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Importance of state of methylation of *oriC* GATC sites in initiation of DNA replication in *Escherichia coli*

Douglas W.Smith, Adella M.Garland, Gary Herman, Robert E.Enns, Tania A.Baker[1] and Judith W.Zyskind[2]

Department of Biology, C-016, University of California, San Diego, La Jolla, CA 92093, [1]Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305, [2]Department of Biology, San Diego State University, San Diego, CA 92182, USA

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***In vivo* and *in vitro* evidence is presented implicating a function of GATC methylation in the *Escherichia coli* replication origin, *oriC*, during initiation of DNA synthesis. Transformation frequencies of *oriC* plasmids into *E. coli dam* mutants, deficient in the GATC-specific DNA methylase, are greatly reduced compared with parental *dam*$^+$ cells, particularly for plasmids that must use *oriC* for initiation. Mutations that suppress the mismatch repair deficiency of *dam* mutants do not increase these low transformation frequencies, implicating a new function for the Dam methylase. *oriC* DNA isolated from *dam*$^-$ cells functions 2- to 4-fold less well in the *oriC*-specific *in vitro* initiation system when compared with *oriC* DNA from *dam*$^+$ cells. This decreased template activity is restored 2- to 3-fold if the DNA from *dam*$^-$ cells is first methylated with purified Dam methylase. Bacterial origin plasmids or M13-*oriC* chimeric phage DNA, isolated from either base substitution or insertion *dam* mutants of *E. coli*, exhibit some sensitivity to digestion by *Dpn*I, a restriction endonuclease specific for methylated GATC sites, showing that these *dam* mutants retain some Dam methylation activity. Sites of preferred cleavage are found within the *oriC* region, as well as in the ColE1-type origin.**

Key words: E. coli dam mutants/GATC methylation/*oriC*/*in vitro* initiation/transformation

Introduction

The *Escherichia coli* origin for DNA replication, *oriC*, has been localized to a region of 238 – 246 bp at 85.5 min on the *E. coli* genetic map (Oka *et al.*, 1980). The origins of replication from five other Gram-negative bacteria have been cloned into *E. coli*, and all five function as *dnaA*-dependent replication origins using *E. coli trans*-acting factors (Zyskind *et al.*, 1979,1983; Harding *et al.*, 1982). Nucleotide sequence comparison of these origins shows regions of complete homology interspersed by 'spacer' regions of constant length (no relative insertions/deletions) but variable choice of nucleotide, and has led to a consensus sequence for the bacterial origin (Zyskind *et al.*, 1983). The observed pattern of homology, reminiscent of regulatory sites such as promoters, suggests that the *oriC* region is a highly complex regulatory site. Four 9-bp direct and inverted repeats, the 'R regions', found in *oriC* are almost totally conserved between all six squenced bacterial origins (Zyskind *et al.*, 1981, 1983). These repeats are essential sites for the binding of DnaA protein to DNA (Fuller and Kornberg, 1983; Fuller *et al.*, 1984).

Each bacterial origin contains 12 – 14 GATC sequences within the minimal 246-bp *oriC* region, eight of which are conserved between all six origins, although any given 4-bp sequence is expected to occur at random only once every 256 bp. The *E. coli dam* DNA methylase specifically recognizes this sequence, catalyzing methylation of both adenine residues (Geier and Modrich, 1979). *E. coli dam* mutants are highly sensitive to growth in the presence of 2-aminopurine (Marinus and Morris, 1973) and exhibit a variety of phenotypes consistent with involvement of the Dam methylase in mismatch repair (Marinus and Morris, 1975; Goze and Sedgwick, 1978; Bale *et al.*, 1979; McGraw and Marinus, 1980). The proposal of Wagner and Meselson (1976) that the completely methylated parental DNA strand is recognized as the 'correct' strand for repair of mismatches introduced during replication has been substantiated using genetically marked hemimethylated DNA substrates (Pukkila *et al.*, 1983). Many of the phenotypic properties of *dam* mutants are suppressed by *recL, uvrD, uvrE, mutH, mutL,* and *mutS* mutations, suggesting that these genes code for mismatch repair enzymes (McGraw and Marinus, 1980; Marinus, 1980; Glickman and Radman, 1980). The *recL, uvrD,* and *uvrE* mutations appear to be in the same gene (Kushner *et al.*, 1978). One possible function of the high number of GATC sites found in *oriC* may be to conserve the nucleotide sequence of this region by efficiently 'attracting' the mismatch repair system to it (Zyskind and Smith, 1980); however, this does not explain the conserved positions of the eight totally conserved GATC sites. Although insertion mutations of the *dam* gene have been constructed (Marinus *et al.*, 1983), deletions of this gene have not yet been isolated, even though deletions of the *E. coli dcm* gene, coding for a cytosine methylase, are rather easily isolated (Bale *et al.*, 1979). Here we present several lines of evidence indicating that the Dam methylase and the state of methylation of GATC sites are directly involved in the initiation process at *oriC*, and that specific GATC sites, some within *oriC* and in the ColE1 replication origin, remain methylated in *E. coli dam* mutants.

Results

To determine if a relationship between methylation state of GATC sites and *oriC* function exists, *E. coli dam* mutants were transformed with *oriC* plasmids. The transformation frequency itself is a key observable property of a given plasmid, although a complete explanation for an observed frequency is complex. For each of the several types of plasmids examined, transformation frequencies for several nonisogenic *E. coli dam*$^+$ strains varied considerably (Tables I and II). We have thus constructed an isogenic set of *E. coli dam* mutants in the highly transformable strain *E. coli* LE392; transformation results are shown in Table IB. Reduction in transformation frequency occurs at three levels, namely, those of (i) *dam*$^-$ mutants by ColE1-type plasmids, (ii) *dam*$^+$ cells by *oriC* plasmids relative to ColE1-type plasmids, and (iii) *dam*$^-$ mutants by *oriC* plasmids. At level one, ColE1-type origin plasmids (pMK2004, pJZ37, pJZ34) appear to transform *dam* mutants and the least transformable wild-type *dam*$^+$ strain (Table IA) equally well. However, when isogenic strains are used, these

Table I. Transformation of *E. coli* strains

A. Non-isogenic *E. coli* strains	Plasmid DNA			
	pJZ37 ColE1	pJZ34 ColE1; *oriC* (St)	pJZ15 *oriC* (St)	pOC15 *oriC* (Ec)
	[8.9×10^{-1}]	[1.0×10^{0}]	[1.2×10^{-2}]	[9.7×10^{-3}]
JC12 *dam*$^+$	3.3×10^{6}	3.7×10^{6}	4.6×10^{4}	3.6×10^{4}
	(1.0×10^{0})	(1.0×10^{0})	(1.0×10^{0})	(1.0×10^{0})
C600 *dam*$^+$	2.4×10^{5}	1.4×10^{5}	5.1×10^{3}	1.5×10^{4}
	(7.3×10^{-2})	(3.8×10^{-2})	(1.1×10^{-1})	(4.2×10^{-1})
GM81 *dam-3*	1.7×10^{5}	1.1×10^{5}	2.1×10^{2}	9.7×10^{2}
	(5.2×10^{-2})	(3.1×10^{-2})	(4.6×10^{-3})	(2.7×10^{-2})
GM130 *dam-4*	1.3×10^{5}	1.8×10^{5}	1.4×10^{2}	7.7×10^{2}
	(3.9×10^{-2})	(4.9×10^{-2})	(3.0×10^{-3})	(2.1×10^{-2})
GM204 △(*dcm*)	1.7×10^{5}	1.9×10^{5}	2.5×10^{4}	5.9×10^{3}
	(5.3×10^{-2})	(5.1×10^{-2})	(5.4×10^{-1})	(1.6×10^{-1})
GM153 *dam-4 recL153*	2.0×10^{5}	1.6×10^{5}	2.4×10^{2}	7.6×10^{2}
	(6.1×10^{-2})	(4.3×10^{-2})	(5.2×10^{-3})	(2.1×10^{-2})
B. Isogenic *E. coli* strains	**Plasmid DNA**			
	pMK2004 ColE1	pJZ34 ColE1; *oriC* (St)	pJZ15 *oriC* (St)	pOC15 *oriC* (Ec)
	[9.9×10^{-1}]	[1.0×10^{0}]	[1.4×10^{-2}]	[4.0×10^{-3}]
LE392 *dam*$^+$	1.82×10^{6}	1.84×10^{6}	2.6×10^{4}	7.3×10^{3}
	(1.0×10^{0})	(1.0×10^{0})	(1.0×10^{0})	(1.0×10^{0})
DS1310 *dam-3*	1.96×10^{5}	1.34×10^{5}	5.0×10^{2}	2.2×10^{2}
	(1.1×10^{-1})	(7.3×10^{-2})	(1.9×10^{-2})	(3.0×10^{-2})
DS1312 *dam-4*	1.3×10^{5}	6.2×10^{4}	5.3×10^{2}	3.4×10^{2}
	(7.1×10^{-2})	(3.4×10^{-2})	(2.0×10^{-2})	(4.6×10^{-2})
DS1313 *dam-12*	1.6×10^{5}	1.9×10^{5}	3.6×10^{2}	3.0×10^{2}
	(8.8×10^{-2})	(1.03×10^{-1})	(1.4×10^{-2})	(4.1×10^{-2})
DS1315 *dam-13*	8.2×10^{4}	4.0×10^{4}	1.9×10^{2}	1.5×10^{2}
	(4.5×10^{-2})	(2.2×10^{-2})	(7.3×10^{-3})	(2.1×10^{-2})

Values given are transformation frequencies per ug plasmid DNA. Numbers in brackets: frequencies for each plasmid relative to pJZ34 of JZ12 in A, and of LE392 in B. Numbers in parentheses: frequencies for each plasmid relative to those of JC12 in A, and to those of LE392 in B. Plasmid replication origins are given. *oriC*(St): *Salmonella typhimurium* origin *oriC*; *oriC*(Ec): *Escherichia coli* origin *oriC*.

plasmids clearly transform *dam* mutants only 5 – 10% as well as they do the parental *dam*$^+$ strain (Tables IB and II) independent of state of methylation of transforming DNA (Table II). These transformation frequencies are independent of the presence of a bacterial origin (compare pMK2004 and pJZ37 with pJZ34 and pJZ101).

At level two, in contrast to ColE1-type plasmids, plasmids whose only origin is a bacterial origin (pJZ15 and pOC15, two comparably sized origin plasmids) transform each of the *dam*$^+$ strains used (Tables I and II) at comparable frequencies. These frequencies, however, are much reduced (~1%) from those observed for plasmids harboring a ColE1-type origin, with or without an additional bacterial origin. Further, at level three, these plasmids transform *E. coli dam* strains ~3% as well as they do *dam*$^+$ strains (Tables I and II). Thus, their transformation frequencies are reduced ~30-fold in *E. coli dam*$^-$ strains. The full 30-fold (or more) decrease is observed only for bacterial origin plasmids that harbor no ColE1-type origin (pJZ15 and pOC15). Thus, to observe this 30-fold reduced frequency, the plasmid must not just harbor a bacterial origin (pJZ34, pJZ101, pJZ15 and pOC15), it must be using this origin to initiate rounds of replication (pJZ15 and pOC15). When compared with the maximal transformation frequency observed, that for ColE1-type plasmids, transformation of bacterial origin plasmids into *dam* cells is reduced by about four orders of magnitude. Little difference in transformation frequency is seen between the point mutants *dam-3* and *dam-4* and the insertion mutants *dam-12* and *dam-13*. In contrast, transformation of a *dcm* deletion mutant (GM204, Table IA) occurred at nearly the same frequency as for wild-type strains. All *dam* transformants retained the sensitivity to 2-aminopurine typical of *dam* mutants (data not shown). Hence, the observed transformation was not into rare *dam*$^+$ 'revertants'.

To determine if this reduced transformation frequency is due to a role of the Dam methylase at the bacterial origin similar to its proposed strand discrimination role in mismatch repair (Wagner and Meselson, 1976), transformation frequencies of *dam* mutants suppressed by second site mutations in the *recL, uvrD, uvrE, mutH, mutL,* and *mutS* loci were determined. Neither ColE1-type plasmids (pJZ37 and pJZ34) nor *oriC*-type plasmids (pJZ34, pJZ15 and pOC15) could transform GM153, a *dam-4 recL* strain related to JC12 *dam*$^+$, at a higher frequency than they could non-suppressed *dam* mutants (Table IA). Thus, the *recL* mutation, which suppresses a number of phenotypic properties of *dam* mutants (Marinus, 1980), is unable to suppress either level 1 or level 3 reduction in transformation frequency observed with these plasmids.

To confirm and extend this result to other suppressors (*mutH, mutL, mutS, uvrD, uvrE*) of phenotypic properties of *dam* mutants (McGraw and Marinus, 1980; Marinus, 1980; Glickman and Radman, 1980), the *dam-13* insertion mutation was transduced into an isogenic set of *mut* and *uvr* strains, obtained from B. Glickman; transformation properties of these strains are shown in Table II. Using plasmid DNA isolated from *dam*$^+$ cells (methylated DNA), the results from each of the *mut* and *uvr* mutations tested were similar. All four plasmids transformed the *dam*$^+$ *mut* and *uvr* parental single mutants with frequencies com-

Table II. Transformation of isogenic suppressed *E. coli dam*$^+$ strains[a]

Isogenic *E. coli* strains:	Plasmid DNA from *dam*$^+$ cells				Plasmid DNA from *dam*$^-$ cells			
	pJZ34 ColE1; *oriC*(St)	pJZ101 ColE1; *oriC*(Ec)	pJZ15 *oriC* (St)	pOC15 *oriC* (Ec)	pJZ34 ColE1; *oriC*(St)	pJZ101 ColE1; *oriC*(St)	pJZ15 *oriC* (St)	pOC15 *oriC* (Ec)
	[1.0 x 10^0]	[1.4 x 10^0]	[4.5 x 10^{-2}]	[1.0 x 10^{-2}]	[1.0 x 10^0]	[1.4 x 10^0]	[8.4 x 10^{-3}]	[4.3 x 10^{-2}]
NR3835 *dam*$^+$	1.3 x 10^6	1.8 x 10^6	5.8 x 10^4	1.3 x 10^4	8.0 x 10^5	1.1 x 10^6	6.7 x 10^3	3.4 x 10^4
	(1.0 x 10^0)	(1.0 x 10^0)	(1.0 x 10^0)	(1.0 x 10^0)	(1.0 x 10^0)	(1.0 x 10^0)	(1.0 x 10^0)	(1.0 x 10^0)
NR3744 *dam-3*	1.8 x 10^5	1.4 x 10^5	1.7 x 10^3	8.3 x 10^3	7.2 x 10^4	7.7 x 10^4	2.7 x 10^2	<1.1 x 10^1[b]
	(1.4 x 10^{-1})	(7.8 x 10^{-2})	(2.9 x 10^{-2})	(6.4 x 10^{-2})	(9.0 x 10^{-2})	(7.0 x 10^{-2})	(4.0 x 10^{-2})	(<3.2 x 10^{-4})
NR3939 *mutH*	1.7 x 10^6	9.1 x 10^5	1.1 x 10^4	1.6 x 10^4	7.2 x 10^5	1.1 x 10^6	3.0 x 10^3	3.0 x 10^3
	(1.3 x 10^0)	(5.1 x 10^{-1})	(1.9 x 10^{-1})	(1.23 x 10^0)	(9.0 x 10^{-1})	(1.0 x 10^0)	(4.5 x 10^{-1})	(8.8 x 10^{-2})
DS1334 *mutH dam-13*	2.0 x 10^5	1.0 x 10^5	6.8 x 10^2	8.5 x 10^2	8.1 x 10^4	5.4 x 10^4	<2.5 x 10^1	<1.1 x 10^1
	(1.5 x 10^{-1})	(5.6 x 10^{-2})	(1.2 x 10^{-2})	(6.5 x 10^{-2})	(1.0 x 10^{-1})	(4.9 x 10^{-2})	(<3.7 x 10^{-3})	(<3.2 x 10^{-4})
NR3940 *mutL*	2.1 x 10^6	8.6 x 10^5	7.0 x 10^3	1.6 x 10^4	5.2 x 10^5	6.2 x 10^5	4.5 x 10^3	2.5 x 10^3
	(1.6 x 10^0)	(4.8 x 10^{-1})	(1.2 x 10^{-1})	(1.23 x 10^0)	(6.5 x 10^{-1})	(5.6 x 10^{-1})	(6.7 x 10^{-1})	(7.4 x 10^{-2})
DS1336 *mutL dam-13*	1.9 x 10^5	7.4 x 10^4	4.4 x 10^2	1.0 x 10^3	5.7 x 10^4	5.6 x 10^4	1.9 x 10^2	<1.1 x 10^1
	(1.5 x 10^{-1})	(4.1 x 10^{-2})	(7.6 x 10^{-3})	(7.7 x 10^{-2})	(7.1 x 10^{-2})	(5.1 x 10^{-2})	(2.8 x 10^{-2})	(<3.2 x 10^{-4})
NR3996 *mutS*	1.7 x 10^6	1.2 x 10^6	5.8 x 10^4	1.6 x 10^4	6.8 x 10^5	7.2 x 10^5	1.5 x 10^3	5.0 x 10^3
	(1.3 x 10^0)	(6.7 x 10^{-1})	(1.0 x 10^0)	(1.23 x 10^0)	(8.5 x 10^{-1})	(6.5 x 10^{-1})	(2.2 x 10^{-1})	(1.5 x 10^{-1})
DS1338 *mutS dam13*	1.6 x 10^5	9.2 x 10^4	6.8 x 10^2	5.8 x 10^2	5.6 x 10^4	7.1 x 10^4	<2.5 x 10^1	2.2 x 10^1
	(1.2 x 10^{-1})	(5.1 x 10^{-2})	(1.2 x 10^{-2})	(4.5 x 10^{-2})	(7.0 x 10^{-2})	(6.5 x 10^{-2})	(<3.7 x 10^{-3})	(6.5 x 10^{-4})
NR3992 *uvrD*	1.3 x 10^6	1.5 x 10^6	9.6 x 10^3	2.4 x 10^4	6.4 x 10^5	6.5 x 10^5	1.8 x 10^3	2.6 x 10^3
	(1.0 x 10^0)	(8.3 x 10^{-1})	(1.7 x 10^{-1})	(1.85 x 10^0)	(8.0 x 10^{-1})	(5.9 x 10^{-1})	(2.7 x 10^{-1})	(7.6 x 10^{-2})
DS1330 *uvrD dam-13*	1.7 x 10^5	8.4 x 10^4	2.0 x 10^2	<1.1 x 10^1	5.2 x 10^4	7.3 x 10^4	1.3 x 10^2	<1.1 x 10^1
	(1.3 x 10^{-1})	(4.7 x 10^{-2})	(3.4 x 10^{-3})	(<8.5 x 10^{-4})	(6.5 x 10^{-2})	(6.6 x 10^{-2})	(1.9 x 10^{-2})	(<3.2 x 10^{-4})
NR3993 *uvrE*	6.7 x 10^5	1.2 x 10^6	4.0 x 10^3	6.5 x 10^4	7.6 x 10^5	7.6 x 10^5	1.2 x 10^3	4.3 x 10^3
	(5.2 x 10^{-1})	(6.7 x 10^{-1})	(6.9 x 10^{-2})	(5.0 x 10^0)	(9.5 x 10^{-1})	(6.9 x 10^{-1})	(1.8 x 10^{-1})	(1.3 x 10^{-1})
DS1332 *uvrE dam-13*	1.2 x 10^5	1.3 x 10^5	6.7 x 10^1	3.6 x 10^2	9.1 x 10^4	5.4 x 10^4	<2.5 x 10^1	1.8 x 10^2
	(9.0 x 10^{-2})	(7.2 x 10^{-2})	(1.2 x 10^{-3})	(2.8 x 10^{-2})	(1.1 x 10^{-1})	(4.9 x 10^{-2})	(<3.7 x 10^{-3})	(5.3 x 10^{-3})

[a]Values given and nomenclature used are as in Table I. Numbers in brackets: frequencies of NR3835 for each plasmid relative to those for pJZ34. Numbers in parentheses: frequencies relative to those of NR3835 for each plasmid.
[b]<: No colonies were observed for the amount of plasmid DNA used.

parable with those of the parental strain NR3835, showing that the level 2 transformation frequency reduction is not altered by these mutations. Further, none of the four plasmids transformed any of the *dam uvr* and *dam mut* double mutants at higher frequencies than for the *dam* single mutant, demonstrating no suppression of level 1 or level 3 transformation frequency reduction by any of these mutations. Thus, the reduced ability to transform (i) *dam*$^-$ mutants by ColE1-type plasmids, (ii) *dam*$^+$ cells by *oriC* plasmids relative to ColE1-type plasmids, and (iii) *dam*$^-$ mutants by *oriC* plasmids is not suppressible by any of the mutations which have been shown to suppress the mismatch repair functions of the Dam methylase. This suggests that the function of the Dam methylase in bacterial origin function, and possibly also in ColE1-type origin function, is different from that of its role in mismatch repair.

Table II also shows transformation results using plasmid DNA isolated from DS1310 *dam-3* (non-methylated DNA). The ColE1-type plasmids showed a slight, but consistent (a factor of 2–3) reduction in transformation ability for all strains tested, still with an ~5–10% ability in the *dam* derivative of the corresponding *dam*$^+$ strain. This reduction was more pronounced (up to 10-fold) for the origin plasmids with no ColE1-type origin. The ability of these plasmids to transform *dam* strains remained at 1–5% that for the corresponding *dam*$^+$ strain. Further, relative to the parental strain, the transformation frequency of the *mut* and *uvr* derivatives was consistently reduced, rather than increased as would be expected if these mutations suppressed the effects of the *dam* mutation. Thus, the results using non-methylated DNA are similar, albeit accentuated, to those using methylated DNA, reducing the overall transformation frequency as much as 4–5 orders of magnitude.

Stable inheritance of bacterial origin plasmids propagated in these strains was examined, as a measure of their segregation properties (data not shown). In contrast to large differences in transformation frequencies, once established, both pOC15 and pJZ15 exhibit nearly the same degree of stability in all strains tested (50% loss of plasmids in 2–5 generations). This is true also for both plasmids isolated from DS1310 *dam-3* cells, and retransformed into these strains. Hence, the state of methylation of these plasmids alters the degree of their stability very little if at all, and argues that the transformation properties do not reflect segregation properties of these plasmids. The instability of the *E. coli* plasmid pOC15 and the *S. typhimurium* plasmid pJZ15 in various *dam* mutants demonstrates that the plasmids have not recombined into the *E. coli* chromosome.

Although transformation frequencies are greatly reduced, transformants were nevertheless obtained in nearly all the above experiments. Further, no chromosomal deletions of the *dam* gene have yet been isolated. Thus, some residual Dam methylase activity may be present in these *dam* mutants. The methylation state of the *E. coli oriC* plasmid pJZ101 and the *oriC* phage M13mpRE85 was determined *via* sensitivity to cleavage by the restriction endonucleases *Sau*3A, *Mbo*I and *Dpn*I. *Mbo*I cleaves only at GATC sites with neither adenine methylated (Lacks and Greenberg, 1977), *Dpn*I cleaves only at GATC sites with both adenines methylated (Geier and Modrich 1979), and *Sau*3A cleaves at any GATC site (Sussenbac *et al.*, 1976). For pJZ101 DNA isolated from either *dam*$^+$ or *dam-3* cells (Figure 1), uncut DNA migrates mainly as closed circular DNA with some open circular species (lanes A and H) and *Sau*3A cleaves at all GATC sites (lanes E and L). For pJZ101 DNA isolated from *dam*$^+$ cells, the pattern of digestion by *Dpn*I (lane M) is iden-

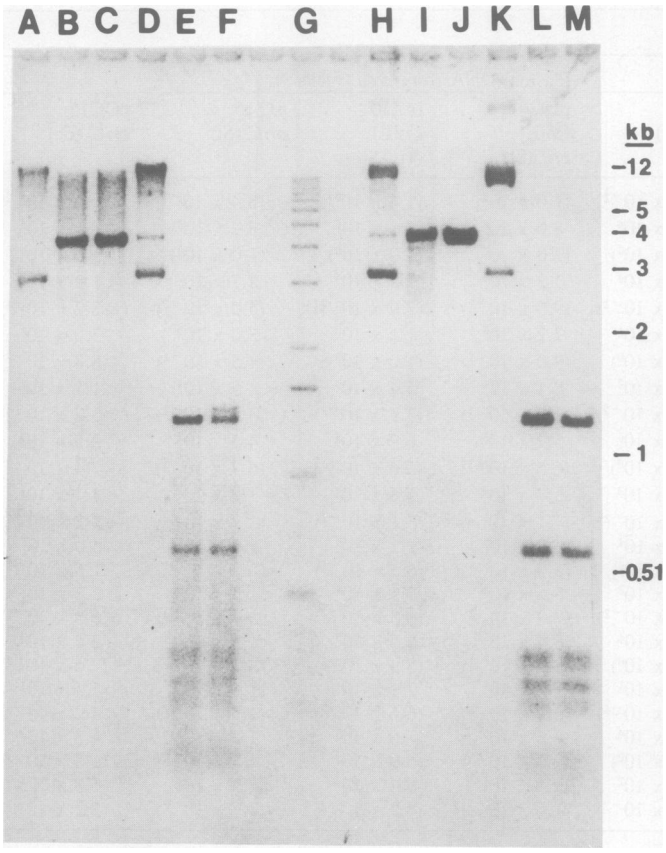

Fig. 1. Horizontal agarose gel electrophoresis of restriction enzyme digested pJZ101 DNA isolated from LE392 *dam*$^+$ and from DS1310 *dam-3* cells. **Lanes A–F,** pJZ101 from *dam-3* cells: **A,** uncut; **B,** *Dpn*I and *Eco*RI; **C,** *Eco*RI; **D,** *Dpn*I; **E,** *Sau*3A; **F,** *Mbo*I. **Lanes H–M,** pJZ101 from *dam*$^+$ cells. **H,** uncut; **I,** *Mbo*I and *Eco*RI; **J,** *Eco*RI; **K,** *Mbo*I; **L,** *Sau*3A; **M,** *Dpn*I. **Lane G,** marker DNA, the '1 kb ladder' from BRL. Sizes of the marker DNA fragments are indicated.

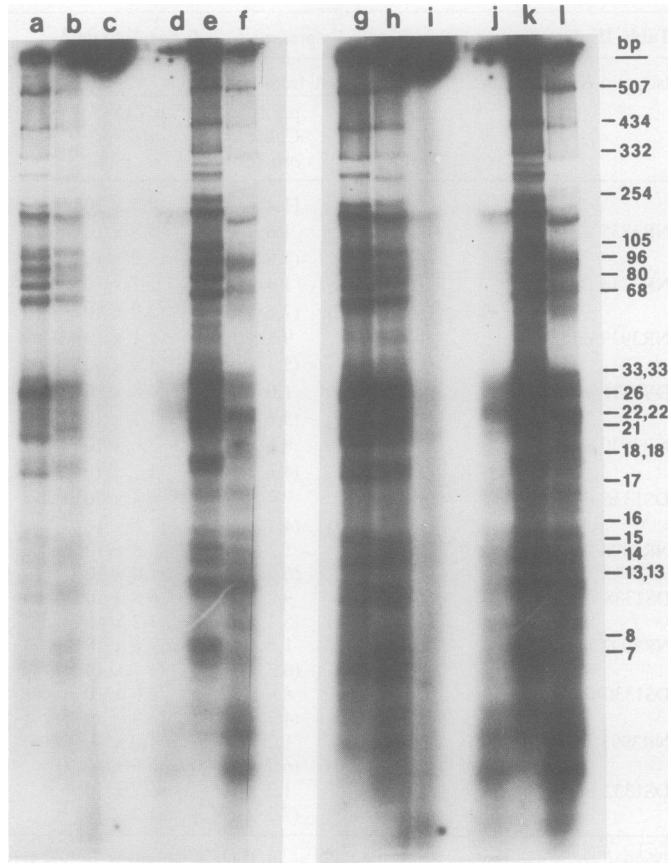

Fig. 2. Autoradiogram of ^{32}P-labeled restriction fragments of M13mpRE85 RF I DNA separated on a 20% polyacrylamide gel. RF I DNA was isolated from JM109 *dam*$^+$ and from DS4113 *dam-13* cells. **Lanes A–F:** 9 h film exposure; **Lanes G–L:** 24 h film exposure. **Lanes A–C** and **G–I:** DNA from *dam*$^+$ cells; **lanes D–F** and **H–L:** DNA from *dam-13* cells. **Lanes A, D, G,** and **J:** *Dpn*I digest; **lanes B, E, H,** and **K:** *Sau*3A digest; **lanes C, F, I** and **L:** *Mbo*I digest. Indicated fragment sizes are based on the nucleotide sequence of M13mpRE85 and positions of marker dyes.

tical to that for *Sau*3A (lane L), and that for *Mbo*I (lane K) is indistinguishable from uncut DNA (lane H). In contrast, for pJZ101 DNA isolated from *dam-3* cells, digestion with *Dpn*I (lane D) converts a significant fraction of the DNA into unit length linear molecules, indicating that many plasmid molecules have one, or more than one closely adjacent, GATC site(s) that remain methylated (*Dpn*I-sensitive). Conversely, digestion with *Mbo*I (lane F) yields a pattern similar to that of digestion with *Sau*3A (lane E), except that a distinct additional band at ~1.4 kb is present.

To determine the location(s) of the *Dpn*I-sensitive sites on these molecules, double digestions with *Dpn*I and *Eco*RI, or with *Dpn*I and *Pst*I, were performed (Figure 1), since pJZ101 has unique *Eco*RI and *Pst*I sites. Each double digest will localize a single *Dpn*I-sensitive site (or short region) per molecule to one of two unique locaitons. For plasmid DNA from *dam*$^+$ cells, double digestion with both *Eco*RI and *Mbo*I (lane I) shows a series of faint bands migrating somewhat faster than the unit length linear band, suggesting that some molecules may possess *Mbo*I-sensitive, non-methylated GATC sites at varying distance from the single *Eco*RI site. Such has been observed for ColE1-type plasmids grown under amplification conditions (Szyf *et al.*, 1984). For plasmid DNA from *dam-3* cells digested with both *Eco*RI and *Dpn*I (lane B), faint bands migrating faster than the unit length linear band are also observed, in particular, near 3.9 kb and near 3.2 kb. The band near 3.9 kb is consistent with a *Dpn*I-sensitive site(s) arising from the *oriC* region, and the *Dpn*I-*Pst*I experiment confirmed this location (data not shown). For this location, no change in the *Mbo*I digestion pattern should be seen, due to the large number of GATC sites within *oriC*. When compared with lane C, the band near 3.2 kb is unlikely to be residual uncut closed circular DNA. Further, such a band is predicted if a *Dpn*I-sensitive site were present in the ColE1 origin region (*rep*) of pJZ101; this location is confirmed by the *Dpn*I-*Pst*I double digest experiment (data not shown). Using the *oriC* numbering system (see Figure 4), if the GATC site at position 2883 in *rep* were methylated, and hence insensitive to *Mbo*I digestion, a new band of 1374 + 75 = 1449 bp should be present in the *Mbo*I digest (lane F); such is in fact observed. Thus, some pJZ101 molecules from *dam-3* cells retain specific GATC methylation within the ColE1-type origin region.

RFI DNA of the *oriC* phage M13mpRE85 was similarly analyzed, using 5′-labeled DNA and polyacrylamide gels, to localize and identify specific *Dpn*I-sensitive sites from within the *oriC* region of DNA isolated from *dam*$^-$ cells. M13mp vectors are particularly useful for these studies, since the phage DNA contains very few GATC sites. Since the nucleotide sequence of M13mp8 is known, the sizes of all expected GATC fragments, from complete and partial digests, are known. Two exposure times for M13mpRE85 RFI DNA isolated from JM109 *dam*$^+$ cells and from DS4113 *dam-13* cells are shown in Figure

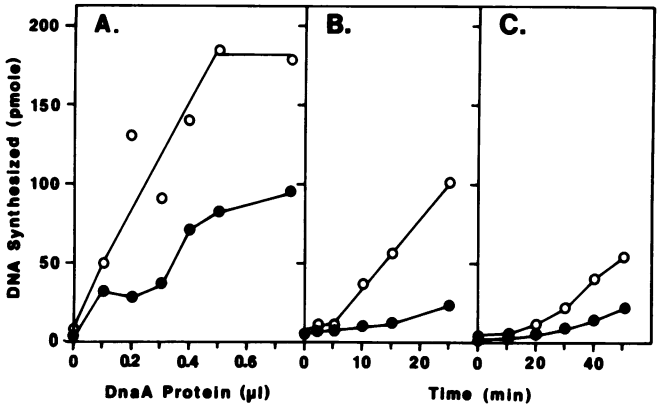

Fig. 3. Template activity of M13mpRE85-*oriC* DNA isolated from JM109 *dam*$^+$ and from DS4113 *dam-13* cells in the *dnaA*-dependent *oriC*-dependent *in vitro* initiation system. **A:** DnaA protein titration, using the Fraction II assay system. Incubation was for 20 min at 30°C. ○, DNA from *dam*$^+$ cells; ●, DNA from *dam-13* cells. **B:** Time course of *in vitro* DNA synthesis, using the Fraction II assay system. 0.5 µl DnaA protein was added; incubation was at 30°C. ○, DNA from *dam*$^+$ cells; ●, DNA from *dam-13* cells. **C:** Effects of *in vitro* methylation of *dam*$^-$ DNA on template activity. M13mpRE85 DNA isolated from DS4113 *dam-13* cells was methylated to completion with purified *E. coli* Dam methylase. Time course of *in vitro* DNA synthesis using the reconstituted enzyme assay system at 30°C with 0.5 µl DnaA protein added. ○, S-adenosylmethionine included in the Dam methylase reaction prior to assay for template activity; ●, S-adenosylmethionine omitted from the Dam methylase reaction prior to assay for template activity.

2, to show the major and minor labeled DNA bands. The pattern of digestion by *Sau*3A of either RFI DNA is similar (lanes b,e,h,k), and the mol. wts. of the observed bands agree well with those predicted from the DNA sequence. The pattern of digestion of the DNA from *dam*$^+$ cells by *Dpn*I (lanes a and g) is nearly identical with that of *Sau*3A (lanes b and h) and *Mbo*I shows no activity on the *dam*$^+$ DNA (lanes c and i). Thus, RFI DNA isolated from *dam*$^+$ cells has essentially completely methylated GATC sites. For M13mpRE85 DNA from *dam-13* cells, if no GATC sites were methylated, the *Mbo*I digestion pattern would be identical to that of *Sau*3A, and *Dpn*I would exhibit no activity with such DNA. However, the *Mbo*I digestion profile (lanes f and e) differs significantly from that for *Sau*3A (lanes e and k), and *Dpn*I exhibits considerable activity against this DNA (lanes d and j). These two results argue strongly that this DNA, isolated from an insertion mutation of the *dam* gene, still retains some completely methylated GATC sites. Since many of the bands resulting from the *Dpn*I digestion are the lower mol. wt. bands from the *oriC* region, many of these residual methylated GATC sites are found within *oriC*.

In vitro experiments support the *in vivo* evidence presented here for a role of methylation of GATC sites in initiation function of the *oriC* region. Using M13mpRE85 RFI DNA isolated from JM109 *dam*$^+$ and from DS4113 *dam-13* (Figure 3A and B), the *dam*$^-$ DNA is 2- to 4-fold less effective as a template than is the DNA isolated from *dam*$^+$ cells in the *dnaA*-dependent *in vitro* initiation system specific for *oriC* (Fuller *et al.*, 1981). This is true for both the relatively crude Fraction II system (Fuller *et al.*, 1981) as well as for a reconstituted purified enzyme system (Kaguni and Kornberg, 1984). Further, *in vitro* activity of M13mpRE85 RFI DNA from *dam-13* cells is enhanced 2- to 3-fold when methylated *in vitro* with purified Dam methylase prior to use as template in the *in vitro* initiation system (Figure 3C).

Discussion

Several lines of evidence indicate that the Dam methylase and the state of methylation of GATC sites are directly involved in initiation of rounds of DNA replication in *E. coli*. First, plasmids having only *oriC* as an origin transform *E. coli dam* mutants ~30-fold less efficiently than they do *dam*$^+$ strains (Tables I and II). This decrease in transformation frequency is less pronounced in plasmids containing *oriC* but using a ColE1-type origin. Thus, *oriC* must not only be present but must be functioning as an origin to observe the maximal decrease in transformation frequency. The net decrease in transformation frequency is 4–5 orders of magnitude, but, in contrast to the accompanying paper by Messer *et al.*, some transformants were obtained in nearly all cases. This difference in results may be due in part to the different *E. coli* strains used. Second, this reduced transformation frequency is not observed in *E. coli dcm* mutants, thus demonstrating specificity for Dam methylation. Third, the reduced transformation frequency is still observed in double mutants where the second mutation (*mutH, mutL, mutS, recL, uvrD* and *uvrE*) suppresses mismatch repair phenotypic properties of *dam* mutations (Table II). If anything, introduction of these second mutations accentuates the low transformation frequency phenotype. This suggests that the role of the GATC sites and the Dam methylase in *oriC* function is different from that in mismatch repair. Fourth, *oriC* plasmid DNA isolated from *dam* cells again exhibits the 30-fold reduction (or more) in transformation frequency of *dam* mutants, arguing against modification of the plasmid DNA in the *E. coli dam* mutants. Transformed cells themselves remain *dam*$^-$, as determined by sensitivity of growth to 2-aminopurine. Fifth, using sensitivity to cleavage by the restriction enzymes *Sau*3A, *Mbo*I, and *Dpn*I, *oriC* plasmid or phage DNA isolated from *dam* point or insertion mutants retains some completely methylated GATC sites, and some of these are in the *oriC* region. Sixth, M13mpRE85 RFI DNA isolated from *dam*$^-$ cells functions 2- to 4-fold less well as *oriC* template DNA than does such DNA from *dam*$^+$ cells in the *dnaA*-dependent *in vitro* initiation system specific for *oriC*. Kohiyama and coworkers (Hughes *et al.*, 1984) have recently made similar observations, as have Messer and co-workers (see accompanying paper). Further, ability to function as *oriC* DNA template in this *in vitro* system is partially restored if the M13mpRE84 RFI DNA from *dam*$^-$ cells is first methylated *in vitro* with purified Dam methylase, directly demonstrating that the undermethylation of GATC sites in this DNA is the reason for its reduced ability to serve as template *in vitro*.

Digestion profiles of pJZ101 isolated from *dam-3* cells (Figure 1) show that some molecules retain methylation of one or more GATC sites within the ColE1 origin region and within the *oriC* region. If both regions had methylated GATC sites in the same pJZ101 molecule, a band at 2661 bp should be observed. Since such a band is not apparent (Figure 1, lane b), the pJZ101 population appears to be mainly non-methylated, with <10% retaining one or more methylated GATC sites. The 1450-bp new band in the *Mbo*I digestion profile (Figure 1, lane f) strongly suggests that at least the GATC site at position 2883 in the ColE1 origin region is methylated in some of the pJZ101 molecules. However, the data do not rule out additional methylation at one or more of the GATC sites at positions 2894, 2902, 2980, 2992 or 3097. The GATC site at position 2883 is of particular interest to regulation of ColE1 initiation events, since it resides in the DNA coding for both the primer RNA and the negatively regulating RNA I species (Tomizawa *et al.*, 1981),

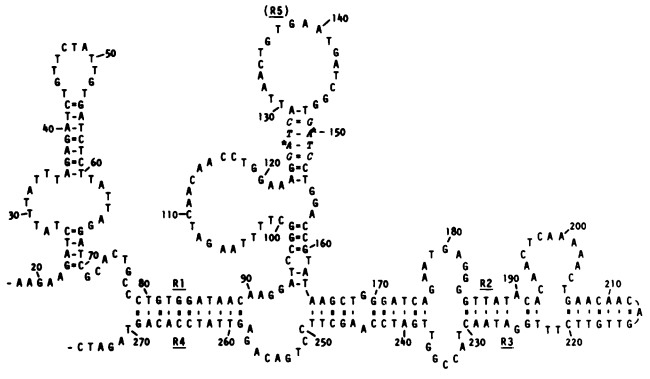

Fig. 4. Intrastrand secondary structure of lowest total free energy (−82.8 Kcal/mol) for the *E. coli* minimal origin region, using the Ooi and Takanami DNASEQ2B computer program with minimal helix length of 4 bp and a maximal loop size of 300 bp. Position 1 is from the *Bam*HI site adjacent to *oriC*. The direct/inverted 9-bp repeats R1, R2, R3, and R4 are indicated; a possible fifth such repeat called R5, of homology 7 of 9 is shown. Italicized GATC sites near positions 125 and 150: the two sites shown in this work to remain methylated in *dam-13* cells. *, the methylated adenines in these two GATC sites.

and is transcribed into the stem of arm II of RNA I (Lacatena and Cesareni, 1981). Methylation of this GATC site may be important, perhaps essential, for initiation from the ColE1 origin, perhaps *via* inhibition of RNA I synthesis or of its function as a repressor of these initiation events.

The data of Figure 2 provides initial evidence for the state of methylation of specific GATC sites within and near *oriC*. From the nucleotide sequence of M13mp8 (van Wezenbeek *et al.*, 1980; Messing and Vieira, 1982) and of the *E. coli oriC* region, the sizes of all GATC fragments, beginning with chromosomal DNA in the orientation *atp-oriC-asn* and proceeding sequentially around the circular molecule, are as follows:

-80-105-21-16*-15*-13*-26*-14*-18*-17*-7*-22*-68*-33*-8-33-22-18-13-254-96-434-1696-332-507-4021-

Fragments containing the breakpoints between chromosomal DNA and the vector are underlined. Nucleotide 1 at the *oriC* *Bam*HI site (see Figure 4) is at the junction between the 105-bp and 21-bp fragments. Fragments found within the minimal origin are indicated with stars. The profile of the *Dpn*I-digested M13mpRE85 DNA from *dam-13* cells shows a dominant band at ~24 bp. Such a band can arise only if *both* GATC sites bracketing the 24-bp fragment are methylated. If the 17-bp and 7-bp contiguous fragments were the source of this band, then the *Mbo*I profile should exhibit new 18 + 17 = 35 bp and 7 + 22 = 29 bp fragments; such are indeed present. This suggests that the GATC sites at positions 125 and 149 (see Figure 4) within *oriC* are methylated, and that the site between them is not. Similarly, weak bands appear in the *Dpn*I track at positions corresponding to the 13-bp, 15-bp, and 16-bp bands. These bands also appear in the *Mbo*I track, but are less intense than expected when compared with the *Sau*3A bands, and a strong *Mbo*I band is present at ~91 bp. Taken together, this suggests that the four GATC sites bracketing the contiguous 16-bp, 15-bp, and 13-bp fragments at positions 23, 39, 54, and 67 (Figure 4) are fully methylated in some RFI DNA molecules in the population, and not in others. Because of these four nearly equally spaced GATC sites, essential for origin function and fully conserved between all six analyzed bacterial origins (Zyskind *et al.*, 1983), several potential intrastrand and interstrand secondary structures of low free energy exist; one example of these is shown in Figure 4.

Outside the minimal origin, the 105-bp and 80-bp bands are absent from the *Mbo*I track (Figure 2, lanes F and L), but a new band at ~180 bp is present, suggesting that the GATC site between the 105-bp and 80-bp fragments is methylated. This GATC site is part of the *Bam*HI site toward the *atp* operon which borders the 105-bp fragment adjacent to *oriC* recently shown to contain sequences essential to origin function (J.W.Zyskind, in preparation). State of methylation of this GATC site may be important in the function of this fragment in initiation. Similar analysis suggests that the sites bounding the 8-bp and 33-bp, the 33-bp and 22-bp and the 18-bp and 13-bp fragments found just outside the minimal origin region toward the *asnA* gene, are methylated in some of the RFI molecules isolated from the *dam-13* host. These sites are within the gene coding for the 16-kd protein adjacent to *oriC*, a region exhibiting incompatibility phenomena (Yamaguchi *et al.*, 1982) and possibly important for normal origin function; again, correct methylation of GATC sites may be essential for such function.

Many intrastrand secondary structures are possible within the minimal *oriC* region, as determined visually and by computer analysis using several available secondary structure programs. All of these predict a pairing of the totally conserved and perfect inverted regions termed R1 and R4 (Zyskind *et al.*, 1981). The origin is thereby separated into two regions, that containing the four equally spaced GATC sites to the *atp* side of R1, and the stem-loop structure enclosed by R1 and R4. The 'loop' so-formed is large, and capable itself of a variety of intrastrand base pairing. Figure 4 shows the prediction of lowest total Gibb's free energy using the Ooi and Takanami (1981) program. This structure, and those of comparable free energies, predict that the two GATC sites at positions 125 and 149, indicated by the evidence of Figure 2 to be methylated in *dam-13* cells, are base-paired together in a stem structure. Thus, methylation of these two GATC sites may be essential to origin function, possibly either to form or to maintain an essential secondary structure such as that of Figure 4.

The structure of Figure 4 is consistent with the sequences of the six analyzed bacterial origins functional in *E. coli* (Zyskind *et al.*, 1983), although the specific stem-loop structures found between the R1:R4 stem vary. This structure is also in general consistent with the mutagenesis data from Takanami and coworkers (Takanami *et al.*, 1983; Oka *et al.*, 1984). More than twice as many (156/233) of the base changes present in Ori^+ point mutants are found in loop regions than in stem regions. Of those in stem regions, half (37/77) are found in the end intrastrand base pairs of a given stem region. Two-thirds (14/17) of the Ori^- mutations are found in stem regions, mostly near the middle of the stem. Thus, changes which severely disrupt the predicted intrastrand base pairings of Figure 4 are in general more detrimental (Ori^- phenotype) than those found in the loop regions.

What might be the function(s) of specific methylated GATC sites within *oriC* during initiation? First, this methylation may be important for correct binding to the *oriC* region of specific initiation protein(s) or other transacting molecules. Such interaction may be necessary to stabilize a 'pre-initiation' complex of initiation molecules with *oriC* in an intrastrand secondary structure such as that of Figure 4. Possible *trans*-acting molecules certainly include RNA species, which could function either to stabilize a 'pre-replication' structure, or as a pre-primer for DNA

Table III. *E. coli* strains used in this study

Strain	Partial Genotype[a]	Source or reference
C600	F⁻ *thr-1 leuB6 thi-1 lacY1 tonA2 supE44*	CGSC[b]
JC12	Hfr *purF1 metB1 gal-6 lacY1,Z4 mtl-2 xyl-7 tonA2 tsx-1*	A.J.Clark; CGSC[b]
GM81	As JC12 but *dam-3*	Marinus and Morris, 1975
GM130	As JC12 but *dam-4*	Marinus, 1980
GM204	F⁻ △*(supD-dcm-flaI,II) gal tsx bfe rpsL* (λ*CI857*)	Bale *et al.*, 1979
GM153	F⁻ *argE3 his-4 ara-14 galK2 lacBK1 thi-1 tsx-33 supE44 recL152 dam-4*	Marinus, 1980
GM659	Hfr △*(lac-pro)*XIII *rpsL dam-12*::Mud*(lac*,Ap^r)	Marinus *et al.*, 1983
GM1987	F⁻ *thr-1 leuB6 argE3 his-4 proA2 thi-1 lacY1 galK2 uvrB5 mtl-1 xyl-5 ara-14 rpsL31 tsx-33 supE44 dam-13*::Tn9(Cm^r)	Marinus *et al.*, 1983
LE392	F⁻ *metB1 trpR55 lacY1 galK2 galT22 supE44 supF58 hsdR514*	N.Grindley
DS1310	As LE392 but *dam-3*, Sm^r, P1^s	This study
DS1312	As LE392 but *dam-4*, Sm^r, P1^s	This study
DS1313	As LE392 but *dam-12*, Ap^r, P1^s	This study
DS1315	As LE392 but *dam-13*, Cm^r, P1^s	This study
NR3835	F′ *pro*$^+$ *lacI*^{q} *lacPL8 lac*$^+$ *trpE9777 thi ara* △*(pro-lac)*	Glickman, 1979
NR3744	As NR3855 but *trp*$^+$ *dam-3*	Glickman, 1979
NR3992	*uvrD3* Derivative of NR3835	B.W.Glickman
NR3993	*uvrE502* Derivative of NR3835	B.W.Glickman
NR3939	*mutH101* Derivative of NR3835	B.W.Glickman
NR3940	*mutL101* Derivative of NR3835	B.W.Glickman
NR3996	*mutS101* Derivative of NR3835	B.W.Glickman
DS1330	As NR3992 but *dam-13*, Cm^r, P1^s	This study
DS1332	As NR3993 but *dam-13*, Cm^r, P1^s	This study
DS1334	As NR3939 but *dam-13*, Cm^r, P1^s	This study
DS1336	As NR3940 but *dam-13*, Cm^r, P1^s	This study
DS1338	As NR3996 but *dam-13*, Cm^r, P1^s	This study
JM109	F′ *proA*$^+$,*B*$^+$ *lacI*^{q} △*(lacZ)*M15 *traD36*/△*(lac-pro) endA1 gyrA96 thi-1 hsdR17 supE44 relA1 recA1*	J.Messing
JM103	F′ *proA*$^+$,*B*$^+$ *lacI*^{q} △*(lacZ)*M15 *traD36*/△*(lac-pro) supE thi*	J.Messing
DS4113	As JM103 but *dam-13*, Cm^r, P1^s	This study
JZ279	As LE392 but *recA56*, Tc^s, P1^s	J.W.Zyskind

[a]Standard bacterial genetic nomenclature is used.
[b]CGSC: Coli Genetic Stock Center.

synthesis, or as an inhibitor similar to the RNA I of the ColE1 origin. The ColE1 origin region coding for RNA I and the RNA primer may also require a methylated GATC site for function (Figure 1), and a function for such methylation in initiation at *oriC* similar to that in initiation at the ColE1 origin is feasible. Second, Figure 4 predicts that the two methylated GATC sites within *oriC* base pair together in an intrastrand manner forming a stem region. If such a structure exists just after initiation, both GATC sites in the stem of the parental strand would be methylated, whereas both GATC sites in the stem of the daughter strand would be non-methylated. Since the Dam methylase most efficiently converts a hemimethylated GATC site in double-stranded DNA to a completely methylated GATC site (Geier and Modrich, 1979), the GATC sites in the daughter strand would tend to remain non-methylated until the *oriC* region returned to the normal double-stranded DNA secondary structure. Subsequent methylation of these two GATC sites could then be required for stabilization of a new 'pre-initiation' complex of *oriC* in its intrastrand secondary structure with stabilizing initiation molecules. In such a scheme, methylation of these two GATC sites would provide a timing mechanism separating consecutive initiation events, and may account for the time lag observed between initiation events (Evans and Eberle, 1975; Helmstetter and Krajewski, 1982); some evidence for this possibility is presented by Messer *et al.* (accompanying paper). These possible functions of GATC methylation in initiation, as well as others, are of course not mutually exclusive, and the state of methylation of different GATC sites may be important, or essential, in more than one step in DNA initiation.

Materials and methods

Bacterial strains, plasmids and phage

Bacterial strains used are described in Table III. For construction of the isogenic set of LE392 derivatives, the *dam-3* allele from GM81 and the *dam-4* allele from GM130 were transduced *via* P1vir, selecting streptomycin-resistant colonies and screening for sensitivity to 2-aminopurine. Co-transduction was found to be ~14%. The *dam-12* allele was transduced from GM659, selecting ampicillin-resistant colonies and screening for sensitivity to 2-aminopurine; co-transduction was >90%. The *dam-13* allele was transduced from GM1987, selecting chloroamphenicol-resistant colonies and screening for sensitivity to 2-aminopurine; co-transduction was nearly 100%. Both the *dam-12* and *dam-13* alleles are unstable in our hands, and require antibiotic selection. Even so, growth and properties of the *dam-12* strains were highly variable. Plasmid pMK2004 has the pBR322 ColE1-type origin, and confers resistance to ampicillin, kanamycin and tetracycline (Kahn *et al.*, 1979). Plasmids pJZ34, which has both a minimal *Salmonella typhimurium* bacterial origin and the pBR322 origin, and pJZ37, a deletion derivative of pJZ34 with a non-functional *S. typhimurium* origin, have been described (Zyskind and Smith, 1980). Plasmid pJZ15 has only the *S. typhimurium* origin and confers resistance to kanamycin (Zyskind *et al.*, 1979). Plasmid pOC15 has only the *E. coli* origin and confers resistance to ampicillin (Messer *et al.*, 1979). Plasmid pJZ101 is an origin plasmid with the *E. coli oriC* region from the *Bam*HI site at −105 to the *Xho*I site at position +417 (Meijer *et al.*, 1979) cloned into the *Bam*HI and *Sal*I sites of pBR322 (J.W.Zyskind, unpublished results). Phage M13mpRE85 is an M13mp8 derivative containing the *E. coli oriC* region from the *Hinc*II site at position −189 to the *Pst*I site at position +448 (R.E.Enns, unpublished results).

General procedures

Growth conditions, selection of antibiotic resistance, determination of plasmid stability, transformation, P1 transduction and other genetic procedures were as described (Zyskind *et al.*, 1979). Close to 100 ng DNA (saturating amount) was used in all transformation experiments. Sensitivity to 2-aminopurine was scored by comparing growth in a 'mini-dilution series' of 10 μl of four sequential 10-fold dilutions of exponentially growing cells spotted on supplemented M9 minimal

salts-glucose plates with or without 200 μg/ml 2-aminopurine (CalBiochem). Techniques for M13 growth, assay, DNA and phage isolation were as described (Maniatis *et al.*, 1982).

Recombinant DNA procedures

Conditions for plasmid DNA isolation, agarose and polyacrylamide gel electrophoresis, restriction endonuclease digestions and ligation using phage T4 DNA ligase were as described (Zyskind *et al.*, 1979), with some minor modifications. Restriction endonucleases were from Bethesda Research Laboratories, Boehringer-Mannheim, or New England BioLabs; DNA ligase was from Bethesda Research Laboratories. The 5′ ends of restriction fragments were radioactively labeled using phage T4 polynucleotide kinase (P-L Biochemicals) and [γ-^{32}P]ATP prepared from [^{32}P]phosphate as previously described (Zyskind and Smith, 1980).

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