reprobing with a chromosomal (*galK*) probe was carried out after boiling the membrane in 0.02× SSC–0.01% SDS for 20 min.

Labeled probes. The probes were the 3-kb *Eco*RI-to-*Hin*dIII fragment encompassing the ampicillin resistance region from pOC15, the 1.8-kb *Pst*I fragment encompassing the chloramphenicol resistance region from pSP102, and the 1-kb *Sna*BI-to-*Bst*BI fragment encompassing the *E. coli galK* gene. The DNA fragments were gel isolated and labeled with [α-^{32}P]dCTP by using a NEBlot kit (New England Biolabs) to a specific activity of 1 to 1.6 × 10^9 dpm/μg.

Media, enzymes, buffers, special chemicals, and other techniques. Media, enzymes, buffers, chemicals, and other techniques were described previously (2). Western blotting (immunoblotting) to determine the levels of DnaA protein was carried out as described elsewhere (36) with antiserum to DnaA kindly supplied by Jon Kaguni.

RESULTS

A high-copy-number P1 miniplasmid can transform Dam^- strains. A plasmid construct driven by the wild-type P1 plasmid replicon is unable to establish itself in Dam^- strains (2). However, the mini-P1 plasmid pSP102, which lacks the P1 copy control element *incA* (33), did give some transformants (Table 1). This plasmid retains all the elements essential for replication, including the origin with its multiple GATC sites. However, in wild-type hosts, pSP102 is maintained at an 8- to 10-fold-higher copy number than wild-type mini-P1 plasmids (33) (Fig. 2). In this respect, its behavior resembles that of *oriC* plasmids whose copy number is similarly elevated relative to that of the *E. coli* chromosome from which they are derived (39) (Fig. 2).

TABLE 1. Frequency of transformation of Dam$^-$ and Dam$^+$ strains by P1 *oriR* and *oriC* miniplasmids

Transforming plasmid[a]	No. of plasmid-containing colonies/μg of DNA in transformed strain		Dam$^-$/Dam$^+$ ratio (no. of plasmid-containing colonies/μg of DNA)
	Dam$^+$	Dam$^-$	
pALA109 (Meth$^+$)	7×10^4	<10[b]	$<1 \times 10^{-4}$
pOC15 (Meth$^+$)	2×10^5	2×10^2	1×10^{-3}
pSP102 (Meth$^+$)	5×10^5	4×10^2	1×10^{-3}
pSP102 (Meth$^-$)[c]	8×10^5	1×10^6	1
pSP102 (Meth$^+$)[d]	2×10^5	2×10^1	1×10^{-4}

[a] The strains used for pOC15 and pSP102 transformations were MC1061 and its Dam$^-$ derivative, S1540. Very similar results were obtained with the Dam$^+$ strain AB1157 (20) and its Dam$^-$ derivative GM3819 (34) (data not shown). Plasmid pALA109 (Km^r) transformations were done with the kanamycin-sensitive strains AB1157 (Dam$^+$) and RS5033 (Dam$^-$) (28). The terms Meth$^+$ and Meth$^-$ indicate that the transforming DNA was prepared from Dam$^+$ and Dam$^-$ hosts, respectively.

[b] A total of 17 colonies was recovered from this transformation. However, none of these colonies contain free plasmid DNA: when DNA was prepared from these cells by a standard minipreparation procedure (36), none of the samples gave a plasmid band on agarose gel electrophoresis and ethidium bromide staining.

[c] The DNA used for these transformations was minipreparation DNA recovered from the few *dam* mutant transformants. Its concentration was 0.01 μg/μl or less.

[d] The DNA used for these transformations was minipreparation DNA recovered from the *dam*$^+$ transformants that had been transformed with the DNA from the *dam* mutants to show that the increased transformation frequencies found with the *dam* mutant DNA was not due to accumulation of mutations.

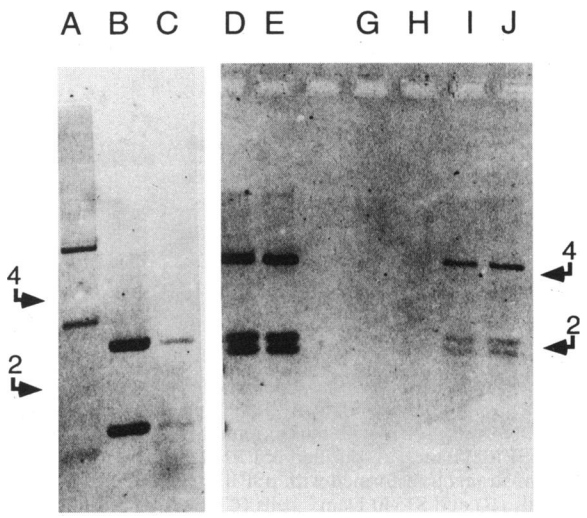

FIG. 2. The copy numbers of the P1 *oriR* and *oriC* plasmids are low in Dam$^-$ strains. Relative plasmid DNA contents of cells carrying the P1 *oriR* plasmid pSP102 (lanes B and C) and the *oriC* plasmid pOC15 (lanes D through J) are shown. The wild-type mini-P1 plasmid pALA109 (lane A), which is present at 1 to 2 copies per host chromosome, serves as a standard for quantitation. Minipreparation DNA was prepared from 5-ml overnight cultures grown in the presence of antibiotic. The DNA was resuspended in 30 μl of Tris-EDTA. Five microliters of the preparation was used for each digest in lanes A through C, and 8 μl was used for lanes D through J. The lanes contain the following: pALA109 (Meth$^+$) (methylated) digested with *Hin*dIII (A), pSP102 (Meth$^+$) digested with *Eco*RI (B), pSP102 (Meth$^-$) (unmethylated) digested with *Eco*RI (C), pOC15 (Meth$^+$) digested with *Hin*dIII (D and E), and pOC15 (Meth$^-$) digested with *Hin*dIII (G through J). Bent arrows indicate the positions and sizes (in kilobase pairs) of marker bands.

Transformation frequencies in Dam$^+$ and Dam$^-$ strains. A key observation for the understanding of the *oriC* methylation control system was made by Russell and Zinder (35). They showed that methylated *oriC* plasmid DNA transformed strains lacking DNA adenine methyltransferase activity (Dam$^-$ strains) very inefficiently. These plasmids were replicated once, but further replication of most of the copies was blocked at the hemimethylated stage. However, fully unmethylated *oriC*-driven plasmid DNA was able to transform Dam$^-$ strains relatively efficiently and could be propagated in the plasmid state.

We carried out similar experiments using the P1-derived plasmid pSP102. This plasmid transformed a wild-type host readily, and plasmid DNA recovered from these cells was fully methylated, as judged by digestion with methylation-sensitive restriction endonucleases (data not shown). Methylated DNA extracted from these cells transformed a Dam$^-$ strain some 10^3-fold less efficiently than an isogenic Dam$^+$ strain (Table 1). A similar effect was seen with the *oriC* plasmid pOC15 (Table 1), in conformity with the original observation (35). The few pSP102 colonies that arose from the Dam$^-$ host contained low levels of plasmid DNA when propagated under selection (Fig. 2 and 3), and this DNA proved to be fully unmethylated when analyzed with methylation-sensitive restriction endonucleases (data not shown).

The small quantities of unmethylated mini-P1 DNA recovered from the Dam$^-$ strain were used to retransform the Dam$^-$ strain. This DNA was some 1,000-fold more efficient at transforming the Dam$^-$ strain than fully methylated DNA (Table 1). As the unmethylated DNA used in these experiments had been subjected to selection for replication in a Dam$^-$ host, we were concerned that its properties might be due to spontaneous mutation rather than to the methylation state of the DNA. However, this possibility was ruled out; further passage through a Dam$^+$ strain fully restored the properties of the derivatives of the unmethylated DNA pool to those of methylated DNA (Table 1). We conclude that unmethylated mini-P1 DNA is able to bypass a block to plasmid establishment which normally occurs when a methylated plasmid is introduced into a Dam$^-$ strain. This is a property shared with *oriC* plasmids (35).

Mini-P1 plasmid copy number is low and maintenance efficiency is poor in Dam$^-$ cells. The plasmid content of the Dam$^-$ cells transformed with mini-P1 pSP102 is much less than that of the equivalent Dam$^+$ strain when grown under selection for plasmid retention (Fig. 2). From examination of a number of independent plasmid-containing clones, we estimate that Dam$^-$ cells contain some fivefold fewer copies of pSP102 than Dam$^+$ cells. The lowered copy number was also seen in Dam$^-$ cells examined by hybridization of a plasmid probe to whole-cell DNA extracts (Fig. 3). As this plasmid lacks an active plasmid-partitioning system, its maintenance stability should be roughly proportional to plasmid copy number (13). In the absence of selection, the plasmid was relatively stable in Dam$^+$ cells but was rapidly lost from Dam$^-$ cells (Table 2). Thus, although pSP102 can be established in a Dam$^-$ strain without continuous methylation, replication is inefficient under these conditions, so that the average copy number of the plasmids is low and loss of the plasmid during cell division is high. We presume that the wild-type mini-P1 also replicates inefficiently in Dam$^-$ strains but that the combination of this inefficiency and the stringent copy control imposed by the P1 *incA* locus results in a plasmid that is so unstable that its establishment is difficult to demonstrate.

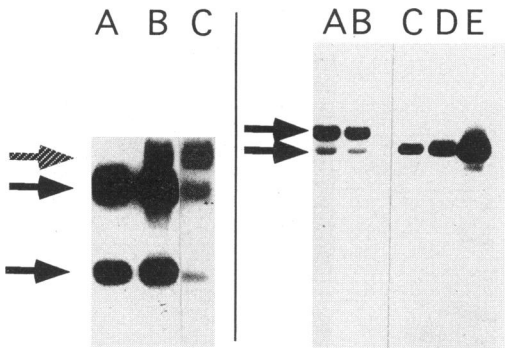

FIG. 3. Southern blots of total cell DNA. (Left panel) Blot showing that Dam$^-$ cells contain relatively few copies of pSP102. *Eco*RI-digested DNA was probed with the chloramphenicol resistance gene of plasmid pSP102. Lanes: A, purified pSP102 DNA; B and C, total cell DNA from strains transformed with pSP102, extracted from MC1061 Dam$^+$ cells (B) and S1540 Dam$^-$ cells (C). Solid arrows indicate the 1.5- and 2.8-kb *Eco*RI fragments of pSP102 and the hatched arrow indicates an unidentified cross-reacting chromosomal fragment of approximately 4 kb. On reprobing with DNA from the chromosomal *galK* gene, each lane proved to have equivalent amounts of chromosomal DNA (data not shown). (Right panel) Blot showing that plasmid pOC15 frequently integrates into the chromosome of Dam$^-$ cells. *Nde*I-digested DNA was probed with the *Eco*RI-*Hin*dIII fragment from the ampicillin resistance region of pOC15. Lanes: A through D, total cell DNA from independent clones transformed with pOC15, extracted from S1540 Dam$^-$ cells (A and B) and MC1061 Dam$^+$ cells (C and D); E, purified pOC15 DNA. Arrows mark the 8.8-kb *Nde*I plasmid fragment and the ca. 18-kb *Nde*I fragment generated when pOC15 integrates via its *oriC* sequences into the *oriC* region of the host chromosome.

Poor maintenance of mini-P1 in Dam$^-$ cells is not due to insufficient DnaA protein. There are several *dam* methylation sites in the vicinity of the promoter of the *E. coli dnaA* gene, and Dam$^-$ strains synthesize less DnaA protein than their Dam$^+$ counterparts (12, 25). As replication of mini-P1 plasmids requires DnaA protein (19), we were concerned that the poor maintenance of the plasmid in Dam$^-$ strains was due to a lack of sufficient DnaA. However, this possibility was ruled out. Overproduction of DnaA protein in the Dam$^-$ host from a plasmid (pDS596) carrying an arabinose-inducible *dnaA* gene did not improve either the poor transformation of a Dam$^-$ strain by pSP102 (data not shown) or the stability of the plasmid once established (Table 2). It has previously been shown that DnaA overproduction does not help *oriC* plasmids to transform Dam$^-$ strains (25).

Both *E. coli* and phage T4 DNA adenine methylases support efficient mini-P1 replication. The maintenance of pSP102 in a Dam$^-$ strain was fully restored when the strain was transformed with a high-copy-number plasmid carrying the *E. coli dam* gene (Table 2). Thus, the replication defect in the Dam$^-$ strain used is specifically due to the lack of the methylase rather than to some other fortuitous mutation. When a similar plasmid that expresses the phage T4 DNA adenine methylase was used, the same result was obtained (Table 2), showing that the T4 enzyme can fully substitute for its host counterpart. The amino acid sequence of the T4 Dam protein (29) shows only 25% homology with the *E. coli* Dam sequence (14). Thus, it is unlikely that the host methylase protein is a required component of the P1 replication complex. Rather, DNA methylation per se appears to be sufficient. This is consistent with experiments that show that methylation of the P1 origin GATC sequences is the only required role for Dam methylase in the in vitro system (2).

TABLE 2. Plasmid maintenance in Dam$^+$ and Dam$^-$ strains

Strain	Phenotype	Retention of plasmid in 25 generations of unselected growth (%)[a]	
		pSP102	pOC15
MC1061	Dam$^+$	44	12
S1540	Dam$^-$	<1	69[b]
MC1061/pDS596[c]	Dam$^+$	82	NT[d]
S1540/pDS596[c]	Dam$^-$	<2	NT
S1540/pDAM118	Dam$^+$	51	NT
S1540/pSSH6	Dam$^+$	44	NT

[a] Retention of pSP102 or pOC15 was estimated by scoring retention of antibiotic resistance as described in Materials and Methods. Resident plasmids pDS596, pDAM118, and pSSH6 supply the *E. coli* DnaA protein and the *E. coli* and T4 *dam* methylases, respectively.

[b] This high level of retention does not reflect stable maintenance of free plasmids. Rather, the strains grow poorly under selection and there is a strong selection for integration of the plasmid into the chromosome. When representative colonies scored as ampicillin resistant were recovered and retested, all were stably antibiotic resistant after a further 25 generations of growth without selection. Such clones all have integrated plasmid copies (Fig. 3).

[c] These cells were continually induced for DnaA production from pDS596 by growth in the presence of 0.7% arabinose. Essentially the same results were found when the experiment was carried out with 0.1% arabinose or with no arabinose. Western blotting with antiserum specific for DnaA (36) showed that the presence of pDS596 in cells without arabinose gives no detectable increase in DnaA over the level normally present in plasmid-free host cells. Continuous induction with 0.7% arabinose gave at least a 50-fold increase in DnaA protein content (data not shown).

[d] NT, not tested.

The copy number and maintenance efficiency of an *oriC* plasmid in a Dam$^-$ host are low. The similarity of the properties of mini-P1 and *oriC* plasmids on transforming Dam$^-$ cells prompted us to ask whether *oriC* plasmids were also poorly replicated and maintained once established in Dam$^-$ strains. Several pOC15 Dam$^-$ transformants (Table 1) were grown under ampicillin selection, and plasmid DNA was prepared from them. Approximately one-half of the clones contained small amounts of plasmid DNA, giving about eightfold less DNA than was recovered from the Dam$^+$ clones propagated under the same conditions (Fig. 2). The rest of the Dam$^-$ clones showed little or no evidence of plasmid DNA (Fig. 2). Southern analysis of these clones shows that additional copies of the plasmid are integrated into the chromosome (Fig. 3).

Analysis of the maintenance stability of pOC15 in the Dam$^+$ and Dam$^-$ hosts is shown in Table 2. As evidenced by loss of the ampicillin resistance marker, the Dam$^+$ cells retained pOC15 rather poorly when free of selection. The high loss rate observed is typical of *oriC* plasmids in wild-type hosts (39). In contrast, the Dam$^-$ cells appear to retain pOC15 rather well (Table 2). However, the actual rate of loss of free plasmid from these clones appears to be much higher than indicated. In marked contrast to Dam$^+$ clones, these Dam$^-$ clones grow very poorly under selection, and the resulting colonies contain relatively few ampicillin-resistant cells (data not shown). The apparent retention of plasmid in the standard stability test is due to the generation of subclones whose ampicillin resistance is completely stable because of chromosomal integration (Fig. 3). We conclude that the *oriC* plasmid is replicated very inefficiently in the Dam$^-$ strain. This leads to a much lower average copy number and very poor plasmid maintenance. These cells have a severe growth disadvantage when grown in the presence of ampicillin, and frequently variants are selected

TABLE 3. Efficient in vitro replication from P1 *oriR* requires methylated or hemimethylated DNA[a]

Plasmid DNA used[b]	Methylation in vitro[c]	pmol of [^{3}H]dTTP incorporated in reaction mixture	
		Without RepA	With RepA
Vector (f1h$_0$)	No	11	11
f1P1 (Meth$^+$)	No	10	174
f1P1 (Meth$^+$)	Yes	8	152
f1P1 (Meth$^-$)	No	12	39
f1P1 (Meth$^-$)	Yes	8	210
f1P1 (Hemimeth)[d]	No	10	110
f1P1 (Hemimeth)[d]	Yes	12	103

[a] The in vitro system used is described in Materials and Methods. The f1P1 template has the P1 origin region (bp 373 to 606) cloned into the *Eco*RI site of the f1h$_0$ vector. Replication was measured as pmol of [^{3}H]dTTP incorporated into a trichloroacetic acid-precipitable fraction by using the *E. coli* in vitro replication system (2). The reaction mixtures contained 2 pmol of added DnaA protein, 0.04 pmol of DNA, and 6 pmol of RepA (when present).

[b] Meth$^+$, methylated; Meth$^-$, unmethylated; Hemimeth, hemimethylated.

[c] The DNA was methylated in vitro for 30 min at 30°C with purified Dam and *S*-adenosylmethionine. RepA, DnaA, and FII extract were then added, and replication was measured as before.

[d] Hemimethylated DNA was prepared as described in Materials and Methods.

in which a copy of the plasmid (or at least the ampicillin resistance gene) is integrated into the chromosome. These clones tend to lose the free plasmid copies, even under selection, because the plasmid is no longer required for ampicillin resistance. They eventually give rise to cells which contain no plasmid DNA but never lose ampicillin resistance in the standard test.

The effect of methylation state on replication in vitro. Unmethylated, hemimethylated, and fully methylated DNAs act as templates in a standard *oriC* in vitro replication system (10, 27). However, the fully methylated template is superior to the other forms by a factor of 2 or 3 (10, 21, 27). Replication of hemimethylated DNA is specifically blocked when a membrane fraction is added to the in vitro system (27).

We have previously described an in vitro replication system for P1 plasmid DNA and used it to show that origin methylation of the template is required for detectable initiation in vitro (2). In Table 3, we give results obtained with a more sensitive version of this in vitro system (3) (see Materials and Methods), which was used to probe methylation effects further and to investigate the activity of hemimethylated DNA in the system. The template DNA differs from that used previously (2). It consists of the minimal P1 origin introduced into the *Eco*RI site of the vector f1h$_0$. This vector is a modified f1 phage that has no *dam* methylation sites (26). Thus, any effects of Dam methylation on the template can be attributed directly to effects on the inserted P1 origin DNA. Incorporation in each assay was shown to originate from the P1 origin by demonstrating dependence on addition of the P1 initiator protein RepA.

In contrast to the original system (2), the more sensitive system gives a low, but significant, level of incorporation with fully unmethylated DNA templates (Table 3). This was not, apparently, due to endogenous methyltransferase activity in the wild-type *E. coli* extract, because extracts from *dam* mutant hosts gave a similar result (data not shown). Detection of this background activity may be due to the increased sensitivity of the improved system. Unlike our previous experiments (2), those described in Table 3 were carried out with exogenously added DnaA protein. It is possible that the absolute requirement for *dam* methylation previously described is partially alleviated by an excess of DnaA protein. In any case, the lack of origin methylation still severely limits P1 origin function. When this unmethylated DNA was treated with *dam* methylase in vitro prior to being used in the system, a greater-than-sixfold increase in incorporation was seen (Table 3). Thus, efficient activity in the in vitro system is dependent on template methylation. As the only adenine methylation sites present are in the P1 origin region, we conclude that methylation of the P1 origin is required for efficient initiation. In addition to the group of four GATC sequences nested in the 7-bp repeats of the origin core, there are two more present in the origin fragment as used here. Neither of these two is important for origin function, as one (bp 461 to 464; Fig. 1) can be mutated without effect (13) and the other was created at the junction with the vector DNA to facilitate cloning and is not normally present in active P1 replicons.

Hemimethylated, supercoiled DNA was constructed as described in Materials and Methods. As shown in Table 3, the hemimethylated template was active in vitro. Although the incorporation achieved with this DNA was somewhat less than that achieved with fully methylated template, this is probably due to some trivial deficiency or damage to the hemimethylated DNA. This was shown by pretreating the hemimethylated DNA with *dam* methylase. No further stimulation of template activity was seen (Table 3), despite the fact that the DNA was now fully methylated on both strands (data not shown). Thus, at least one of the hemimethylated forms normally produced by replication (that with the methyl groups on the upper portion of the conventional map shown in Fig. 1) is fully competent for replication in vitro. Preliminary evidence suggests that the other hemimethylated form is also active (data not shown).

DISCUSSION

The current in vivo results suggest that the methylation controls of the *oriC* and P1 *oriR* plasmids are very similar. Plasmids driven by *oriC* are subject to a well-documented negative-control system acting at the hemimethylated level. The results presented here strongly suggest that P1 *oriR* is subject to a similar type of regulation. However, an additional level of control was detected. A mini-P1 plasmid can be maintained in a Dam$^-$ strain, but the resulting replication is inefficient; the plasmid is unable to maintain its normal copy number and is frequently lost. We demonstrate that *oriC* replication shows a similar response. The *oriC* plasmids in Dam$^-$ cell clones have much lower copy numbers than those in Dam$^+$ cells grown under the same conditions. Plasmid maintenance under Dam$^-$ conditions is very poor, although the production of stable variants, apparently by integration into the host chromosome, makes quantitation difficult. We conclude that both P1 *oriR* and *oriC* origins function poorly when unmethylated. It has been shown that methylation can enhance both bending (24) and unwinding (40) of *oriC*. These physical effects on the origin might directly affect origin efficiency. Alternatively, some protein factor involved in initiation might recognize the origin in a methylation-specific manner.

Our in vivo results show that the two types of origin work inefficiently in the absence of any methylation. In the presence of methylation, hemimethylation causes a temporary block to reinitiation, presumably due to membrane sequestration. The first effect is paralleled by the behavior of the plasmids in vitro, where unmethylated templates of both plasmid types are severalfold less efficient than their methylated counterparts. The second effect is not expected to be evident in the in vitro system, because membranes are absent. This explains why the

hemimethylated P1 origin was found to be an efficient template in vitro.

Although methylation appears to act on mini-P1 and *oriC* plasmids at two different levels, the two effects could formally be manifestations of the same mechanism. For example, if membrane sequestration brings the origin sequences into a cell compartment that is particularly favorable for subsequent initiation events, a complete lack of methylation might be disadvantageous. However, we favor models in which the two levels of control are due to independent mechanisms. For example, in addition to recognition and sequestration of the hemimethylated origin DNA by membrane sites, some factor might exist that is required for optimum origin efficiency and binds to the origin only in its fully methylated state. Our in vitro results with P1 *oriR* pertain to this question. The P1 in vitro system does not contain membranes and shows no discrimination against hemimethylated DNA. Yet, the system shows a strong discrimination against the unmethylated P1 *oriR* origin. Thus, the unmethylated origin appears to be inherently inefficient, even when no hemimethylation block is operating. This ability of the in vitro system to uncouple the two types of effect caused by *dam* methylation strongly suggests that they are due to separate mechanisms.

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