

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

II. Mutations affecting subunit assembly

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Introduction

Type I restriction endonucleases are complex multimeric enzymes comprising three subunits. HsdM and HsdS are sufficient to produce an active methylase and have the stoichiometry M₂S (Taylor *et al.*, 1992; D. Dryden, personal communication). To produce an active endonuclease the HsdR subunit is also required along with the co-factors ATP, Mg²⁺ and *S*-adenosyl methionine (Kuhnlein *et al.*, 1969). The stoichiometry of the endonuclease is less well defined (Eskin and Linn, 1972) and may be influenced by the level of production of the individual subunits (personal observation).

Classically mutations within the *hsdR* gene of type I R-M systems produced a R⁻M⁺ phenotype, while those in the *hsdM* or the *hsdS* produced a R⁻M⁻ phenotype (Hubacek and Glover, 1970). However, recently we have described temperature-sensitive mutations within the *hsdS* and *hsdM* genes of *EcoK* that are altered in their restriction phenotype (Zinkevich *et al.*, 1990, 1992). These mutations appear to affect the ability of the HsdS or HsdM subunits to interact with the HsdR subunit.

In this paper we describe a number of observations that suggest the assembly of these multi-subunit enzymes may be controlled by the concentration of the individual subunits. This type of control is shown to be correct it represents a novel

method for genetic control of enzyme function, beyond that of the control of transcription and translation. We also propose a testable model of how this control may function within type I R-M systems.

Materials and methods

JM109(DE3) was obtained from Promega, Madison, WI; C600 is as described by Appleyard (1954), λ vir was as in Jacob and Wollman (1954), other plasmids used are described in the text. Isolation and manipulation of DNA was as described in Maniatis *et al.* (1982). Restriction and modification, and the growth of bacteriophage lambda are as described in Hubacek and Glover (1970). Misincorporation mutagenesis was as described in Chapter 19. 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.

Results and discussion

Construction of recombinant plasmids used in this study

Construction of pKF600. The *EcoR*124 R-M system was subcloned from the plasmid pCP1005 (Firman *et al.*, 1985) into *Bam*HI/*Hind*III digested pACYC184 following *Bgl*II/*Hind*III digestion of pCP1005. This produced the plasmid pKF600 which is compatible with "pBR-related" plasmids and expresses *EcoR*124 from the natural promoters.

Construction of pVM241 and pVM Δ C23. The *hsdM* + *HsdS*₁₂₄ under the control of *PMOD*, were cloned into pBR322 on a *Bam*HI/*EcoR*I fragment isolated from F'-90-XC (Hubacek *et al.*, 1989) to produce the plasmid pZH9 (Zinkevich *et al.*, 1992). To produce the plasmid pVMC23 the equivalent fragment of the wild-type R-M system was cloned from the chromosome of C600. The appropriate recombinant fragment was identified by *in situ* hybridization with radioactively labelled pZH9 DNA. pVM241 and pVM Δ C23 were produced by removing the internal *Sma*I/*Bam*HI fragment of pZH9 and pVMC23 respectively.

Construction of pVM27. The *Hind*III fragment from pBG3 (Sain and Murray, 1980), isolated from an agarose gel, was further digested with *Sma*I, the *Hind*III cohesive end was filled in using the Klenow fragment of *E. coli* DNA polymerase I, and the resulting blunt ends were ligated to pACYC184 (Chang and Cohen, 1978) cut at the *Hind*III site and blunt-ends produced by treatment with the Klenow fragment.

The influence of over-production of HsdS on restriction

The over-production of the *HsdS*(R124) subunit, from the plasmid pJS491 (Patel *et al.*, 1992), in the presence of the *EcoR*124/3 restriction endonuclease leads to a

lower level of *EcoR124/3* restriction (see Chapter 19; Patel *et al.*, 1992). This is likely to be due to competition between HsdS(R124) and HsdS(R124/3) for the HsdR and hsdM subunits. However, over-production of HsdS(R124) in the presence of the *EcoR124* endonuclease, produced from pKF600, also leads to a reduction in the level of restriction (Table 20.1). Two possible explanations exist for this observation. The first is that the HsdS subunit can influence the level of expression of the *hsdR* gene, and thereby, reduce the level of production of the endonuclease. The second is that the HsdS subunit can interact with the HsdR subunit to produce an inactive complex, and this reduces the level of active endonuclease produced.

Table 20.1. The effect of over-production of HsdS(R124), produced by pJS491, on the level of *EcoR124* restriction, produced by pKF600

Phage	Plating efficiency of bacteriophage lambda				
	JM109(DE3)	JM109(DE3) [pKF600]	JM109(DE3) [pKF600] [pJS491]	C600	C600 [pKF600]
λ .C600	1.0	10^{-4}	10^{-2}	1.0	10^{-4}
λ .[pKF600]	1.0	1.0	1.0	1.0	1.0

Table 20.2. The influence of the level of production of HsdS on the chromosomal R-M system *EcoK* of *Escherichia coli* C600 and the temperature-sensitive phenotype of HsdS_{ts}-I (Zinkevich *et al.*, 1990).

Plasmid	Temperature °C	Restriction $\lambda_{vir.0}$	Modification	R-M phenotype
-	30	0.0001	0.9	R ⁺ M ⁺
-	42	0.0006	1.0	R ⁺ M ⁺
pMS _{ts} 64(PR->S)	30	0.0004	1.0	R ⁺ M ⁺
pMS _{ts} 64	42	0.003	1.0	R ⁺ M ⁺
pVM241(MS _{ts})	30	0.0001	1.0	R ⁺ M ⁺
pVM241	42	0.0002	1.0	R ⁺ M ⁺
pVM Δ C23 (MS)	30	0.0001	0.9	R ⁺ M ⁺
pVM Δ C23	42	0.0001	1.0	R ⁺ M ⁺
pVM30(PR->S _{ts})	30	0.0006	1.0	R ⁺ M ⁺
pVM30	42	0.5	0.1	R ⁻ M ⁻
pVM30(PR->S _{ts})	30	0.00002	0.9	R ⁺ M ⁺
pVM27(M)				
pVM30	42	0.002	1.0	R ⁺ M ⁺
pVM27				
HfrH-90-XC	30	0.003	1.0	R ⁺ M ⁺
	42	0.9	0.08	R ⁻ M ⁻

PR indicates expression from the temperature-sensitive, rightward promoter of lambda.

S_{ts} and S are the HsdS_{ts}-I subunit and HsdS respectively.

M is the HsdM subunit expressed from its natural promoter.

Table 20.2, lines 1–4 show the influence of over-production of the *EcoK* HsdS subunit on the level of the K-12 chromosomal R-M system. At 42°C the powerful rightward promoter of lambda drives the production of HsdS on pMS_K64 (Zinkevich *et al.*, 1982) in the presence of the chromosomal *EcoK* system. As with *EcoR124*, over-production of HsdS leads to a decrease in the level of restriction but by approximately one order of magnitude.

Increased production of the ts-HsdS subunits of EcoK

Table 20.2, lines 5 and 6 show the effect of increased production of the HsdS_{*ts-1*} mutant on the temperature-sensitive phenotype (Table 20.2, lines 15 and 16 show the normal *ts*-phenotype observed with a chromosomal copy of the mutant; Hubacek *et al.*, 1989). pVM241 carried the *hsdM-hsdS_{ts-1}* genes on the multicopy plasmid pBR322. The temperature-sensitivity is not observed when the genes are present on pBR322. DNA sequence analysis reveals the mutation is still present (data not shown). It appears from this data that the level of production of the temperature-sensitive HsdS subunit influences the *ts*-phenotype. It is difficult to explain how such a subtle change in the level of production of HsdM and HsdS_{*ts-1*} can affect the level of transcription of HsdR. The temperature-sensitive mutation may affect the choice of pathway used. Subtle changes in the concentration of the HsdS subunit could reverse this choice of pathway and hence remove the *ts*-phenotype. At 42°C the *hsdS_{ts-1}* gene on plasmid pVM30 (Zinkevich *et al.*, 1990) is expressed from the powerful rightward promoter of bacteriophage lambda (Table 20.2, lines 9 and 10). This produces a substantial increase in the level of production of HsdS_{*ts-1*}. This plasmid restores the *ts*-phenotype in the strain C600[pVM30] giving further support to the hypothesis that these effects reflect the influence of the subunit concentrations on the function of the endonuclease. This effect supports the hypothesis that HsdS can interact with either HsdM or HsdR in two alternate assembly pathways and the choice between which pathway of assembly is used is finely balanced.

This model is further supported by the data shown in Table 20.2, lines 11–14. Increased production of HsdM, from pVM27, in the presence of the over-produced HsdS_{*ts-1*} (pVM30) removes the *ts*-phenotype (although restriction shows a slight reduction at 42°C). The only possible explanation is that the *ts*-phenotype reflects the control of function by means of subunit–subunit interactions, and that subtle changes in subunit concentration can lead to different subunit interactions, and thereby, different functions.

"Hyper-restriction" mutants of EcoR124

In Chapter 19 we have described the isolation of mutations within the *hsdS* gene of *EcoR124* using misincorporation mutagenesis. Two unexpected types of mutants were obtained by this technique. Figure 20.1 shows the level of restriction obtained for these mutants (nos 50 and 56), both show elevated restriction by an order of magnitude. Both mutants are frameshift mutants (Figs 20.1 and 20.2) that result in the introduction of a stop codon shortly after the frameshift. This effectively deletes the last third of the HsdS subunit. We propose that this deletion removes a region of the HsdS subunit that is involved in the initial HsdS-HsdR interactions, and that

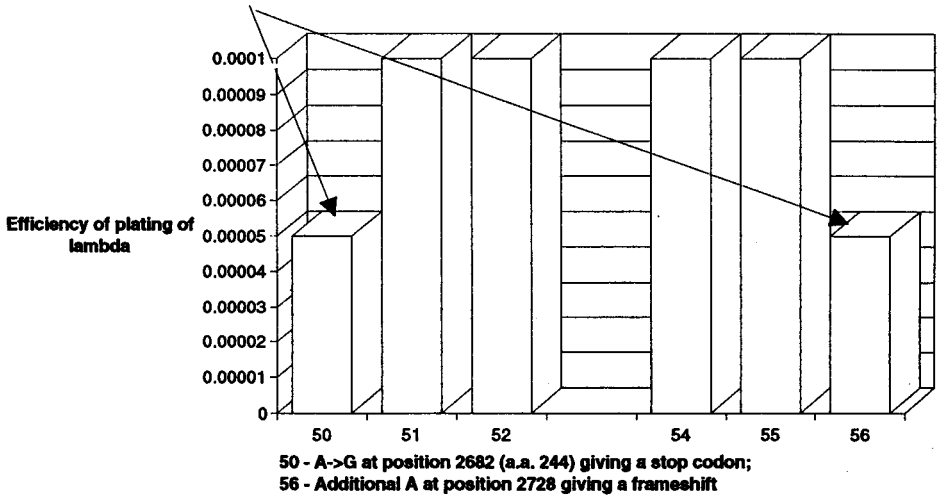


Figure 20.1. "Hyper-restriction" produced by the frameshift mutants HsdSA50 and HsdSA56.

these interactions normally lead to an inactive assembly. The higher level of restriction observed with these mutants may be due to the mutant subunits being able to follow the pathway involving HsdM as the initial interaction, and then subsequent interaction of the methylase with HsdR produces an active endonuclease (Fig. 20.3). More endonuclease is produced when only this pathway is followed and, therefore, there is a higher level of restriction.

Solubility of HsdS requires

Over-production of the *EcoR*124 HsdS subunit in JM109(DE3)[pJS4912] leads to an insoluble protein (Fig. 20.4). Variation in temperature, growth conditions and

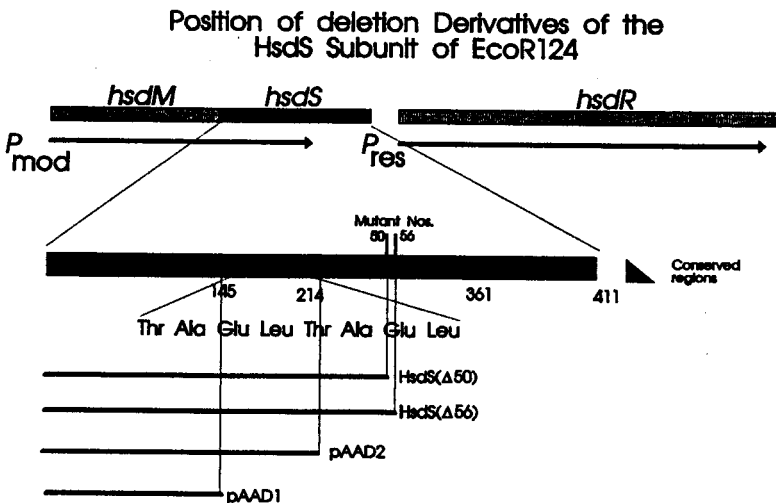


Figure 20.2. The frameshift mutants HsdSA50 and HsdSA56 result in deletion of the C-terminal conserved region of HsdS.

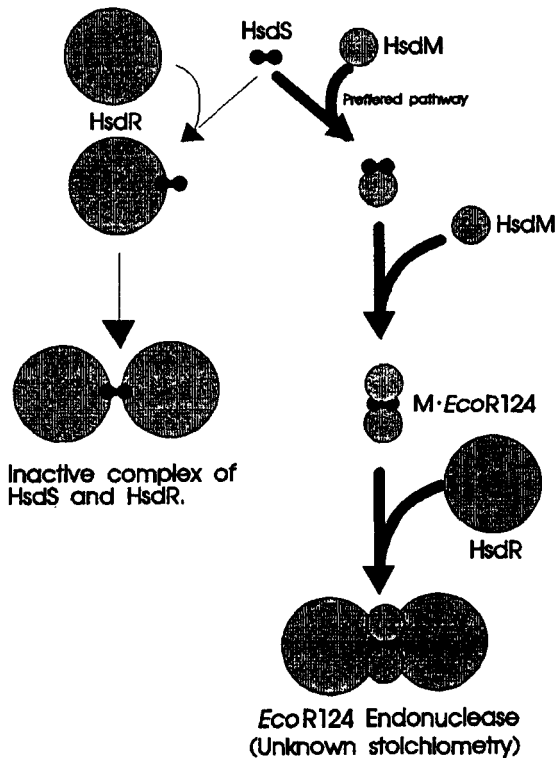


Figure 20.3. Proposed model for subunit interactions between the subunits of type I restriction endonucleases.

promoter used failed to produce any observable soluble protein (data not shown). However, in the presence of an excess of the HsdM subunit the HsdS subunit forms part of a soluble methylase (see Chapter 19). It seems likely that the HsdS subunit has a large hydrophobic surface, which can make protein–protein contacts with either HsdM or HsdR, and that this surface must be covered for HsdS to be soluble.

pAAD1 and pAAD2 are over-producing plasmids carrying the N-terminal variable domain of HsdS(R124) (Fig. 20.2). These proteins are also insoluble when produced in JM109(DE3). When they are produced in the presence of an excess of HsdM they still remain insoluble (data not shown). While this may be due to the proteins being truncated (many truncated proteins are insoluble, D. Fox, personal communication) it is also possible that these proteins lack a domain responsible for interaction with HsdM. This model is supported by the isolation of pAAD3. This plasmid over-produces the N-terminal of HsdS up to amino acid 273. This domain was found to be soluble in the presence of HsdM, but insoluble when produced alone. This lack of interaction between HsdM and HsdS may leave the N-terminal domain insoluble. Thus HsdM may act as a molecular chaperone for the correct folding of HsdS. The “hyper-restriction” mutants previously described are also deletions of the C-terminal region of HsdS but produce soluble, active *M.ecoR124* (data not shown).

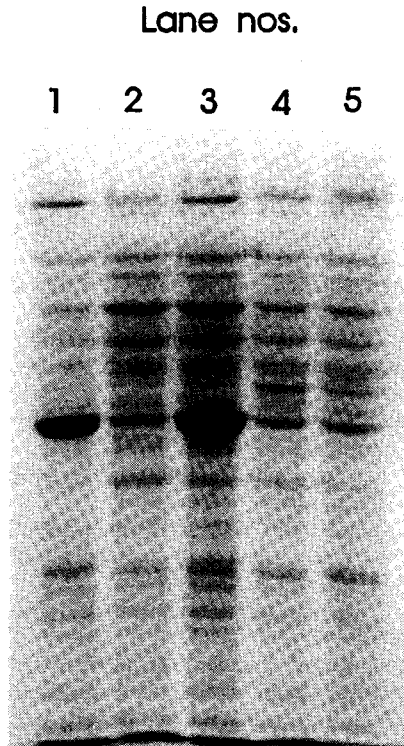


Figure 20.4. SDS-PAGE gel showing the insolubility of HsdS. *Lanes:* 1, Pellet fraction of induced JM109(DE3)[ppJS491]; 2, soluble fraction of JM109(DE3)[ppJS491]; 3, whole-cell extract of JM109(DE3)[ppJS491] (induced); 4, uninduced JM109(DE3)[ppJS491]; 5, whole-cell extract of JM109(DE3)[ppJS491] (uninduced) (Patel *et al.*, 1992).

The proposed model for subunit interactions predicts that a complex will be formed between HsdS and HsdR. The truncated polypeptides HsdSA50 and HsdSA56 will fail to produce this complex. We are currently investigating these interactions and attempting to determine the minimum polypeptide that can interact with both hsdM and HsdR.

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