

Generation of new DNA binding specificity by truncation of the type IC *EcoDXXI hsdS* gene

Maria P. MacWilliams and Thomas A. Bickle¹

Department of Microbiology, Biozentrum, Basel University,
Klingelbergstrasse 70, CH-4056 Basel, Switzerland

¹Corresponding author

The *hsdS* subunit of a type IC restriction-modification enzyme is responsible for the enzyme's DNA binding specificity. Type I recognition sites are characterized by two defined half-sites separated by a non-specific spacer of defined length. The *hsdS* subunit contains two independent DNA binding domains, each targeted towards one DNA half-site. We have shown previously that the 5' half of *hsdS* can code for a functional substitute of the full-length *hsdS*. Here we demonstrate that the 3' half of the gene, when fused to the appropriate transcriptional and translational start signals, also codes for a peptide which imparts DNA binding specificity to the enzyme. About half the natural *hsdS* size, the mutant peptide contains a single DNA recognition domain flanked by one copy of each internal repeat found in the full-length *hsdS*. Deletion of either repeat sequence results in loss of activity. Like the 5' *hsdS* mutant, the 3' mutant recognizes an interrupted palindrome, GAAYN₂RTTC, suggesting that two truncated subunits participate in DNA recognition. Co-expression of the 5' *hsdS* mutant and the 3' *hsdS* mutant along with *hsdM* regenerates the wild-type methylation specificity. Thus, there is a free assortment of subunits in the cell.

Keywords: DNA modification/DNA recognition/DNA restriction/gene duplication/type I restriction systems

Introduction

Classical DNA restriction and modification (R-M) systems can be broken down into three groups based on subunit composition and co-factor requirements. Type I R-M systems have been found in several species of the eubacterial kingdom, including Gram-negative enterics such as *Escherichia coli*, *Salmonella typhimurium*, *Citrobacter freundii* and *Klebsiella pneumoniae* (Daniel *et al.*, 1988; Barcus *et al.*, 1995; Valinluck *et al.*, 1995) as well as in such diverse species as *Mycoplasma pneumoniae* and *Bacillus subtilis* (Dybvig and Yu, 1994; Xu *et al.*, 1995). The better characterized type II systems are more widespread, being found throughout the Eu- and Archaeobacterial kingdoms. Type III systems, like the type Is, are, so far, more restricted in their distribution, with only four characterized systems to date (for reviews, see Wilson and Murray, 1991; Bickle and Krüger, 1993).

The two main (competing) activities of all R-M systems are methylation of the bacterial DNA at a specific sequence

and the endonucleic cleavage of DNA which is not methylated at that sequence. Type II R-M systems accomplish these tasks with two independent enzymes, a DNA methyltransferase and a restriction endonuclease. Type I systems differ from their type II cousins in that a single, multisubunit enzyme accomplishes both DNA modification and restriction. This enzyme is composed of three subunits, *hsdM*, *hsdS* and *hsdR*. A complex of *hsdM* and *hsdS* alone can catalyse methylation, but all three subunits are required for restriction. The type I group can be subdivided further into three families, IA, IB and IC, based on sequence similarities, antigenic cross-reactivity and genetic complementation (Bickle, 1987).

The mode of DNA recognition by the type I enzymes is interesting in that they generally recognize non-palindromic sequences composed of two defined half-sites, 3-5 bp in length, separated by a non-specific spacer of defined length. For example, the *EcoDXXI* system, the subject of this study, recognizes TCAN₇RTTC (where N = any base and R = G or A). The spacer arranges the half-sites on the DNA such that there is approximately one helix turn length between the two adenines to be methylated.

A single peptide, *hsdS*, is responsible for recognition of both DNA half-sites as well as for spacer length determination. In addition, *hsdS* must also form inter-subunit contacts with the other peptides of the enzyme complex. It has been shown previously that *hsdS* is composed of two independent DNA binding domains (Fuller-Pace *et al.*, 1984; Nagaraja *et al.*, 1985; Fuller-Pace and Murray, 1986; Cowan, 1989; Gubler *et al.*, 1992). The amino-proximal domain (ARD; Chen, *et al.*, 1995) recognizes the 5' half-site, and the carboxy-proximal domain (CRD) the 3' half site. These variable regions which constitute the DNA binding domains are separated by amino acid segments that are conserved between members of a particular type I family (Figure 1; reviewed in Kneale, 1994). Since the *hsdS* subunits within a family are interchangeable, the conserved regions are thought to mediate interactions with the other subunits of the enzyme complex (Fuller-Pace and Murray, 1986; Abadjieva *et al.*, 1994; Cooper and Dryden, 1994). Sequence comparisons amongst the IC enzymes reveal three conserved regions, one at either end of the peptide and a third, centrally located region (Meister *et al.*, 1993; Tyndall *et al.*, 1994). Within the central conserved region, there is a tandem repeat of a tetraamino acid sequence, TAEL. It has been shown that the number of repeats governs the length of the recognition site spacer: two repeats result in a 6 bp spacer while three repeats result in a 7 bp spacer (Price *et al.*, 1989; Gubler and Bickle, 1991; Gubler *et al.*, 1992).

When the amino acid sequence of a type IC *hsdS* subunit is compared with itself, another level of sequence conservation is revealed (Meister *et al.*, 1993; Abadjieva

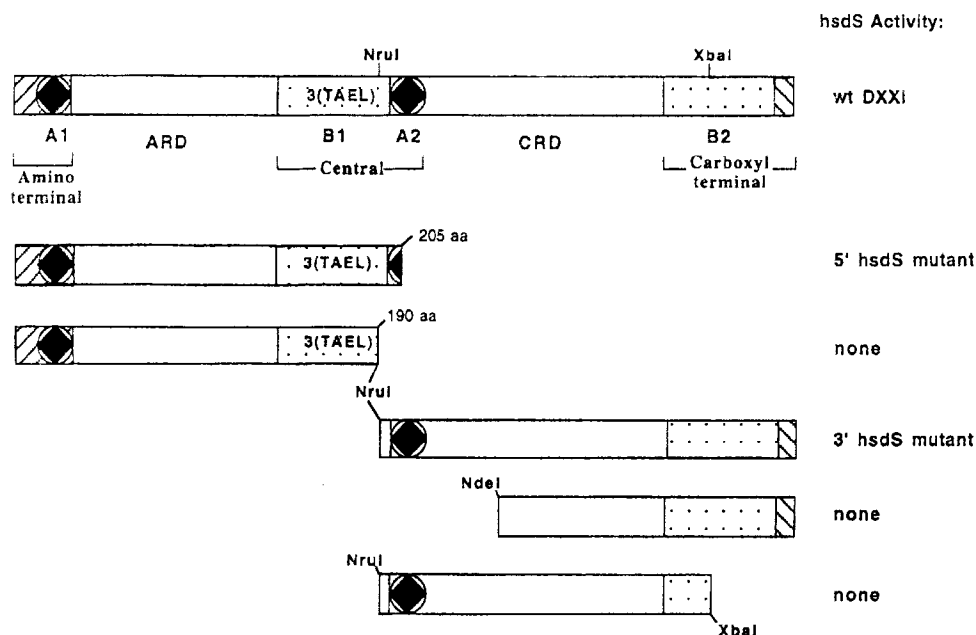


Fig. 1. Generation of new DNA binding specificities by truncation of the *hsdS* gene. The wild-type *EcoDXXI* *hsdS* peptide can be subdivided into two DNA binding domains (ARD and CRD, white areas) which are flanked by regions conserved amongst the IC family members. The three intrafamily conserved regions, amino-terminal, central and carboxy-terminal, are indicated in the figure. A portion of the amino-terminal region, A1, has a high degree of similarity to part of the central conserved region, A2 (diamonds within circles). The remainder of the central region, B1, is similar to part of the carboxy-terminal region, B2 (stippled rectangles). Deletion analyses have shown that each half of the *EcoDXXI* *hsdS* can substitute separately for the full-length *hsdS* protein (Meister *et al.*, 1993; this study). At least one copy of each of the internally repeated regions (A and B) is required for *hsdS* function. Indicated at the right is the DNA recognition specificity for each construct. The 3' *hsdS* mutant peptide is coded by pMMW81. The last two non-functional constructs shown are expressed by pMMW82 and pMMW88, respectively.

et al., 1994; Kneale, 1994; Tyndall *et al.*, 1994). Within the central region conserved between type IC *hsdS* subunits can be found a 13 amino acid stretch which, for *EcoDXXI*, is 91% similar to part of the amino-terminal conserved region (A1 and A2, Figure 1). The remaining portion of the central conserved region appears to be duplicated at the carboxy-terminal portion of the protein (also 91% similarity; B1 and B2, Figure 1). Such an arrangement has given rise to the proposal that the present day *hsdS* genes arose from a gene duplication event (Argos, 1985; Gann *et al.*, 1987). The hypothesis is supported by the observation that a truncated *hsdS* peptide composed of only the amino-terminal half of the wild-type peptide can still function as a recognition subunit (Abadjieva *et al.*, 1993; Meister *et al.*, 1993). The shortest truncated *hsdS* mutant is composed of a single copy of both repeated regions flanking a single DNA binding domain. In the case of the *EcoDXXI* 5' *hsdS* mutant, the mutant enzyme recognizes TCAN₈TGA, an interrupted palindrome of the wild-type 5' half site (Meister *et al.*, 1993). The recognition sequence suggests that the 5' mutant methylase utilizes two truncated *hsdS* subunits for recognition. Thus, the amino-terminal half of the peptide can substitute for the carboxyl half.

We were interested in whether the carboxyl half of the *EcoDXXI* *hsdS* protein could also substitute for a full-length *hsdS* to form a functional enzyme. Although the carboxyl half of the protein contains a copy of each conserved region as well as a DNA binding domain, it lacks an exact copy of the TAEL repeat. The results presented here demonstrate that the 3' half of the *EcoDXXI* *hsdS* gene, when fused to the proper transcriptional and translational start sequences, can code for a functional

hsdS subunit. In addition, co-expression of the 5' and 3' truncated *hsdS* genes, along with *hsdM*, resulted in the reconstitution of the wild-type *EcoDXXI* methylation activity. These cells also expressed both the 5' and the 3' methylation specificities. Thus, there is a free assortment of *hsd* subunits in the cell.

Results

The 3' half of hsdS codes for a functional hsdS subunit

We have demonstrated previously that the amino-terminal half of the *EcoDXXI* *hsdS* peptide can function as a specificity subunit. The mutant enzyme recognizes a novel sequence that is an interrupted palindrome of the 5' half-site of the wild-type enzyme. It is thought that two truncated subunits substitute for the full-length *hsdS* in the enzyme complex. This result suggests that the two halves of *hsdS* can code independently for the DNA binding and multimerization functions of a full-length *hsdS*.

To test this hypothesis further, we examined the ability of the carboxyl half of *hsdS* to substitute for the full-length *hsdS*. The 3' half of the *EcoDXXI* *hsdS* gene first was fused in-frame to the *LacZ* sequences of pBluescript II KS- (pKS-). The choice of fusion point was guided by sequence analysis of the *hsdS* gene. The *hsdS* gene appears to have arisen by a duplication event so that part of the amino-terminal conserved region of *hsdS* (A1, Figure 1) is very similar to the region just downstream of the middle of the peptide (A2). In addition, a central portion of the peptide (B1) bears a high similarity to the carboxyl end (B2). The repeated regions are separated from each other

by variable regions thought to function as the DNA binding domains for the peptide. The *NruI* site at 569 bases from the start of the gene lies at the extreme end of the B1 repeat. The *hsdS NruI* site was ligated to the *HincII* site of the pKS-polylinker to create an in-frame fusion (pMMW81, Figure 1). The fusion provided the *lac* regulatory sequences, promoter and transcriptional and translational start sites for the gene. The resulting peptide should be composed of 243 amino acids, 216 of which are derived from *hsdS*.

The 3' *hsdS* construct was analysed for its ability to provide a functional *hsdS* subunit by co-expression in cells along with *hsdM* or *hsdM* and *hsdR*. The methylation and restriction activities of the cells were monitored using bacteriophage λ restriction assays. The experiments were done as follows. Unmodified λ lysates (λ_0) were prepared by growing the phage on DH1 cells lacking any additional R-M-encoding plasmids. Bacteriophage bearing a specific type IC methylation pattern were prepared by growth on DH1 cells expressing that particular R-M system. Lysate dilutions subsequently were plated on cells lacking any IC R-M system, cells bearing the wild-type *EcoDXXI* system (DH5 α /pMMW68) or cells which co-expressed *hsdM*, *hsdR* and the 3' *hsdS* fusion (DH5 α /pHJ8+pMMW81). The efficiency of plating (EOP; ratio of the phage titre on restricting host to the titre on the non-restricting host) served as a measure of the restriction activities of the cells. The results are shown in Table I.

As expected, growth of λ_0 on cells expressing the wild-type *EcoDXXI* system (DH5 α /pMMW68) was restricted (EOP = 3×10^{-6}). Co-expression of the 3' *hsdS* fusion along with *hsdM* and *hsdR* (DH5 α /pHJ8+pMMW81) also led to restriction of λ_0 (EOP = 2.3×10^{-4}). Neither the cells expressing only the 3' *hsdS* fusion (DH5 α /pMMW81) nor cells expressing *hsdM* and *hsdR* (DH5 α /pHJ8) restricted λ_0 growth. These results demonstrate that the 3' *hsdS* can interact with *hsdM* and *hsdR* to assemble a functional restriction endonuclease.

λ_{vir} phages modified by passage through cells expressing the wild-type *EcoDXXI* system (λ_{DXXI}) were protected from restriction by DH5 α /pMMW68 cells but were restricted by the 3' mutant system (Table I). Likewise, phage grown on cells expressing *hsdM* and the 3' *hsdS* fusion ($\lambda_{3'mut}$) were protected against the 3' mutant R-M system but were restricted by the wild system. Thus, the 3' *hsdS* R-M system has a DNA binding specificity different from that of wild-type *EcoDXXI*.

The *NruI* 3' *hsdS* fusion of pMMW81 (and pMMW84) contains one copy of each repeated region flanking a variable region. To determine whether or not both conserved regions play a role in *hsdS* function, shorter derivatives of the 3' *hsdS* fusion were constructed (Figure 1). pMMW82 lacks the last 132 bp of *hsdS*, including a portion of repeat B, while still retaining the identical 5' fusion point of pMMW81. The 5' fusion point of pMMW88 is 78 bp downstream of that of pMMW81, while the rest of the gene is identical to that of pMMW81. Thus pMMW88 lacks the A repeat. Neither of these plasmids could provide a *hsdS* activity to participate in the formation of a functional methylase or restriction endonuclease *in vivo* (data not shown). These results suggest that intact copies of both conserved regions are necessary for *hsdS* function.

Regeneration of the wild-type *EcoDXXI* methylase

Both the 3' and the 5' halves of the *hsdS* gene can code independently for peptides which functionally substitute for the full-length *hsdS*. The palindromic recognition sequence of the 5' mutant suggests that two truncated 5' *hsdS* subunits participate in DNA recognition. We were interested in whether the co-expression of the 5' and 3' truncated *hsdS* genes along with *hsdM* could regenerate the wild-type methylation activity *in vivo*.

To investigate this possibility, modified λ phages were prepared on cells expressing the 5' mutant methylase from pMMW62 ($\lambda_{5'mut}$), the 3' methylase from pMMW84 ($\lambda_{3'mut}$) or in cells which co-express the 5' *hsdS* and *hsdM* from pMMW62 and the 3' *hsdS* from pMMW81 ($\lambda_{5'+3'mut}$). The modified lysates subsequently were plated on indicator strains expressing the different R-M systems. The ability to plaque on cells expressing a particular R-M system indicates that the phage bears that particular modification. Phages which had been passaged through cells expressing the 5' mutant methylase were restricted when plated on cells expressing the wild-type or 3' mutant R-M systems (Table II). Similarly, the 3' mutant modification protected $\lambda_{3'mut}$ from the analogous R-M system but not against the other two systems. Lysates grown on cells which expressed both the 5' and 3' mutant *hsdS* genes along with *hsdM* escaped restriction by not just the individual 5' and 3' systems but also by the wild-type system. Thus the wild-type specificity is reconstituted.

Identification of the 3' mutant recognition sequence

Previously, hybrid *hsdS* genes were constructed by recombination within the central region conserved amongst the IC family members (Gubler *et al.*, 1992). These swapping experiments demonstrated that the *EcoDXXI* carboxy-terminal DNA binding domain recognizes the *EcoDXXI* 3' half-site, RTTC. The fact that the 5' *hsdS* mutant recognizes a palindromic sequence (in which the *EcoDXXI* 5' half-site is repeated) suggested that the 3' mutant recognition sequence would be an interrupted palindrome of the *EcoDXXI* 3' half-site. Since the carboxyl portion of the *EcoDXXI* gene lacks a definite T A E L repeat, we were unable to estimate the non-specific spacer length between the two half-sites of defined sequence. Thus, the proposed recognition sequence for the 3' mutant was GAAYN_xRTTC (where Y = C or T and R = G or A). In addition, all previously characterized type IC recognition sequences (natural and synthetic) have a trinucleotide 5' half-site so that we were unsure as to whether the 'Y' position would remain as a defined base.

Coincidentally, the recognition sequence of the hybrid *hsdS* R-M system, *EcoRD2* (RD2), is very similar to the proposed 3' mutant site (Gubler *et al.*, 1992). In an effort to identify the 3' mutant recognition sequence *in vivo*, we analysed the ability of the 3' mutant methylation to protect λ phage against the RD2 system and vice versa. The RD2 modification completely protected λ against restriction by the 3' mutant system (EOP = 0.80). On the other hand, $\lambda_{3'mut}$ was restricted, but had a 200-fold higher EOP on the RD2-expressing strain compared with λ_0 (EOP = 5.56×10^{-2} for $\lambda_{3'mut}$ versus 2.77×10^{-4} for λ_0). These results suggested that the RD2 system methylated all the

Table I. Restriction of λ_{vir} by wild-type *EcoDXXI* and 3' hsdS mutant R-M systems^a

Plating lawn ^b		Bacteriophage modification ^c		
Plasmid	Restriction system	λ_0	λ_{DXXI}	$\lambda_{3' mut}$
None	none	1.0	1.0	1.0
pMMW68	<i>EcoDXXI</i>	3.0×10^{-6}	1.6	2.1×10^{-6}
pHJ8	hsdM + hsdR 0.86	0.81	0.85	
pMMW81	3' hsdS 0.86	0.99	0.74	
pMMW81 + pHJ8	3' hsdS + hsdM, hsdR	2.3×10^{-4}	6.8×10^{-4}	0.83

^aEOP values reported are averages of at least four independent assays.

^bPlating lawns were DH5 α containing the designated plasmids.

^cLysates were prepared on DH1 cells containing the following plasmids: λ_0 none; λ_{DXXI} pMMW68; $\lambda_{3' mut}$ pMMW84.

Table II. Regeneration of the wild-type *EcoDXXI* specificity^a

Plating lawn ^b		Bacteriophage modification ^c				
Plasmid	Restriction specificity	λ_0	λ_{DXXI}	$\lambda_{5' mut}$	$\lambda_{3' mut}$	$\lambda_{5'+3'}$
None	none	1.0	1.0	1.0	1.0	1.0
pMMW68	<i>EcoDXXI</i>	3.0×10^{-6}	1.6	7.0×10^{-7}	2.1×10^{-6}	1.20
pES14::Tn5	5' mutant	5.6×10^{-4}	1.1×10^{-3}	0.83	ND	0.93
pMMW81 + pHJ8	3' mutant	2.3×10^{-4}	6.8×10^{-4}	4.3×10^{-4}	0.83	0.56

^aEOP values are averages of at least three independent assays.

^bPlating lawns were DH5 α containing the designated plasmids.

^cLysates were prepared on DH1 cells containing the following plasmids: λ_0 none; λ_{DXXI} pMMW68; $\lambda_{5' mut}$ pMMW62; $\lambda_{3' mut}$ pMMW84; $\lambda_{5'+3' mut}$ pMMW62 + pMMW81.

3' mutant sites on λ while the 3' mutant system modified only a subset of the RD2 sites. This partial protection by the 3' mutant suggested overlapping specificities between the 3' mutant and RD2 systems.

The RD2 system recognizes GAAN₆RTTC (Gubler *et al.*, 1992). A potential candidate for the 3' mutant recognition sequence would be GAAYN₅RTTC, in which the first base of the RD2 spacer would be replaced by a Y in the 3' mutant site. The 3' mutant spacer would then be correspondingly 1 bp shorter. Indeed, λ contains 11 RD2 sites, of which eight have a Y at the first spacer position and three have an R.

Other potential DNA substrates were examined for RD2 sites. M13mp18 contains four RD2 sites, all of which have a T or C at the first spacer position. *In vivo* restriction assays were performed with modified M13mp18 phage to analyse further the overlapping sequence specificities of the RD2 and 3' mutant systems. M13mp18 modified by the 3' mutant was protected completely against restriction by the RD2 R-M system and RD2-modified M13mp18 was also protected against the 3' mutant system (Figure 2A).

To verify further the 3' mutant recognition sequence, two M13mp18 derivatives were constructed. M13mp18.2c contains two additional RD2 sites of the type GAARN₅RTTC, while M13mp18.141 has an extra GAAYN₅RTTC site. Results of restriction assays of these phages modified by the RD2 or 3' mutant methylases are shown in Figure 2B and C. The introduction of the GAARN₅RTTC sequence into M13mp18 rendered the M13mp18.2c phage sensitive to RD2 restriction even when the phage DNA had been modified previously by the 3' mutant system (Figure 2B). On the other hand, M13mp18.141, with the additional RD2 site of the GAAYN₅RTTC type, was still protected against RD2 restriction by prior 3' mutant modification (Figure 2C).

Thus, the 3' mutant recognizes GAAYN₅RTTC but not GAARN₅RTTC.

Confirmation of the 3' mutant recognition sequence and identification of the methylated adenine

There are eight predicted 3' mutant recognition sites in phage λ . Two of these sites overlap *SspI* sites (AATATT) such that the first and second As of the *SspI* site respectively correspond to the first and second As of the 3' mutant GAAY half-site (Figure 3A). We decided to exploit this overlapping arrangement to determine the nucleotide modified by the 3' mutant methylase. The type IC enzymes are adenine specific so that one of the As in the GAAY sequence was the probable methylation target. For *SspI*, methylation on the first A of the recognition site does not inhibit cleavage.

Bacteriophage λ was grown on cells containing the 3' mutant methylase, the RD2 methylase or no type IC system. DNA was prepared from the phage particles and subjected to *SspI* digestion. The cleavage products were analysed by agarose gel electrophoresis. The results are shown in Figure 3C.

Cleavage of the *SspI* site at 33 572 produces two fragments, 1431 and 4056 bp in size, while protection from *SspI* cleavage at this site should result in a single 5487 bp fragment (Figure 3B). Lack of *SspI* cleavage at the 42 822 site results in a 1358 bp fragment, while sensitivity to *SspI* at this position is indicated by the presence of 438 and 920 bp fragments. λ_0 DNA was cleaved at both positions, as shown by the presence of the diagnostic fragments (Figure 3C). Conversely, λ DNA modified by either the 3' mutant or the RD2 enzymes were resistant to *SspI* cleavage at these positions, as indicated by the 5487 and 1358 bp fragments. Since *SspI*

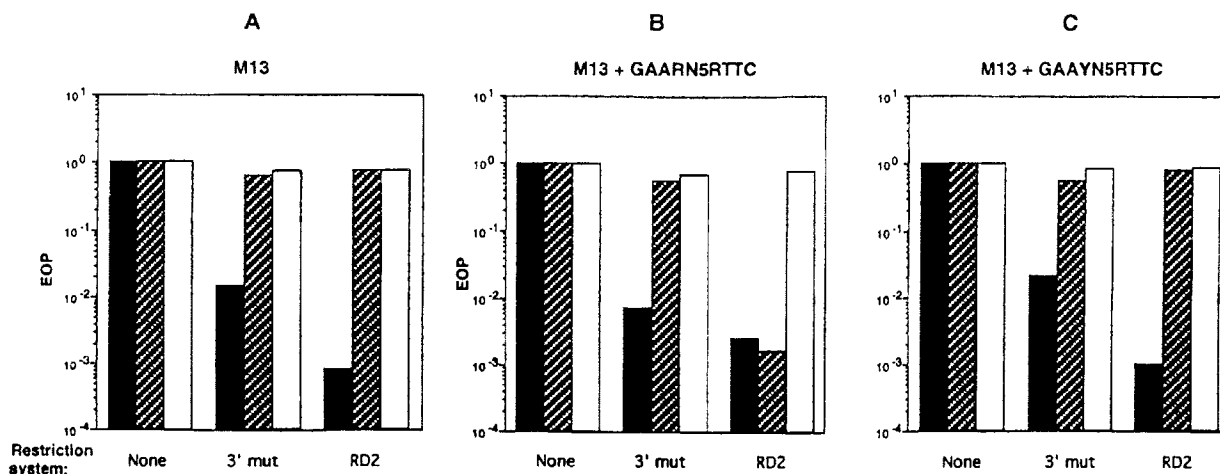


Fig. 2. The *EcoRD2* and 3' *hsdS* mutant R-M systems have overlapping DNA recognition specificities. (A) Bacteriophage M13mp18 lysates were prepared on XL1-Blue cells expressing no type IC system (black bars), cells expressing the 3' mutant system (striped bars) or cells expressing the RD2 methylase (white bars). These lysates subsequently were plated on XL1-Blue cells expressing the indicated restriction systems. The EOP values reported are the means of four independent assays. (B) and (C) Same as (A) except that the M13mp18 phage derivatives used contain additional RD2 recognition sites. The results in (B) are for M13mp18.2c which contains two additional sites of the class GAARN₅RTTC. The phage used in (C) was M13mp18.141 which contains an additional GAAYN₅RTTC site.

is not inhibited by methylation on the first A of its recognition sequence and the middle A is a variable position for the overlapping type I site, the second A of the *SpyI* site must be the one that is modified. Thus, the 3' mutant methylase (as well as RD2) methylates the second A of the GAAY half-site. As the 3' mutant site is an interrupted palindrome, it is reasonable to assume that the same position is methylated on the opposite strand.

Discussion

R-M systems serve as a barriers to protect the host from foreign DNA and viruses. The ability quickly to evolve a new DNA binding specificity could be advantageous to the cell in that a bacteriophage that had managed to avoid initial restriction and acquire the old methylation specificity would still be restricted by the few cells with an altered recognition specificity. Type I R-M systems are ideally suited to such a defence strategy because the *hsdS* subunit is a separate entity from both the methylation and restriction subunits. When assembled into a complex, *hsdS* confers DNA specificity for both the methylation and restriction activities of the complex. In contrast, the type II systems are composed of separate enzymes which carry out the methylating and restricting activities independently. Each must specify the identical recognition sequence independently. For such systems to change their DNA binding specificity requires a minimum of two independent mutations. With type I systems, hypothetically only a single change in *hsdS* need occur. The modular arrangement of the *hsdS* gene also lends itself well to producing altered specificity mutants. It has been shown previously that altered specificity mutants have arisen due to both homologous and non-homologous recombination as well as to gene truncation (Bullas *et al.*, 1976; Fuller-Pace *et al.*, 1984; Gann *et al.*, 1987; Gubler and Bickle, 1991; Gubler *et al.*, 1992; Abadjieva *et al.*, 1993; Meister *et al.*, 1993).

To understand further what is required for type I DNA recognition, we have tested the ability of the carboxyl

half of the *EcoDXXI* *hsdS* protein to function as a specificity protein. The results presented here demonstrate that the carboxyl half can functionally substitute for the wild-type *hsdS* in both the restriction and modification reactions. The 3' mutant recognizes the sequence GAAYN₅RTTC, with the second A of each half-site as the methylation target. This is the first type I enzyme whose recognition sequence has a 4 bp 5' half-site. Like the previously characterized 5' truncated *hsdS* mutant enzymes (Abadjieva *et al.*, 1993; Meister *et al.*, 1993), the palindromic nature of the recognition sequence suggests that the 3' mutant enzyme is composed of two mutant *hsdS* subunits.

The 5' and 3' mutant results show that the two DNA binding domains of *hsdS* do not need to be covalently linked as in the wild-type enzyme. In fact, the wild-type specificity can be regenerated upon co-expression of the 5' and 3' truncated *hsdS* genes along with *hsdM*. These cells not only methylated the wild-type DXXI recognition sequence but also the 5' and 3' mutant sequences. This demonstrates that there is a free assortment of subunits within the cells.

Both the wild-type and the 5' mutant methylases specify a 10 bp distance between the methylatable adenines, while the 3' mutant recognizes a sequence with 9 bp separation. The reconstituted enzyme seemed to acquire the spacer distance of the 5' enzyme, but we were interested in whether the 3' mutant spacer distance was also represented in the hybrid expression system. We were able to demonstrate that some methylation did indeed occur at the sequence TCAN₆RTTC, because an overlapping *EcoRI* site was partially protected from *EcoRI* cleavage by the 3'-5' co-expressing cells (data not shown). This alternate methylation pattern might be due to a relaxed specificity of the enzyme. Alternatively, it might reflect the arrangement of S subunits in the methylase complex. The two half positions in the enzyme may not be equivalent, with one position responsible for dictating the distance between the two half-sites of the recognition sequence.

It might be thought that the covalent association of the