Cloning and analysis of the genes encoding the type II restriction–modification system HphI from Haemophilus parahaemolyticus

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ABSTRACT

The genomic region encoding the type II restriction–modification (R-M) system HphI (enzymes recognizing the asymmetric sequence 5′-GGTGA-3′/5′-TCACC-3′) from Haemophilus parahaemolyticus were cloned into Escherichia coli and sequenced. Sequence analysis of the R-M HphI system revealed three adjacent genes aligned in the same orientation: a cytosine 5 methyltransferase (gene hphIMC), an adenine N6 methyltransferase (hphIMA) and the HphI restriction endonuclease (gene hphIR). Either methyltransferase is capable of protecting plasmid DNA in vivo against the action of the cognate restriction endonuclease. hphIMA methylation renders plasmid DNA resistant to HindIII at overlapping sites, suggesting that the adenine methyltransferase modifies the 3′-terminal A residue on the GGTGA strand. Strong homology was found between the N-terminal part of the m6A methyltransferase and an unidentified reading frame found between the N-terminal part of the m6A methyltransferase and an unidentified reading frame

INTRODUCTION

The majority of restriction endonucleases operate within short symmetrical nucleotide sequences (1). A subclass of the type II enzymes, so-called type IIS ENases (2) recognize asymmetrical nucleotide sequences and cleave DNA at a specified distance from it. The spatial separation of the DNA recognition and cleavage sites suggests that the type IIS ENases are likely organized as two-domain enzymes, where one domain is responsible for DNA recognition and the other one carries the catalytic center that makes a double strand break in DNA. This suggestion was supported experimentally by analysis of the domain structure of the FokI restriction endonuclease (3). This finding opens up new prospects for efforts to change the specificity of restriction enzymes that might be more feasible than in the case of type II restriction endonucleases, which are organized as single domain homodimeric proteins (4). However, until now the sequences of only three type IIS restriction–modification (R-M) systems, FokI, MboII and SstI (5–7), have been published. Here we report cloning and sequence analysis of the genomic region encoding the type IIS R-M system HphI from Haemophilus parahaemolyticus (EMBL accession no. X85374). This R-M system recognizes an asymmetrical sequence, 5′-GGTGA-3′/5′-TCACC-3′. The restriction endonuclease makes a staggered cut at the eighth base pair downstream of its recognition sequence on the upper strand, producing single base protruding ends (8,9). Earlier studies of this system reported the identification of only one type of methylation within the recognition sequence, namely modification of the inner C on the lower strand, 5′-Tm5CACC-3′ (10). Our results provide evidence for the presence of a second methylase that modifies an A base within the complementary strand, yielding 5′-GGTGm6A-3′.

MATERIALS AND METHODS

Bacterial strains, plasmids and phages

Haemophilus parahaemolyticus (ATCC no. 49700) was used as a source of DNA for cloning of the HphI R-M system. Escherichia coli strain ER2267 of genotype e14(mcrA−)endA1 supE44 thi-1 Δ(mcrC-mrr)114:IS10 Δ(argF-lac)U169 recA1/F ΔrfaA B ΔlacZΔ15 ΔlacZΔM15 zff::mini-Tn10 (Km8) was used as host for all cloning and subcloning procedures. Citrobacter freundii RFL9 (Ap8Cm8) isolated in this laboratory is a wild-type strain producing the Cfr9I restriction endonuclease (11). It was used as host for the construction and propagation of the positive selection vector pBR-R. The T7 expression system (12) employed for expression of R.HphI was purchased from Novagen and includes E.coli BL21(ΔDE3) (E.coli B strain F−ompT [lon] hsdSB F− mrg− with a λ prophage DE3 carrying the T7 RNA polymerase gene) and plasmid pET-21b. Plasmids pBR322 (13), pBR329 (14), pUC19 (15) and pACYC184 (16) were used as

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vectors in cloning and subcloning experiments. pCfr9IRM2.3 (17) was used as the source of the gene of the Cfr9I restriction endonuclease. Phage λvir stocks were prepared according to Sambrook et al. (18).

Media and transformation
Haemophilus parahaemolyticus cells were grown as described by Kleid et al. (8). Escherichia coli and C.freundii cells were grown in LB medium containing ampicillin (Ap, 60 µg/ml), kanamycin (Km, 50 µg/ml) and chloramphenicol (Cm, 30 µg/ml) as required. Cells were transformed using the CaCl2–heat shock method (18). Transformants were selected by plating onto LB agar supplemented with appropriate antibiotics.

Enzymes and chemicals
Restriction enzymes, T4 DNA ligase, DNA polymerase I large fragment (Klenow), Bal31, bacterial alkaline phosphatase (BAP), the ExoIII/S1 Deletion Kit, the DNA Labeling Kit (version 2.0), HindIII or EcoRI-digested and BAP-dephosphorylated pBR322 and oligonucleotides were products of MBI Fermentas. The DNA Sequencing Kit was purchased from Pharmacia. All enzymes and oligonucleotides were products of MBI Fermentas. The DNA preparation and manipulation of pBR322. Plasmid DNA of the resulting 19 individual transformants was then screened for resistance to R.HphI digestion.

Mapping and cloning of the H.parahaemolyticus genomic R-M locus
Electrophoresis of the H.parahaemolyticus DNA fragments obtained after digestion with various restriction enzymes (single and double digests) on agarose gels and Southern transfer were performed as described (18). A DNA Labeling Kit was used for the preparation of two radioactive DNA probes (1.05 kb, EcoRI and 1 kb, EcoRI–BglII fragments) from the previously cloned 2 kb fragment which encodes the functionally active HphI methyltransferase (MTase) (Fig. 1B). Fragments overlapping with the 2 kb insert were identified and used to construct a partial genomic map. The cloned hphIM gene mapped to a 7.2 kb HindIII fragment of the H.parahaemolyticus genomic DNA, which seemed large enough to contain the complete R-M system. This fragment was cloned into pBR322 as follows. Genomic DNA was digested with HindIII and the reaction products resolved on an agarose gel, where 6–8 kb DNA fragments were isolated. Aliquots of 3 µg of the fragments were digested at 16°C for 16 h in a 50 µl reaction volume with 3 µg HindIII-cleaved and BAP-dephosphorylated pBR322. Plasmid DNA isolated from the 5000 pooled Ap R Km R transformants was subjected to digestion with R.HphI, followed by re-transformation back into ER2267. Eighty five transformants were obtained, which were tested for resistance to R.HphI digestion. Twenty four of these clones were picked randomly for individual screening of their plasmid DNA with the hphIM gene marker and part of the HphI MTase gene (hphIMC, marked by a dotted arrow) has been cloned into pBR-R at the Eco47III site. 3 h and then transformed back into ER2267. Plasmid DNA of the resulting 19 individual transformants was then screened for resistance to R.HphI digestion.

Analysis of the HphI endonuclease and methylase activities
To determine restriction activity of individual clones in vivo, plating efficiency of λvir on these clones was measured and compared with that of E.coli ER2267 cells carrying the pBR322

Figure 1. Restriction maps of the cloning vector pBR-R (A) and recombinant plasmid pPphIM2.0 (B). The thin line represents DNA from pBR329, the bold line DNA subcloned from pCfr9IRM2.3 (17). The arrows in the circular map indicate genes for the Cfr9I ENase (cfr9IR) and ampicillin and chloramphenicol resistance (ApR and CmR respectively), ori, origin of replication. Direction of transcription from the P tet promoter is shown as a bold arrow. (B) Plasmid clone of hphIM. The H.parahaemolyticus DNA fragment (shaded line) encoding the HphI methyltransferase (MTase) (Fig. 1B). Fragments overlapping with the 2 kb insert were identified and used to construct a partial genomic map. The cloned hphIM gene mapped to a 7.2 kb HindIII fragment of the H.parahaemolyticus genomic DNA, which seemed large enough to contain the complete R-M system. This fragment was cloned into pBR322 as follows. Genomic DNA was digested with HindIII and the reaction products resolved on an agarose gel, where 6–8 kb DNA fragments were isolated. Aliquots of 3 µg of the fragments were digested at 16°C for 16 h in a 50 µl reaction volume with 3 µg HindIII-cleaved and BAP-dephosphorylated pBR322. Plasmid DNA isolated from the 5000 pooled ApR KmR transformants was subjected to digestion with R.HphI, followed by re-transformation back into ER2267. Eighty five transformants were obtained, which were tested for resistance to phage infection in vivo (see below). Twenty four of these clones were picked randomly for individual screening of their plasmid DNA with the HphI restriction endonuclease in vitro.
vector alone (22). Screening of restriction-proficient cells was carried out by replicating the transformants onto top-layer agar containing $10^6$ phage particles per plate. The endonuclease activity in vitro was tested by incubating serial dilutions of cell-free extracts prepared as described (23) with 1 µg λ DNA at 37°C for 1 h in a 40 µl reaction mixture containing 10 mM Tris–HCl, pH 7.5 at 37°C, 10 mM MgCl$_2$ and 0.1 mg/ml bovine serum albumin. Reaction products were analyzed by electrophoresis in 1% agarose gels. To determine the Hphl-specific modification in vivo, plasmid DNA was challenged with an excess of the Hphl restriction endonuclease followed by agarose gel electrophoresis.

**Determination of the base modified by the Hphl m6A MTase**

To test for methylation by the hphlMA gene product, we first designed a plasmid, pBR-H, which contains the Hphl recognition sequence (bold letters) overlapping by 1 bp with the recognition sequence of HindIII (boxed). The double-stranded linker

\[
\begin{align*}
5' & - \text{CGGTAGAGCTG} - 3' \\
3' & - \text{GTACGGCACTGCAAAGCTG} - 5'
\end{align*}
\]

was introduced into SalI/PstI-digested pBR322. The resulting construct was verified by DNA sequencing. To determine the effect of Hphl methylation on cleavage of the overlapping HindIII site by R.HindIII, the plasmid pBR-H was transformed into E.coli ER2267 harboring plasmid pAC-HphlA3 (Fig. 2). The plasmid DNA isolated from Ap$^R$Cm$^R$Km$^R$ transformants was tested with Hphl and HindIII endonucleases.

**Expression system for R.Hphl**

To provide the genes for Hphl methylases in trans, these genes (hphlMA and hphlIMC) were subcloned into a compatible vector, pACYC184. The 2.75 kb Psp1406I–VspI fragment of pHphIMM1, containing both methylase genes, was isolated (Fig. 2) and blunt-ended by treatment with Klenow polymerase. The fragment was ligated to Eco32I-digested and dephosphorylated pACYC184. After transformation of E.coli ER2267 several of the Cm$^R$Km$^R$ transformants were tested for the presence of Hphl-specific methylation and for the desired orientation of the subcloned fragment. The resulting 7.0 kb plasmid, designated pAC-HphlMM, was introduced into the recipient cells and was expressed in trans during subsequent endonuclease expression experiments. Subcloning of the hphlIR gene into the expression vector pET-21b was carried out as follows. A 1.3 kb TaqI fragment of pHphIMM1 was gel purified, treated with Klenow polymerase and ligated to XbaI-digested, blunt-ended and dephosphorylated pET-21b. The ligate was used to transform E.coli ER2267 cells that contained the methylation-proficient plasmid pAC-HphlMM. Individual Ap$^R$Cm$^R$Km$^R$ clones were screened for the correct size and orientation of the hphlIR gene. One isolate (pET-R1) was used in the expression experiments. Expression of the hphlIR gene was induced by adjusting the culture [E.coli BL21(DE3) pre-transformed with plasmids pET-R1 and pAC-HphlMM; see Fig. 2] to 1 mM isopropyl–β-D-thiogalactoside (IPTG) at an OD$_{600}$ of $\sim$0.5. After 3 h induction the cells were harvested by centrifugation and lysed (23). Crude cell extracts were used for determination of R.Hphl activity.

**DNA sequence determination**

DNA sequencing was done in both directions from a series of nested deletions generated using the ExoIII/S1 or Bal31 methods (24). Sequencing reactions were carried out using a DNA Sequencing Kit, [α-33P]dATP, MI3/pUC (direct, reverse) or pBR322 (SalI site, ccw) standard sequencing primers and double-stranded, supercoiled plasmid DNA as templates. The reaction products were resolved by electrophoresis on wedge-shaped polyacrylamide gels.

**Comparison of deduced amino acid sequences**

The comparison of deduced amino acid sequences with the EMBL and GenBank databases translated in six reading frames was done using the BLAST (25) program to identify similar, potentially related sequences.

**RESULTS AND DISCUSSION**

**Construction of the positive selection vector pBR-R**

The lethality of a restriction endonuclease that is expressed without its accompanying methylase has been used as the basis for the construction of positive selection systems in several
laboratories (26,27). The pBR-R vector described in this work (Fig. 1A) permits cloning of any blunt-ended fragments (EcoRIIII site) as well as fragments generated by the restriction endonucleases BamHI, BglII, BstYI, MboI, Sau3A (BglII site), NsiIII, NspI or PaeI (PaeI site). Our system possesses certain advantages over the other known systems: (i) propagation of this plasmid in *C.frieduni* RFL9, which lacks any natural plasmids, does not require subsequent separation of a companion methylase-producing plasmid, as in the case of pKG2 (27); (ii) pBR-R is considerably smaller than the positive selection vectors pLV57 and pLV59 and it does not require a temperature shift for pBR-R is considerably smaller than the positive selection vectors plasmids, does not require subsequent separation of a companion plasmid in *Ap* and *Cm*. Expression of the pKG (27) is carried out. Two antibiotic resistance genes are located on pBR-R (Ap and Cm). Expression of the *fr9IR* gene from the constitutive P~0~ promoter allows transformation of common *E.coli* strains and, in contrast with pKGW and pKGS, requires no addition of IPTG (27). The survival frequency of cells transformed with this plasmid was found to be 10^-4 as compared with that of cells transformed with a control vector, pBR322. The efficiency of pBR-R as a cloning vector was demonstrated by the construction of a *H.parahaemolyticus* library using DNA fragments generated by sonication.

**Cloning of the genes encoding *HphI* methyltransferases**

Selection of the gene coding for M.HphI was based on the resistance of self-modifying recombinant plasmids to digestion by *R.HphI* (28). Nineteen transformants obtained after the selection procedure described in Materials and Methods were further screened for the presence of the *HphI*-specific modification. Eight modification-positive clones contained the same plasmid with a 2 kb insert (designated pHphIM2.0; Fig. 1B). None of these clones showed any *HphI* endonuclease activity as assayed both in *vivo* and in *vitro*. The 2.5 kb *Eco*III fragment from pHplM2.0 was subcloned into pUC19 for sequence determination (not shown). Sequence analysis revealed the presence of a complete ORF potentially encoding a m6A methylase. This conclusion was based on the analysis of conserved amino acid motifs typical for DNA MTases (29). The ORF corresponding to the presumed m6A methylase is flanked by two truncated ORFs. The upstream ORF (Fig. 1B) contained conserved motifs VI–X characteristic of m5C methylases (30), while the downstream ORF did not show any similarities with known protein sequences. As restriction endonucleases usually share little conservation, we assumed that the latter ORF might encode the N-terminal part of the *HphI* ENase. It therefore seemed likely that the entire *HphI* R-M system is located in DNA regions extending in both directions around the cloned fragment.

**Cloning of the entire R-M system**

To select for a DNA fragment large enough to include the entire m5C methylase and *HphI* restriction endonuclease genes a restriction map of the *H.parahaemolyticus* genomic R-M locus was determined (not shown). Two DNA fragments prepared from pHphIM2.0 (1.05 kb *Eco*III–EcoRI and 1 kb *Eco*RI–BglII) were used as probes in Southern hybridization. The 7.2 kb genomic HindIII fragment was cut with *R.EcoRI* into two pieces. The 2.2 kb subfragment was cut with the 0.65 kb probe, while the 5.0 kb subfragment hybridized with the 1 kb probe (not shown). This indicated that the HindIII fragment includes the cloned 2 kb region of pHphM2.0 and flanking sequences. Cloning of the 7.2 kb HindIII fragment and selection of *R.HphI*-resistant clones was carried out as described in Materials and Methods. Eighty five transformants were analyzed, but none of them demonstrated resistance to *λ* infection. Nine out of 24 clones tested were resistant to cleavage in *vitro* with *R.HphI*. Restriction mapping revealed the presence of two types of recombinant plasmids that differed in the orientation of the same insert (designated pHphIM1 and pHphIM2; Fig. 2). These clones were tested for endonuclease activity both in *vivo*, using the plating efficiency test (Materials and Methods) and in *vitro*. In neither case was endonuclease activity detected, indicating that the *R.HphI* gene was either incomplete or inactive in *E.coli* cells. To analyze the regions surrounding the MTase genes, sequencing of the 4.8 kb HindIII–MluI fragment from pHphIM1 and pHphIM2 (Fig. 2) was carried out.

**Gene structure of the *HphI* system and flanking DNA sequences**

A 4790 bp region of the cloned 7.2 kb HindIII DNA fragment encompassing the genes for *HphI* MTases as well as adjacent regions was sequenced on both strands. Five ORFs (three complete, one truncated and one partially sequenced), all oriented in the same direction, were identified (Fig. 2). The first ORF (ORF1), 258 bp long (nt 3–260, termination codon included) is truncated and may represent the 3′-terminal part of an unknown gene. The next three ORFs encode the *HphI*R-M enzymes: ORF2 (hphIMC), m5C MTase; ORF3 (hphIMA), m6A MTase; ORF4 (hphIR), *HphI* restriction endonuclease (see next paragraph). The position of a translation initiation codon in hphIMC could not be unambiguously inferred from the nucleotide sequence alone due to the presence of four potential initiation sites upstream of the first conserved motif of the m5C MTase (30) (ATG at position 562, ATG at position 634, TTG at position 640 and ATG at position 688); none of the start codons is preceded by a putative Shine–Dalgarno sequence either. This ORF (hphIMC) ends with a translation termination codon TAA at position 1680. ORF3 (hphIMA) is 1011 bp long and extends from nt 1673 to 2683 (336 amino acids). This ORF overlaps by 8 nt with the preceding ORF and by 1 nt with the downstream one. The fourth ORF, 1140 bp long (nt 2683–3822, the hphIR gene), encodes the *HphI* ENase of 379 amino acid residues. The last ORF (menB) was not completely sequenced; it extends from nt 3983 to 4789 and represents the 5′-terminal part of the menB gene (see below). A putative Shine–Dalgarno sequence was found only upstream of the last gene, menB (AAGGA at positions 3969–3973).

Further analysis of the sequence revealed two copies of a 56 bp stretch that flanked the *HphI* R-M system on both sides (Fig. 3). Restriction–modification systems, although not essential for cell viability, are widespread in bacteria (1). Dissimilar systems are often present in related bacterial strains and, *vice versa*, homologous systems can be found in taxonomically distant microorganisms (17,31,32). These observations suggest that many R-M genes may have enjoyed a certain degree of mobility, at least in the recent evolutionary past. Our finding of a 56 bp long direct repeat flanking the *HphI* R-M genes may be important in this context. Its presence suggests models for R-M mobility involving recombination of a circular DNA containing the 56 bp sequence and the *HphI* R-M gene cassette with a corresponding (although
somewhat divergent) 56 bp sequence in the chromosome. One class of models involves site-specific recombination, such as with a temperate phage (i.e. λ) (33) and with the 56 bp sequence constituting (part of) the attachment site; a related model would be one based on ‘integrons’, a recently discovered class of mobile DNA segments that underlie site-specific insertion of certain antibiotic resistance genes into new plasmids or transposons (34). There is no obvious similarity between the resistance genes into new plasmids or transposons (34). There is no possible ‘integron’ involving the 56 bp repeat element and the HphI R-M system would differ in specificity from the drug resistance integrons described to date. It is of course formally possible that the 56 bp repeats are not components of a site-specific recombination system. Rather, since the repeats exceed the minimum length needed for RecA protein-mediated DNA pairing (35), this suggests generalized recombination for both insertion of the HphI R-M genes into particular chromosomal sites or their excision and loss from the cell. Of note, two sequences highly related to the HphI repeats (Fig. 3) are present in intergenic regions of H.influenzae strain Rd, whose genome has been entirely sequenced (36).

Expression of the HphI R-M system in E.coli

The deletion and subcloning experiments were carried out to investigate the expression of HphI R-M genes (Fig. 2). We found that both MTases are expressed in E.coli and protect plasmid DNA against R.HphI when cloned together or subcloned separately. Moreover, a similar degree of DNA protection was detected when the plasmids carrying the MTase genes in different orientations were isolated, treated with R.HphI and analyzed. These findings indicate that the endogenous promoters operate efficiently in E.coli. No activity of the restriction endonuclease was detected in crude extracts from cells carrying the initial plasmids, pHphIMM1 and pHphIMM2. We subcloned the presumed hphIR gene (ORF4) into the expression vector pET-21b using cells harboring the methylase genes in trans as recipient. In this system, the restriction endonuclease activity was readily detectable upon induction with IPTG, indicating that translation initiation functions normally and that there are no strong transcription termination signals immediately upstream of the hphIR gene. Apparently, expression of this gene from its own promoter in E.coli is so weak that it could not be detected by the methods used.

Analysis and comparison of deduced amino acid sequences

No amino acid sequences similar to that of R.HphI were found in the EMBL and GenBank databases. The sequence motif PD(X)\_17EGK was found in the central part of R.HphI (amino acids 140–161), which matches well the consensus P(E/D)/X\_18/E/D/XX motif essential for catalytic activity and Mg\_2\+ binding in the EcoRI, EcoRV and PvuII restriction endonucleases (4).

Similar analysis of hphMC revealed the presence of all 10 motifs common to m5C MTases (30). This MTase shares the greatest degree of similarity with DdeI (37) MTase (28% identical and additionally 11% similar amino acids) and AquI (38) MTase (33% identical and 11% similar amino acids). The translation product of hphDMA contains conserved motifs typical for N6-methyladenine MTases. This MTase belongs to the D1 class of m6A MTases (29), a group where the conserved motifs appear in the order ‘F–G–G’ and then ‘DPPY’. Similarly to some other members of the D1 group, the HphI m6A MTase contains a slightly scrambled version of the first motif: F–GG instead of F–G–G (29). This MTase shares the greatest, although marginal, degree of similarity with the N-terminal part of FokI and SstI MTases (21 and 20% identity was observed, which rises to 38 and 36% respectively when conservative substitutions are taken into account). In addition, strong homology was found between the N-terminal part of this HphI MTase and an unidentified ORF located upstream of the truncated copy of the galE gene in Neisseria meningitidis (39,40) (67% identity; Fig. 4). Curiously, the conservation is not only observed within the uncharacterized ORF, but extends downstream in the same reading frame after the termination codon. The N-terminal part of the HphI-like MTase of N.meningitidis and the C-terminal part of the truncated GalE protein overlap by the KMPYT pentapeptide, downstream of which no further homology is observed. This suggests that the HphI-like MTase in N.meningitidis was disrupted during duplication of the galE locus.

We found that the protein encoded by the first ORF matched the C-terminal part of the (p)ppGpp 3’-pyrophosphohydrolase (gene spoT) from H.influenzae Rd (36) and E.coli (41) and contains 29% identical and 24% functionally compatible amino acids in the case of SpoT from H.influenzae Rd and 26% identical and 29% functionally compatible amino acids with SpoT from E.coli. Such a degree of homology was insufficient to predict a function for the truncated protein, so this ORF was designated orfX. The last ORF hit two entries in the database, dihydroxynaphthoate synthases (gene menB) from H.influenzae Rd (36) and E.coli (42) (89 and 88% identity respectively). This suggests that the ORF is possibly the 5’-end of the menB gene in H.parahaemolyticus. The sequence context surrounding the HphI R-M system differs from those in the other three R-M systems identified in Haemophilus strains. Namely, HindIII and HinP1I from, respectively, H.influenzae Rd and H.influenzae P1 and HpaII from H.parahaemolyticus, all being found located in the neighborhood of the valS gene (43,44).
Specificity of the \textit{Hphi} MTases

Many sequence-specific MTases, a constituent part of the type II R-M systems, are able to modify bases of a certain single type (either A or C) positioned symmetrically in the complementary strands of a palindromic recognition sequence (10). More complex and poorly investigated are the type IIS enzymes, which interact with asymmetrical sequences. In such a case two different sequences in complementary DNA strands are recognized. There have been only a few reports where specificity of type IIS MTases were analyzed in detail (5,45,46). These studies have been only a few reports where specificity of type IIS DNA methylases were analyzed in detail (5,45,46). These methylases yielding m6A or m5C on complementary strands in the reaction catalyzed by a monomeric enzyme modifying adenine residues on both strands of the target DNA (HgaI) (5); two separate enzymes or two separate m6A and m5C MTases (46); J. Bitinaite, personal communication). The \textit{Hphi} R-M system is likely to belong to the latter type. Sequence analysis of p_phiIIMM1 revealed two separate genes encoding proteins resembling, respectively, m5C and m6A DNA modification methyltransferases. \textit{hphi}IMC is most likely responsible for the reported earlier (10) methylation of the 5'-proximal C base in the \textit{hphi}I MTase (\textit{Hphi}I) encoded by pBR322. To verify this assumption, we constructed a plasmid, pBR-H (see Materials and Methods), incorporating the overlapping \textit{Hphi}–HindIII site GGTTAAAGCCTT. It was reported that the methylated sequence 5'AGCTT-3' is resistant to R.HindIII, while this site is readily cleaved in the unmodified state (10). We found that R.HindIII was unable to cleave the diagnostic site when pBR-H was isolated from ER2267[pAC-HphiIM3] cells, while the cleavage of a second HindIII site originating from pBR322 occurred as expected (not shown). These results indicate that methyladenine-specific \textit{M.Hphi} modifies the 3'-terminal A base in the upper (5'-GGTGA-3') strand. Additional experiments are necessary to define whether this methylation is specific for the upper strand or whether modification of the bottom strand (5'-TCACC-3') also occurs.

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