

The domain structure of the DNA specificity subunit of type I restriction endonucleases

I. Cloning, mutagenesis and over-production of the *EcoR124* DNA methyltransferase

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Introduction

Type I restriction endonucleases consist of three subunits encoded by the genes *hsdR*, *hsdM* and *hsdS*. The *hsdS* gene product is responsible for DNA specificity and together with the *hsdM* gene product is sufficient for modification (methylation) of the DNA recognition sequence (Boyer and Roulland-Dussoix, 1969; Glover and Colson, 1969; Hubacek and Glover, 1970); *hsdR* is required for endonuclease activity. The endonuclease requires ATP, SAM and Mg^{2+} as co-factors while the DNA methyltransferase requires only SAM and Mg^{2+} . Evidence has been presented to support the hypothesis that the HsdS protein has two domains responsible for DNA recognition, separated by a spacer region (Fuller-Pace and Murray, 1986; Price *et al.*, 1989).

The *EcoR124* R-M system is of particular interest in that an alternative DNA specificity (*EcoR124/3*) has been identified (Firman *et al.*, 1983) which differs from the *EcoR124* DNA specificity by the presence of an extra non-specific nucleotide:

EcoR124 – GAANNNNNNRTCG (or GAAN₆RTCG)
EcoR124/3 – GAANNNNNNRTCG (or GAAN₇RTCG)

EcoR124 and its variant form *EcoR124/3* have been cloned producing the recombinant plasmids pCP1005 and pUNG31 respectively, their transcripts mapped, the genes sequenced and low levels of the endonuclease have been purified (Firman

et al., 1983, 1985; Glover *et al.*, 1983; Price *et al.*, 1987, 1989). These data show that they are members of a new sub-class of type I R-M systems: type IC. The only difference between *EcoR124* and *EcoR124/3* lies within their *hsdS* genes. The *EcoR124/3 hsdS* gene has an extra copy of a 12-bp repeat within the predicted spacer region (repeated twice in *EcoR124* and three times in *EcoR124/3*) (Price *et al.*, 1989).

The *EcoR124* endonuclease has not been purified at sufficiently high levels for detailed *in vitro* studies of this enzyme. Studies to date on the intact M-*EcoR124* DNA methyltransferase have been limited to expression from the natural promoter (Gubler and Bickle, 1991), and there have been no studies on the individual subunits. Our primary aim was to purify the DNA methyltransferase for enzymological studies, DNA binding assays, analysis of the stoichiometry and production of DNA binding mutants. We report here the successful over-expression of these genes and the production of a variety of DNA binding mutants.

Materials and methods

JM109(DE3) was obtained from Promega, Madison, WI; C600 is as described in Appleyard (1954), λ_{vir} was as in Jacob and Wollman (1954), pET3A is described in Rosenberg *et al.* (1987); other plasmids used are described in the text. Isolation and manipulation of DNA was as described in Maniatis *et al.* (1982).

Protein production was induced with 1 mM IPTG followed by growth for 7–8 hours. Cells were lysed by sonication in 25% sucrose, 50 mM EDTA, 3 mM DTT, 1 mM benzamine, 0.1 mM PMSF. The soluble fraction was obtained following centrifugation for 30 min at 18,000 rpm in a Sorvall SS34 rotor.

Oligonucleotide-directed mutagenesis was carried out as described by Nakamaye and Eckstein (1986) using mutagenic oligonucleotides prepared on a Cruachem P250 DNA synthesiser.

ssDNA isolation was as described in the Amersham oligonucleotide-directed mutagenesis kit (Cat. No. RPN 1523) except that a DNase I + RNase digestion step was included after re-suspending the PEG precipitated phage. This was followed by another round of PEG precipitation.

Uracil containing single strand DNA was prepared in a similar manner to that described for thymidine ("normal") containing DNA except that *E. coli* strain CJ236 was used instead of JM109 to maintain the phagemid.

Misincorporation mutagenesis was as described by Singh *et al.* (1986) except that a variety of limiting concentrations of dCTP were used and the size of the "mutagenic window" determined using ³⁵S-dATP and analysis on a 6% sequencing gel.

Results

Over-production of M-EcoR124 DNA methyltransferase

The *EcoR124 hsdM* gene was cloned from pCP1005 (Firman *et al.*, 1985) into *Sma*I digested pUC118 (Vieira and Messing, 1987) following digestion of pCP1005 with

*Hinf*I. The *hdsS* gene was cloned into *Sma*I digested pTZ19R (Mead *et al.*, 1986) following digestion of pCP1005 with *Ava*I. *Nde*I restriction target sites were introduced at the start codons of both genes and an internal *Nde*I site removed from *hdsS* using oligonucleotide directed mutagenesis (Patel *et al.*, 1992). This produced the two plasmids pJS491 and pUM120*. HsdS produced from pJS491 was found to be insoluble and could not be used for further studies (see Chapter 20). To construct

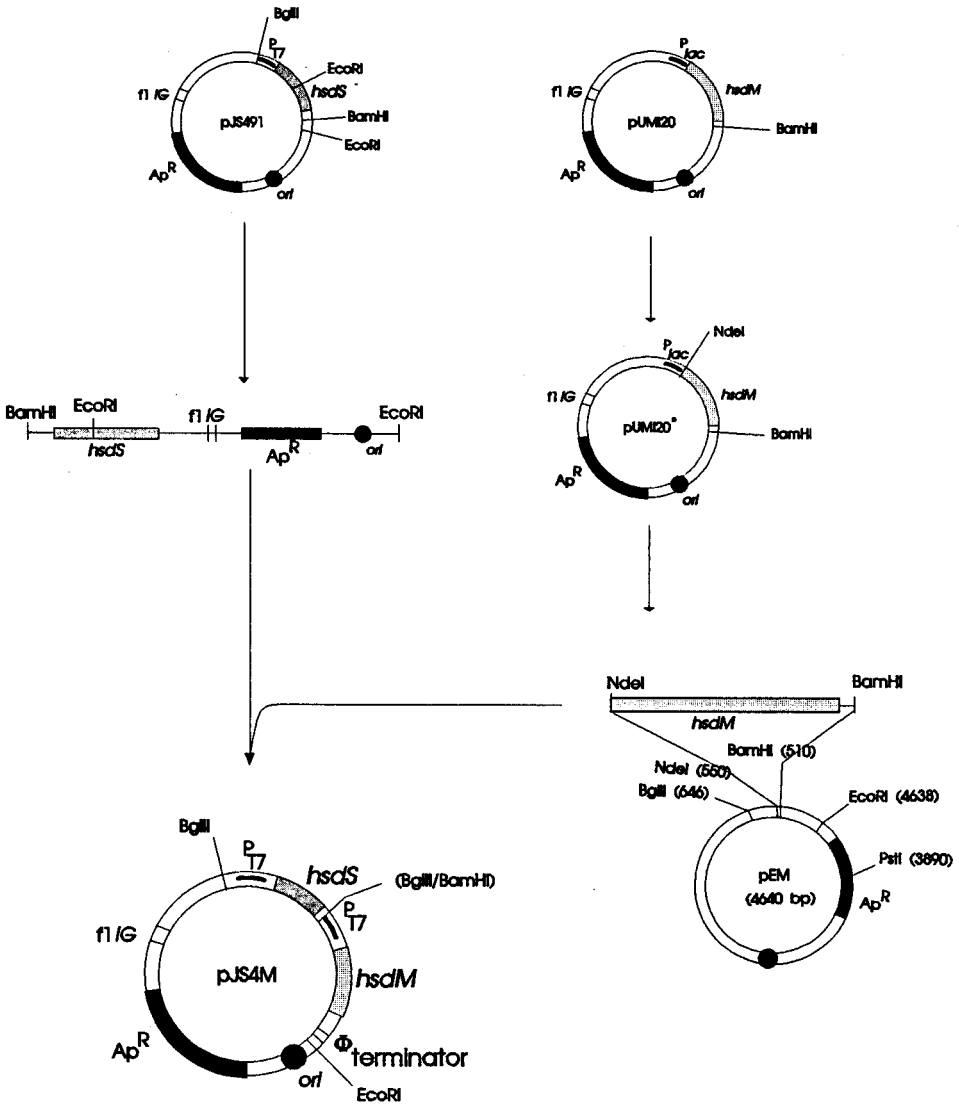


Figure 19.1. Construction of the co-expression vector pJS4M which produces M-*Eco*R124. Plasmid pUM120 (Patel *et al.*, 1992) ssDNA was subjected to site-directed mutagenesis to introduce an *Nde*I site at the start codon of *hsdM*. This produced pUM120*. The 1560-bp *Nde*I-*Bam*HI fragment containing *hsdM* was isolated and ligated to *Nde*I-*Bam*HI-digested pET3A (Rosenberg *et al.*, 1987). The resulting recombinant plasmid, pEM, was digested with *Eco*RI + *Bgl*II and ligated to a fragment containing *hdsS* produced by digesting pJS491 completely with *Bam*HI and partially with *Eco*RI.

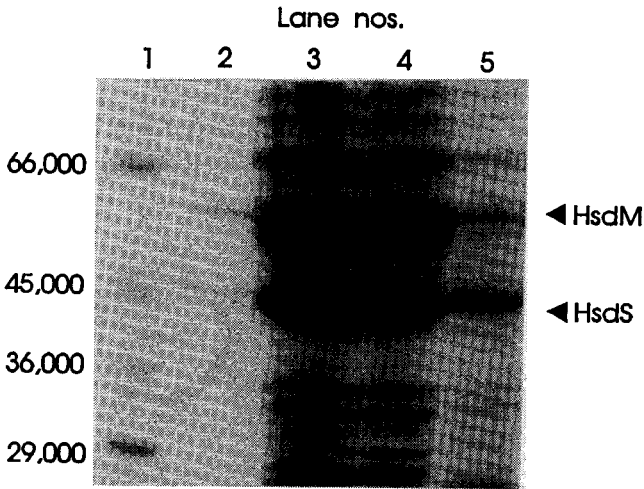


Figure 19.2. 0.1%–SDS–12.5% PAGE of bacterial lysates showing co-production of soluble HsdM and HsdS subunits. Induction was with 1 mM IPTG for 4 h. *Lane 1*, size markers; *Lane 2*, JM109(DE3)[pJS4M] pellet fraction (induced); *Lanes 3–5*, JM109(DE3)[pJS4M] soluble fraction induced, undiluted, diluted 1:10 and diluted 1:100 respectively.

an over-producing strain for the methylase the two genes were expressed from separate T7 promoters on a single plasmid (Fig. 19.1). In the resulting plasmid (pJS4M) the HsdS protein was produced from a single T7 promoter while the HsdM protein was produced from both its own T7 promoter and the T7 promoter expressing HsdS. This allows an excess of the HsdM protein to be produced which helps to solubilize the HsdS protein. Proteins isolated from both the soluble and insoluble fractions were visualized on 12.5% SDS-PAGE (Fig. 19.2). As can be seen the bulk

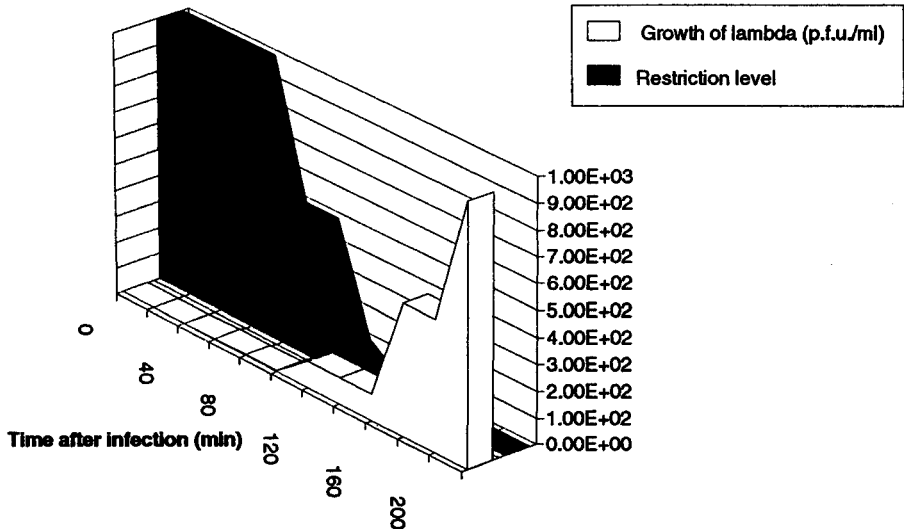


Figure 19.3. Effect of *in vivo* methylation by pJS4M on the susceptibility of bacteriophage lambda to restriction by EcoRI24.

of the HsdS subunit is present in the soluble fraction with all of the HsdM subunit, however, a small fraction of HsdS remains insoluble.

In vivo methylation

JM109(DE3)[pJS4M] was infected with lambda virulent bacteriophage, during early logarithmic growth, at a multiplicity of infection (MOI) of 1/10. This ensures that every bacteriophage infects a cell and will be subject to DNA modification. Samples were removed at 20 minute intervals, mixed with chloroform to lyse the cells, and centrifuged. The modified lambda was assayed by plating on C600 and C600[pCP1005]. The results in Fig. 19.3 show that while the rate of growth of the bacteria, and the consequent modification of lambda, are slowed by the methylase production (by approximately one-third, data not shown) eventually full modification of the bacteriophage does occur.

Effect of high-level production of HsdS on EcoR124/3 – complementation assay

Over-production of HsdS(R124) reduces the level of *EcoR124/3* restriction *in vivo*. This may reflect competition between HsdS(R124) and HsdS(R124/3) for production of endonuclease (Table 19.1). Mutations in the *hdsS* gene that result in restriction-deficiency could be due to either conformational changes that prevent subunit assembly or changes in the DNA binding properties of the subunit. The ability of a mutant subunit to compete with HsdS(R124/3) for production of the endonuclease would be a strong indicator that the restriction-deficiency was due to a failure to bind DNA and not due to conformational changes of the mutant HsdS(R124). Mutant HsdS proteins can be produced in the presence of pKF650 (Patel *et al.*, 1992) and restriction of both λ_{vir} .R124 and λ_{vir} .R124/3 measured. Mutants showing a reduced level of restriction against λ_{vir} .R124/3 are scored as restriction-deficient; those that show a reduced level of restriction against λ_{vir} .R124 are scored as proteins capable of protein-protein interactions.

Isolation of DNA binding mutants of EcoR124

A 17-mer oligonucleotide that hybridizes to bases 973–989 of the *hdsS*(R124) gene was used to prime mis-incorporation mutagenesis of pJS491 covering a region immediately upstream (700 bp to 900 bp) to this primer. The limiting nucleotide used was dCTP. 60 colonies were picked and ssDNA isolated for DNA sequencing. Single-track (C-track) sequencing was performed and those isolates showing changes in the DNA sequence were noted.

DNA from the same 60 isolates was transformed into JM109(DE3)[pKF650] and the level of restriction determined against λ_{vir} .R124 and λ_{vir} .R124/3. Table 19.1 shows the plating efficiency of bacteriophage lambda on the 60 isolates. 39 of the isolates were found to be wild-type, 2 mutants (50 and 56) gave elevated levels of restriction and are described in Chapter 20 and 17 potential DNA binding mutants were identified. Figure 19.4 shows the location of these mutations within the C-terminal variable domain.

Table 19.1. Complementation by the over-produced HsdS protein with the *EcoR124/3* R-M system. Phage λ plaques modified against each indicated restriction system were isolated from bacterial lawns and cycled twice on the same bacteria to produce working stocks of the modified bacteriophage. The plating efficiency of bacteriophage λ was obtained as the ratio of the titre on the test culture to that on C600. Plasmid pJS491 over-produces HsdS(R124). Plasmid pKF650 produces *EcoR124/3* restriction endonuclease (Patel *et al.*, 1992).

Modified phage	Plating efficiency of bacteriophage λ					
	JM109(DE3)	JM109(DE3) [pJS491]	JM109(DE3) [pJS491] [pKF650]	C600	C600 [pCP1005]	C600 [pKF650]
λ vir.C600	1.0	1.0	10^{-5}	1.0	10^{-4}	10^{-4}
λ vir.[pKF650]	1.0	1.0	10^{-4}	1.0	10^{-4}	1.0
λ vir.[pJS491][pKF650]	1.0	1.0	1.0	1.0	1.0	1.0
λ vir.[pCP1005]	1.0	1.0	10^{-2}	1.0	1.0	10^{-4}

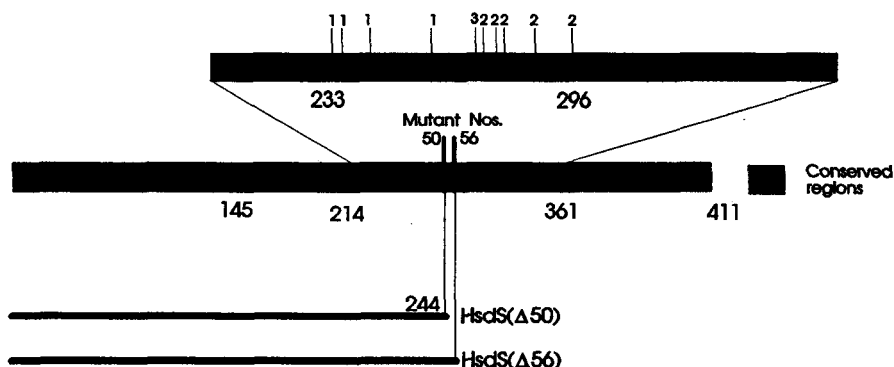


Figure 19.4. Location of the potential DNA binding mutants identified by the complementation assay. The mutants lie between amino acids nos 233 and 296 (the numbers above the lines indicate the number of mutants obtained at each location).

All the mutants that exhibited changes in DNA sequence were associated with changes in the restriction-phenotype (data not shown). That is the competition assay is sufficiently sensitive to detect mutations within the *hsdS* gene. Isolate numbers 15 and 53 did not to give a positive competition assay and are, therefore, altered in their protein configuration and cannot assemble with the other subunits. The bulk of the mutants obtained in this region are capable of protein-protein interactions, but show reduced levels of restriction. This strongly suggests these mutants are altered in their ability to bind DNA. We are currently investigating these mutants by means of gel retardation following production of the mutant DNA methyltransferases.

Discussion

The HsdS subunit isolated from the over-producing strain JM109(DE3)[pJS491] was found to be insoluble and could not be used directly to study DNA binding. To overcome this problem *M.EcoR124* DNA methyltransferase was over-produced from the T7 promoter. Excess HsdM protein produced by the plasmid pJS4M was found to solubilize HsdS and, therefore, produce a soluble, functional methylase. The DNA binding characteristics and stoichiometry of this methylase have been determined (Patel *et al.*, 1992; Taylor *et al.*, 1992).

The over-produced HsdS(R124) subunit was found to compete with the HsdS(R124/3) subunit, produced by the compatible plasmid pKF650, for production of an intact endonuclease. This allowed us to develop an *in vivo* complementation assay for mutant HsdS subunits that could potentially determine whether the mutation affects protein-protein interaction or DNA binding.

A number of mutations were produced, using misincorporation mutagenesis, within the putative C-terminal DNA binding domain of *EcoR124*. Both types of mutants were identified using the complementation assay – two mutants failed to show any competition with the wild-type HsdS(R124/3) subunit, and are presumably unfolded or partially folded mutants. The remaining mutants are all capable of protein-protein interaction but show reduced levels of restriction and are, therefore, potential DNA binding mutants.

We have shown, therefore, that the *in vivo* complementation assay is an extremely sensitive assay for detection of potential DNA binding mutants within the *EcoR124* R-M system. We intend to utilize this assay to map the location of the DNA binding domains of *EcoR124* in more detail.

Acknowledgements

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