The EMBO Journal vol.4 no.5 pp.1327-1332, 1985

Effect of *dam* methylation on the activity of the *E. coli* replication origin, *oriC*

Walter Messer, Ute Bellekes and Heinz Lother

Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 63-73, D-1000 Berlin 33, FRG

Communicated by T.A.Trautner

Methylation of GATC sites by the *dam* methylase is required for efficient initiation of DNA replication at the replication origin, *oriC*, of *Escherichia coli*. This is demonstrated by the inability of minichromosomes to be maintained in *dam* mutant strains. The requirement for methylated GATC sites is less stringent *in vitro* than *in vivo*. The time required for complete methylation of the origin region apparently determines the minimal spacing of replication forks on the chromosome.
Key words: dnaA/initiation of replication/minichromosomes/replication *in vitro*/transformation

Introduction

The chromosomal replication origin, *oriC*, of *Escherichia coli* K12 is the most complex regulatory region analyzed so far in prokaryotic cells. A striking feature is that *oriC* contains 21 GATC sites (Meijer *et al.*, 1979; Sugimoto *et al.*, 1979), 12 of them are located within the 246 bp of the minimal replication origin (Oka *et al.*, 1980). Nine GATC sites are found in the region between the minimal origin and the promoter for a 16-kd polypeptide (Buhk and Messer, 1983), which is apparently a regulatory component of *oriC* (Lother *et al.*, 1984). In replication origins of five other Gram-negative bacteria, eight of the 12 GATC sequences within the minimal origin are conserved (Zyskind *et al.*, 1983).

The *E. coli dam* DNA methylase methylates the adenine in GATC sequences (Lacks and Greenberg, 1977; Geier and Modrich, 1979). All GATC sites in *E. coli* are methylated (Razin *et al.*, 1980). Missense (Marinus and Morris, 1973) and insertion mutants (Marinus *et al.*, 1983) in the *dam* gene have been isolated. These mutants show increased sensitivity to u.v. irradiation, increased generalized mutability, and are sensitive to 2-aminopurine (Marinus and Morris, 1973). Combinations of *dam* with *recA, recB, recC, polA* or *lexA* are lethal (Marinus and Morris, 1975).

Methylated GATC sites have been implicated to be responsible for strand recognition in mismatch repair (Wagner and Meselson, 1976; Glickman *et al.*, 1978; Glickman and Radman, 1980; Pukkila *et al.*, 1983; Radman and Wagner, 1984). The abundance of GATC sites within the replication origin suggests that the state of methylation is important for the function of *oriC*, in addition to a possible role in repair processes. A role of methylation in the replication process has been suggested (Lark, 1968), and unmethylated *oriC* DNA was shown to be inefficient in replication *in vitro* (Hughes *et al.*, 1984). Methylated GATC sites may be important in the interaction of initiation proteins with the *oriC* site: *dnaA* protein (Chakrabory *et al.*, 1983; Fuller and Kornberg, 1983; Fuller *et al.*, 1984). DNA gyrase (Lother *et al.*, 1984), protein HU (Dickson and Kornberg, 1984), DNA topoisomerase I (Fuller *et al.*, 1983), RNase H (Ogawa *et al.*, 1984) and other factors involved in initiation. In this paper we correlate *oriC* function with the state of methylation of GATC sites by the *dam* methylase. Complementing results are reported in the accompanying paper by Smith *et al.* (this issue).

Results

Transformation of dam mutant strains with minichromosomes

The ability of minichromosomes to replicate in *dam* mutant strains was analyzed by transforming different *dam* mutants with minichromosomes. pBR322, which contains only few GATC sites in the origin region was used as a control.

Minichromosomes, i.e., plasmids containing only *oriC* as their replication origin, were transformed into *dam* and *dam*$^+$ strains. No transformants were found in *dam* mutants using the small minichromosome pOC84. With the larger minichromosomes pOC81 and pOC15 the transformation frequency was more than three orders of magnitude lower in *dam* mutants as compared with *dam*$^+$ (Table I). Fourteen and nine transformants, respectively, were obtained. However, in none of these transformants could we detect free plasmid DNA by the procedure of Kado and Liu (1981). This shows that minichromosome DNA was taken up by the cells and was stable for a sufficient amount of time to allow recombination of plasmid DNA into the chromosome. This interpretation is corroborated by the transduction of minichromosome-carrying strains with *dam* reported below. Replication as free plasmid is apparently not possible in *dam* mutants or occurs with such a low efficiency that plasmids cannot be detected in the transformant colonies.

These results are qualitatively in agreement with results reported in the accompanying paper by Smith *et al.*. However, the reduction in transformation efficiency of minichromosomes in *dam versus dam*$^+$ cells is not as pronounced in their experiments.

pBR322 transformed *dam* mutants with an efficiency of 5 – 10% as compared with the *dam*$^+$ control (Table I). Similar

Table I. Transformation of *dam* mutant strains

	Transformants per μg DNA in[a]		
	C600 (*dam*$^+$)	GM82 (*dam*-3)	GM2199 (*dam*-13::Tn9)
pBR322	1.4×10^5	7.9×10^3	1.4×10^4
pOC84	1.9×10^5	$<5 \times 10^0$	$<5 \times 10^0$
pOC81	6.3×10^5	3.2×10^1[b]	3.2×10^1[b]
pOC15	1.4×10^5	–	8.6×10^1[c]
pOC42 (methylated)	3.6×10^5	6.3×10^3	1.3×10^3
pOC42 (not methylated)	2.0×10^4	2.6×10^4	6.9×10^3

[a]Average for 2 – 4 experiments.
[b]This corresponds to a total of 14 transformants, none of which contained free plasmids.
[c]This corresponds to nine transformants none of which contained free plasmids.

Table II. Transformation of *dam* mutants with unmethylated DNA

	Transformants per μg ligated DNA in	
	C600 (*dam*$^+$)	GM2199 (*dam*::Tn9)
Methylated DNA (from HB101);		
Km^r,Tc^s	2.2 x 10^5	5 x 10^1[a]
Tc^r	2.8 x 10^3	1.2 x 10^4
Km^r + Tc^r	5.4 x 10^2	1.6 x 10^3
Unmethylated DNA (from GM82):		
Km^r,Tc^s	2.4 x 10^5	<5 x 10^1
Tc^r	5.4 x 10^4	4.4 x 10^4
Km^r + Tc^r	1.5 x 10^3	6.5 x 10^3
Unmethylated DNA (from GM2199):		
Km^r,Tc^s	2.2 x 10^5	<5 x 10^1
Tc^r	1.9 x 10^5	1.6 x 10^5
Km^r,Tc^r	8.4 x 10^4	3.2 x 10^4

[a]This was one transformant which did not contain free plasmid DNA.

results were obtained with pOC42 (Messer *et al.*, 1980), a joint replicon which contains the pBR322 replication origin and *oriC* (Table I). The reduced transformation frequency in *dam* cells is apparently not due to a reduced activity of the pBR322 replication origin since the yield of plasmids, once established, was similar for *dam*$^+$ and *dam* strains (data not shown). In addition, this lower transformation efficiency in *dam* mutant cells was not observed when relaxed pBR322 DNA from a ligation was used (Table II, see below). pOC42 DNA with unmethylated GATC sites, isolated from *dam* strains, also showed a reduced transformation efficiency when re-introduced into *dam*$^+$ cells as compared with methylated pOC42 DNA (Table I).

The state of methylation of the joint replicon pOC42 was verified by digestion with *Mbo*I, *Dpn*I and *Sau*3A. The digestion pattern was very similar to the one documented in the accompanying paper by Smith *et al.* DNA isolated from *dam* mutant strains (GM82 and GM2199) was resistant to digestion by *Dpn*I and was digested completely by *Mbo*I, except for a minor band of 1450 bp (data not shown).

It might be possible that the replication system in *dam* mutants is altered in such a way that unmethylated DNA is preferentially replicated. To determine whether *dam* mutant strains can be more efficiently transformed with unmethylated minichromosomes, the following experiment was performed. pOC89, a small minichromosome with a single *Eco*RI site and a kanamycin-resistance determinant, was joined with pBR322 at the *Eco*RI site. This composite plasmid was introduced into HB101 (*dam*$^+$, control) and into GM82 (*dam*-3) and GM2199 (*dam*::Tn9). Plasmid DNA from all three strains was digested with *Eco*RI and ligated at low DNA concentration to obtain separate rings of pBR322 and pOC89, respectively. This ligation mixture was used to transform C600 (*dam*$^+$) and GM2199 (*dam*::Tn9), respectively. Selection was for tetracycline resistance (pBR322) and for kanamycin resistance (pOC89). Kanamycin-resistant colonies were replica plated onto tetracycline plates (Km^r+Tc^r, pOC89::pBR322). Both methylated and unmethylated pOC89 were unable to transform the *dam* strain GM2199 (Table II), demonstrating that unmethylated minichromosomes as well cannot be established in *dam* strains. The relatively low transformation efficiency of *dam* methylated pBR322 (isolated from strain HB101) in strain C600 is due to *Eco*K restriction. pOC89 does not contain an *Eco*K recognition site.

Transduction of minichromosome-carrying strains with dam::Tn9

The inability of *dam* mutants to be transformed with minichromosomes could be due to a special sensitivity of minichromosomes during transformation and establishment or to a general inability of the plasmids to replicate. To analyze the replication of minichromosomes independent of the transformation procedure, we introduced *dam* into a strain already carrying a minichromosome. Minichromosomes can integrate into the chromosome, unless they are maintained in *recA* strains. However, *dam* is incompatible with *recA*. To circumvent this difficulty we have used a *recA*$^+$ strain with a deletion of the chromosomal replication origin region (CM1671, von Meyenburg and Hansen, 1980), with or without the minichromosome pOC81. In this deletion strain there is no sequence homology between pOC81 and the chromosome.

The generalized transducing phage T4GT7 (Wilson *et al.*, 1979) was grown on GM2199 (*dam*::Tn9). Strains CM1671 and WM1373, the pOC81-containing derivative of CM1671, were transduced with this T4GT7 lysate, and chloramphenicol-resistant or chloramphenicol- and kanamycin-resistant transductants, respectively, were selected. *dam*::Tn9 transductants arose with high efficiency in both strains. Selection for the transposon and the plasmid marker in the minichromosome-containing strain gave approximately half as many transductants as the control, *dam*::Tn9 transduction into strain CM1671. Of the transductants, 18 were analyzed for plasmid content using the rapid detection procedure (Kado and Liu, 1981). None of them contained free plasmid DNA. This suggests that selection for the plasmid marker forced recombination of the minichromosome into the bacterial chromosome, although the region homologous to pOC81 is deleted from the host strain. CM1671 is an Hfr strain, the integrated F factor serving as origin for chromosomal replication. In a mating between the WM1373 *dam*::Tn9 transductants and the F$^-$ strain CSH57B the kanamycin determinant of the minichromosome was transferred to the recipient strain, giving positive proof for its integration into the chromosome.

These results demonstrate that minichromosomes with *oriC* as the only replication origin are unable to replicate in *dam* mutant strains, or at least replicate very inefficiently.

In vitro replication of dam methylated and unmethylated DNA in dam$^+$ *and dam extracts*

The replication of minichromosomes was analyzed in the *dnaA*-dependent *in vitro* replication system (Fuller *et al.*, 1981). Replication extracts were prepared from the *dam* strain GM119, *dam*$^+$ controls were prepared from strain GM119 carrying the *dam*$^+$ plasmid pdam118 (Brooks *et al.*, 1983) and from C600. *Dam* methylated template DNA was pOC51, which consists of the *oriC*-containing and the *asnA*-containing *Pst*I fragments from the chromosomal origin region (Buhk and Messer, 1983). DNA devoid of *dam* methylation was pBP8, a joint replicon between the *Bacillus subtilis* plasmid pPL603 and an *oriC*-carrying *Pst*I fragment from *E. coli* (Ceglowski and Trautner, 1984), propagated in *B. subtilis*, in which no adenine methylation occurs.

Dam$^-$ extracts gave about half as much DNA synthesis *in vitro* with methylated DNA as template as did extracts prepared from *dam*$^+$ cells, and the concentration of extracts required for optimal DNA replication was ~2-fold higher with the *dam*$^-$ extracts (Figure 1A). This shows that minichromosome replication occurs *in vitro* in the *dam*$^-$ extract, although with a reduced efficiency. We do not know whether the reduced efficiency of the *dam*$^-$ extract is due to the suboptimal concentration of components of the extract, or is a reflection of possible multiple in-

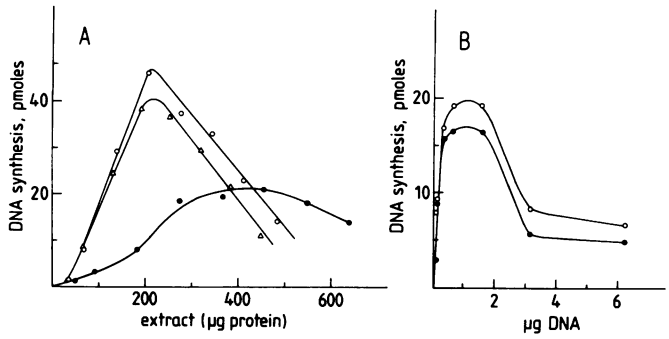

Fig. 1. (A) *In vitro* replication of methylated DNA in *dam*$^+$ and *dam*$^-$ extracts. Extracts were prepared from: *dam*$^-$ (●): GM119; *dam*$^+$ (△): C600; (○): GM119/pdam 118. Methylated DNA was pOC51 added at 245 ng DNA per assay. A constant amount of DNA and varying amounts of extract were used. (B) *In vitro* replication of unmethylated DNA in *dam*$^+$ and *dam*$^-$ extracts. *dam*$^-$ extracts (from GM119) contained 274 µg protein/assay, *dam*$^+$ extracts (from GM119/pdam 118) contained 206 µg protein/assay. Unmethylated DNA was pBP8 grown in *B. subtilis*. Constant amounts of extract were incubated with varying amounts of DNA. Symbols as in A.

initiations in the *dam*$^+$ extract. The results also show that the *dcm* mutation in strain GM119 has no effect on DNA replication *in vitro*.

Unmethylated DNA was replicated *in vitro* in *dam*$^-$ and *dam*$^+$ extracts. The optimal concentrations of extracts for the replication of methylated pOC51 DNA (see Figure 1A) were used and the amount of DNA was varied. Both extracts, *dam*$^+$ and *dam*$^-$, replicated unmethylated DNA with nearly equal efficiency (Figure 1B). However, at optimal DNA concentration unmethylated DNA was only about half as effective as a template as methylated DNA (compare Figure 1A and B).

The replication of unmethylated DNA *in vitro* was dependent on *dnaA*. No replication occurred in extracts from *dnaA* mutants. These extracts could be complemented with an extract from a *dnaA* overproducing strain (data not shown). These experiments corroborate results obtained by Hughes *et al.* (1984) and Smith *et al.* (this issue).

Contrary to the results obtained *in vivo*, *dnaA*- and *oriC*-dependent replication was possible *in vitro* with both methylated and unmethylated template DNA in *dam*$^-$ and *dam*$^+$ extracts. However, unmethylated DNA as a template reduced the efficiency of replication.

Does constitutive stable DNA replication occur in dam mutants?

The observation that *oriC* on minichromosomes is inactive or very inefficient in *dam* mutants poses the question how chromosomal replication occurs in *dam* mutant strains. Constitutive stable DNA replication (*sdrA*) mutants of *E. coli* can initiate chromosomal DNA at sites different from *oriC* (Kogoma and von Meyenburg, 1983). Subsequently it has been shown that *sdrA* mutants are mutations in *rnh*, the gene for RNase H (Ogawa *et al.*, 1984). *Dam* methylation-dependent expression of the *rnh* gene would, therefore, be a straight-forward possibility to induce an initiation pathway which is independent of *oriC* and thus of *dam* methylation. Therefore, we analyzed constitutive stable DNA replication (Kogoma, 1978) in GM82 (*dam*-3) and in C600 and W3110 *rnh* as controls. DNA synthesis was measured before and after the addition of chloramphenicol. As shown in Figure 2, neither the *dam* mutant strain nor the *dam*$^+$ control showed continuing DNA synthesis in the absence of protein synthesis, contrary to the *rnh* mutant. Therefore, constitutive stable DNA

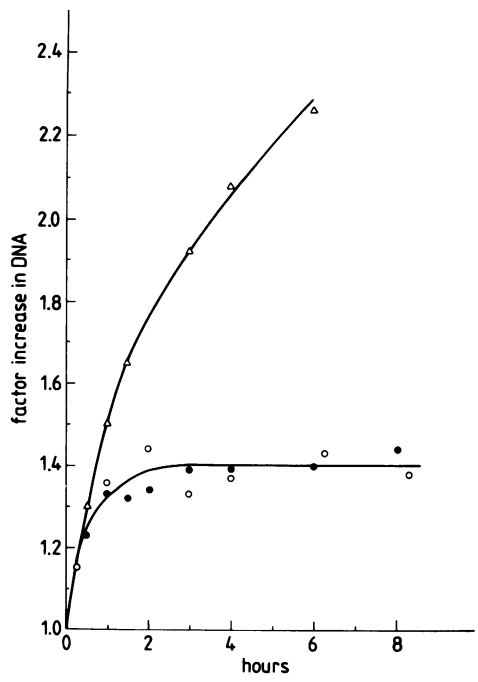

Fig. 2. DNA synthesis after addition of chloramphenicol. (○) *dam*$^+$ (C600); (●) *dam* (GM82); (△) *rnh* (W3110 *rnh*).

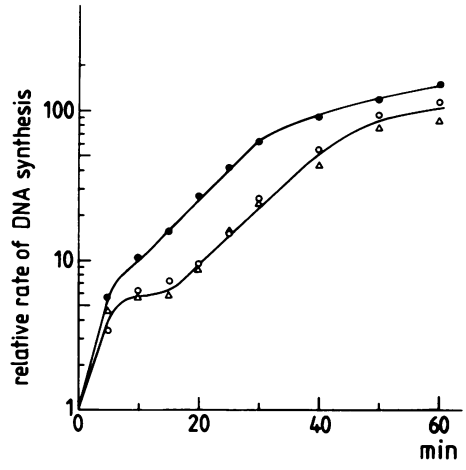

Fig. 3. Rates of DNA synthesis after re-initiation. Strains WM494 (*dnaA*5) and its plasmid-containing derivatives were incubated at 42°C for 120 min. After shift to 30°C (time 0) the rate of DNA synthesis was measured with 5 min pulses of [^{3}H]thymidine. (○) WM494 without plasmid; (△) WM494 with pBR322; (●) WM494 with pdam 118.

replication does not occur in *dam* mutant strains. In agreement with this result, RNase H activity was unaffected by the presence of the *dam* mutation (data not shown).

dam methylation and the spacing of replication forks

A number of observations suggests that replication forks cannot follow each other immediately. When re-initiation occurs after a period of inhibition of initiation, e.g., when conditional lethal initiation mutants are incubated at non-permissive temperature for more than one generation, and subsequently incubation is continued at permissive temperature, the rate of DNA replication increases in two waves which show a spacing of ~20 min (Evans and Eberle, 1975; Eberle *et al.*, 1982; Helmstetter and Krajewski, 1982; LaDuca and Helmstetter, 1983; Figure 3). Since at the

moment of re-initiation all necessary protein molecules have been accumulated (Hanna and Carl, 1975; Tippe-Schindler *et al.*, 1979), the reason for this spacing must be independent of protein synthesis. It is also independent of the rate of RNA and DNA synthesis (Helmstetter and Krajewski, 1982). It might be possible that the delay of the second round of initiation is due to the state of methylation of the replication origin and that the extent of the delay reflects the time required for complete methylation of *oriC*.

To compare the spacing of replication forks in strains with normal and with excessive *dam* methylation, we determined the pattern of re-initiation in a *dnaA* mutant strain containing the *dam*$^+$ methylase gene on a high copy number plasmid, in the plasmid-free *dnaA* strain and in the *dnaA* strain containing the vector plasmid pBR322. As shown in Figure 3, the rate of DNA synthesis increased in a stepwise manner after the cultures were shifted to permissive temperature following a 2 h pre-treatment at the non-permissive temperature. The time interval between the two steps was ~20 min in the control cells.

In the *dnaA* mutant, containing the pdam$^+$ plasmid, the second wave of initiation occurred ~10 min earlier than in the control cells (Figure 3). This suggests that an increased supply of *dam* methylase allowed an earlier second initiation due to a more efficient methylation of the origin region. A similar experiment was done using a *dnaC* mutant. However, in this case, the presence of the pdam$^+$ plasmid did not result in an earlier second initiation, presumably because *dnaC* acts later in initiation than *dnaA* (Zyskind *et al.*, 1977; Kung and Glaser, 1978).

Discussion

Minichromosomes which use *oriC* as their only replication origin were not stably maintained in a free form in *dam* mutant strains of *E. coli*, contrary to plasmids containing a ColE1 type origin. Minichromosome markers could only be recovered when integration into the host chromosome occurred. This was true when *dam* mutants were transformed with minichromosome DNA and when minichromosome containing strains were made *dam*$^-$.

Qualitatively similar results were obtained by Smith *et al.* (accompanying paper). The smaller difference in transformation efficiencies between *dam*$^+$ and *dam* strains observed by them, and the fact that these authors were able to isolate minichromosome DNA from *dam* cells, might be attributable to a residual *dam* methylation activity in the strains they used. However, positive proof for this assumption is missing, and both groups used the same *dam* alleles, albeit in a different genetic background. Smith *et al.* (accompanying paper) could also demonstrate that the low transformation efficiency of minichromosomes in *dam* mutants was unrelated to the effects of *dam* methylation on mismatch repair.

In contrast to the results obtained *in vivo*, the replication of unmethylated minichromosomes *in vitro* was only reduced to ~50%, in agreement with published results (Hughes *et al.*, 1984; Smith *et al.*, accompanying paper). The most likely interpretation for these results is that the *E. coli* replication origin, *oriC*, functions only inefficiently in *dam* mutants, presumably more inefficiently *in vivo* than *in vitro*. Possibly a control mechanism is affected which is less important or not operative *in vitro*.

The missing or inefficient initiation at *oriC* on minichromosomes might suggest that chromosomal initiation in *dam* mutants occurs *via* a different pathway which is independent of *oriC*. *oriC*-dependent initiation of chromosomes and minichromosomes requires *de novo* protein synthesis and is dependent on a functional *dnaA* gene (Hirota *et al.*, 1968; von Meyenburg *et al.*, 1979; Lother *et al.*, 1981). Stable DNA replication mutants (*sdrA, rnh*) can initiate chromosomal DNA in the absence of protein synthesis (Kogoma, 1978). This initiation is independent of *oriC* and of a functional *dnaA* gene (Kogoma and von Meyenburg, 1983). However, constitutive stable DNA replication does not occur in *dam* mutants (Figure 2). Of course, this does not rule out the existence of a different *oriC*-independent initiation mode.

An *oriC*-independent initiation mechanism should also be independent of the *dnaA* gene product. However, experiments designed to test the *dnaA* dependence of chromosomal initiation in *dam* mutants have so far given ambiguous results. Therefore, at present, the mode of chromosomal initiation in *dam* mutant strains is still unknown. We can, however, exclude that the initiation system is altered in such a way that only unmethylated origin DNA is accepted, since unmethylated minichromosomes were also unable to transform *dam* mutant strains (Table II).

The observation that initiation at *oriC* does not or inefficiently occur when *oriC* is not methylated, could explain a phenomenon observed many years ago, the spacing of successive replication forks, whose initiation capacity was accumulated in a *dnaA* mutant at 42°C. We have shown here that the spacing was shorter if additional *dam* methylase genes were present in the cell. We suggest that, after accumulation of the potential to initiate two replication rounds, only the first initiation can occur immediately after return to permissive temperature. The second initiation is delayed, although all proteins necessary for initiation have been accumulated, until the newly replicated *oriC* region has been methylated. We have to assume that hemimethylated DNA is as inefficient in initiation as unmethylated DNA. The spacing of the replication forks would thus depend on the efficiency of the methylation of GATC sites in the origin.

The status of methylation of GATC sites in the replication origin seems to be important for an early event in initiation. It may affect the control of two promoters which were identified within *oriC* (Lother and Messer, 1983). These promoters contain GATC sites in their Pribnow boxes. A dependence of gene activity on methylated GATC sites has been shown for the *mom* gene of phage Mu (Kahmann, 1983; Plasterk *et al.*, 1984; Adley and Bukhari, 1984).

The results reported here demonstrate that methylation of adenine in GATC sites by the *dam* methylase of *E. coli* positively regulates the activity of the *E. coli* replication origin, *oriC*. Whether this effect is mainly caused by altering promoter activity or is due to structural alterations by methylation is currently being tested. A regulatory role of methylation in the key process of the bacterial life cycle is particularly interesting in view of the importance of methylation in gene expression in eukaryotic systems (cf. Doerfler, 1984).

Materials and methods

Bacterial strains and plasmids

E. coli strains used are listed in Table III. Minichromosomes pOC81 (3.1 kb, Kunze and Messer, unpublished), pOC84 (1.9 kb, Huhle and Messer, unpublished) and pOC89 (2.1 kb, Huhle and Messer, unpublished) contain the 1.67-kb *oriC*-containing *Hin*cII fragment, the 0.46-kb *oriC Hin*cII-*Acc*I fragment, or the 0.67-kb *oriC Hin*cII-*Pst*I fragment, respectively, from the *oriC* region (Buhk and Messer, 1983). These fragments were ligated with *Eco*RI-linkers to a 1.4-kb *Hae*II fragment containing the kanamycin resistance determinant from Tn903. In pOC89 the *Pst*I/*Eco*RI site at *oriC* coordinate 488 bp is destroyed. pOC51 (4.1 kb) consists of the two *Pst*I fragments from the origin region containing *oriC* and *asnA*, respectively (Buhk and Messer, 1983). pOC15 (8.7 kb) contains DNA from 1.9 kb left to 3.5 kb right of *oriC*, linked to an ampicillin resistance gene (Messer *et al.*, 1979). The joint replicon pOC42 consists of the *oriC Pst*I fragment from pCM959 (von Meyenburg *et al.*, 1979) cloned into the *Pst*I site of pBR322. The

Table III. *E. coli* strains

Strain	Genotype	Reference
C600	*lacY1,leuB6,supE44,thr-1,tonA21*	
HB101	*ara-14,galK2,hsdS20,lacY1,leu,mtl-1, proA2,recA13,rpsL20,supE44,xyl-5*	Maniatis *et al.* (1982)
GM82	*ara-14,dam-3,del(gpt-proA)62,deoB6, galK2,hsdS1,lacY1,leuB6,metB1,supE44, thi-1,thr-1,tsx-33*	Marinus and Morris (1973)
GM119	*dam-3,dcm-6,galK2,galT22,lacY1,metB1, mtl-1?,supE44,thi-1?,tonA32?,tsx-78*	Marinus and Morris (1973)
GM2199	*ara-14,dam-13::Tn9,galK2,galT22,hisG4, lacY1,leuB6,mtl-1,rpsL136,supE44, thi-1,thr-1,tonA31,tsx-78,xyl-5*	Marinus *et al.* (1983)
CM1671	*Hfr,asnA::Tn10,asnB32,del(asnA-oriC-gid)1071,fuc,lysA,relA1,spoT1,thi-1*	von Meyenburg and Hansen (1980)
WM1373	*CM1671 containing pOC81*	
CSH57B	*ara,argG,gal,his,ilv,lac,leu,metA or B, mtl,purE,rpsL,thi,trp,tsx,xyl*	Miller (1972)
WM494	*E.c.B/r;arg-28,deoB23,dnaA5,gal-11, his-47,hsdS-K12,lac-11,leu-19,mal,met-55, pro-19,rbs,rpsL56,sul-1,thyA59,trp-25*	Tippe-Schindler *et al.* (1979)
W3110 rnh	*rnh::Tn3,thyA36*	Horiuchi *et al.* (1984)

joint replicon pBP8 contains the same *oriC PstI* fragment as pOC42, cloned into the *PstI* site of the *B. subtilis* plasmid pPL603 (Ceglowski and Trautner, 1984). pdam118 contains the *dam* methylase gene of *E. coli* cloned in pBR322 (Brooks *et al.*, 1983).

Microbiological techniques

Growth media, cloning techniques and handling of plasmids were as described by Maniatis *et al.* (1982). The rapid procedure for detection of plasmids was used (Kado and Liu, 1981). The limit of detection of free plasmid DNA was determined to be 0.3 copies of a plasmid the size of pOC81 (3.1 kb) per cell. T4GT7-mediated transduction was done as described by Wilson *et al.* (1979).

Radioactive labelling

For the measurement of stable DNA replication, cells were pre-grown for at least four generations in minimal glucose medium (Helmstetter, 1967) in the presence of 100 μg/ml deoxyguanosine, 2 μg/ml thymidine and 10 μCi [^{3}H]thymidine. At time zero 150 μg/ml chloramphenicol were added.

For the measurement of the rate of DNA synthesis in re-initiation experiments, [^{3}H]thymidine pulses were given by adding 50 μg/ml uridine (Womack, 1977) and 10 μCi/ml [^{3}H]thymidine. Pulses were terminated after 5 min with 10% trichloroacetic acid (TCA).

All radioactive samples were spotted on glass fibre filters (50 μl), washed once with 10% TCA containing thymidine, followed by four washes with 5% TCA and two washes with ethanol. They were counted in a liquid scintillation counter.

In vitro replication

The *dnaA*-dependent *in vitro* replication system described by Fuller *et al.* (1981) was used. Assays were done in 25 μl. The system was optimized by varying either the amount of template DNA or the amount of cell-free extract (see Figure 1). Incubation was at 30°C for 60 min.

Acknowledgements

We thank J.E.Brooks, T.Horiuchi, M.G.Marinus and K.von Meyenburg for strains, and our whole department, in particular T.A.Trautner, for continuing stimulating discussions. The technical assistance of M.Hearne is gratefully acknowledged. Part of this work was supported by grant Me 659/2-3 of the Deutsche Forschungsgemeinschaft.

References

Adley,C.C. and Bukhari,A.I. (1984) *Nucleic Acids Res.*, **12**, 3535-3550.
Brooks,J.E., Blumenthal,R.M. and Gingeras,T.R. (1983) *Nucleic Acids Res.*, **11**, 837-851.
Buhk,H.-J. and Messer,W. (1983) *Gene*, **24**, 265-279.
Ceglowski,P. and Trautner,T.A. (1984) *Mol. Gen. Genet.*, **197**, 522-524.
Chakraborty,T., Yoshinaga,K., Lother,H. and Messer,W. (1982) *EMBO J.*, **1**, 1545-1549.
Doerfler,W. (1984) *Curr. Top. Microbiol. Immunol.*, **108**, 79-98.
Dixon,N.E. and Kornberg,A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 424-428.
Eberle,H., Forrest,N., Hrynshyn,J. and Van Knapp,J. (1982) *Mol. Gen. Genet.*, **186**, 57-65.
Evans,I.M. and Eberle,H. (1975) *J. Bacteriol.*, **121**, 883-891.
Fuller,R.S. and Kornberg,A. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5817-5821.
Fuller,R.S., Kaguni,J.M. and Kornberg,A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7370-7374.
Fuller,R.S., Bertsch,L.L., Dixon,N.E., Flynn,J.E.,Jr., Kaguni,J.M., Low,R.L., Ogawa,T. and Kornberg,A. (1983) *UCLA Symp. Mol. Cell. Biol., New Series*, **10**, 275-288.
Fuller,R.S., Funnell,B.E. and Kornberg,A. (1984) *Cell*, **38**, 889-900.
Geier,G.E. and Modrich,P. (1979) *J. Biol. Chem.*, **254**, 1408-1413.
Glickman,B., van den Elsen,P. and Radman,M. (1978) *Mol. Gen. Genet.*, **163**, 307-312.
Glickman,B.W. and Radman,M. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1063-1067.
Hanna,M.H. and Carl,P.L. (1975) *J. Bacteriol.*, **121**, 219-226.
Helmstetter,C.E. (1967) *J. Mol. Biol.*, **24**, 417-427.
Helmstetter,C.E. and Krajewski,C.A. (1982) *J. Bacteriol.*, **149**, 685-693.
Hirota,Y., Ryter,A. and Jacob,F. (1968) *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 677-693.
Horiuchi,T., Maki,H. and Sekiguchi,M. (1984) *Mol. Gen. Genet.*, **195**, 17-22.
Hughes,P., Squali-Houssaini,F.-Z., Forterre,P. and Kohiyama,M. (1984) *J. Mol. Biol.*, **176**, 155-159.
Kado,C.I. and Liu,S.-T. (1981) *J. Bacteriol.*, **145**, 1365-1373.
Kahmann,R. (1983) *Cold Spring Harbor Symp. Quant. Biol.*, **47**, 639-646.
Kogoma,T. (1978) *J. Mol. Biol.*, **121**, 55-69.
Kogoma,T. and von Meyenburg,K. (1983) *EMBO J.*, **2**, 463-468.
Kung,F.C. and Glaser,D.A. (1978) *J. Bacteriol.*, **133**, 755-762.
Lacks,S. and Greenberg,B. (1977) *J. Mol. Biol.*, **114**, 153-168.
LaDuca,R.J. and Helmstetter,C.E. (1983) *J. Bacteriol.*, **154**, 1371-1380.
Lark,C. (1968) *J. Mol. Biol.*, **31**, 401-414.
Lother,H. and Messer,W. (1981) *Nature*, **294**, 376-378.
Lother,H., Buhk,H.-J., Morelli,G., Heimann,B., Chakraborty,T. and Messer,W. (1981) *ICN-UCLA Symp. Mol. Cell. Biol.*, **22**, 57-77.
Lother,H., Koelling,R., Kuecherer,C. and Schauzu,M. (1984) *EMBO J.*, **4**, 555-560.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
Marinus,M.G. and Morris,N.R. (1973) *J. Bacteriol.*, **114**, 1143-1150.
Marinus,M.G. and Morris,N.R. (1975) *Mutat. Res.*, **28**, 15-26.
Marinus,M.G., Carraway,M., Frey,A.Z., Brown,L. and Arraj,J.A. (1983) *Mol. Gen. Genet.*, **192**, 288-289.
Meijer,M., Beck,E., Hansen,F.G., Bergmans,H.E.N., Messer,W., von Meyenberg,K. and Schaller,H. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 580-584.
Messer,W., Meijer,M., Bergmans,H.E.N., Hansen,F.G., von Meyenburg,K., Beck,E. and Schaller,H. (1979) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 139-145.
Messer,W., Heimann,B., Meijer,M. and Hall,S. (1980) *ICN-UCLA Symp. Mol. Cell. Biol.*, **19**, 161-169.
Miller,J.H. (1972) *Experiments in Molecular Genetics*, published by Cold Spring Harbor Laboratory Press, NY.
Ogawa,T., Pickett,G.G., Kogoma,T. and Kornberg,A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1040-1044.
Oka,A., Sugimoto,K., Takanami,M. and Hirota,Y. (1980) *Mol. Gen. Genet.*, **178**, 9-20.
Plasterk,R.H.A., Vollering,M., Brinkman,A. and Van de Putte,P. (1984) *Cell*, **36**, 189-196.
Pukkila,P.J., Peterson,J., German,G., Modrich,P. and Meselson,M. (1983) *Genetics*, **104**, 571-582.
Radman,M. and Wagner,R. (1984) *Curr. Top. Microbiol. Immunol.*, **108**, 23-28.
Razin,A., Urieli,S., Pollack,Y., Gruenbaum,Y. and Glaser,G. (1980) *Nucleic Acids Res.*, **8**, 1783-1792.
Sugimoto,K., Oka,A., Sugisaki,H., Takanami,M., Nishimura,A., Yasuda,Y. and Hirota,Y. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 575-579.
Tippe-Schindler,R., Zahn,G. and Messer,W. (1979) *Mol. Gen. Genet.*, **168**, 185-195.
von Meyenburg,K. and Hansen,F.G. (1980) *ICN-UCLA Symp. Mol. Cell. Biol.*, **19**, 137-159.
von Meyenburg,K., Hansen,F.G., Riise,E., Bergmans,H.E.N., Meijer,M. and Messer,W. (1979) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 121-128.
Wagner,R. and Meselson,M. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 4135-4139.
Wilson,G.G., Young,K.K.Y. and Edlin,G.J. (1979) *Nature*, **280**, 80-82.

Womack,J.E. (1977) *Mol. Gen. Genet.*, **158**, 11-15.
Zyskind,J.W., Deen,L.T. and Smith,D.W. (1977) *J. Bacteriol.*, **129**, 1466-1477.
Zyskind,J.W., Cleary,J.M., Brusilow,W.S.A., Harding,N.E. and Smith,D.W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1164-1168.

Received on 5 February 1985