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

Kai-Fai Lee, Kai-Man Kam and Pang-Chui Shaw

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

Received September 22, 1994; Revised and Accepted November 24, 1994

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 Eael 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 recognize specific DNA sequences. In most of these systems, the ENase and MTase have a large and partially dispensable variable region (6); MAqul 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 HK31I was isolated in a local hospital. E.coli K802 (HsdRK⁺, HsdMk⁺, McrA⁺, McrB⁺) and GM2163 (HsdRk⁻, Hsdmk⁻, McrA⁺, CmrB⁺, dam+, dcm+) were obtained from New England Biolabs Inc., USA. BL21 (DE3) (HsdRk⁺, HsdMk⁺), BL21 (DE3) [plysS], BL21 (DE3) [plyS] 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 [pEcoHK31E] were grown in Luria-Bertani medium at 37°C according to standard procedures. 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–Sephalac 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
The complete EcoHK311 R–M system was subcloned into pUC19 to generate pEcoHK31E 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 Ndel site overlaps with the start codon of polypeptide β. Digestion with Ndel and BglII ENases generated a 0.8 kb fragment encoding the polypeptide β gene. This fragment was ligated to a Ndel and BamHI cleaved pET3a vector. Expression was in BL21 (DE3) [pLysS] host. Overlapping PCR was used to clone the polypeptide α encoding gene pET3a and expressed in BL21 (DE3) [pLysE] (Fig. 1). Protein expression was according to ref. 14.

RESULTS
Enzyme characterization
Purified EcoHK311 was used to digest various DNA with known sequences (Fig. 2A). The sizes and numbers of fragments produced are identical to those cleaved by Eael which recognizes 5′- (C/T)GGCC(A/G)-3′ (15). Similar to Eael, EcoHK311 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), 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 EcoHK311 was determined by comparing the restriction patterns with other known enzymes and by double digestion with other enzymes on known DNA sequences. 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 methylation 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 µl $\Phi$ EcoHK311 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.

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.

Cloning and sequencing of EcoHK311 R–M system
E.coli HK311 chromosomal DNA was isolated as described (11), followed by CsCl ultracentrifugation. Plasmid DNA was prepared by the alkaline-SDS procedure (8).

E.coli HK311 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 Eael (New England Biolabs) and re-transformed into E.coli GM2163. The survival clones were selected to check in vitro restriction and methylation activities.
Figure 1. Construction of pET3a-M38 for over-expressing polypeptide α by overlapping mutagenic PCR (13). Sequences of the primers are: Primer 1; \[NdeI\] 5'-AAGCCCAEAIOAAAAAGAAACCA-3', Primer 2; 5'-TTTTGCGTATGTATAAAA-3', Primer 3; 5'-TTTTATAC[\text{BamHI}]AAGAA-3', Primer 4; 5'-CATCGGATCCACCACGTC-.V. 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 [\text{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 EcoHK311 restriction and methylase activities.

To analyze the organization of the EcoHK311 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.
Two proteins are needed for methylation

The 0.8 kb NdeI and BglII fragment which encoded for polypeptide \( \beta \) was cloned directly into pET3a digested with NdeI and BamH1 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 \( \alpha \) after induction. The crude extract was polypeptide p. Overlapping PCR was done to construct a gene encoding polypeptide \( \alpha \) only (Fig. 1) and the sequence was confirmed by DNA sequencing. Protein was translated from nt 541 (Fig. 5). The latter ORF encodes a 176 aa polypeptide which was translated at nt 231 while that of the small protein (23 Kd) was found to be matched with that predicted from the nt sequence of the position 541. Two proteins are needed for methylation activity. The small protein polypeptide p. exists separately or together to protect non-methylated lambda DNA from R.EcoHK31I digestion (Fig. 6). Protection was only shown when two extracts were mixed together. This indicated that both proteins were needed for in vitro methylation. The collaboration of polypeptides \( \alpha \) and \( \beta \) was further confirmed by the experiment on the incorporation of tritiated methyl groups to substrate DNA (Table 1).

Table 1. Incorporation of \([\text{H}]\text{AdoMet}\) to non-methylated lambda DNA

<table>
<thead>
<tr>
<th>Extract</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>711</td>
</tr>
<tr>
<td>pET3a</td>
<td>772</td>
</tr>
<tr>
<td>pET3a-M38 (encodes polypeptide ( \alpha ))</td>
<td>1687</td>
</tr>
<tr>
<td>pET3a-C23 (encodes polypeptide ( \beta ))</td>
<td>987</td>
</tr>
<tr>
<td>pET3a-M38 + pET3a-C23</td>
<td>19 691</td>
</tr>
</tbody>
</table>

Crude extracts of expressed host cells harboring the mentioned plasmid DNA were diluted and mixed with 1 \( \mu \)g of non-methylated lambda DNA in the presence of 1 \( \mu \)M \([\text{H}]\text{AdoMet}\) in 50 \( \mu \)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 dried for radioactivity counting.

Figure 3. Purification profile of M.EcoHK31I. 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 EcoHK31I R-M system and the location of M.EcoHK31I. Restriction fragments were subcloned to pUC19 or pBluescript and crude extract was tested for restriction activity. Plasmid DNA was purified and digested with R.EcoHK31I to study in vitro methylation capability. The smallest clone which carries methylase in vivo was used separately or together to protect non-methylated lambda DNA (Fig. 4). Affi-gel heparin pool. (2) Crude extract after ultracentrifugation. (3) DEAE-Sephacel pool. (4) Affi-gel heparin pool.

In vivo In vitro
In vivo In vitro
M R MTase
Unknown protein

Table 2. Activity of M.EcoHK31I.

<table>
<thead>
<tr>
<th>Extract</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>711</td>
</tr>
<tr>
<td>pET3a</td>
<td>772</td>
</tr>
<tr>
<td>pET3a-M38 (encodes polypeptide ( \alpha ))</td>
<td>1687</td>
</tr>
<tr>
<td>pET3a-C23 (encodes polypeptide ( \beta ))</td>
<td>987</td>
</tr>
<tr>
<td>pET3a-M38 + pET3a-C23</td>
<td>19 691</td>
</tr>
</tbody>
</table>

Crude extracts of expressed host cells harboring the mentioned plasmid DNA were diluted and mixed with 1 \( \mu \)g of non-methylated lambda DNA in the presence of 1 \( \mu \)M \([\text{H}]\text{AdoMet}\) in 50 \( \mu \)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.
on 16 September 2017

by guest

Downloaded from https://academic.oup.com/nar/article-abstract/23/1/103/2400321/A-bacterial-methyltransferase-M-Eco-HK31I-requires

The Mspl are Hhal methylation and hybrid DNA of and PAll and HpaW such as DNA can complement for M.BsuRI M.BspRI in vivo or (18,19). Detached parts of certain monospecific mC5-MTases be crossed over to generate novel target specificities methylases, DNA recognizing domains in the variable region can provide us with valuable information in the structure-function relationship of mC5-MTase and insight on the organization plan of M.EcoHK31I. M.Hhal 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.EcoHK31I, 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 GKGQGER in the variable region of M.Hhal (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.EcoHK31I and to purify the two polypeptides to homogeneity for biochemical characterization.

ACKNOWLEDGEMENTS

Thanks are due to Dr X. Cheng for providing us coordinates of M.Hhal and its enzyme–substrate complex and Dr T. B. Ng for critical reading of the manuscript. The screening of E.coli HK31 was supported by a contract grant from New England Biolabs Inc., USA.

REFERENCES


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 μl 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).