DNA Methylation Inhibits the Transfecting Activity of Replicative-Form ϕ X174 DNA

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Replacement of virtually all the cytosine residues with 5-methylcytosine residues in the complementary strand of the replicative form (RF) of ϕ X174 DNA caused a 300- to 500-fold loss in its transfecting activity. Similar results were obtained with analogously methylated M13 RF. Transfection experiments with ϕ X RF hemimethylated in only part of the molecule, as assessed by analysis with restriction endonucleases, indicated that gene A of ϕ X, which needs to be nicked at a specific site by the gene A protein for RF replication, was not the main target for this inhibition by DNA methylation. We propose that the loss of transfecting activity was due to hemimethylation of the ϕ X RF interfering with the processively catalyzed movement of the replication fork.

5-Methylcytosine (m^5C) is found commonly as a minor base in procaryotic DNA, invariably as a minor component of the genomes of mammals and vascular plants (10), and rarely as a major base in bacteriophage DNA (9, 21). In procaryotes in which m^5C is present at low levels in the DNA, the only known function of DNA-cytosine methylation is prevention of restriction of host DNA (29). DNA methylation in mammalian cells may be involved in the control of transcription, as well as in other processes exclusive of restriction (7, 10, 24, 31). To gain insight into the effects of methylation on the biological activity of DNA, we examined the transfecting activity of extensively methylated replicative-form (RF) DNA from ϕ X174.

The single-stranded viral DNA from this *Escherichia coli* phage was used as a template for in vitro DNA synthesis in the presence of *E. coli* DNA polymerase I, primer, and a deoxynucleoside triphosphate mixture containing 5-methyl-deoxycytidine triphosphate (m^5dCTP) instead of dCTP (12) to yield DNA methylated only in the complementary strand. We show that such hemimethylated ϕ X RF had less than 1% the transfecting activity of the comparable nonmethylated DNA. Similar results were obtained from the analogously hemimethylated RF of phage M13. We have also studied the transfecting activity of ϕ X RF methylated to different extents and in different regions of the genome to elucidate the mechanism by which methylation was interfering with its biological activity.

MATERIALS AND METHODS

Preparation of methylated DNA. To obtain covalently closed circular RF molecules in which virtually the entire complementary strand was methylated, 1 μ g of ϕ X174 or M13mp8 (25) viral-strand DNA was used as a template, and 30 ng of an oligonucleotide mixture derived from DNase I-treated RF I was the primer in a reaction catalyzed by *E. coli* DNA polymerase I and T4 DNA ligase (S. Shenoy and M. Ehrlich, manuscript in preparation). The nucleoside triphosphates in this reaction mixture were 75 μ M dTTP, dGTP, [^{32}P]dATP (13 Ci/mmol), and m^5dCTP , as well as 1 mM ATP. The reaction mixtures also contained 1 U of dUTPase (4) from HeLa cells (6). Covalently closed circular double-stranded DNA was isolated from these reaction

mixtures by alkaline denaturation, neutralization, and then filtration through a nitrocellulose membrane (Shenoy and Ehrlich, in preparation) or, where indicated, by centrifugation in an ethidium bromide-cesium chloride density gradient (8). The control DNA preparations are referred to as non-methylated because they were neither substituted with m^5C residues in vitro nor methylated with *HpaII* methylase (see below). However, these DNA molecules do contain a small number of sites with m^5C or N^6 -methyladenine residues introduced in vivo by host modification enzymes (5, 13, 14, 17). For example, by assessing the sensitivity of in vivo-derived M13 RF I and in vitro-synthesized M13 RF IV to *MboI* and *DpnI* (37), we showed that M13 RF I and viral DNA are methylated at the adenine residues of GATC sites (5).

Lower extents of ϕ X RF methylation were obtained by including both m^5dCTP and dCTP in various ratios in the reaction mixture or by methylating in vivo-derived RF I with *HpaII* methylase (42), which symmetrically methylates only CCGG sites. In addition, we prepared ϕ X DNA molecules hemimethylated only outside of the gene A region by primer extension with the Klenow fragment of *E. coli* DNA polymerase I (18), with [3H]dTTP as the source of radiolabel. For this reaction, the largest fragment in a *HpaII* digest was purified by electrophoresis, denatured, and then annealed to ϕ X viral strands as described by Baas and Jansz (3), except that sodium chloride was used instead of sodium citrate.

Transfection assays. Unless otherwise noted, spheroplasts of *E. coli* BD1213 (*ung-1 thyA*) or BD1212 (*ung⁺ thyA*) from H. Warner (40) were used as host cells for transfection with ϕ X RF. The ϕ X phage stock was supplied by P. Weisbeek. Standard transfection techniques were used (15, 19). These bacterial strains are resistant to infection by intact ϕ X; phage-sensitive *E. coli* C122 from P. Weisbeek was used as the indicator. The phenotype of *ung* and *ung⁺* cells was verified by assaying crude extracts for enzymatic activity by thin-layer chromatographic detection of the release of uracil from [^{14}C]uracil-containing DNA (38). For transfection with M13 RF, calcium-shocked (22) *E. coli* JM101 (43) was used as the host. The same strain served as the indicator. Unless otherwise stated, transfection was monitored by infective-center assays in which spheroplasts or calcium-shocked cells were diluted and plated for plaque assay within 30 min after the phage DNA and the bacteria were mixed.

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Restriction analysis. To monitor in vitro methylation of cytosine residues, hemimethylated phage [32 P]DNA or [3 H]DNA was treated with one of various restriction endonucleases (4 U/ μ g of DNA) for 1 h under standard conditions specified by the manufacturer (Bethesda Research Laboratories or New England BioLabs). In each reaction mixture, unlabeled ϕ X RF I was included as an internal control, and separate reactions were also conducted with analogously prepared, nonmethylated, radiolabeled DNA. Digests were analyzed by agarose gel electrophoresis followed by visualization of the DNA by autoradiography, fluorography, or ethidium bromide-induced fluorescence. In all cases, control DNA samples were digested as expected.

RESULTS

Preparation and characterization by restriction analysis of hemimethylated ϕ X RF. To study the effect of DNA methylation on transfection, we prepared relaxed, covalently closed circular DNA (RF IV) molecules with essentially complete replacement of cytosine residues by m^5 C residues in the complementary strand of ϕ X174 RF and of M13 RF. 32 P-labeled hemimethylated RF and control nonmethylated RF molecules were synthesized by extension of a heterogeneous mixture of oligonucleotide primers present in a 1:33 ratio (wt/wt) of primer to viral DNA template and purified as described above. The m^5 C-substituted RF was shown to be hemimethylated by its resistance to digestion by *Hpa*II and *Hha*I (23), in contrast to the normal cleavage of the internal control, nonmethylated ϕ X RF I. However, a small amount (<10%) of the hemimethylated RF IV was converted to the open circular form (RF II) by *Hpa*II and to an approximately equimolar mixture of RF II and full-length linear molecules (RF III) by *Hha*I. This limited conversion of RF IV to RF II could have been due to the nicking reaction not being completely inhibited at hemimethylated recognition sites, as for *Sau*3A (33), or to contamination of the restriction enzyme with a nonspecific endonuclease. *Hha*I has about four times as many cleavage sites on ϕ X RF as does *Hpa*II. The slight extent of linearization of the hemimethylated RF IV by *Hha*I suggests that most but not all of the random collection of unmethylated, priming oligonucleotides were removed by the 5'→3' exonucleolytic activity of DNA polymerase I. The m^5 C-substituted M13 RF IV was similarly shown to be hemimethylated by its resistance to *Hpa*II and *Hha*I.

We also examined the ability of *Tha*I, *Sst*II, *Cfo*I, *Acc*I, and *Dde*I to cleave hemimethylated DNA. The sensitivity of these enzymes to methylation of cytosine residues had not been described previously. We found that each of these enzymes was inhibited by hemimethylation of ϕ X DNA, although incubation with the first four enzymes gave a slight amount (<10%) of RF II. In the *Dde*I reaction mixture, ~30% of the hemimethylated RF IV was converted to RF II, and in both the *Dde*I and *Cfo*I reaction mixtures, ~5% of this DNA was converted to RF III. Formation of RF III was probably due to there being a large number of restriction sites for these enzymes on ϕ X DNA RF, so that ~5% of the molecules had unmethylated primer remaining at one of the *Dde*I or *Cfo*I restriction sites. It had previously been reported that *Hpa*I and *Hinf*I could cleave hemimethylated ϕ X RF DNA (12). However, we reproducibly showed that *Hpa*I was unable to cleave this DNA except for a very small amount of nicking and that although *Hinf*I cleaves hemimethylated RF IV, it does so very slowly. Similarly, *Hinf*I cleaves the naturally m^5 C-rich (20, 21) phage XP12 DNA abnormally slowly (16).

Transfection by ϕ X RF containing essentially complete substitution of m^5 C for cytosine in the complementary strand. Purified ϕ X RF DNA with the complementary strand having virtually complete replacement of cytosine residues with m^5 C residues was compared with analogously prepared, nonmethylated RF IV DNA in transfection assays. In both infective-center assays (Table 1) and progeny-phage assays with *E. coli* BD1212 spheroplasts, the transfecting activity of uniformly hemimethylated ϕ X RF IV DNA was ca. 0.2% that of the corresponding nonmethylated DNA. In the experiment for Table 1, the transfecting activities of in vivo-derived ϕ X RF I and viral DNA were 1.1×10^8 and 2.1×10^8 infective centers per μ g of DNA.

A similar decrease in transfecting activity upon hemimethylation was observed in three different preparations of M13 RF IV (Table 1). Mixing experiments showed that no inhibitory contaminants were present in either hemimethylated ϕ X or M13 RF IV preparations. Also, nuclease S1-treated preparations of hemimethylated ϕ X RF IV and nonmethylated RF IV gave the same decrease in transfecting activity due to hemimethylation.

To avoid the possibility of artifactual decreases in transfecting activity due to contamination of polymerization mixtures with dUTP, dUTPase was present during in vitro DNA synthesis (4). However, the same results were obtained with or without dUTPase in the M13 RF polymerization mixture. Even replacing 1% of the dTTP with dUTP in the triphosphate mixture during the polymerization had relatively little effect on the transfecting activity of the resulting M13 RF IV, whether or not it was substituted with m^5 C residues. Furthermore, transfection of *E. coli* BD1212 (*ung*⁺) and BD1213 (*ung*) spheroplasts with ϕ X RF IV molecules purified either by denaturation and nitrocellulose filtration or by cesium chloride-ethidium bromide density gradient centrifugation gave the same results, that is, a 300- to 500-fold lower transfection efficiency of hemimethylated ϕ X RF than of nonmethylated ϕ X RF.

Transfection by ϕ X RF with different ratios of m^5 C to cytosine in the complementary strand. Given the large decrease in transfecting activity of ϕ X RF due to uniform hemimethylation, we tested the effect on transfection of varying the proportions of m^5 C to cytosine residues in the ϕ X RF. First, m^5 C was only partially substituted for cyto-

TABLE 1. Transfection by ϕ X RF or M13 RF with virtually all of their cytosine residues in the complementary strand methylated

DNA sample	Hemimethylated	Transfecting activity ^a (infective centers/ μ g of DNA)
ϕ X RF IV ^b	+	1.2×10^5
	—	5.6×10^7
M13 RF IV	+	8.4×10^3
	—	3.7×10^6

^a For transfection with ϕ X DNA, *E. coli* BD1212 spheroplasts were the recipients and *E. coli* C was the indicator. With M13 RF IV, calcium-shocked *E. coli* JM101 cells were the recipients and the same untreated strain was the indicator.

^b RF IV (relaxed, covalently closed double-stranded circular DNA) was synthesized with mixed oligonucleotides, viral DNA template, and either m^5 dCTP or dCTP in the polymerization mixture as described in the text. From restriction analysis it can be concluded that ~0.3% of the hemimethylated RF IV DNA sequences were symmetrically unmethylated due to residual primer.

sine in randomly determined positions on the complementary strand by including both m^5dCTP and $dCTP$ in different ratios in the RF IV synthesis mixture. Replacing half of the cytosine residues on the complementary strand with m^5C residues gave a 20-fold decrease in transfection activity, whereas substitution of $\sim 1/10$ of these cytosine residues resulted in a loss of only $\sim 60\%$ of the transfecting activity (Table 2). When only $\sim 1\%$ of the cytosine residues on the complementary strand were methylated, there was no decrease in transfecting activity.

The second type of partially methylated ϕX RF used in these experiments was in vivo-derived RF I which had been methylated with *HpaII* methylase. This RF was shown to be symmetrically methylated at all CCGG sites by retention of 100% of its transfecting activity after incubation with *HpaII* restriction endonuclease. Like a low extent of methylation of cytosine residues in random positions on the complementary strand, symmetrical methylation at the *HpaII* sites of ϕX RF I did not affect its transfecting activity (Table 2).

Transfection by ϕX RF with hemimethylation of only part of the chromosome. Almost half of the genome, excluding the origin for RF and viral-strand DNA replication in gene A and the three transcriptional promoters, was hemimethylated by extension in the presence of m^5dCTP , $dTTP$, $dATP$, and $dGTP$ of a large *HpaII* fragment that was ~ 3 kilobase pairs (kb) (Fig. 1) annealed to ϕX viral DNA. The syntheses of the previous hemimethylated RF molecules employed the intact *E. coli* DNA polymerase I. To synthesize RF for this experiment, the Klenow fragment (18) was used to avoid polymerization proceeding into the 5' end of the primer by nick translation and thereby hemimethylating gene A. However, commercial preparations of the Klenow fragment are often contaminated with residual 5' $\rightarrow$ 3' exonuclease activity. Restriction analysis (Fig. 2) indicated that such contami-

TABLE 2. The effect of varying the ratio of cytosine to m^5C residues in the complementary strand on the transfecting activity of ϕX RF

DNA sample	Ratio of m^5C : cytosine in the minus strand	Transfecting activity ^a (infective centers/ μg of DNA)	% Transfecting activity remaining after methylation
RF IV ^b	0:1	3.5×10^7	
RF IV	1:100	3.5×10^7	100
RF IV	1:10	1.5×10^7	43
RF IV	1:1	1.8×10^6	5
RF IV	1:0	1.2×10^5	0.3
<i>HpaII</i> -methylated ^c RF I DNA	1:300	3.6×10^7	113
Mock-methylated ^d $\phi X174$ RF I DNA	0:1	3.2×10^7	
Untreated $\phi X174$ RF I ^d	0:1	4.5×10^7	

^a Transfection activity was determined on *E. coli* BD1213 spheroplasts with *E. coli* C as the indicator strain.

^b The RF IV samples were prepared as described in footnote b, Table 1, except with various ratios of m^5dCTP to $dCTP$ in the polymerization mixture.

^c This sample is the only one tested which was symmetrically methylated, that is, also methylated in the viral strand. Its transfecting activity is to be compared with that of mock-methylated RF I, which was prepared identically, except in the absence of *S*-adenosylmethionine.

^d The minus strand of $\phi X174$ RF I actually contains $\sim 0.2\%$ of its cytosine residues methylated when ϕX is propagated on *E. coli* C (14).

FIG. 1. Relevant restriction map of $\phi X174$ DNA. The *HpaII*-digested fragment used as a primer during in vitro DNA synthesis is indicated by the hatched region (see below). The replication origin (O), three promoters (P), and the gene A region of ϕX are also shown. From a comparison of our ϕX RF preparation, a commercially obtained $\phi X am3$ RF preparation (Bethesda Research Laboratories), and the known sequence of $\phi X174$ DNA (30), two alterations in the restriction pattern of our DNA were noticed. One was at the junction of *HpaII*-digested fragments 1 and 4, which resulted in the largest fragment in the *HpaII* digest of our ϕX RF preparation, with a length of 3,096 base pairs instead of 2,748 base pairs (30). Therefore, we used a fused fragment (fragment 1 plus fragment 4) as the primer for DNA synthesis in the experiment described in Table 3. The other missing restriction site was at the junction of *AluI* fragments 3 and 9. This gave rise to a fragment with a length of 808 base pairs, which migrated very close to the *AluI* fragment 2 (Fig. 2). Both missing restriction sites were in the gene H region but resulted in no noticeable change in the phenotype of the phage propagated on the host cells used in this study.

nating activity was present in the Klenow fragment preparation which we used; however, a sufficiently large fraction of the RF molecules synthesized were bifilarly unmethylated in the gene A region, which allowed us to examine the effect of regional hemimethylation. The partially hemimethylated RF molecules labeled with [3H]dTTP during their in vitro synthesis were digested with *HhaI* in the presence of unlabeled, in vivo-derived RF I and then subjected to gel electrophoresis. The RF I and a nonmethylated in vitro-synthesized RF preparation were completely digested with *HhaI*, in contrast to the partially hemimethylated RF. The distribution of radioactivity in the gel (Fig. 2) indicated that slightly more than one-third of the latter RF molecules had no m^5C residues in ca. 60% of their length, including the gene A region (Fig. 1). The rest of the labeled molecules in this *HhaI* digest were full length due to nick translation through the primer.

This partially hemimethylated RF preparation on the average had only $\sim 2\%$ of the transfection activity found in an analogous preparation of nonmethylated RF (Table 3); a mixing experiment indicated that no inhibitors of transfection were present. If the RF molecules which retained intact primer had normal transfecting activity, there should have

FIG. 2. Restriction analysis of the in vitro-synthesized, regionally hemimethylated ϕ X174 RF DNA used for transfection. Samples of nitrocellulose-purified ^3H -labeled ϕ X RF (4×10^4 cpm; 2×10^6 cpm/ μg), which was prepared by extension of a ~ 3 -kb primer in the presence of $[\text{H}]$ dTTP as described in the legend to Table 3, were each mixed with $2 \mu\text{g}$ of unlabeled ϕ X RF I DNA as an internal control for complete digestion. These DNAs were digested with four U of the indicated restriction endonuclease per μg of DNA at 37°C for 2 h. Upon electrophoresis in 2% agarose gels, the unlabeled DNA was observed to be completely digested. This figure shows the fluorograph of the gel containing the following $[\text{H}]$ RF digests: lanes 1 through 4, m^5C -substituted, partially hemimethylated DNA; lanes 5 through 8, nonmethylated DNA. The enzymes used for digestion were as follows: lanes 1 and 5, no enzyme control; lanes 2 and 6, *Hpa*II; lanes 3 and 7, *Hha*I; lanes 4 and 8, *Alu*I. On the right are indicated the molecular lengths (in base pairs) of the restriction fragments from nonmethylated DNA, and on the left are indicated the positions of RF II and RF III; RF IV coelectrophoresed with RF II under the conditions used for electrophoresis. The only portions of the RF which are visualized in this fluorograph are those that contain sequences which were replicated by extension of the intact primer or by nick translation involving $5' \rightarrow 3'$ exonucleolytic removal of primer residues. The lowest molecular weight band from *Hha*I-digested, partially hemimethylated RF (lane 3) was calculated to be ~ 2.9 kb long, and therefore it represents a large fragment composed of *Hha*I fragments 2, 3, 5b, 7b, 8a, 5a, 6, 9, 11, 13, 10, and 12 (Fig. 1). This large fragment was derived from RF molecules in which the primer was intact, that is, in which somewhat more than half of the RF remained nonmethylated. The size and relative amount of the smallest *Alu*I fragment from *Alu*I-digested, partially hemimethylated RF (~ 2.9 kb; lane 4) is consistent with this conclusion. The RF II and RF III molecules in lanes 3 and 4 probably arose from complete removal of the primer by $5' \rightarrow 3'$ exonucleolytic activity contaminating the Klenow fragment (see the text). Bands from the samples analogous to those of lanes 3 and 4 in another, identical gel were excised, and their radioactivity was determined. The percentage of molecules in the three bands was determined by normalizing for the different size of the ~ 2.9 -kb fragments compared with the full-length RF II and RF III.

been no more than a 66% loss in transfecting activity due to methylation. Therefore, even methylation only outside the replication origin of the viral strand and gene A, the only early gene (35), caused more than an order of magnitude decrease in transfecting activity.

In another set of experiments, only a short region (~ 600 nucleotides) of ϕ X DNA was replicated in the presence of m^5dCTP by using the same large *Hpa*II fragment as a primer. This DNA could not be separated from the unreplicated template, so that nonmethylated viral strand was present as

a contaminant. Nonetheless, the transfecting activity of this DNA, as well as that of fully replicated, but not purified, uniformly hemimethylated RF II molecules, was reproducibly half that of a nonmethylated ϕ X DNA molecule synthesized analogously. Similar results were obtained when *Hpa*II fragment 2 (30) was used as a primer for the synthesis of only ~ 300 nucleotides. Therefore, substitution of ~ 5 to 10% of the ϕ X RF in two widely separated regions exclusive of the gene A region caused at least a 50% reduction in transfecting activity.

DISCUSSION

Both ϕ X174 RF and M13 RF lost $>99\%$ of their transfecting activity when they were hemimethylated throughout the DNA (Table 1). If the hemimethylated, transfecting RF DNA is replicated normally during its first round of replication, it would probably escape from any major effects of methylation on the subsequent phage yield. Of the final yield of progeny RF molecules, all but one will contain the normal base composition, and so these should be replicated and transcribed normally. Furthermore, it is highly unlikely that these hemimethylated RF molecules are selectively and efficiently restricted during uptake into the host cells. The only known precedent for a bacterial restriction system requiring DNA methylation is *Dpn*I, which is specific for GATC sequences methylated at position 6 of the adenine residue (37). However, this enzyme, like most type II restriction endonucleases, will not fragment DNA that is hemimethylated in its recognition sites (37). We conclude that the loss of transfecting activity in the hemimethylated ϕ X RF and M13 RF was due to inhibition of DNA replication during synthesis of the first two progeny RF molecules. This inhibition could have resulted from interference with the functioning of the replication machinery or from blocking production of the replication-associated, phage-encoded gene A protein in the case of ϕ X (35) or gene II protein in the case of M13 (27).

We analyzed the transfecting activity of ϕ X RF molecules hemimethylated only in the half of the ϕ X RF distant from the gene A region, which contains the origin of RF replication and viral-strand synthesis. The transfecting activity of these molecules was also greatly reduced (Table 3). We conclude that the loss of normal transfecting activity upon methylation is not simply due to extensive modification of the complementary strand opposite the site which is specifically nicked for primer formation by the gene A protein and later ligated by this protein during RF replication (11, 28, 36). We can similarly conclude that the loss of biological activity is not primarily due to inhibition of transcription of gene A, the only phage gene necessary for ϕ X RF replication (41). Priming of viral-strand synthesis during RF replication was, therefore, not the main target for the biological effects of hemimethylation. Also, this hemimethylation should not have affected priming of complementary-strand synthesis because such priming occurs on the single-stranded form of the newly synthesized viral strand (2), which in this experiment had the normal base composition. It is unlikely that DNA methylation causes a drastic decrease in the in vivo polymerization rate catalyzed by DNA polymerase III and thereby is responsible for the decrease in transfecting activity, since $\text{m}^5\text{C} \cdot \text{guanine}$ base pairs in DNA are even more stable than cytosine $\cdot$ guanine base pairs (9), and m^5dCTP was shown to be incorporated in vitro as efficiently as is dCTP by another *E. coli* polymerase, DNA polymerase I (39). From the relationship between the decrease in transfecting activity and the percentage of substituted cytosine

TABLE 3. Transfection with ϕ X RF with m^5C substitution at sequences outside the gene A region

Expt no.	Transfecting activity ^a			
	Infective centers/ μ g of DNA		Progeny phage/ μ g of DNA ^b	
	Methylated RF	Nonmethylated RF	Methylated RF	Nonmethylated RF
1	2.3×10^5	8.9×10^6	1.2×10^7	7.6×10^8
2	2.7×10^5	1.2×10^7		
3	2.0×10^5	8.1×10^6		

^a These transfection assays were done with partially hemimethylated or nonmethylated ϕ X RF prepared by extension of the largest denatured *Hpa*II fragment (~3 kb; Fig. 1 and 2) by the Klenow fragment with either m^5dCTP or $dCTP$ in the triphosphate mixture. As explained in the text, slightly more than one-third of the hemimethylated RF molecules had about 60% of the molecule nonmethylated due to the persistence of the unmethylated primer. The RF molecules used in these experiments were prepared in the presence of DNA ligase and were purified by nitrocellulose filtration but without a prior denaturation step, so that they consisted of RF IV and RF II (without extensive gaps). The transfection efficiency of in vivo-derived ϕ X RF I on the batch of *E. coli* BD1213 spheroplasts which was used for these experiments was 2.2×10^7 infective centers per μ g of DNA.

^b Progeny phage obtained after 90 min of incubation of transfected spheroplasts at 37°C were released by the addition of 0.1 volume of chloroform and then titered on *E. coli* C.

residues (Table 2), it appears that the RF does not contain a specific target sequence for this inactivation, which has only one or two cytosine residues.

We propose that ϕ X RF replication and the somewhat similar replication of M13 RF (26, 32) were inhibited by extensive DNA methylation due to impaired movement of the replication fork. This could have resulted from perturbations in normal protein-DNA interactions or from direct retardation of the localized denaturation which accompanies DNA replication. In the first case, m^5C -rich regions might be recognized abnormally by the *E. coli* *rep* protein (helicase), which catalyzes the separation of strands in M13 RF (26, 34) or by the *rep* protein-gene A protein complex, which catalyzes unwinding of the ϕ X RF (1, 2, 11). Alternatively, *E. coli* single-stranded DNA-binding protein, which facilitates strand separation (1, 26), may bind poorly to denatured regions of m^5C -rich DNA. Also, enrichment of DNA with m^5C might impede movement of the replication complex by the known stabilizing effect of DNA cytosine methylation on the secondary structure of DNA (9). Either way, it is possible that short, naturally occurring m^5C -rich sequences in various procaryotic or eucaryotic DNAs (10) might modulate rates of DNA replication in vivo.

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