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Complementation by detached parts of GGCC-specific DNA methyltransferases

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ABSTRACT

Individually inactive N- and C-terminal fragments of the m^5C-methyltransferase M.*Bsp*RI can complement each other resulting in specific, *in vivo* methylation of the DNA. This was shown by cloning the coding regions for N- and C-terminal parts of the enzyme in compatible plasmids and co-transforming them into *E.coli* cells. The enzyme could be detached at several different sites, producing either non-overlapping or partially overlapping fragments capable of complementation. Reconstitution of the active methyltransferase from inactive fragments was demonstrated *in vitro*, as well. Another GGCC-specific methyltransferase, M.*Bsu*RI, showed a similar complementation phenomenon. Moreover, interspecies complementation was observed between appropriate fragments of the two closely related enzymes M.*Bsp*RI and M.*Bsu*RI. Fragments of structurally and functionally more different methyltransferases were unable to complement each other.

INTRODUCTION

DNA modification methyltransferases (MTases) recognize short, specific nucleotide sequences and transfer a methyl group from S-adenosyl-methionine (AdoMet) to the DNA. MTases can be classified in three groups according to the methylated base they produce: m^5C-, m^4C- and m^6A-MTases. Although there is certain similarity at the amino acid (aa) level within each group (1,2,3), the most pronounced homologies are found in the family of m^5C-MTases. The genes of more than two dozen m^5C-MTases have been cloned and sequenced (4). Sequence comparisons revealed a common building plan for the enzymes, presumably reflecting a common evolutionary origin. m^5C-MTases share 10 homologous blocks in their aa sequence (2). One of these blocks, carrying a Pro-Cys dipeptide has been proposed to be part of the catalytic center (5). Based on point mutations and domain swapping experiments with phage coded multispecific MTases (6,7), it has been suggested that the so-called variable region between the 8th and 9th homologous blocks contains the target recognizing domain responsible for the sequence specificity of these enzymes.

No direct three-dimensional structural data are available to date for MTases. To understand the structural and functional organization of these enzymes we attempted to construct chimaeras of different m^5C-MTases. In the case of the closely related M.*Bsp*RI (8) and M.*Bsu*RI (9) enzymes this approach produced active chimaeras (10). In the course of this work we found that physical continuity of the gene coding for M.*Bsp*RI is not a necessity for activity. We hypothesized that detached parts of the enzyme might complement each other. A similar phenomenon was observed in a few cases where fragments of a monomeric protein could reconstitute enzymatic activity by complementation (11). Moreover, it was demonstrated that a m^5C-MTase, M.*Aqu*I consists of two peptides coded by two, partially overlapping reading frames (12). The basic structure of these two peptides corresponds to the N-terminal and C-terminal halves of the prototype m^5C-MTase.

To explore the complementation phenomenon we chose a way of modifying the genes rather than the proteins. The limited availability of purified MTases, as well as the broader possibilities of producing truncated enzymes at the DNA level made this approach feasible.

MATERIALS AND METHODS

Strains, plasmids and media

All experiments were done in *E.coli* strains JM107MA2 (*mcrB*$^-$) (13) or HB101 (*mcrB*$^-$) (14). Bacteria were grown in LB medium at 37°C. The antibiotics ampicillin and tetracyclin were used at concentracions of 50 and 15 μg/ml, respectively. Plasmids pUC18 and pUC19 (15), pACYC184 (16,17) and pKK223-3 (18) were used in subcloning experiments. The sources for MTase genes were plasmids pES2(8), pSU11 (9), pSU21 (19), pSI4 (20) and pSau5 (21), expressing the MTases M.*Bsp*RI, M.*Bsu*RI, M.SPR, M.*Sin*I and M.*Sau*96I, respectively. Plasmid pBE carries a 1300 bp insert, poor in GC-rich restriction sites, cloned in pUC19. This plasmid was used as DNA substrate in restriction protection tests *in vitro*, for better visualization of partial digestion patterns.

Enzymes and chemicals

Restriction endonucleases were purchased from New England Biolabs or were prepared in this institute. DNA polymerase large fragment, mung bean nuclease and BAL31 were from New England Biolabs. T4 DNA ligase was prepared in this institute.

All enzymes were used in accordance with the manufacturer's specifications. Tritiated S-adenosyl-methionine (81.5 Ci/mmol) was purchased from Amersham.

Recombinant DNA techniques

Transformation of *E.coli*, isolation of plasmid DNA, agarose gel electrophoresis and SDS polyacrylamide gel electrophoresis were done by standard procedures (22).

Methyltransferase assays

In vivo MTase activity was detected by isolating the plasmids from induced cells and subjecting them to 20 to 100-fold overdigestion by the appropriate restriction endonuclease. Resistance against cleavage indicated methylation of the relevant sites on the plasmids.

MTase activity was also determined in crude extracts. Cells were grown overnight, induced by 1 mM IPTG, harvested by centrifugation, suspended in 1/10 volume 50 mM Tris (pH 8.0) buffer and disrupted by french press. Cell debris was removed by centrifugation in an Eppendorf-centrifuge at 4°C. The supernatant was then subjected to DNaseI digestion (25 μg/ml, 10 min, 20°C). The reaction was stopped by the addition of 10 mM EDTA. MTase activity of the extract was then tested by measuring the transfer of ^{3}H-labelled methyl groups from AdoMet to DNA, as described in an earlier paper (23). Alternatively, plasmid DNA was methylated by the extract, protecting it from digestion by the corresponding restriction endonuclease. Typical conditions for the latter test were: 5 μl crude extract, 1 μg plasmid DNA (pBE), 80 μM AdoMet, 50 mM Tris pH 8.0, 10 mM EDTA pH 8.0. Incubation was at 37°C. After phenol extraction and ethanol precipitation, samples were digested by an excessive amount of the appropriate restriction endonuclease.

RESULTS

Construction of compatible plasmids coding for N- and C-terminal fragments of M.*Bsp*RI

In the course of subcloning experiments a plasmid construct carrying a pair of detached gene segments, representing partially overlapping N- and C-terminal fragments of M.*Bsp*RI showed partial resistance against *Bsp*RI cleavage. Based on preliminary experiments we hypothesized that separate protein products are synthesized from the 5'-and 3'-end fragment, and the two peptides produce active enzyme by complementation. To test this hypothesis, two series of plasmids were constructed.

The first set of plasmids (Fig.1) carried 3'-end fragments of different length of the *bspRIM* gene inserted in the polylinker region of pUC19 or pUC18. The orientation of the insert was such that the transcription of the truncated MTase gene was under control of the *lacZ* promoter-operator. Translation of the fragment was ensured by fusing it to a short N-terminal segment of the β-galactosidase. In cases the MTase fragment was not in frame with the β-galactosidase, the plasmid was linearized by cutting it at a unique site in the polylinker, upstream of the insert. The reading frame was then appropriately adjusted either by filling in the recessed ends by DNA polymerase large fragment or by digestion of the protruding ends by mung bean nuclease.

The second set of plasmids (Fig.1) was constructed in two steps. First, the 5'-end fragments of the *bspRIM* gene were inserted in pUC19 or pUC18. To obtain proper translation termination of the truncated gene, the polylinker region at the 3'-end of the gene segment was manipulated similarly as above, resulting in a translation stop signal downstream of the insert. The whole region, together with the *lacZ* promoter-operator was then cut out by *Bsp*RI (pUC19 coordinates of the cleavage points: 390/647) and inserted between the two *Pvu*II sites of pACYC184. Transcription of the insert was ensured by the *lacZ* promoter and the gene's natural promoter. Translation was initiated at the original start site of M.*Bsp*RI.

In vivo complementation by N- and C-terminal fragments of M.*Bsp*RI

The two sets of compatible plasmids were pairwise co-transformed into JM107MA2 cells and Ap^{R}Tc^R double-transformants were selected. Plasmids were isolated from IPTG induced overnight cultures and subjected to excessive *Bsp*RI digestion. Partial or complete protection against cleavage indicated the *in vivo* presence of *Bsp*RI specific MTase activity. Results, summarized in Fig.2, indicate that various combinations

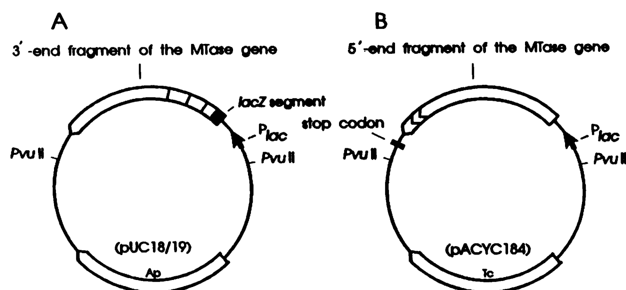

Figure 1. Schematic diagram of two sets of plasmids carrying 3'- or 5'-end gene segments of *bspRIM*.

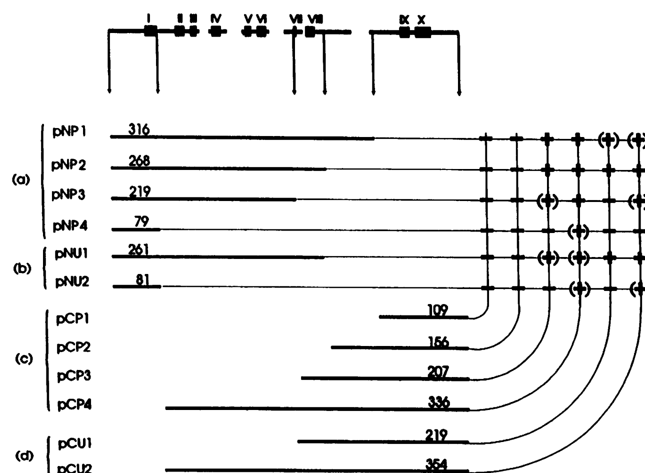

Figure 2. Schematic representation of the complementation ability of MTase fragments. On the top the general structure of m^{5}C-MTases is shown with conserved blocks I–X. Vertical arrows mark the boundaries of the fragments with respect to the MTase structure. Horizontal thick lines represent the relevant MTase fragments. The numbers above the thick lines show the number of amino acid residues in the MTase fragments. The pNP (a) and the pNU (b) plasmid series codes for N-terminal fragments of M.*Bsp*RI and M.*Bsu*RI, respectively. The pCP (c) and the pCU (d) plasmid series codes for C-terminal fragments of M.*Bsp*RI and M.*Bsu*RI, respectively. The effectiveness of *in vivo* methylation by the products of co-transformed plasmid pairs is shown on the right. Full or nearly full methylation of the M.*Bsp*RI sites on the plasmids is marked by +, partial methylation by (+), absence of methylation by –, as judged from *Bsp*RI digestion patterns.

of individually inactive N- and C-terminal fragments are capable of methylation by complementation. All four N-terminal fragments were active in complementation, even the one carrying only the first homologous block. Among the C-terminal fragments, those reaching to at least block VII were able to complement. MTase activity was produced by pairs of N- and C-terminal fragments which were either partially overlapping or were non-overlapping but ended at the same break point. No complementation was observed if there was a 'gap' between the N- and C-terminal fragments, and none of the complementing partners alone was able to methylate (for illustration see Fig.3).

To exclude the possibility that a complete *bspRIM* gene might have been formed by homologous recombination between the two plasmids, and the observed MTase activity might have originated from the intact M.*Bsp*RI molecule, the following experiment was done: Plasmids pNP2 and pCP4 (Fig.2) were isolated from double-transformant cells. This plasmid mixture is completely resistant to *Bsp*RI cleavage. The plasmids were transformed in JM107MA2 cells at low DNA concentration to avoid double-transformants, and the cells were grown in LB medium supplemented with either Ap or Tc. Plasmids were again prepared, and 1 μg of them was excessively digested by *Bsp*RI. The digestion mixture was again transformed in JM107MA2 cells. After two such rounds no transformants were obtained, indicating the absence of a plasmid molecule with a recombinant, intact *bspRIM* gene, which would have otherwise survived the repeated *Bsp*RI digestion due to self-protection.

Further observations also exclude recombination: A similar though somewhat lower MTase activity was observed if the *recA* HB101 was used as host (data not shown). Moreover, when a frameshift was introduced at the fusion point of the β-galactosidase segment and the MTase gene 3′-fragment in plasmid pCP4, resulting in plasmid pCP4F, MTase activity was practically abolished in complementation experiments (Fig.3). The weak residual activity might be the result of translation initiation at a secondary start site downstream of the frameshift. In addition, elimination of the stop codon downstream of the MTase gene 5′-fragment in plasmid pNP2, yielding a larger fusion protein, resulted in the loss of complementation ability (data not shown). These alterations would not interfere with a possible recombination, but hinder the formation of a protein fragment active in complementation.

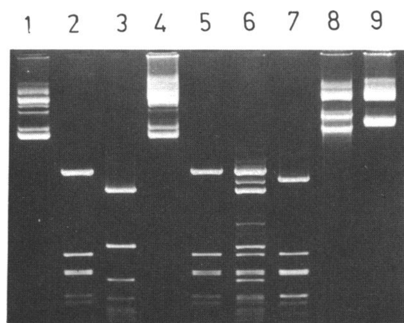

Figure 3. Detection of *in vivo* methylation of *Bsp*RI sites on plasmids coding for complementing M.*Bsp*RI and M.*Bsu*RI fragments. 1 μg plasmid DNA, prepared from single- or double-transformants, was 100-fold overdigested with *Bsp*RI and run on a 1.5% agarose gel, in the following order: lane 1, undigested pNP2+pCP4; lane 2, pCP4; lane 3, pNP2; lane 4, co-transformed pCP4 and pNP2; lane 5, pCP1F; lane 6, co-transformed pCP4F and pNP2; lane 7, pCU2; lane 8, co-transformed pCU2 and pNP2; lane 9, undigested pCU2+pNP2.

Detection of the complementing protein fragments

For further analysis we chose a pair of plasmids (pNP2/pCP4) the protein products of which ensure complete *in vivo* methylation of the DNA by complementation. Total extract from JM107MA2 cells harboring plasmid pCP4 was run on a denaturing polyacrylamide gel (Fig.4). Upon induction a 42 kDa protein band appears on the gel, corresponding to the C-terminal fragment of the MTase (calculated M_r: 40861). In addition, a stronger band of a 38 kDa protein is detected, which might be a degradation product of the C-terminal peptide.

Since the N-terminal MTase fragment is not detectable in total extracts of cells harboring plasmid pNP2, probably due to the low copy number of the pACYC184-derived plasmid, we constructed an overproducer (pKNP2) by inserting the gene fragment in a pKK223-3 plasmid vector. Upon induction, a protein band of 32 kDa appears on the gel, in good agreement with the calculated M_r (31033) of the N-terminal MTase fragment (Fig.4).

In vitro methylation by the complementing proteins

Crude extract was prepared from JM107MA2 cells harboring the plasmid pair pNP2/pCP4. The extract was then used to methylate plasmid pBE DNA. Under our experimental conditions the pNP2/pCP4 extract completely methylated the substrate in 30 min, as judged by *Bsp*RI digestion of the samples (Fig.5).

Crude extracts were also prepared from single-transformant cells, harboring either pNP2 or pCP4. Neither the pNP2 nor the pCP4 extract alone showed any *Bsp*RI specific MTase activity. However, when the two extracts were combined, methylation was detectable after 4 hours of incubation (Fig.5).

Comparison of the methylation potential of the extract from double-transformant cells with the combined extracts of single-transformant cells reveals a marked difference in their activity. Association of the N- and C-terminal MTase fragments seems to be more effective *in vivo* than *in vitro*.

The same extracts were used in another MTase assay, where ^{3}H-labelled methyl group transfer was measured. Results reinforced the observations above (Table 1). In addition, methylation by the complementing MTase fragments proved to

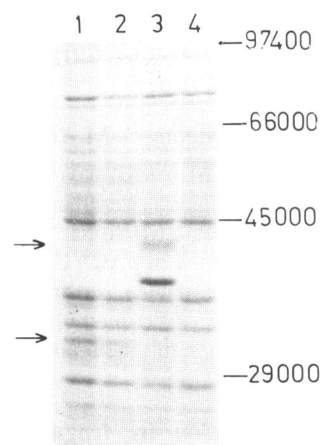

Figure 4. Detection of M.*Bsp*RI fragments in crude extracts. Total extract from induced (lanes 1 and 3) and uninduced (lanes 2 and 4) overnight cultures harboring plasmid pKNP2 (lanes 1 and 2) or pCP4 (lanes 3 and 4) were run on a 10% SDS/polyacrylamide gel. Arrows point to the Mtase fragments.

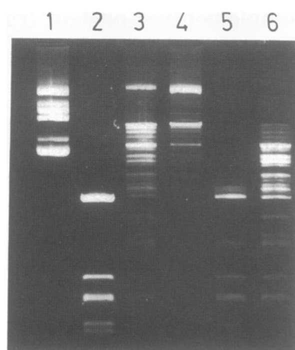

Figure 5. *Bsp*RI digestion of plasmid DNA methylated *in vitro* by crude extracts containing M.*Bsp*RI fragments. Extracts used for methylation were prepared from cells carrying plasmids pCP4 and pNP2 simultaneously (lanes 3 and 4) or were a mixture of extracts containing the two plasmids individually (lanes 5 and 6). Incubation time was 10 min (lane 3), 30 min (lanes 4 and 5), 4 h (lane 6). Methylated plasmids were 100-fold overdigested with *Bsp*RI, and run on a 1.5% agarose gel. Controls were undigested pBE (lane 1) and pBE digested by *Bsp*RI (lane 2).

Table 1. Methyl group incorporation into plasmid DNA substrates by crude extracts carrying M.*Bsp*RI fragments. The pNP2/pCP4 extract was from cells containing both plasmids. The pNP2+pCP4 extract was a mixture of extracts from cells containing the two plasmids separately. The extract from cells harboring pUC19 was a control. Incorporation of tritiated methyl groups into 5 μg DNA by 5 μl extract was measured by procedures described earlier (23) and is shown in cpm. Incubation time was 1 h. The DNA substrate pES2 carried M.*Bsp*RI specific methylation, pBE was unmethylated.

		DNA substrate	
		pBE	pES2
extract	pUC19	1800	1550
	pNP2	1850	1650
	pCP4	1950	1600
	pNP2+pCP4	3250	1800
	pNP2/pCP4	12500	1750

be strictly M.*Bsp*RI specific, since no methyl group incorporation was detected in the DNA substrate carrying *Bsp*RI specific modification.

Complementation by fragments of M.*Bsu*RI

M.*Bsu*RI is closely related to M.*Bsp*RI, having identical sequence specificity (GGCC) and sharing an overall aa homology of 64%. Similarly to the experiments with M.*Bsp*RI, 5′- and 3′-fragments of the *bsuRIM* gene were cloned in pACYC184 and pUC18/19, respectively. Following pairwise co-transformation into JM107MA2 cells, GGCC-specific methylation was observed indicating that complementation of the N- and C-terminal MTase fragments occured (Fig.2).

Interspecies complementation by fragments of different MTases

The similar structure of the m^{5}C-MTases led us to the idea of testing the complementation potential of fragments of different MTases. Plasmids carrying the coding region for N-terminal peptides of M.*Bsp*RI were co-transformed with those coding for the C-terminus of M.*Bsu*RI, and vice versa. DNA isolated from double-transformants showed a various degree of methylation (Fig.2). In general, M.*Bsp*RI and M.*Bsu*RI fragments can complement each other (Fig.3), though the effectiveness of this complementation is somewhat lower than within the M.*Bsp*RI or M.*Bsu*RI system, as judged by the *Bsp*RI digestion patterns.

A series of pUC18/19-derived plasmids were constructed carrying the coding region for the C-terminus of the following MTases: *Sau*96I (GGNCC), *Sin*I (GGA/TCC), SPR (GGCC, CCGG, CCA/TGG). These plasmids were tested for complementation with the constructs coding for the N-terminus of M.*Bsp*RI. None of the combinations gave any detectable methylation, as checked by digestion of plasmids obtained from double-transformant cells by either corresponding restriction endonuclease (data not shown).

DISCUSSION

Studies on bovine pancreatic ribonuclease A (24,25), β-galactosidase (26), staphylococcal nuclease (27,28), cytochrome *b5* reductase (29), thioredoxin (30,31) and *Salmonella typhimurium* alanine racemase (32) demonstrated that fragments of a monomeric enzyme could combine by non-covalent interactions to reconstitute an active enzyme. A similar observation is reported here in the case of the m^{5}C-MTase M.*Bsp*RI. Detached N- and C-terminal fragments of the enzyme complement each other retaining the GGCC-specificity. Fragments of M.*Bsu*RI showed a similar behaviour. The common structure of the m^{5}C-MTases, as well as the finding that M.*Aqu*I is in it's natural state a combination of two peptides (12), suggest that this phenomenon might be a common feature of m^{5}C-MTases.

Our approach of manipulating the genes made the construction of several peptide fragments of the MTases possible. The M.*Bsp*RI molecule can be detached at several different sites still giving actively complementing fragments, none of which showing any activity alone. Both non-overlapping and partially overlapping protein fragments produce enzyme activity. Even a peptide carrying only the first conserved block, which is suggested to be the AdoMet binding site (3), is capable to complement the rest of the protein. The relative independence of the conserved blocks as structural units is underlined not only by the complementation experiments, but also by the finding that a mammalian m^{5}C-MTase contains the same blocks but in an altered sequential order (33).

Complementation was achieved not only *in vivo*, but also *in vitro* by using crude extracts of cells carrying the complementation partners. However, the extract from double transformants was always significantly more active than the mixture of the extracts from single transformants. Simultaneous *in vivo* presence of the complementing partners seems to stabilize them. Whether this is a result of mutual protection against proteases or the two halves promote each other's correct folding or other factors are responsible, remains to be seen.

Our original objective was to construct chimaeric MTases by transferring the domain determining the recognition specificity from one enzyme to another. The complementation phenomenon gave a potential new tool: instead of precisely fusing different gene pieces resulting in a fusion protein, it could be assumed that complementation allows more flexibility between two peptides of different origin. In fact, interspecies complementation was demonstrated between fragments of M.*Bsp*RI and M.*Bsu*RI.

The two enzymes share a high degree of homology and have identical sequence specificity (GGCC). However, fragments of evolutionally more distant MTases with different recognition specificity did not show complementation with fragments of M.*Bsp*RI. Not excluding the negative role of protein instability, incorrect folding, etc., the reason for this is probably the larger difference in the structure of the enzymes. When counting the identical aa-s in the conserved blocks of the MTases, the identity scores are 83% for M.*Bsp*RI/M.*Bsu*RI, 48% for M.*Bsp*RI/M.*Sin*I, 46% for M.*Bsp*RI/M.*Sau*96I and 38% for M.*Bsp*RI/SPR, which suggest that an identity score somewhere between 48% and 83% is needed for active complementation. The same limitation seems to exist when constructing chimaeric genes: M.*Bsp*RI/M.*Bsu*RI chimaeras work well (10), while M.*Bsp*RI/SPR, M.*Bsp*RI/M.*Hha*I and M.*Bsp*RI/M.*Sau*96I hybrids were inactive (unpublished results).

The complementation ability of MTase fragments provides a new approach to study the structural organization of these enzymes. Specific MTase functions can possibly be assigned to smaller units of the enzymes, and their evolutionary relatedness could be investigated by the complementation phenomenon.

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