Effect of Altering GATC Sequences in the Plasmid ColE1 Primer Promoter

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Plasmid ColE1 has three recognition sites for the *Escherichia coli* DNA adenine methylase in the immediate upstream region of the primer promoter. Two of these sites are conserved among all plasmid relatives of ColE1 and constitute parts of an inverted repeat that can conceivably form a cruciform structure. Recent experiments have indicated that hemimethylated ColE1-type plasmids are inefficiently replicated after transformation (D. W. Russell and N. Zinder, Cell 50:1071–1079, 1987). By mutating the three methylation sites, we found that disruption of all three GATC sites was necessary for complete relief of the hemimethylation-mediated inhibition of replication in vivo. We also found that these three methylation sites acted in a position-specific manner. The putative cruciform, if present, did not play a regulatory role in the hemimethylation-mediated inhibition of replication.

Methylation at GATC sites in DNA by DNA adenine methylase is known to play a role in the replication of certain plasmids in *Escherichia coli*. For example, replication of the unit-copy plasmid P1 has been shown to be dependent on Dam methylation in vitro (1). Russell and Zinder (23) have suggested that Dam methylation affects replication of the multicopy plasmid ColE1 in vivo. They observed that methylated and unmethylated plasmids transformed dam^+ strains with high effciency; in contrast, methylated DNA transformed dam strains poorly. The inability to efficiently transform dam strains was not observed if the DNA was unmethylated. In addition, hemimethylated plasmid DNA was observed to accumulate after one round of replication if fully methylated plasmid DNA was used to transform dam cells. Taken together, these results suggested that whereas methylated and unmethylated DNA could replicate efficiently in dam^+ cells, hemimethylated DNA was inefficiently replicated.

Initiation of replication of a ColE1-type plasmid is thought to occur after the hybridization of a specific primer precursor RNA (also called RNA II) to its template DNA strand, in the vicinity of the origin (21). The resulting RNA-DNA hybrid is a substrate for RNase H, which cleaves the hybridized RNA at the origin. The cleaved RNA serves as a substrate for leading-strand DNA synthesis mediated by DNA polymerase I. Negative control of initiation is exerted by a small countertranscript to primer RNA, called RNA I. RNA I is complementary to the 5'-terminal region of the primer precursor. By annealing to the primer precursor, RNA I induces conformational changes that preclude hybridization of the primer to the DNA template strand and thereby prevents formation of a substrate for leading-strand synthesis (26).

An intriguing feature of the ColE1 primer promoter region is the existence of three GATC methylation sites. These occur at positions −32, −43, and −71 relative to the start of primer transcription. We designate these sites the α,

β, and γ GATC sequences, respectively. A comparative sequence analysis of the primer promoter region of plasmid ColE1 and related plasmids shows that the α and β GATC sites are conserved in all members of the ColE1 plasmid family, while a third, and sometimes a fourth, GATC site occurs in the near vicinity (Fig. 1). Since other promoters with GATC sites are regulated by methylation (22), and since primer RNA plays a critical role in initiation, we investigated the idea that the methylation effect on plasmid replication previously observed (23) was mediated specifically by the GATC sequences in the primer promoter, as opposed to the numerous GATC sequences elsewhere in the plasmid.

In addition to their roles as methylation sites, the α and β GATC sequences in the primer promoter make up part of a 9-base-pair (bp) inverted repeat that could conceivably form a cruciform structure. Since cruciform formation in DNA could play a regulatory role in promoter activity, it was possible that methylation played a role in replication by affecting its formation. In has recently been shown that methylation-induced local destabilization of the DNA double helix can enhance that rate of formation of a cruciform in vitro (19). Thus, the GATC sequences might affect promoter function by altering DNA structure and consequent interaction with RNA polymerase.

By mutating all three methylation sites in the primer promoter, either singly or in all possible combinations, we have identified the primer-associated GATC sequences as being collectively responsible for the apparent hemimethylation-mediated inhibition of replication. Furthermore, we show that these sequences act in a position-specific manner and that their ability to form a cruciform is not obligatory for the mediation of the methylation-related phenomenon. We have also measured the effect of altering the GATC sequences on the activity of the primer promoter in vivo and on plasmid copy number and stability.

MATERIALS AND METHODS

Strains and media. All *E. coli* strains used were derivatives of K-12 (Table 1). Bacteria were grown in 2× YT medium

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FIG. 1. Nucleotide sequence comparison of the primer promoter regions of ColE1 and related plasmids. The sequence of the ColE1 L strand is shown aligned with the corresponding regions of CloDF13, p15A, pBR322, and ColA. The numerical coordinates are those of the ColE1 sequence, with +1 indicating the start site of primer RNA transcription. All Dam methylation sites are boxed. Outside the boxes, positions where the sequence is identical with that of ColE1 are marked with a dot. Positions where the sequences differ from that of ColE1 are marked with the appropriate nucleotide. Absence of a dot or a nucleotide at any particular position indicates either that the sequence is not known or that the construction of a particular plasmid introduced entirely unrelated sequences that were considered inappropriate to compare. The sequences for CloDF13, p15A, and pBR322 are from Selzer et al. (24), and that of ColA is from Morlon et al. (17).

unless otherwise stated. Ampicillin and tetracycline were used at concentrations of 100 and 30 μg/ml, respectively.

Construction of pMet1111. The 4.2-kilobase-pair (kb) plasmid pNOP42-II contains a wild-type ColE1 replication origin and has been described elsewhere (18). It was linearized at the unique *Eco*RI site, and *Pvu*I linkers were attached. It was then cut with *Pvu*I, and the 2.6-kb fragment bearing the ColE1 origin of replication was isolated. This fragment was ligated to the 1,046-bp *Pvu*I fragment from plasmid pTZ18U, containing the single-stranded bacteriophage f1 origin and part of the ampicillin resistance gene. Successful ligation in one of the two possible orientations reconstituted the Amp^r gene and provided selection. The plasmid obtained was called pSTK131.

pMet1111 (Fig. 2) was derived from pSTK131 by oligonucleotide-directed mutagenesis (see below). To facilitate subcloning of the primer promoter and additional manipulations, we introduced unique restriction sites in the immediate upstream region of the primer by site-directed mutagenesis. These sites consisted of a *Kpn*I site at position −2, a *Bam*HI site at −39, a *Hin*dIII site at −52, an *Sst*II site at −75, and a *Sal*I site at −94 relative to the start site of primer transcription. These changes had no effect on any of the plasmid-associated phenomena studied here, i.e., copy number, promoter strength, stability, or methylation-mediated discrimination (data not shown).

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was carried out as described by Kunkel et al. (9). Single-stranded template DNA was isolated from the *dut ung* strain CJ236, using the helper phage M13K07. Sequence alterations were confirmed by DNA sequencing.

Copy number analysis. A cleared lysate preparation (3) prepared from equal numbers of plasmid-bearing cells was applied to a nitrocellulose membrane. Known amounts of pMet1111 DNA were also applied to serve as DNA stan-

dards. The membrane was probed with nick-translated pMet1001 DNA, which hybridizes specifically to the ColE1-type plasmid. Hybridization and washing conditions were as described previously (12). To normalize for recovery and loading errors, the initial probe was washed off and the membrane was reprobed with nick-translated pRK248 DNA.

Construction of the promoterless *galK* vector pKO-1c. pKO-1c was derived from the promoterless *galK* vector pKO-1 (15) by inserting a synthesized polylinker at the *Sma*I site of pKO-1. The polylinker contained sites for the restriction enzymes *Sma*I, *Sal*I, *Sst*II, *Hin*dIII, *Bam*HI, and *Kpn*I.

Insertion of primer promoter into pKO-1c. A 1.8-kb *Kpn*I-*Pst*I fragment from pMet1111 containing the primer promoter
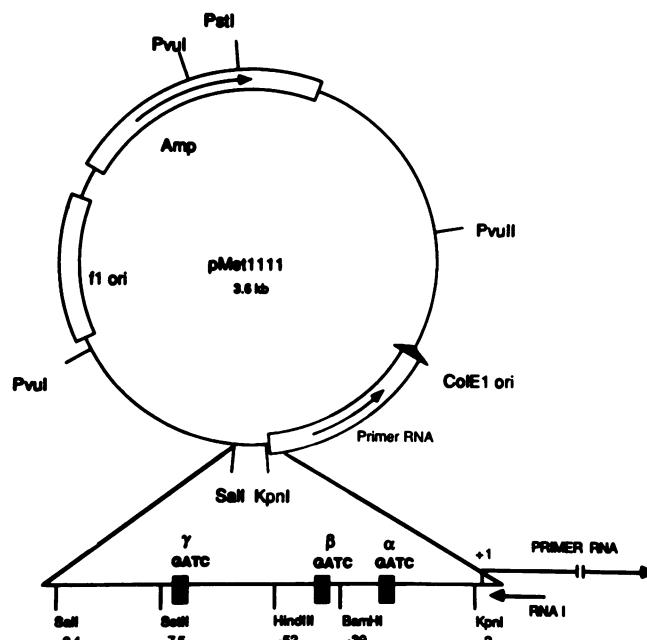

FIG. 2. Map of the parent plasmid pMet1111. The gene encoding for ampicillin resistance, the region encompassing the phage f1 origin of replication, and the section coding for primer RNA are boxed. The orientation of transcription units is indicated by arrows. All relevant restriction sites are shown. The immediate upstream region of the primer promoter has been expanded to show the position of the three Dam methylation sites (GATCs) and the installed restriction sites. The numerical coordinates are with reference to the start site of primer RNA transcription.

TABLE 1. Genotypes of *E. coli* strains used

Cell line	Genotype
CJ236	*dut-1 ung-1 thi-1 relA1*(pCJ105) (Cm^r)
DH5αF′	F′ *endA1 hsdR17* (r$_K^-$ m$_K^+$) *supE44 thi-1 recA1 gyrA96 relA1* φ80 *dlac* ZΔM15 Δ(*lacZYA-argF*)
GM3819	F$^-$ *dam-16* (deletion) *thr-1 leuB6 thi-1 argE3 hisG4 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 rpsL31 tsx-33 glnV44 rfbD1 kdgK51* Km^r
AB1157	*dam$^+$* parent of GM3819

with extensive upstream sequences was ligated to the 2.8-kb *Pst*I-*Kpn*I fragment of pKO-1c to create pGKK1111. Sequences downstream of the primer start site were introduced by ligating a 110-bp *Kpn*I-*Hae*III fragment containing the entire RNA I gene without its promoter into *Kpn*I-*Sma*I-cut pGKK1111. The resulting plasmid, called pGKH1111, has the wild-type primer promoter driving *galK* expression. The promoterless RNA I gene is oriented in the opposite direction. Equivalent plasmid constructs containing mutant primer promoters were constructed by exchanging the 1.8-kb *Kpn*I-*Pst*I fragment of pGKH1111 with the 1.8-kb *Kpn*I-*Pst*I promoter-bearing fragments from other pMet constructs.

Galactokinase assays. Galactokinase assays were performed as described by McKenney et al. (15). *dam*+ (AB1157) and *dam* (GM3819) cell lines harboring the promoter insertion constructs were grown overnight in 2× YT medium with ampicillin.

Stability assays. Wild-type or mutant plasmid constructs were transformed into the AB1157 (*dam*+) or GM3819 (*dam*) cell lines. Colonies from selective plates were transferred into liquid culture and grown overnight under selection. After overnight growth, 5 μl of this sample was reinoculated into 20 ml of fresh medium, selective pressure was removed, and cells were allowed to grow to saturation. This process was repeated three times. Cells were plated on selective and nonselective media to determine the percentage of cells bearing plasmids.

Transformation. Transformation was carried out as described by Mandel and Higa (11) as modified by Cohen et al. (7).

Construction of pMet1000pI. The 92-bp *Sal*I-*Kpn*I fragment from pMet1111 containing the three GATCs from the upstream region of the primer promoter was gel purified. Ends were filled in by using deoxyribonucleotides and T4 DNA polymerase as described by Maniatis et al. (12). The blunt-end fragment was then inserted into the *Pvu*II site of pMet1000. Plasmids were obtained with the insert in both orientations. These were called pMet1000pIa and pMet1000pIb.

RESULTS

Alteration of the methylation sites in the primer promoter. To facilitate site-directed mutagenesis of the DNA sequence upstream of the primer transcription start, we constructed a plasmid containing the ColE1 origin of replication, the genes encoding primer RNA and RNA I, and extensive upstream and downstream regions (nucleotides 2 to 1652 of ColE1 [6]). We inserted the replication origin of the single-stranded phage f1 to facilitate sequencing and oligonucleotide-directed mutagenesis. Plasmids described here have been numbered in a binary manner that designates whether the methylation sites in the primer promoter region are wild type or mutant. For example, pMet1111 has three wild-type GATC sites, whereas pMet1110 (described below) is mutant only in the α GATC site. (For details of construction, see Materials and Methods.)

Methylation site mutants were obtained by a series of steps of site-directed mutagenesis, using synthesized oligonucleotides. Table 2 shows the various methylation site mutants and the steps by which they were obtained. The first mutant obtained, designated pMet1110, has a single T-to-C transition at position −30 with respect to the start of primer transcription which eliminates the α GATC methylation site in the −35 region of the promoter. Furthermore, pMet1110 was expected to have reduced the stability of the putative

TABLE 2. Methylation site mutants and the parent plasmids from which they were derived by site-directed changes[a]

[a] *, Sequence alteration at the GATC site. The rest of the sequence (—) is identical in all plasmids.

cruciform structure in the primer promoter region by creating an A-C mismatch in the stem of the cruciform (Fig. 3).

Using pMet1111 DNA, pMet1110 DNA, and oligonucleotides that directed single changes to the β or γ methylation site, we constructed additional methylation site mutants. The change at the β methylation site was designed to complement the one at the α site by Watson-Crick base pairing, so that the αβ double mutant would have the potential to restore the cruciform structure. In this manner, the three methylation sites in the region immediately upstream of the primer were mutated in all possible combinations. We observed that all of the mutants were replication proficient and none required the presence of the single-stranded phage origin for plasmid maintenance.

Copy number analysis. We measured the copy number for each of the mutants in the cell line AB1157 (*dam*+) by slot blot hybridization. Samples were removed from cultures at logarithmic and stationary phases of growth (Fig. 4).

On the basis of copy number, the mutants could be classified into three groups. Group 1 consisted of plasmids pMet1011 (γ−), pMet1101 (β−), and pMet1001 (β−γ−), whose copy numbers in exponential phase were essentially identical to that of the wild-type plasmid pMet1111. Like the wild-type plasmid, their copy numbers showed no amplification at stationary phase. Copy numbers of the plasmids pMet1110 (α−) and pMet1010 (α−γ−) were twofold below those of the wild type. These constituted group 2 mutants. Group 3 mutants, consisting of plasmids pMet1100 (α−β−) and pMet1000 (α−β−γ−), showed a 10- to 12-fold-lower copy number during the logarithmic phase of growth in comparison with the wild-type plasmid. Furthermore, these plasmids underwent a three- to fourfold amplification in copy number in stationary phase.

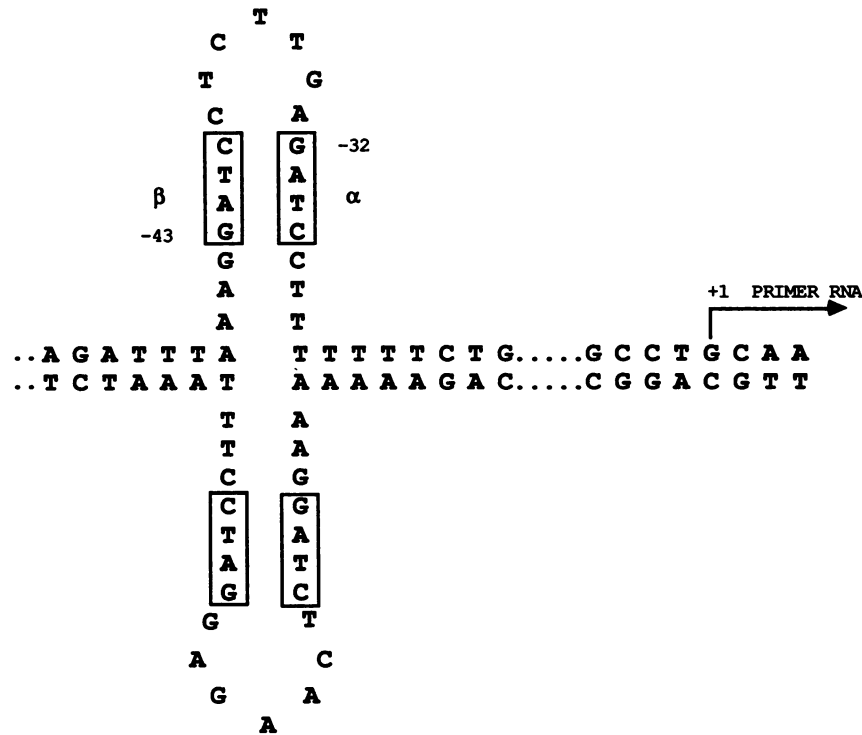**Promoter activity assay.** The copy number data were

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                        T
              C         T
              T         G
              C         A
             [C]       [G]  -32
        β    [T]       [A]  α
             [A]       [T]
        -43  [G]       [C]
              G         C
              A         T
              A         T
    ..A G A T T A   T T T T C T G.....G C C T G C A A
    ..T C T A A A T A A A A A G A C.....C G G A C G T T
              T         A
              T         A
              C         G
             [C]       [G]
             [T]       [A]
             [A]       [T]
             [G]       [C]
              G         T
              A         C
              G         A
                        A
```
(+1 PRIMER RNA)

FIG. 3. Putative cruciform structure postulated for the upstream region of the ColE1 primer promoter. The four Dam methylation sites, two on each strand, are boxed.

consistent with the idea that the mutations did not affect primer promoter strength except in the case of the α mutants. To assess promoter activity, we cloned the primer promoter region of each of the mutants, including extensive upstream and downstream sequences, into a promoterless galK vector, pKO-1c (see Materials and Methods). Promoter activities were determined by measuring galactokinase ac-
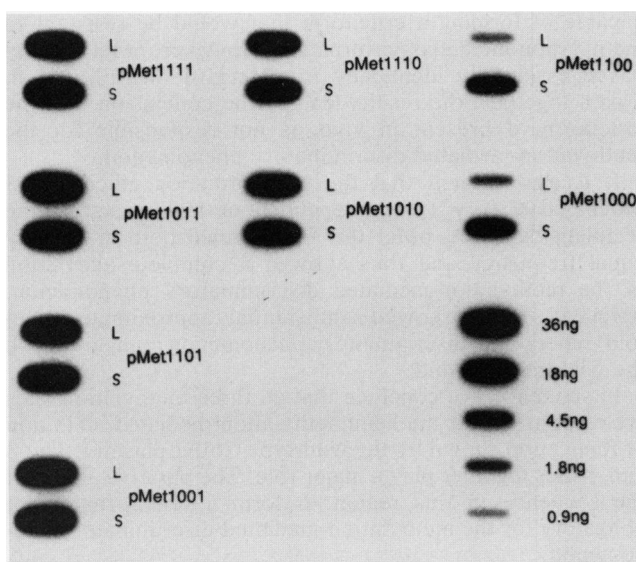

FIG. 4. Slot blot hybridization for determination of copy number of wild-type and mutant plasmid constructs at exponential (L) and stationary (S) phases of growth. Copy numbers were determined by comparison with the standards blotted onto the same membrane.

tivity in isogenic dam⁺ and dam cells containing the resulting plasmid constructs (Table 3).

Background levels of galK from the construct pGKS130, which has upstream sequences of the wild-type primer promoter but lacks the proximal SalI-to-HaeIII fragment present in the other plasmids, indicated no readthrough transcription from adventitious upstream promoters. Hence, galactokinase units were considered a direct measure of primer promoter strength for each of the mutants.

Promoter activities closely paralleled the copy number data. All of the α⁻ mutants showed lower promoter strength, with the α⁻β⁻ and α⁻β⁻γ⁻ mutant promoters being especially low. Strikingly, promoter strength did not change appreciably between a dam⁺ and a dam background. The approximately twofold difference measured between dam⁺ and dam cells for all promoters is a consequence of the fact that dam cells grown to the same optical density as dam⁺ cells have twofold fewer cells, as determined by plating (data not shown). This result is in general agreement with previous data (14). Note that both the galK promoter used as a control and the primer promoter from pMet1000, with no GATC sequences, showed the same twofold difference.

Stability assays. The reduction in promoter strength and consequent lower copy number seen with some of the mutant plasmids could conceivably result in reduced plasmid stability in vivo. To test this possibility, we carried out stability assays in both dam⁺ and dam genetic backgrounds.

Wild-type or mutant plasmids were transformed into AB1157 (dam⁺) and GM3819 (isogenic to AB1157 but dam) cell lines. Colonies were picked and transferred to a liquid culture. After overnight growth under selection, selective pressure was removed. Samples were taken at approximately 12-generation intervals and assayed for the presence or absence of the plasmid by plating on selective and nonselective

TABLE 3. Primer promoter strength of wild-type and mutant primer promoters in *dam*⁺ and *dam* cell lines[a]

PLASMID	PROMOTER INSERT	GALACTOKINASE UNITS	
		dam⁺	dam⁻
pKO-Ic	No promoter —gal K	7	N.D.
pGKS130	P S gal K	6	N.D.
pGKH 1111	P S H gal K	134	90
pGKH 1011	P S H gal K	148	77
pGKH 1101	P S H gal K	101	53
pGKH 1110	P S H gal K	39	19
pGKH 1001	P S H gal K	106	52
pGKH 1010	P S H gal K	36	30
pGKH 1100	P S H gal K	19	10
pGKH 1000	P S H gal K	18	9
pKG 1800	gal K promoter — gal K	166	109

[a] P, S, and H, Restriction enzyme sites for *Pst*I, *Sal*I, and *Hae*III, respectively. Shown are wild-type (■) and mutant (□) Dam methylation sites in the immediate upstream region of the primer promoter. Galactokinase units were calculated as described by McKenney et al. (15).

media. The $\alpha^-\beta^-$ and $\alpha^-\beta^-\gamma^-$ mutants were highly unstable (Fig. 5). Assuming random segregation of plasmid copies at cell division, the rate of loss corresponded to a theoretically calculated plasmid copy number of 3.5 per dividing cell. This value is in good agreement with the copy number empirically determined for these plasmids during the logarithmic phase of growth (Fig. 4). The copy number of the other α^- mutants, although twofold lower than the wild-type value, was apparently sufficiently high to preclude rapid loss.

Efficiency of transformation into *dam* and *dam*⁺ cell lines. We tested the GATC mutants for possible alleviation of the hemimethylation-mediated inhibition of replication. If this inhibition had been relieved in the GATC mutants, we would expect methylated DNA to transform *dam*⁺ and *dam* cell lines with equal efficiency. Transformations were carried out with methylated or unmethylated test DNA and the ColE1-compatible plasmid pRK248. The transformation efficiency of pRK248 showed no sensitivity to the *dam* state of the recipient cell (data not shown), and it was used to normalize the results obtained with the ColE1 derivatives.

Methylated wild-type plasmid pMet1111 transformed the *dam* strain GM3819 16-fold more poorly than it did the isogenic *dam*⁺ strain AB1157. In agreement with Russell and Zinder (23), we observed that unmethylated DNA transformed *dam*⁺ and *dam* cells with equal efficiency (Table 4).

pMet1101 (β^-) and pMet1110 (α^-) showed essentially wild-type behavior with respect to the ratio of their transformation efficiencies into *dam*⁺ or *dam* cell lines. Each of these mutations was expected to have destabilized the putative cruciform structure in the primer promoter region (Fig. 3). Furthermore, pMet1100 ($\alpha^-\beta^-$) is theoretically
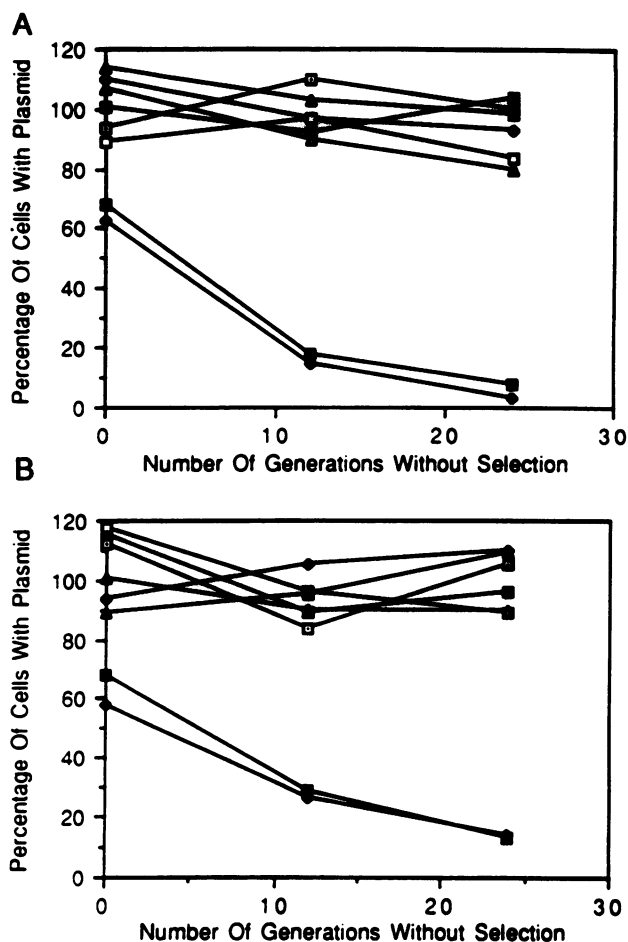

FIG. 5. Stability of wild-type and mutant plasmids in *dam*⁺ (AB1157) (A) and *dam* (GM3819) (B) cell lines. Symbols: ⊡, pMet1111; ◇, pMet1011; ■, pMet1101; □, pMet1110; ▲, pMet1001; △, pMet1010; ▣, pMet1100; ◆, pMet1000.

capable of forming a cruciform that would be resistant to methylation-mediated perturbation. However, pMet1100 behaved essentially identically to pMet1101 and pMet1110. Taken together, the results lead to the conclusion that the cruciform, if present in vivo, is not responsible for the methylation-mediated discriminatory phenomenon.

It is also evident that the transformation efficiency of pMet1000 ($\alpha^-\beta^-\gamma^-$) was independent of the *dam* state of the recipient cell; i.e., pMet1000 transformed both at roughly equal frequency and thus showed a complete alleviation of the methylation-mediated discriminatory phenomenon. pMet1001 ($\beta^-\gamma^-$) showed a substantial (approximately five-fold) relief of the discriminatory phenomenon compared with the wild-type plasmid.

In summary, we conclude that all three methylation sites are responsible for the hemimethylation-mediated inhibition of replication shown by the wild-type ColE1 plasmid. The β and γ sites together play a major role. The putative ability of the sequence in this region to form a cruciform is not obligatory for the methylation-mediated discriminatory phenomenon.

Evidence for position-specific effect of the primer promoter-associated GATCs. The ColE1-type plasmid pMet1111 used in this study has 22 GATC sites. The fact that relief of the hemimethylation-mediated inhibition of replication is

TABLE 4. Ratio of transformation frequencies of wild-type and mutant plasmids into isogenic *dam*⁺ (AB1157) and *dam* (GM3819) cell lines[a]

PLASMID	GATC	GATC	GATC	TRANSFORMATION FREQUENCIES dam⁺ / dam⁻
pMet1111	+	+	+	16.1
pMet1011	−	+	+	24.7
pMet1101	+	−	+	13.5
pMet1110	+	+	−	31.2
pMet1110*	+	+	−	1.9
pMet1001	−	−	+	3.3
pMet1010	−	+	−	24.7
pMet1100	+	−	−	19.2
pMet1000	−	−	−	1.6
pMet1000pI[b]	−	−	−	1.4
pBR322[c]	+	+	+	12.8

[a] CsCl-purified DNA isolated from a *dam*⁺ source was used in the transformations except where indicated (*). The transformation frequency for methylated plasmid pMet1111 transforming the *dam*⁺ host was 10⁷ transformants per μg of DNA. The asterisk identifies DNA isolated from a *dam* source. The ratio has been normalized by using the cotransformed ColE1-compatible plasmid pRK248 in every experiment. Results are averages of 10 independent experiments for pMet1111 and pMet1000 and at least 2 experiments for the remaining plasmids.

[b] pMet1000pIa and pMet1000pIb gave similar results.

[c] The γ GATC of pBR322 is at a different position relative to the start of primer RNA transcription.

achieved by mutating just three GATC sequences in the primer promoter indicates that it is not hemimethylation per se but hemimethylation of these three specific GATC sequences that controls the discriminatory phenomenon.

It could be argued that the spacing between the three methylation sites in the primer promoter is important and that three properly spaced GATC sequences anywhere on the plasmid could mediate the discriminatory phenomenon. To test this hypothesis, we inserted the 92-bp *Sal*I-*Kpn*I fragment of pMet1111, which includes all three methylation sites in the primer promoter, into the *Pvu*II site of pMet1000. The plasmid obtained was designated pMet1000pI.

pMet1000pI was used in a transformation experiment similar to that described above. Like its parent pMet1000, pMet1000pI showed complete alleviation of the discriminatory phenomenon (Table 4). We conclude that neither methylation sites per se nor a proper spacing of three GATC groups elsewhere on the plasmid is sufficient to mediate the discriminatory phenomenon. Rather, the discriminatory behavior is a position-specific effect.

DISCUSSION

The transformation results show that the α, β, and γ methylation sites collectively constitute the methylation switch effecting differential ColE1 plasmid establishment in *dam*⁺ and *dam* cells. Elimination of the β and γ methylation

sites reduces the discriminatory phenomenon substantially. However, elimination of the α site in conjunction with β and γ sites is essential to complete elimination of the discriminatory phenomenon exhibited by pMet1000.

In addition to methylation sites, the α and β GATC sequences in the primer promoter make up part of a 9-bp inverted repeat that could conceivably form a cruciform structure. Both pMet1101 (β⁻) and pMet1110 (α⁻) were expected to have destabilized the putative cruciform structure. Furthermore, the sequence of pMet1100 (α⁻β⁻) was expected to stabilize a methylation-resistant cruciform. Neither the destabilized nor the stable methylation-resistant putative cruciform was substantially different in discriminatory behavior from the wild-type plasmid pMet1111 from each other. This result leads us to conclude that the putative cruciform, if present in vivo, does not play a role in the methylation-mediated inhibition.

The observation that the placement of these sites elsewhere in the molecule does not restore methylation-mediated discriminatory behavior indicates that the sequence acts in a position-dependent manner. This action could conceivably be mediated through an effect on RNA I termination, primer transcription, or both. Mutation at position −32 with respect to primer transcription has been suggested to alter RNA I termination in the closely related plasmid CloDF13, which shows striking conservation of sequence and structural features to ColE1 (27). Although it is formally possible that some of the changes effected in pMet1000 could have altered RNA I termination, we think it unlikely because in vitro transcription experiments indicate no qualitative nor quantitative changes in RNA I produced from the plasmid pMet1000 (data not shown).

Several promoters in *E. coli* with GATC sequences in the vicinity of the RNA polymerase-binding site are known to be sensitive to methylation (2, 5, 13, 22). These include the promoters for *sulA*, *trpS*, *trpR*, *tyrR*, *mioC*, one of the two promoters driving *dnaA* expression, and the promoter for the Tn10 transposase gene. In the case of *mioC* and *dnaA2p*, the fully methylated promoters show a transcription rate twofold higher than those of the unmethylated promoters isolated from a *dam* cell. Methylation of the promoter-associated GATC in the Tn10 transposase gene leads to a significant reduction in the activity of the promoter, as measured in vivo and in vitro (22). Relative promoter strength is nearly 13-fold higher for the unmethylated promoter than the fully methylated one. The activity of the hemimethylated transposase promoter is also significantly above that of the fully methylated one but lower than that of the unmethylated promoter. Thus, transposase promoter activity progressively decreases with methylation. In contrast, the activities of the unmethylated and fully methylated ColE1 primer promoters are approximately equal, as determined from the results of the *galK* fusion experiments.

The activity of the primer promoter for pMet1000 is significantly less than that of the wild-type promoter in both *dam*⁺ and *dam* genetic backgrounds. This reduction has substantial effects on copy number and stability of the plasmid. Reduced promoter strength per se, however, cannot be implicated in the relief of the discriminatory effect for two reasons: (i) the primer promoter of pMet1100 is approximately equal in strength to the pMet1000 promoter yet shows wild-type discriminatory behavior, and (ii) pMet1001 shows a substantial relief of the discriminatory effect, with essentially no change in its primer promoter strength.

Several other hypotheses can be proposed to explain the methylation-mediated discriminatory phenomenon. Possi-

bly, the hemimethylated form of the primer promoter is involved with the binding of plasmid DNA to cell membranes. The replication origin of *E. coli oriC* and those of related bacteria are characterized by an unusually high density of GATC sequences (25, 28, 29). Ogden et al. (20) showed that hemimethylated *oriC* DNA bound to membranes in vitro whereas the fully methylated or unmethylated species did not. The association of plasmid pSC101 with membranes has been observed (8). We do not know whether similar transient binding of ColE1 DNA occurs in vivo. Alternatively, modulation of primer transcription could be achieved by the binding of certain host proteins to the primer promoter that are sensitive to the methylation state of their target sequences. For example, the *E. coli* regulatory protein OxyR is involved in the methylation-mediated regulation of the *mom* gene in bacteriophage Mu (4). OxyR protein binding to unmethylated or hemimethylated GATC sequences in the promoter region of the phage Mu *mom* gene is thought to exclude the positive activator of *mom* transcription, the Mu gene C product (4). This binding has the effect of significantly reducing *mom* gene transcription. Whether a similar process occurs in the ColE1 promoter is unknown.

Regardless of the mechanism, the biological relevance of the methylation-mediated discriminatory phenomenon remains obscure. The inefficiency with which replication is initiated on a hemimethylated plasmid molecule suggests that relief of methylation-mediated discrimination would disrupt timing of initiation (16). Since ColE1-type plasmids have been shown to replicate randomly with respect to the cell cycle (10), it is hard to see how timing could be further disrupted. However, spacing of initiation events in a single plasmid molecule may be advantageous in that it would allow time for proper resolution of newly replicated DNA, a step that might be rate limiting in the production of daughter molecules. Furthermore, by preventing a new initiation event from occurring in a molecule that has recently replicated, methylation-mediated discrimination may promote the maintenance of diversity in a pool of replicating plasmid molecules.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Abeles, A. L., and S. Austin.** 1987. P1 plasmid replication requires methylated DNA. EMBO J. **6:**3185–3189.
2. **Barras, F., and M. Marinus.** 1989. The great GATC: DNA methylation in *E. coli.* Trends Genet. **5:**139–143.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Bolker, M., and R. Kahmann.** 1989. The *Escherichia coli* regulatory protein OxyR discriminates between methylated and unmethylated states of the phage Mu *mom* promoter. EMBO J. **8:**2403–2410.
5. **Braun, R. E., and A. Wright.** 1986. DNA methylation differentially enhances the expression of one of the two *E. coli* dnaA promoters in vivo and in vitro. Mol. Gen. Genet. **202:**246–250.
6. **Chan, P., H. Ohmori, J. Tomizawa, and J. Lebowitz.** 1985. Nucleotide sequence and genome organization of ColE1 DNA. J. Biol. Chem. **260:**8925–8935.
7. **Cohen, S. M., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA

69:2110–2114.
8. **Gustafsson, P., H. Wolf-Watz, L. Lind, K. Johansson, and K. Nordstrom.** 1983. Binding between the *par* region of plasmids R1 and pSC101 and the outer membrane fraction of the host bacteria. EMBO J. **2:**27–32.
9. **Kunkel, T. A., J. Roberts, and R. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotype selection. Methods Enzymol. **154:**367–382.
10. **Leonard, A., and C. E. Helmstetter.** 1986. Cell cycle-specific replication of *Escherichia coli* minichromosomes. Proc. Natl. Acad. Sci. USA **83:**5101–5105.
11. **Mandel, M., and A. Higa.** 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53:**159–162.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Marinus, M. G.** 1987. DNA methylation in *Escherichia coli.* Annu. Rev. Genet. **21:**113–131.
14. **Marinus, M. G., and N. R. Morris.** 1974. Biological function for 6-methyladenine residues in the DNA of *Escherichia coli* K12. J. Mol. Biol. **85:**309–322.
15. **McKenney, K., H. Shimatake, D. Court, U. Schmeissner, C. Brady, and M. Rosenberg.** 1982. A system to study promoter and termination signals recognized by *Escherichia coli* RNA polymerase p. 383–417. In J. Chirikjian and T. Papas (ed.), Gene amplification and analysis, vol. 2. Elsevier/North-Holland Publishing Co., Amsterdam.
16. **Messer, W., and M. Noyer-Weidner.** 1988. Timing and targeting: the biological functions of Dam methylation in *E. coli.* Cell **54:**735–737.
17. **Morlon, J., M. Chartier, M. Bidaud, and C. Lazdunski.** 1988. The complete nucleotide sequence of the colicinogenic plasmid ColA. High extent of homology with ColE1. Mol. Gen. Genet. **211:**231–243.
18. **Muesing, M. A., J. Tamm, H. Shepard, and B. Polisky.** 1981. A single base-pair mutation is responsible for the DNA overproduction phenotype of a plasmid copy-number mutant. Cell **24:**235–242.
19. **Murchie, A. I., and D. Lilley.** 1989. Base methylation and local DNA helix stability. Effect on the kinetics of cruciform extrusion. J. Mol. Biol. **205:**593–602.
20. **Ogden, G. B., M. Pratt, and M. Schaechter.** 1988. The replicative origin of the *E. coli* chromosome binds to cell membranes only when hemimethylated. Cell **54:**127–135.
21. **Polisky, B.** 1988. ColE1 replication control circuitry: sense from antisense. Cell **55:**929–932.
22. **Roberts, D., B. Hooper, W. McClure, and N. Kleckner.** 1985. IS10 transposition is regulated by DNA adenine methylation. Cell **43:**117–130.
23. **Russell, D. W., and N. Zinder.** 1987. Hemimethylation prevents DNA replication in *E. coli.* Cell **50:**1071–1079.
24. **Selzer, G., T. Som, T. Itoh, and J. Tomizawa.** 1983. The origin of replication of plasmid p15A and comparative studies on the nucleotide sequence around the origin of related plasmids. Cell **32:**119–129.
25. **Smith, D. W., A. Garland, G. Heiman, R. Enns, T. Bakes, and J. Zyskind.** 1985. Importance of methylation of *oriC* GATC sites in initiation of DNA replication in *Escherichia coli.* EMBO J. **4:**1319–1326.
26. **Tomizawa, J., T. Itoh, G. Selzer, and T. Som.** 1981. Inhibition of ColE1 RNA primer formation by a plasmid specified small RNA. Proc. Natl. Acad. Sci. USA **78:**1421–1425.
27. **van Putten, A. J., R. de Lang, E. Veltramp, J. Nijramp, J. John, P. van Solinge, and J. van der Berg.** 1986. Methylation-dependent transcription controls plasmid replication of the CloDF13 wp-1(Ts) mutant. J. Bacteriol. **168:**728–733.
28. **Zyskind, J. W., J. M. Cleary, W. S. A. Brusilow, N. E. Harding, and D. W. Smith.** 1983. Chromosomal replication origin from the marine bacterium *Vibrio harveyi* functions in *Escherichia coli: oriC* consensus sequence. Proc. Natl. Acad. Sci. USA **80:**1164–1168.
29. **Zyskind, J. W., and D. Smith.** 1986. The bacterial origin of replication, *oriC.* Cell **46:**489–490.