A bacterial methyltransferase M.EcoHK31I requires two proteins for *in vitro* methylation

Kai-Fai Lee, Kai-Man Kam¹ and Pang-Chui Shaw*

Department of Biochemistry, The Chinese University of Hong Kong and ¹Institute of Pathology, Sai Ying Pun Jockey Club Clinic, Hong Kong

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ABSTRACT

The genes encoding *EcoHK31I* restriction-modification (R-M) system were isolated from a clinically-isolated *Escherichia coli* strain HK31. The entire R-M system of *EcoHK31I* is located in a 2.1 kb fragment. R.*EcoHK31I* is an isoschizomer of *EaeI* which recognizes and cleaves Y↓GGCCR. M.*EcoHK31I* consists of two polypeptides α and β with sizes 309 and 176 aa, respectively. Polypeptide β is encoded within an alternative reading frame of polypeptide α . All the conserved motifs in mC5-MTases can be found in polypeptide α except motif IX which is present in polypeptide β . Polypeptides α and β were separately synthesized in a T7 promoter controlled over-expression system and *in vitro* methylation occurred only when the two extracts were mixed and thus confirms that two polypeptides are required for methylation.

INTRODUCTION

Many bacteria contain restriction-modification (R-M) systems that recognize specific DNA sequences. In most of these systems, the restriction endonuclease (ENase) cuts the DNA at or near the specific recognition sequence, whereas the methylase (MTase) methylates the specific recognition sequence so that it is no longer susceptible to cleavage by the ENase. Because of the presence of these specific R-M systems, unmodified foreign DNA such as a bacteriophage DNA that enters a host cell is rapidly degraded, while the ENase spares the modified host DNA. This protective mechanism for bacteria remains a source for enzyme reagents that are essential for generating recombinant DNA. Up to date, more than 100 type II R-M systems have been cloned and reported (1). Except for a few isoschizomers, the amino acid sequences of ENases are usually unique. On the other hand, MTases can be classified into three groups: mC4-, mC5- and mA6-MTases according to their sites of methylation. Amino acid sequences within class are related (2,3). ENases require Mg²⁺ for cleavage while MTases require S-adenosylmethionine for DNA protection.

A typical mC5-MTase contains a single polypeptide with ten conserved motifs and a variable region between motifs VIII and IX for DNA recognition (2,4). Nevertheless, there are a few

exceptions: *HgaI* modification system consists of two methylases for modification of different strands of the target DNA (5); *AluI* has a large and partially dispensable variable region (6); *M.AquI* consists of two polypeptides with partly overlapping open reading frames at the variable region (7).

In our programme to screen for novel ENases, we came across a clinically-isolated *Escherichia coli* strain which produced R.*EcoHK31I*. This *E.coli* strain was isolated from a patient who had suffered from recurrent urinary tract infection and had been put on various antibiotics. This report describes the cloning and characterization of this R-M system and the unexpected observation that M.*EcoHK31I* requires two polypeptides for *in vitro* methylation of target DNA.

MATERIALS AND METHODS

Bacterial strains, plasmid DNA and chemicals

Escherichia coli HK31 was isolated in a local hospital. *E.coli* K802 (*HsdR*_K⁻, *HsdM*_K⁺, *McrA*⁻, *McrB*⁻) and GM2163 (*HsdR*_K⁻, *HsdM*_K⁺, *McrA*⁻, *McrB*⁻, *dam*⁻, *dcm*⁻) were obtained from New England Biolabs Inc., USA. BL21 (DE3) (*HsdR*_B⁻, *HsdM*_B⁻), BL21 (DE3) [plysS], BL21 (DE3) [plysE] and plasmid pET3a were from Prof F. W. Studier. [³H-methyl]-S-adenosylmethionine (³H-AdoMet, 15 Ci/mmol) was from Amersham. Other plasmid DNA, enzymes and chemicals were from various commercial sources.

Partial purification of *EcoHK31I* ENase and MTase

E.coli HK31 and K802 [p*EcoHK31E*] were grown in Luria-Bertani medium at 37°C according to standard procedures (8). Unless otherwise stated, all purification procedures were carried out at 4°C. Six g of cell paste was resuspended in 20 ml column buffer A [20 mM Tris-HCl (pH 8.3), 5 mM 2-mercaptoethanol, 0.1 mM EDTA and 5% glycerol] and PMSF (phenylmethanesulfonyl fluoride, Sigma) was added to 1 mM. Cells were disrupted by sonication and cell debris was removed by ultracentrifugation at 100 000 × g for 1 h. Supernatant was applied to a DEAE-Sephacel column (Pharmacia, 2.2 × 13 cm) equilibrated with buffer A at a flow rate of 0.3 ml/min. After washing with 100 ml of buffer, proteins were eluted with a 300 ml linear gradient of 0–0.8 M NaCl in buffer A. ENase and MTase were eluted from 0.3 to 0.4 M and 0.25 to 0.35 M NaCl, respectively. The active

* To whom correspondence should be addressed

fractions of ENase were pooled, dialysed with buffer B [20 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.15% Triton X-100 and 5% glycerol] and applied to an Affi-gel Heparin column (Biorad, 1.8 × 10 cm). The enzyme was eluted with a 300 ml linear gradient of 0–1.0 M NaCl in buffer B. Active fractions eluted from 0.6 to 0.7 M were pooled, concentrated by an Amicon concentrator and dialysed against storage buffer [10 mM Tris-HCl; pH 7.5, 0.1 mM EDTA, 1 mM DTT, 200 µg/ml BSA and 50% glycerol]. Methylase after DEAE-Sephacel chromatography was dialysed against buffer C [same as buffer A, except the pH is 7.5] and applied to an Affi-gel Heparin column. Active fractions eluted from 0.4 to 0.5 M NaCl were pooled and dialysed against buffer C. The enzyme was applied to Affi-gel Blue column (Biorad, 2 × 8 cm) and eluted with 300 ml linear gradient of 0–1.0 M NaCl in buffer C. Active fractions eluted from 0.7 to 0.8 M were concentrated and applied to a FPLC-Superose 12 column (Pharmacia). The enzyme was eluted with 150 mM NaCl in buffer C. Active fractions were dialysed against storage buffer. Purified enzymes were stored at –20°C.

Restriction activity assay

Restriction digestion was assayed by incubation of 1 µg DNA in a reaction mixture containing 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 50 mM NaCl and 1 mM DTT for 1 h at 37°C. The cleavage products were analyzed by gel electrophoresis.

Methylation activity assay

The standard assay involved the addition of 1 µl of cell extract to 10 µl methylase reaction buffer [10 mM Tris-HCl; pH 7.5, 50 mM NaCl, 10 mM EDTA, 80 µM AdoMet (Sigma) and 5 mM 2-mercaptoethanol] and 1 µg non-methylated lambda DNA (Sigma) and incubated for 1 h at 37°C. DNA samples after phenol-chloroform extraction were digested with 5 U of *R.EcoHK31I* in reaction buffer [10 mM Tris-HCl; pH 7.9, 10 mM MgCl₂, 50 mM NaCl and 1 mM DTT] for 1 h at 37°C and analyzed by gel electrophoresis.

Determination of recognition and cleavage sites

The recognition site of *EcoHK31I* was determined by comparing the restriction patterns with other known enzymes and by double digestion with other enzymes on known DNA sequences. The cleavage site of *EcoHK31I* was determined according to refs 9 and 10 using pUC19 as template and M13 reverse sequencing primer with sequence 5'-AACAGCTATGACCATGA-3'.

Cloning and sequencing of *EcoHK31I* R-M system

E.coli HK31 chromosomal DNA was isolated as described (11), followed by CsCl ultracentrifugation. Plasmid DNA was prepared by the alkaline-SDS procedure (8).

E.coli HK31 DNA was digested with various 6-bp recognizing ENases and ligated to linearized, dephosphorylated pBR322 and transformed into GM2163. Plasmid DNA was extracted from the transformed cells by Qiagen columns and 1 µg DNA of each library was 150-fold over-digested with *EaeI* (New England Biolabs) and re-transformed into *E.coli* GM2163. The survival clones were selected to check *in vitro* restriction and methylase activities.

The complete *EcoHK31I* R-M system was subcloned into pUC19 to generate p*EcoHK31E* for expression of proteins. DNA fragments subcloned into pUC19 or pBluescript were sequenced by the dideoxy chain termination method (12). Partial purified proteins were resolved by SDS-PAGE and blotted onto a PVDF paper (Millipore) for N-terminal determination.

Polymerase chain reaction

PCR amplification (50 µl) contained 2 µM of each primer, 200 µM of each dNTP, 10 mM KCl, 20 mM Tris-HCl; pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 50 ng of template DNA and 1 U of Vent DNA polymerase. Reaction mixtures were overlaid with mineral oil and reactions were carried out using the Thermolyne Thermal Cycler. Initial template denaturation was programmed for 5 min at 94°C. The cycle profile was programmed as follows: 2 min at 50°C (annealing), 3 min at 72°C (extension) and 1 min at 94°C (denaturation). Thirty-four cycles of the profile was run and the final extension step was increased to 10 min.

Synthesis of polypeptides α and β

To clone the gene encoding polypeptide β, we made use of a unique *NdeI* site overlaps with the start codon of polypeptide β. Digestion with *NdeI* and *BglII* ENases generated a 0.8 kb fragment encoding the polypeptide β gene. This fragment was ligated to a *NdeI* and *BamHI* cleaved pET3a vector. Expression was in BL21 (DE3) [plysS] host. Overlapping PCR was used to clone the polypeptide α encoding gene to pET3a and expressed in BL21 (DE3) [plysE] (Fig. 1). Protein expression was according to ref. 14.

RESULTS

Enzyme characterization

Purified *EcoHK31I* was used to digest various DNA with known sequences (Fig. 2A). The sizes and numbers of fragments produced are identical to those cleaved by *EaeI* which recognizes 5'-(C/T)GGCC(A/G)-3' (15). Similar to *EaeI*, *R.EcoHK31I* was found to cleave the sequence to produce a 4 nt 5'-protruding end (Fig. 2B). Its optimal reaction temperature was at 37°C and optimal buffer contained 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 50 mM NaCl and 1 mM DTT.

Partially purified *M.EcoHK31I* (Fig. 3) was used to protect ΦX174 RFI DNA in the presence of AdoMet. Modification by *M.EcoHK31I* prevented the cleavage of those *HaeIII* sites overlapped with *EcoHK31I* sites (data not shown). Since *HaeIII* cannot cleave the sequence GGCC when the internal cytosine is modified (16), this indicated that *M.EcoHK31I* modified the internal cytosine.

One of the *EcoHK31I* recognition sites 5'-TGGCCA-3' in pBR322 overlaps with dcm site 5'-CCAGG-3' at position 1446. Modification of the internal cytosine residue by dcm methylation blocked the cleavage.

Cloning and sequencing of *EcoHK31I* R-M system

The method we used to clone the *EcoHK31I* R-M system is based on the expression of cloned methylase gene in *E.coli* host rendering the recombinant plasmid resistant to the isoschizomer *EaeI*. Five clones in the *AseI* library were selected. All contained

Figure 1. Construction of pET3a-M38 for over-expressing polypeptide α by overlapping mutagenic PCR (13). Sequences of the primers are: Primer 1; 5'-AAGCCCATATGAAAAAGAAACCA-3', Primer 2; 5'-TTTTCGCGTATG TATAAAA-3', Primer 3; 5'-TTTTCATACATACGCAAAACTGATAG- AAGAA-3', Primer 4; 5-CATCGGATCCACACGTC-3'. Primers 1 and 2, 3 and 4 were used to synthesize a 0.3 and 0.8 kb fragments, respectively. These fragments were used as templates to construct the complete polypeptide α gene by means of overlapping PCR in the presence of primers 1 and 4. The PCR product was gel purified using GeneClean kit (Bio 101), and digested with *NdeI* and *BamHI* and ligated to pET3a which was cleaved by the same enzymes. Bold nt in primers 2 and 3 indicate mutation for the removal of the unique *NdeI* site in the methylase gene. Two stop codons were added in primer 3 to prevent the expression of polypeptide β . These changes did not alter the aa encoded by polypeptide α .

a 2.3 kb insert in the same orientation. Crude extract of these clones contained *EcoHK31I* restriction and methylase activities.

To analyze the organization of the *EcoHK31I* R and M genes encoded in the 2.3 kb fragment, DNA fragments were subcloned and their abilities to confer restriction and modification phenotypes were examined (Fig. 4). We concluded that a minimal of 2.1 kb region is essential for encoding the entire R-M system.

Figure 2. Characterization of recognition and cleavage sites of *EcoHK31I*. (A) *EcoHK31I* digest. Lane: (1) pBR322, (2) non-methylated pBR322, (3) pACYC177, (4) M13mp18, (5) SV40, (6) PhiX174, (7) T7, (8) Ad2, (9) non-methylated lambda, (10) Lambda *HindIII* marker. (B) Determination of *EcoHK31I* cleavage site. Lanes A, C, G and T: the sequence ladder through *EcoHK31I* recognition site of pUC19 DNA. Lane I: the product of the primed-synthesis reaction was cleaved with *EcoHK31I*. Lane II: the cleavage product was treated with T7 DNA polymerase.

The DNA fragment with *in vitro* methylation activity was sequenced. The open reading frame (ORF) from nt 231 to 1157 encodes the 309 aa putative methylase with a A + T content of 54.1% (Fig. 5).

Purification and characterization of M.*EcoHK31I*

The gene encoding putative M.*EcoHK31I* was cloned to pUC19 and attempts were made to purify the protein to homogeneity by using various columns (Fig. 3). After the Affi-gel Heparin column, two proteins with sizes of 38 and 23 Kd were found. Separation of these two proteins by Affi-gel Blue and Superose 12 columns was unsuccessful. The proteins were eluted as a single peak. Nevertheless, when 6 M guanidine-hydrochloride was included in the buffer before passing through Superose 12 column, they dissociated from each other and were eluted separately. This suggested that these two proteins were strongly associated.

In order to clarify the nature of the unknown protein, M.*EcoHK31I* and this unknown protein were separated by SDS-PAGE and blotted onto a PVDF membrane for N-terminal determination. The N-terminal sequence of the M.*EcoHK31I*

Figure 3. Purification profile of *M.EcoHK3II*. Lane: (1) Molecular weight marker. (2) Crude extract after ultracentrifugation. (3) DEAE-Sephacel pool. (4) Affi-gel heparin pool.

Figure 4. Restriction map of the region coding for the *EcoHK3II* R-M system and the location of *M.EcoHK3II*. Restriction fragments were subcloned to pUC19 or pBluescript and crude extract was tested for restriction activity. Plasmid DNA was purified and digested with *R.EcoHK3II* to study *in vitro* methylation capability. The smallest clone which carries *in vivo* methylase activity was located in the 1.1 kb *BamHI*-*AsuII* fragment.

matched with that predicted from the nt sequence of the position 231 while that of the small protein (23 Kd) was found to be translated from nt 541 (Fig. 5). The latter ORF encodes a 176 aa polypeptide. The large protein is designated polypeptide α and the small protein polypeptide β .

Two proteins are needed for methylation

The 0.8 kb *NdeI* and *BglII* fragment which encoded for polypeptide β was cloned directly into pET3a digested with *NdeI* and *BamHI* and transformed to BL21 (DE3) [plysS]. After induction by 0.4 mM IPTG for 3 h at 37°C, about 20% of the total protein was polypeptide β . Overlapping PCR was done to construct a gene encoding polypeptide α only (Fig. 1) and the sequence was confirmed by DNA sequencing. Protein was induced in BL21 (DE3) [pET3a-M38, plysE] and up to 5% of the total protein was polypeptide α after induction. The crude extract was used separately or together to protect non-methylated lambda DNA from *R.EcoHK3II* digestion (Fig. 6). Protection was only

Figure 5. Sequence of the 1.2 kb fragment containing the *EcoHK3II* methylase gene encoding α and β polypeptides. α polypeptide is translated at nt 231 and shown above the DNA sequence. β polypeptide is translated at nt 541 and shown below the DNA sequence. The start and stop codons are bold-typed. The ten highly conserved motifs in mC5-MTases are marked I-X. The invariant aa in the 21 mC5-MTases in ref. 17 are circled. Conserved aa in more than 10 enzymes in ref. 17 are underlined. Motif IX is located in polypeptide β .

shown when two extracts were mixed together. This indicated that both proteins were needed for *in vitro* methylation. The collaboration of polypeptides α and β was further confirmed by the experiment on the incorporation of tritiated methyl groups to substrate DNA (Table 1).

Table 1. Incorporation of [3 H]AdoMet to non-methylated lambda DNA

Extract	c.p.m.
Nil	711
pET3a	772
pET3a-M38 (encodes polypeptide α)	1687
pET3a-C23 (encodes polypeptide β)	987
pET3a-M38 + pET3a-C23	19 691

Crude extracts of expressed host cells harboring the mentioned plasmid DNA were diluted and mixed with 1 μ g of non-methylated lambda DNA in the presence of 1 μ M [3 H]AdoMet in 50 μ l methylase reaction buffer and incubated for 1 h at 37°C. DNA samples after extraction with phenol-chloroform and precipitation with 10% TCA, were spotted onto a glass fibre filter (Whatman). The filters were washed with 10% TCA and ethanol and dried for radioactivity counting.

Figure 6. *In vitro* methylase activity of the crude extracts to protect non-methylated lambda DNA. DNA was treated with 1, 0.5 and 0.25 μ l polypeptide β in lanes (1–3), polypeptide α in lanes (4–6) and mixed crude extract in lanes (7–9) as described in Materials and Methods. No protein extract was added to DNA in lane (10).

DISCUSSION

We have isolated the complete R–M system *Eco*HK31I from a clinically-isolated *E.coli* strain HK31. This isolate is from a patient with recurrent urinary tract infection and serially treated with antibiotics. We intend to use the cloned DNA as a probe to correlate the presence of this R–M system with the occurrence of the infectious *E.coli*. In the course of purification and activity assay for *M.Eco*HK31I, we discovered that two polypeptides, with one translated at an alternative reading frame within another, were needed to protect substrate DNA from the digestion of *R.Eco*HK31I *in vitro* (Fig. 6).

In the strain BL21 (DE3) [pET3a-M38, *plysE*], expression of polypeptide α alone was sufficient to protect the plasmid DNA from the digestion of *R.Eco*HK31I. This apparent discrepancy may be due to a high concentration of polypeptide α in the vicinity of the DNA inside the cell. The possibility that polypeptide α is inefficient in methylation is supported by the evidence that a 2.2-fold increase of methyl group incorporation to non-methylated lambda DNA was found when polypeptide α was used alone as compared to a 25.5-fold increase when both polypeptides α and β were mixed together (Table 1).

Comparison of the aa sequences of various mC5-MTases reveals ten conserved motifs (2). In between motifs VIII and IX, there is a variable region which provides the specificity for DNA interaction. mC5-MTases appeared to be quite flexible in their structural organization. Removal of the amino-proximal third of the variable region of *M.AluI* still retains full *in vivo* MTase activity (6). The naturally occurring *M.AquI* requires two polypeptides with overlapping of a few ORFs at the variable region for full methylase activity (7). In multi-specific phage methylases, DNA recognizing domains in the variable region can be crossed over *in vitro* to generate novel target specificities (18,19). Detached parts of certain monospecific mC5-MTases such as *M.BspRI* or *M.BsuRI* can complement for *in vivo* DNA methylation and hybrid DNA of *HhaI* and *HpaII* or *HpaII* and *MspI* are able to generate functional methylases (20–22). The

work of generating hybrid methylases at either the DNA or protein level revealed that the variable region is an independent domain for DNA recognition and domain IX appears to interact with the variable region.

Recently, the crystal structures of *M.HhaI* and its enzyme–substrate complex have been reported (23,24). These structures provide us with valuable information in the structure–function relationship of mC5-MTase and insight on the organization plan of *M.Eco*HK31I. *M.HhaI* is structurally divided into three parts: large and small domains and a hinge region linking the two together. The first part of motif IX interacts with the variable region and forms part of the small domain while the rest of motif IX and the first part of motif X form the hinge region. This region joins the large and the small domains and forms the bottom of the cleft for the DNA to fit in (18). In *M.Eco*HK31I, motif IX is missed from polypeptide α but found in polypeptide β (Fig. 5) which is translated within the ORF of polypeptide α . Since polypeptide α possesses most of the conserved motifs of a typical mC5-MTase including the FXGXG sequence in motif I for AdoMet binding, Pro–Cys dipeptide in motif IV for catalysis, and contains the target recognition domain DKQGQGER which is similar to the Gly-rich region GKGGQGER in the variable region of *M.HhaI* (24,25), we expect polypeptide α is responsible for the binding of AdoMet, DNA recognition and carries out the actual catalytic process. On the other hand, motif IX in polypeptide β seems to assist the docking of DNA in the correct position and hence in its presence, the activity of polypeptide α is greatly enhanced. Nevertheless, how motif IX in polypeptide β fits into polypeptide α has to be revealed by further structural analysis.

Our discovery provides a more bizarre example showing that motif IX can be detached from a mC5-MTase. Why this motif has moved to another reading frame is unclear, although it seems likely that during evolution, two frameshift mutations, one in the variable region and another between motifs IX and X, have occurred in the ancestral methylase. Hence, in principle, a functional methylase can be re-constituted by fusing polypeptides α and β together. Work is being carried out to generate a fused *M.Eco*HK31I and to purify the two polypeptides to homogeneity for biochemical characterization.

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