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COMMUNICATION

Mutation in the Specificity Polypeptide of the Type I Restriction Endonuclease R · *Eco*K that Affects Subunit Assembly

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We describe the isolation and characterization of a temperature-sensitive mutation within the *hsdS* gene of the type I restriction and modification system *Eco*K. This mutation appears to affect the ability of the HsdR subunit to interact with the HsdS subunit when forming an active endonuclease. We discuss the possibility that this mutant, together with another mutation described previously, may define a discontinuous domain, involved in protein–protein interactions, within the HsdS polypeptide.

Keywords: temperature-sensitive mutations; protein–protein interactions

Considerable advances have been made in our understanding of the structure and function of the genes coding for type I restriction endonucleases and DNA methylases. These enzymes are encoded by three genes, *hsdR*, *hsdM* and *hsdS*‡. The products of all three genes form the multimeric, multifunctional restriction enzyme, while the two genes *hsdS* and *hsdM* are sufficient to encode the DNA methylase (Boyer & Roulland-Dussoix, 1969; Glover & Colson, 1969; Hubácek & Glover, 1970). These enzymes provide a model for the study of protein–protein interactions as well as protein–DNA interaction.

It has been known for some time that a key role for the product of *hsdS* gene is recognition of the target site sequence (Sain & Murray, 1980; Fuller-Pace *et al.*, 1985) and mutations introduced *in vitro* into the *hsdS* gene, for one such restriction and modification system, alter the DNA specificity (Gubler & Bickle, 1991). A temperature-sensitive mutation in the HsdS polypeptide of the R · *Eco*K restriction endonuclease, that converts serine^{340} to phenylalanine, is transdominant, restriction-deficient and temperature-sensitive for modification. It has been suggested that this mutation may impair the assembly of the HsdS–HsdM complex with the HsdR subunit (Hubácek *et al.*, 1989; Zinkevich *et al.*, 1990). To test this suggestion, we designed experiments to isolate mutations in *hsdS* that affected restriction activity without impairing modification.

We report here the isolation, cloning and

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‡ Abbreviations used: *hsd*, host specificity determinant; R · *Eco*K, *Eco*K restriction endonuclease; HsdS, specificity subunit; HsdM, modification subunit; HsdR, restriction subunit; P_R, rightward promoter of bacteriophage lambda; SAM, *S*-adenosyl methionine; bp, base-pair; ATP, adenosine triphosphate; $M^{-/+}$, modification-deficient/proficient; $R^{-/+}$, restriction-deficient/proficient; e.o.p., efficiency of plating.

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Table 1
R-M phenotype of E. coli *QR47(33) and* E. coli *C containing clones* hsd *genes*

Strain	Plasmid†	Temperature (°C)	e.o.p. restriction of phage λ *vir*-0‡	Modification fraction of K-plating phage produced§	R-M phenotype
QR47(33)		30	0·900	0·8000	R^-M^+
		42	0·900	0·0004	R^-M^-
QR47(33)	pZH2	30	0·800	0·7000	R^-M^+
		42	0·900	0·0010	R^-M^-
QR47(33)	pMS_k41	30	1·000	0·5000	R^-M^+
		42	1·000	0·0007	R^-M^-
QR47(33)	pMS_k64	30	0·001	0·5000	R^+M^+
		37	0·040	0·5000	R^+M^+
		42	0·040	0·6000	R^+M^+
C	pZH9	30	1·000	0·6000	R^-M^+
		42	1·000	0·0090	R^-M^-

The bacterial and phage buffers used were as described by Hubácek & Glover (1970). Restriction was measured as described by Hubácek & Glover (1970). Modification was scored using the standard *E. coli* C600 $R_K^+M_K^+$ and *E. coli* C R^-M^-.
† See Fig. 1.
‡ Restriction indicates the e.o.p. of λ*vir*-0 relative to λ*vir*-K at 30°C and 42°C.
§ Modification indicates fraction of λ*vir*-K in lysates of the transformants at 30°C and 42°C.

sequence analysis of a second temperature-sensitive mutation in *hsdS*, which appears to define a protein–protein interaction domain in the HsdS polypeptide. This domain plays an important role in the binding of the HsdR and HsdM polypeptides of HsdS in the formation of the active form of the type I multimeric restriction endonuclease R·*Eco*K.

The *Escherichia coli* strain used for the isolation of temperature-sensitive mutants was QR47 (Signer & Weil, 1968). The method of isolation was essentially the same as that originally devised by Hubácek & Glover (1970). Following mutagenesis with ethylmethane sulphonate, enrichment for restriction-deficient mutants was carried out at 42°C, followed by enrichment for restriction-proficiency at 30°C. Three mutants, which expressed temperature-sensitive restriction/modification phenotypes, were obtained. Complementation tests were carried out on these three mutants by transforming them with plasmids carrying the *hsdS* wild-type gene under the control of lambda P_R promoter (pMS_K64), the *hsdM* wild-type gene under the control of its natural promoter (pMS_K41) and the *hsdR* wild-type gene under the control of its natural promoter (pZH2). In these complementation tests, one of these mutants, QR47(33), was located in the *hsdS* gene (Table 1, lines 3 to 9). This mutation, now designated $hsdS_{ts-2}$, is distinguished from the mutation previously described Zinkevich *et al.* (1990), which is now re-designated $hsdS_{ts-1}$. Two of the mutations analysed in the complementation experiments were found to be located in the *hsdM* gene and will be described elsewhere.

Chromosomal DNA extracted from the strain QR47(33) was digested with *Eco*RI and *Sal*I and the double digested fragments separated by gel electrophoresis; DNA fragments representing the *hsd* region were identified by size selection. The DNA fragments were then ligated to an *Eco*RI/*Sal*I digest of pBR322 vector and introduced into *E. coli* C (Bertani & Weigle, 1953) by transformation. Positive clones were identified by *in situ* hybridization and those giving a strong signal were used for further analysis.

Functional expression of the cloned $hsdS_{ts-2}$ mutation was measured in *E. coli* C by testing for the modification of λ.0 *in vivo* at 30°C and 42°C using plasmid pZH9 (Fig. 1). The results shown in Table 1 (lines 10 and 11) demonstrate the very marked effect of temperature on the level of modification of bacteriophage λ, indicating that the *ts*-mutation was present on the cloned fragment.

To make the task of localizing the position of the *ts*−*2* mutation, within the *hsdS* gene, by DNA sequencing more practial, we constructed a hybrid $hsdS_{ts-2}$ gene joined at an internal *Bgl*II site. Plasmid pZH9 was used as the donor of the *Sal*I–*Bgl*II fragment containing the wild-type *hsdM* gene and the proximal part of the $hsdS_{ts-2}$ gene. Plasmid pMS_K14 carrying the wild-type *hsdS* gene was used as a recipient of the *Sal*I–*Bgl*II fragment (Zinkevich *et al.*, 1981). The purified DNA fragments were ligated and after transformation of *E. coli* C, the transformants were tested for K-specific modification of λ.0 *in vivo* at 30°C and 42°C. The plasmid, designated pZH3 (Fig. 1), showed the same level of modification of phage λ as plasmid pZH9 (data not shown). These results indicated that the temperature-sensitive mutation was located within the proximal (5′) region of the *hsdS* gene (Fig. 1).

The *hsdS* region contains convenient *Bam*HI and *Hin*dIII sites suitable for subcloning into M13 (Fig. 1). The *Hin*dIII and *Bam*HI–*Hin*dIII fragments were cloned into M13mp9 and M13mp19, respectively, and the entire DNA sequence of this region was determined by the dideoxy chain termination method of Sanger *et al.* (1980), using a series of specific oligonucleotides separated by approximately 300 bp. The sequence revealed a single point mutation, a G to A transition, which is predicted to produce a single amino acid substitution in the

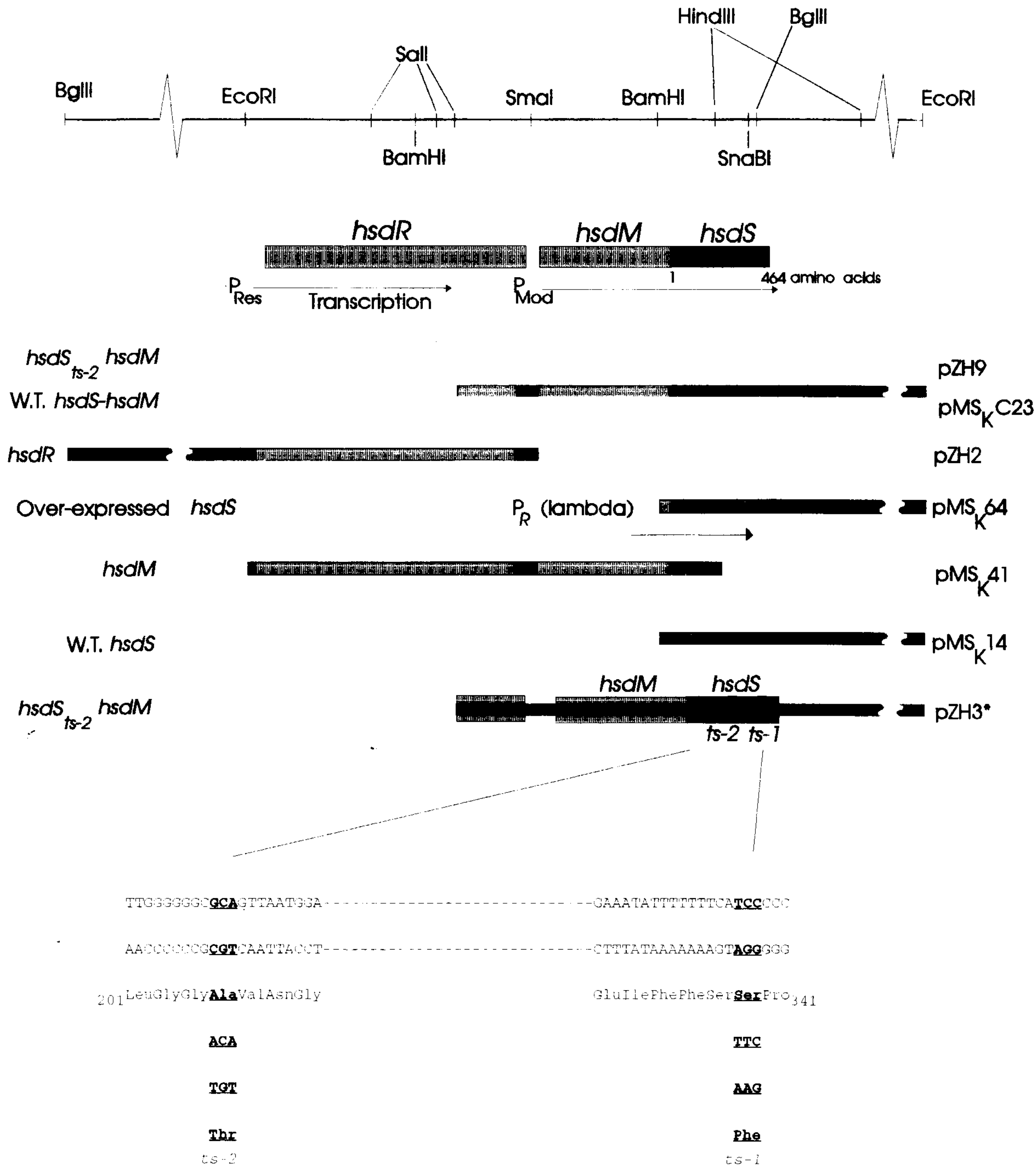

Figure 1. To construct pZH2 the plasmid pBg3 (Sain & Murray, 1980), which carries the *hsdR* and *hsdM* genes of *Eco*K was digested with *Sma*I and *Sna*BI. The large fragment, carrying the *hsdR* gene, was purified from agarose gel as described by Maniatis *et al.* (1982). Following ligation and transformation of *E. coli* WA802 (Raleigh *et al.*, 1988) transformants were screened for K-specific restriction of bacteriophage λ.0. All the other fragments identified above were cloned into pBR322, at the sites indicated by the ends of the fragments, to produce the described recombinant plasmids.

HsdS polypeptide, for alanine to threonine at position 204 (Fig. 1). As further confirmation of this position, the relevant part of the *hsdS* wild-type gene was sequenced and found to be identical to the sequence reported by Gough & Murray (1983).

We conclude that the restriction-deficiency and the temperature-sensitive modification of this mutant are determined by this single base-pair transition in the *hsdS* gene.

It is well established that the HsdR polypeptide is essential for restriction activity and is not required for DNA methylation. It is equally well established that the HsdM polypeptide is essential for restriction activity and for DNA methylation. The HsdS polypeptide plays an essential role in the sequence specificity of these enzymes. The mutation in *hsdS*, that we have described above, leads to complete loss of restriction activity and produces a temperature-sensitive effect on DNA methylation.

The restriction pathway has been analysed in

detail *in vitro* for *Eco*K (Yuan, 1981). *Eco*K is activated by SAM to produce *Eco*K*, which then binds to specific recognition sequences. If the sequence is hemimethylated, the enzyme methylates the second strand. If the sequence is unmethylated, restriction can occur. *Eco*K* undergoes an ATP-dependent conformational change to *Eco*K$^+$ and DNA translocation occurs by a process energized by ATP hydrolysis, ultimately resulting in DNA cleavage at a site distant from the recognition site.

The *Eco*K methylase is a complex of polypeptides coded by the *hsdS* and *hsdM* genes and the methyl donor is SAM, ATP is not required for DNA methylation.

The mutation in *hsdS* that we have analysed, which results in a single amino acid substitution in the HsdS polypeptide from alanine to threonine at position 204, is unlikely to affect the binding of SAM. This is consistent with the report that the binding of SAM is impaired by a mutation in *hsdM* (Buhler & Yuan, 1978). Nor is it likely to affect ATP-binding and the subsequential conformational change of the enzyme, because at the permissive temperature the mutant remains restriction-deficient but is modification-proficient. A temperature-sensitive mutation that affected either ATP binding or SAM binding would be restriction-proficient at the permissive temperature.

A temperature-sensitive mutation that affected binding to the DNA target site would be expected to be restriction-proficient at the permissive temperature, however, it is not. Furthermore, this alanine to threonine substitution is unlikely to affect the target site specificity of the enzyme because it does not lie in the two major variable regions of the polypeptide that have been proposed as domains responsible for recognition of bipartite target sequence by this family of restriction enzymes (Fuller-Pace & Murray, 1986; Loenen *et al.*, 1987; Kannan *et al.*, 1989).

Earlier Work (Hubácek *et al.*, 1989; Zinkevich *et al.*, 1990) identified a temperature-senstitive mutation in the *hsdS* gene that confers a restriction-deficient and modification temperature-sensitive phenotype, and this mutation was shown to be due to a C to T transition resulting in the substitution of a serine^{340} by phenylalanine in the distal variable region of the HsdS polypeptide. This, and the mutation described here defined a new class of mutation within the *hsdS* gene.

These mutations apparently identified a domain (or domains) that is required for restriction and modification at the non-permissive temperature.

While no physical information is available about the domains involved in protein–protein interactions in these complex enzymes, it is clear that protein–protein interactions are involved between HsdS and HsdM to form the active methylase and between HsdS, HsdM and HsdR to form the restriction enzyme complex.

We suggest that this mutation alters a domain in HsdS that is essential for HsdR binding to complex and as a result, no functional restriction enzyme is formed. The mutation additionally creates a temperature-sensitive methylase effect. Thus, the same domain must be involved in the methylase function and we suggest that the mutation results in a weakening of the binding between the HsdS and HsdM, such that at the permissive temperature, a functional methylase is formed, but cannot form at the non-permissive temperature. It has been suggested previously that the conserved regions in HsdS may play a role in interaction of the HsdS and HsdM polypeptides (Fuller-Pace & Murray, 1986).

The isolation of two mutations with this novel phenotype suggests either, that there are two domains in HsdS polypeptide, which are essential for the binding of HsdR in the complex and play a role in the binding of HsdM or, that there is a single but non-linear domain, to which the two regions identified by these two mutations contribute.

It is interesting to note that new mutations have also been identified in *hsdM* that lead to restriction deficiency and at the same time alter the specificity of the methylase (Kelleher *et al.*, 1991). These mutations may also affect the subunit assembly of the endonuclease.

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