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Restriction by *Eco*KI is enhanced by co-operative interactions between target sequences and is dependent on DEAD box motifs

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One subunit of both type I and type III restriction and modification enzymes contains motifs characteristic of DEAD box proteins, which implies that these enzymes may be DNA helicases. This subunit is essential for restriction, but not modification. The current model for restriction by both types of enzyme postulates that DNA cutting is stimulated when two enzyme complexes bound to neighbouring target sequences meet as the consequence of ATP-dependent DNA translocation. For type I enzymes, this model is supported by *in vitro* experiments, but the predicted co-operative interactions between targets have not been detected by assays that monitor restriction *in vivo*. The experiments reported here clearly establish the required synergistic effect but, in contrast to earlier experiments, they use *Escherichia coli* K-12 strains deficient in the restriction alleviation function associated with the Rac prophage. In bacteria with elevated levels of *Eco*KI the co-operative interactions are obscured, consistent with co-operation between free enzyme and that bound at target sites. We have made changes in three of the motifs characteristic of DEAD box proteins, including motif III, which in RecG is implicated in the migration of Holliday junctions. Conservative changes in each of the three motifs impair restriction.

Keywords: DEAD box motifs/DNA translocation/helicase/Rac prophage/restriction enzymes

Introduction

Restriction and modification (R–M) systems are remarkable for their diversity. They are separated commonly into three major types on the basis of their organization and properties (for reviews, see Wilson and Murray, 1991; Bickle, 1993; Bickle and Krüger, 1993; Halford *et al.*, 1993; Heitman, 1993; King and Murray, 1994; Barcus and Murray, 1995). The first R–M systems studied, now designated type I, are the most complex; they comprise three different subunits, HsdR, HsdM and HsdS. Each system recognizes its specific target sequence, the methylation status of which determines whether the R–M complex responds by modifying (methylating) or restricting (cutting) the DNA. Hemimethylated target sequences elicit methylation of the unmodified strand. Restriction, the response to unmodified target sequences, is ATP dependent and involves the cutting of non-specific DNA sequences at variable distances from target sites.

Type I systems, despite their similarities, are sufficiently heterogeneous to warrant their subdivision into different families. Within each family, polypeptide and gene sequences are well conserved, though members that recognize different target sequences have notable differences within HsdS, the single subunit that confers on the multimeric complex specificity for a particular target sequence. These regions of extensive differences, originally referred to as variable regions (Gough and Murray, 1983), identify target recognition domains (TRDs) (see any of the reviews cited above and Noyer-Weidner and Trautner, 1993).

The sequences of the *hsd* genes for representatives of three different families of type I R–M systems have been determined (Loenen *et al.*, 1987; Price *et al.*, 1989; Sharp *et al.*, 1992; Murray *et al.*, 1993) and comparisons of the inferred polypeptide sequences detect quite limited similarities, only 20–35% identities in pairwise interfamily comparisons (Sharp *et al.*, 1992; Murray *et al.*, 1993). The conserved sequences are predicted to include amino acids of key functional importance for the activities common to type I R–M enzymes. In HsdS, the similar sequences include candidate regions for subunit interactions (Kannan *et al.*, 1989; Cooper and Dryden, 1994; Kneale, 1994), or entire TRDs when two HsdS subunits share a common component in their DNA target sequence (Fuller-Pace and Murray, 1986; Cowan *et al.*, 1989). For the HsdM polypeptides two motifs have been implicated in the transfer of methyl groups to the target sequences. Analyses of mutants indicate that one of these motifs is involved in the binding of the methyl donor, *S*-adenosylmethionine, and the other in the methylation reaction itself (Willcock *et al.*, 1994).

The third subunit, HsdR, is essential for restriction, but is not required for modification. The *hsdR* genes for all three families of enzymes encode polypeptides containing motifs characteristic of ATP binding proteins, consistent with the ATP dependence of restriction. In addition, each HsdR subunit includes other motifs previously identified in ATP-dependent helicases or putative helicases (Linder *et al.*, 1989; Gorbalenya and Koonin, 1991; Murray *et al.*, 1993; Fuller-Pace, 1994). One of these, a common variant of the element Asp–Glu–Ala–Asp, or DEAD in a single letter code, has given its name to this family of proteins (Linder *et al.*, 1989).

It has been tempting to speculate that the DEAD box motifs are relevant to the mechanism of restriction, in particular to the ATP-dependent translocation of DNA that precedes the cutting of DNA (Murray *et al.*, 1993).

One attractive model for restriction by type I R–M systems postulates that cutting of DNA is stimulated when two enzyme complexes bound to their target sequences meet as the consequence of ATP-dependent DNA translocation (Studier and Bandyopadhyay, 1988). Type III R–M systems, like type I, are DEAD box proteins (Gorbalenya and Koonin, 1991; Dartois *et al.*, 1993). A common role for DEAD box motifs in the restriction subunits of type I and type III enzymes could be satisfied if ATP-dependent DNA translocation were involved in restriction by both classes of enzymes. It is known that the target necessary for restriction by a type III system consists of two recognition sequences, each of the same six nucleotides but in inverse orientation (Meisel *et al.*, 1992). The distance between the two components of the target sequence may be large, and one obvious means of communication between two distant sites would be ATP-dependent DNA translocation (Murray *et al.*, 1993). Very recent data show that DNA restriction is dependent on the hydrolysis of ATP (Meisel *et al.*, 1995; Saha and Rao, 1995), and translocation is implicated by the finding that Lac repressor bound at an operator site located between the two components of a restriction target inhibits cutting by the type III enzyme *Eco*P15I (Meisel *et al.*, 1995). A role for DNA translocation in restriction by type III systems is readily appreciated where the two components of a restriction target are some distance apart. The target for a type I R–M enzyme, however, is a single bipartite sequence which in itself is sufficient to elicit restriction either *in vivo* (Murray *et al.*, 1973a) or *in vitro* (Horiuchi and Zinder, 1972; Murray *et al.*, 1973b). Here we report evidence from *in vivo* experiments for co-operative interactions between two widely separated targets for the type I restriction enzyme *Eco*KI. We also report the isolation of mutants in which each of three of the so-called 'DEAD box' motifs of the HsdR subunit of the type I system of *Escherichia coli* K-12 (*Eco*KI) have been changed. Conservative changes in each of these three motifs affect restriction activity.

Results

The cloned hsdR gene enhances restriction

Plasmid pBg3 includes the *hsdR* gene of *E.coli* K-12 (Sain and Murray, 1980), but it provides poor amplification of HsdR and is not an ideal substrate for mutational analysis of *hsdR*. A plasmid including the T7 promoter, pT7-7, was chosen for the expression of *hsdR*. The *Bam*HI target in the polylinker of this vector was deleted to permit the subsequent manipulations dependent on the *Bam*HI target in the *hsdR* gene (pT7-7Δ, see Figure 1). The 3.6 kb *Eco*RI–*Sma*I fragment including all but the 5′ end of *hsdR* was cloned in pT7-7Δ (pSB1, Figure 1). The N-terminal sequence of HsdR (D.T.F.Dryden and L.Cooper, personal communication) is consistent with initiation of translation at the second methionine codon of the open reading frame that identified *hsdR* (Loenen *et al.*, 1987). This N-terminal sequence defined the length of the short oligonucleotide linker (Figure 1) which, on insertion into pSB1, completed the coding sequence for HsdR. The resulting plasmid, pSB2, includes the *hsdR* gene with the upstream translational initiation region and promoter of T7 gene 10 and it contains unique *Kpn*I and *Bam*HI sites flanking the three sequences targeted for site-directed mutagenesis.

Plasmid pSB2 was recovered in the *hsd*Δ strain NM679 in the absence of the λDE3 prophage, and consequently in the absence of T7 RNA polymerase. The phenotypic test for a functional HsdR polypeptide requires the presence of HsdM and HsdS to generate the *Eco*KI complex, therefore transformants of $hsdR\Delta 4M^+S^+$ strains were isolated and checked for restriction. Both NM526 and a derivative lysogenic for λDE3, which includes the T7 gene encoding RNA polymerase under the control of the *lac* UV5 promoter, were used. Transformants of both strains were r_k^+, indicating expression of the *hsdR* gene even in the absence of T7 RNA polymerase. The combined presence of the λDE3 prophage and pSB2 resulted in strains that grew slowly even in the absence of inducer. Since T7 RNA polymerase was unnecessary for the expression of *hsdR*, all quantitative phenotypic tests were made in the absence of the λDE3 prophage.

Transformants of a number of hsd^+ and $hsdR^- M^+S^+$ strains were isolated and checked for restriction of λ*vir*.0. All were r_k^+. However, for transformants of either AB1157 or Ymel, hsd^+ strains lacking the Rac prophage, restriction was greatly enhanced.

The Rac prophage present in most *E.coli* K-12 strains reduces the maximal level of restriction by *Eco*KI (Loenen and Murray, 1986; King and Murray, 1995). It is assumed that in rare cells ($\sim 10^{-4}$) the Rac prophage spontaneously induces and, as a consequence, its restriction alleviation gene is expressed and restriction is blocked. In the absence of the Rac prophage, the presence of multiple copies of *hsdR* in strains transformed with pSB2 leads to elevated levels of restriction. In a $Rac^+r_k^+$ strain, the efficiency of plating (e.o.p.) of λ*vir*.0 is 2×10^{-4}, in a Rac^- strain, such as AB1157 or Ymel, it is reduced to 10^{-5} while the presence of pSB2 in a Rac^- strain reduces it to as little as 10^{-8}. We conclude that in wild-type cells restriction is limited by a deficiency of HsdR. This conclusion is consistent with estimates of the relative levels of Hsd subunits in minicells carrying a plasmid encoding *Eco*KI (Weiserova *et al.*, 1993).

Co-operative interactions between widely separated targets

The realization that the restriction alleviation function of the Rac prophage obscures the maximal level of restriction led us to re-examine the correlation between target number and level of restriction. The Rac^- strains AB1157 and AB1157 transformed with pSB2 were used.

Hybrid phages ($h^{80}imm^{\lambda}$) with zero, one or two targets for restriction for *Eco*KI (Murray *et al.*, 1973a; Brammar *et al.*, 1974) were grown on a non-modifying strain (NM679) and their e.o.p. on a $Rac^-r_k^+$ strain (AB1157) determined relative to a congenic $hsdR^-$ derivative (NM795). Derivative phages that lacked targets for *Eco*KI were used to monitor any other factors affecting e.o.p. In the absence of co-operative effects between targets, the expected restriction of a phage with two targets was calculated as the product of the values found for each of the two phages with single targets. Two phages with two targets were tested; one has two λ targets (skλ1 and skλ2) separated by ~12 kb and the other, a *trp*-transducing

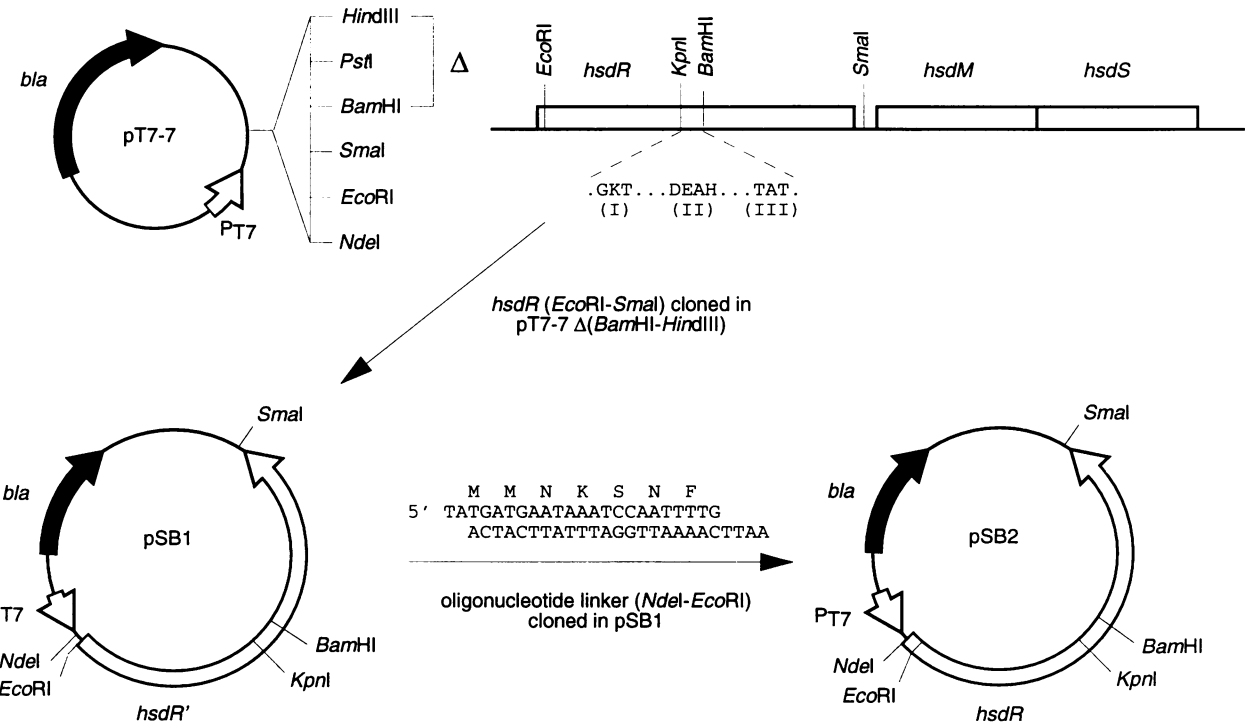

Fig. 1. Origin of pSB2. A derivative of the expression vector pT7-7 was made which lacks the *Bam*HI target in the polylinker (pT7-7Δ). The 3.6 kb *Eco*RI–*Sma*I fragment containing most of *hsdR* was cloned in pT7-7Δ (pSB1), and the 5′ end of *hsdR* was added as an *Nde*I–*Eco*RI synthetic linker (pSB2). The only *Kpn*I and *Bam*HI targets in pSB2 are those that flank the sequence encoding the DEAD box motifs I–III. *bla*, gene coding for β-lactamase; pT7, the promoter and upstream translational initiation region from phage T7 gene 10; GKT, DEAH and TAT denote the positions of the DEAD box motifs I, II and III, respectively. The maps are not drawn to scale.

Table I. *Eco*KI targets co-operate to enhance restriction

Targets in $h^{80}imm^{\lambda}$ phages	Host	No. of experiments	'Enhanced restriction' = Observed value[a] / Expected value[b]		
			Minimum	Maximum	Average
skλ1 + skλ2 (targets ~12 kb apart)	AB1157	10	7	18	15
sk*trp* + skλ2 (targets ~2 kb apart)	AB1157	9	8	20	13
skλ1 + skλ2	AB1157 + pSB2	5	0.6	1.6	1.1
skλ*trp* + skλ2	AB1157 + pSB2	4	1.4	1.6	1.5

[a]Observed value determined from e.o.p. of phage with two targets. The e.o.p. on AB1157 was determined relative to NM795, the *hsdR* derivative of AB1157, and on AB1157 + pSB2 relative to NM795 harbouring the vector plasmid pT7-7.
[b]Expected value based on the product of the values for the two phages with one target.

phage, has skλ2 and a second target in *trpE* (sk*trp*) separated by only a few kilobases.

In all experiments using AB1157, restriction of phages with two targets was much in excess over the value predicted in the absence of co-operative interactions between targets. The lowest estimate of enhanced restriction for AB1157 was 7-fold, the highest 20-fold (Table I). In contrast, in the presence of the plasmid pSB2, when restriction of phages with even single targets is greatly elevated (400-fold rather than 20-fold in AB1157), the observed restriction for two targets was as expected in the absence of co-operative interactions between targets (Table I).

Mutations in the DEAD box motifs

The sequences in which the mutations were made are shown in Figure 2. Motifs I and II are as identified by Gorbalenya and Koonin (1991). These authors, however, noted that motif III was difficult to identify in the *hsdR* gene of a type IA system. The recently corrected nucleotide sequence for *hsdR* of *E.coli* K-12 (Blattner *et al.*, unpublished, GenBank accession No. U14003) introduces a short frame-shifted segment within which motif III is readily recognized. This sequence, TAT, aligns with TAT and TGT respectively, in type IB and IC HsdR polypeptides (Figure 2). TAT was the third target for mutagenesis.

Mutations in *hsdR* were made by site-directed mutagenesis using PCR. The 550 bp fragment, generated by PCR, was cut with *Bam*HI and *Kpn*I, or its neoschizomer *Asp*718, and substituted for the wild-type sequence of pSB2. The nine mutations shown in Figure 2 were identified by DNA sequence. All plasmids contain the expected sequence in the PCR-derived region and no other changes.

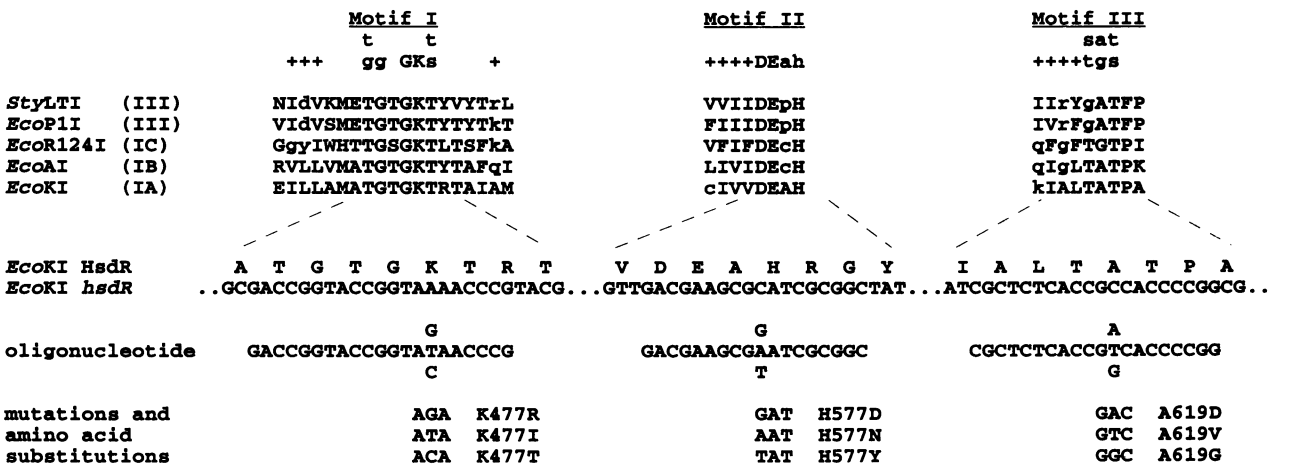

Fig. 2. Alignment of DEAD box motifs in type I and III R–M systems and identification of the substitutions made in *Eco*KI. The consensus sequence for DEAD box proteins is shown above an alignment of the predicted amino acid sequences of *Sty*LTI, *Eco*P1I (type III R–M systems), *Eco*R124I, *Eco*AI and *Eco*KI (type IC, IB and IA R–M systems, respectively) in the regions of motif I, II and III. Shown below is the nucleotide sequence of *hsdR* from *E.coli* K-12 in the region of each motif, the sequence of one of each pair of complementary oligonucleotides used for the site-directed mutagenesis, and the amino acid substitutions generated. The numbers identify the location of the amino acid in the HsdR polypeptide. In the DEAD box consensus, upper case letters indicate invariant residues, lower case letters denote preferred residues and the symbol '+' is for hydrophobic residues. In the alignments, lower case letters are used to indicate a deviation from the consensus sequence.

Table II. The effects of changes in the DEAD box motifs on the restriction phenotype

Motif	Sequence	Mutation on plasmid		Mutation on chromosome[b]
		in *hsdR*$^-$ host[a]	in *hsdR*$^+$ host[a]	
(I)	GTG**R**T	r_k^-	r_k^-	r_k^-
GTG**K**T	GTG**I**T	r_k^-	r_k^-	nt
	GTG**T**T	r_k^-	r_k^-	nt
(II)	DEA**D**	r_k^-	r_k^-	r_k^-
DEA**H**	DEA**N**	r_k^-	r_k^-	nt
	DEA**Y**	r_k^-	r_k^+	r_k^-
(III)	T**D**T	r_k^-	r_k^-	nt
T**A**T	T**V**T	r_k^-	r_k^-	r_k^-
	T**G**T	r_k^+	r_k^+	r_k^{+c}

[a]The *hsdR*$^-$ strain, NM795, is a derivative of the *hsdR*$^+$ strain, AB1157.
[b]The mutations were transferred to the chromosome of AB1157 via a λ*hsdR*$^-$ phage.
[c]See Table III for quantification.
r_k^- is inferred from an e.o.p. of ~1 relative to the r_k^- strain NM795; nt = not tested.

Phenotypes of mutants

Tests dependent on multicopy plasmids. The parent plasmid, pSB2, when maintained in an *hsdR* strain, compensates for the chromosomal *hsdR* mutation; the restriction-proficient phenotype was recognized by the low e.o.p. of λ*vir*.0. The phenotypes associated with the presence of the mutant derivatives of pSB2 were tested in the same way; a low e.o.p. of unmodified phages would be indicative of a functional HsdR polypeptide. On the basis of this test, only one mutation (TAT to TGT in motif III) fails to inactivate the HsdR polypeptide (Table II).

This test, however, is not ideal for at least two reasons. First, any cell lacking the plasmid will fail to restrict λ*vir*.0 and progeny phages will be modified by the *Eco*KI methyltransferase. It is, therefore, critical that the vast majority of cells retain the plasmid, otherwise a false indication of restriction deficiency will result. Second, the level of HsdR polypeptide produced by the multicopy plasmid will be very different from that found in a cell with only a chromosomal copy of the *hsdR* gene. The first of these problems is avoided if the mutant polypeptide can compete with wild-type and, particularly when present in excess, can convert an *hsd*$^+$ host into an r_k^- phenocopy.

Each of the nine plasmids carrying a mutant *hsdR* gene was used to transform the r_k^+ strain AB1157, and the restriction phenotypes of the transformed strains were tested. Seven of the transformed strains were clearly r_k^- (see Table II), a phenotype that requires the presence of an inactive HsdR polypeptide. Two were r_k^+, consistent with either a functional HsdR polypeptide, or an inactive polypeptide that fails to displace the wild-type HsdR subunit.

Test dependent on mutations in the bacterial chromosome. Quantification of the restriction phenotype of any *hsdR* mutant is only possible if the mutation is stably maintained in single copy on the bacterial chromosome, preferably in its normal location under the control of its resident promoter. Five of the mutations have been transferred to the chromosome of the Rac$^-$ strain AB1157 and restriction quantified using λ*vir*.0. All but one of the mutants tested, including the conservative change of DEAH to DEAD,

Table III. Changing motif III from TAT to TGT reduces restriction

Phage (targets for *Eco*KI)	Efficiency of plating (e.o.p.)		Restriction[b]	
	Wild-type[a]	Mutant (TGT)[a]	Wild-type	Mutant
λNM105	2.7×10^{-4}	1.1×10^{-2}	3227 ± 1007	121 ± 51
(skλ1 + skλ2)	2.9×10^{-4}	9.6×10^{-3}		
	5.7×10^{-4}	1.1×10^{-2}		
	2.5×10^{-4}	5.1×10^{-3}		
λNM175	1.6×10^{-2}	8.6×10^{-2}	38 ± 23	8.3 ± 2.4
(skλ1)	1.6×10^{-2}	1.2×10^{-1}		
	7.7×10^{-2}	1.4×10^{-1}		
	3.8×10^{-2}	1.1×10^{-1}		
	4.3×10^{-2}	1.9×10^{-1}		
λNM176	2.3×10^{-2}	3.1×10^{-1}	33 ± 10	3.5 ± 1.5
(skλ2)	2.8×10^{-2}	4.8×10^{-1}		
	2.9×10^{-2}	3.1×10^{-1}		
	5.0×10^{-2}	1.8×10^{-1}		

[a]The wild-type strain AB1157 and the congenic mutant derivative were used. The e.o.p. was calculated relative to an r_K^- derivative of AB1157. In all experiments a hybrid phage with no targets for *Eco*KI gave an e.o.p. of ~1.
[b]A value for restriction was estimated from the inverse of each e.o.p. The means and standard deviations are shown.

were completely deficient in restriction. The remaining mutant (TAT to TGT) gave reduced levels of restriction, only readily detected for phages with one or two targets (Table III), rather than for λ*vir* with its complement of five targets (Table II). All of the nine mutations affect restriction, eight of them severely, and seven of these make polypeptides that compete with wild-type for interaction with the methyltransferase. As expected, the chromosomal *hsdR* mutations affected only restriction and not modification.

Discussion

Many enzymes containing the purine NTP binding motif have been identified as members of a 'super family' sharing a number of motifs including GxGKS/T, an abridged version of the 'A' component of the ATP binding motif (Linder *et al.*, 1989). Within this super family of 'DEAD box' proteins, there are subgroups which include either the motif 'DEAD' or 'DExH', (where x is commonly A or C), and at least five other conserved regions (Linder *et al.*, 1989). Some of these proteins are known to be helicases, the remainder are regarded as putative helicases (Schmid and Linder, 1992).

Mutational analyses of DEAD box proteins have been reported, the most extensive being for an RNA helicase, the mammalian translation initiation factor eIF-4A (Pause and Sonenberg, 1992). The results suggest that the highly conserved regions in the DEAD box proteins are critical for helicase activity. Mutations in motifs I and II affect ATP binding and ATP hydrolysis respectively, while those in motif III can affect helicase activity in the absence of any major effect on ATP hydrolysis.

Some members of the DEAD box protein family have been shown to be DNA, rather than RNA, helicases. These include RAD 3 of yeast (Sung *et al.*, 1988), and RecG (Sharples *et al.*, 1994) and RecQ (Umezu *et al.*, 1990) of *E.coli*, all involved in recombination and repair processes. RecG includes all seven motifs characteristic of the DExH family (Lloyd and Sharples, 1991) and catalyses branch migration of Holliday junction intermediates in a reaction that requires ATP hydrolysis. A mutation in motif III, in which the sequence TAT was changed to TVT, was shown to reduce ATP hydrolysis and to block branch migration (Sharples *et al.*, 1994). For RecG, it is concluded that branch migration of Holliday junctions is related to an ATP-dependent helicase activity.

A search of the inferred HsdR polypeptide sequences of *Eco*KI (type IA) and *Eco*R124I (type IC) for the seven motifs identified in one family of DEAD box proteins identified six of the seven in each HsdR polypeptide (Gorbalenya and Koonin, 1991). The counterpart to motif III was only identified with any confidence in *Eco*R124I and that to motif IV only in the HsdR polypeptide of *Eco*KI. More recently, two items of sequence data add further support to justify the identification of type I R–M systems as DEAD box proteins. First, within the inferred sequences of the HsdR polypeptides of *Eco*AI and *Eco*EI, two members of another family (type IB) of type I R–M systems, all seven motifs were identified (Murray *et al.*, 1993). Second, the correction to the sequence of the *hsdR* gene of *E.coli* K-12, made by the Blattner group (GenBank accession No. U14003), introduces a short frame-shift within which is a convincing motif III, one of the targets of our analysis. The 'DEAD box' motifs I, II and III in the HsdR polypeptides of three type I systems are shown in Figure 2, together with those identified in two type III R–M systems (Gorbalenya and Koonin, 1991; Dartois *et al.*, 1993).

Comparisons of the amino acid sequences of the HsdR polypeptides of the three families of type I R–M systems with those of the restriction subunits of type III enzymes emphasize the DEAD box motifs. It has been suggested that the implicated helicase activity may be involved in the local unwinding of DNA at the cleavage site (Gorbalenya and Koonin, 1991; Dartois *et al.*, 1993). There are some type II enzymes (type IIs) which do not cut DNA within their target sequence. These enzymes are not ATP dependent, they are not DEAD box proteins, and DNA translocation is not implicated. Rather than a role for DEAD box motifs in local unwinding of DNA at a cleavage site removed from the target sequence, we favour a role in the ATP-dependent translocation that has been proposed to precede DNA cutting, by both type I and type

III R–M enzymes. Recent data for type III enzymes (Meisel *et al.*, 1995), and the experiments reported in this paper, emphasize DNA translocation as the feature common to type I and type III R–M systems.

The model proposed for *Eco*KI (Studier and Bandyopadhyay, 1988), in which the cutting of DNA is stimulated when two *Eco*KI enzyme complexes bound to their target sequences meet as the result of ATP-dependent DNA translocation, can be adapted for type III systems (Murray *et al.*, 1993; Meisel *et al.*, 1995). The target sequence necessary to elicit restriction by the type III enzyme *Eco*P15I comprises two identical, but inversely oriented, sequences separated by as much as 3 kb; DNA translocation would provide the necessary means of communication between the two components of the target.

Early experiments showed that *Eco*KI remains bound to its target sequence but cuts the DNA elsewhere (Bickle *et al.*, 1978). The model of Studier and Bandyopadhyay (1988) was based on the demonstration that, under appropriate conditions, DNA cutting does not occur randomly, but mid-way between two target sites. Although this model is well supported by the analysis of the products of *in vitro* reactions, previous quantification of restriction *in vivo* has not indicated the predicted co-operative interactions between pairs of target sequences (Murray *et al.*, 1973a). Furthermore, the observation that a single target within a phage genome is sufficient to elicit restriction requires significant modification of the model. The *in vivo* experiments presented in this paper now document the anticipated co-operative interactions between pairs of well separated target sequences. They also show that at higher concentrations of enzyme co-operative interactions are less evident, in agreement with the suggestion that unbound enzyme can co-operate with bound complex and cut DNA substrates that have only one target sequence (Studier and Bandyopadhyay, 1988). The signal necessary to elicit restriction by *Eco*KI *in vivo*, when a phage genome has only a single target, could be dependent on the substrate being a covalently closed, circular molecule and the consequent constraints imposed on DNA translocation. *In vitro*, cutting linear λ genomes with only single targets for *Eco*KI was inefficient and needed 40-fold more enzyme than the cutting of substrates with two or more targets (Murray *et al.*, 1973b).

It has been suggested that the DNA translocation mediated by *Eco*KI is associated with topoisomerase activity, but the nicking and joining of DNA strands by *Eco*KI has not been documented (Yuan *et al.*, 1980). Similarly type I and III R–M enzymes remain *putative* DNA helicases; our genetic data indicate that the motifs characteristic of DEAD box proteins are relevant to restriction but their role, even if indicative of helicase activity, remains unknown. Of particular interest is the change of the TAT sequence to TGT, the sequence found in the type IC enzyme *Eco*R124I. This motif has been correlated with helicase activity in other systems (Sharples *et al.*, 1994). For *Eco*KI, as is the case for RecG, the change from TAT to TVT leads to the loss of activity, whereas the very conservative change to TGT leads to only a modest reduction in the efficiency of restriction without a loss in co-operativity. Conservative changes in motifs I and II (GTGKT to GTGRT and DEAH to DEAD) resulted in subunits that appear to compete with wild-type HsdR for the other components of the complex and yield enzymes defective in restriction. Mutations in the HsdR polypeptide should facilitate the dissection of the complex, ATP-dependent steps that intervene between the specific binding of *Eco*KI to its target sequence and the cutting of DNA at sites remote from the target sequence.

The expression of *hsdR* in pT7-7 did not provide effective yields of HsdR, as assayed by immunological tests of polypeptides following their separation on polyacrylamide gels (data not shown), although the restriction phenotype associated with the presence of pSB2 in the *hsd*$^+$ strain AB1157 indicated enhanced levels of *Eco*KI. Even the levels of HsdR provided by pSB2 were detrimental to the bacterial cell in the presence of the other subunits needed to make the restriction enzyme. This suggests that an excess of modification enzyme is normally required to maintain viability of the bacteria. Since the endonuclease is active only on unmethylated DNA, some newly synthesized DNA, perhaps the product of DNA repair, may be the target for restriction.

Materials and methods

Bacterial strains, phages and plasmids

Three restriction-proficient (r_k^+), modification-proficient (m_k^+) *E.coli* K-12 strains, C600 (Appleyard, 1954), Ymel (Rickenberg and Lester, 1955) and AB1157 (Low, 1973), were used. C600 generally has been the standard $r_k^+m_k^+$ host, but both Ymel (King and Murray, unpublished observations) and AB1157 (Kaiser and Murray, 1979) are preferred hosts for quantifying restriction because they lack the restriction alleviation function associated with the Rac prophage. The phenotypes of the *hsdR* mutants were first examined following transformation of *hsdR*(r_k^-) derivatives of Ymel (NM526), and AB1157 (NM795), with plasmids carrying mutated *hsdR* genes. NM526 has an extensive deletion (Δ4) in the *hsdR* gene; NM795 is a missense mutant. Checks for negative complementation were made following transformation of AB1157. NM526 lysogenic for λDE3 (Studier and Moffatt, 1986; King and Murray, 1995) was used to elicit transcription of *hsdR* by T7 RNA polymerase.

DNA from λNM1050, a phage including the complete coding sequence for *Eco*KI (Sain and Murray, 1980), was the template for amplification by the PCR. λ virulent (laboratory collection), either unmodified (λ*vir*.0) following propagation on NM679 (Δ*hsdRMS*; King and Murray, 1995), or *Eco*KI-modified (λ*vir*.K) by growth on C600, was used to measure restriction activity. The plasmid pBg3 (Sain and Murray, 1980) was the source of the truncated *hsdR* gene cloned in the expression vector pT7-7 (Tabor, 1990). Phages λNM1265 (Whittaker *et al.*, 1988) and λNM1347, a *c*I857 derivative of λNM1266 (Whittaker *et al.*, 1988), were used to clone mutant *hsdR* genes and facilitate their transfer from the plasmid to the host chromosome.

Hybrid phages with the left arm of *phi*80 substituted for that of λ have reduced numbers of targets (sk) for *Eco*KI (Franklin and Dove, 1969). Hybrid phages with either one or two targets were used as substrates to monitor restriction *in vivo*. Phages of the genotype h^{80} att^{λ} *int*29 skλ2$^+$ *c*I857 skλ1$^+$ and mutant derivatives that have lost skλ1, skλ2 or both targets were those described by Murray *et al.* (1973a). Hybrid *trp*-transducing phages with a target in *trpE* (sk*trp*) and skλ2 together with mutant derivatives lacking either one or the other site have been described by Brammar *et al.* (1974).

DNA manipulation

Preparation and manipulation of DNA, nucleotide sequencing and site-directed mutagenesis using the PCR were performed as described previously (King and Murray, 1995). The origin of the plasmid, pSB2, that encodes the HsdR polypeptide is shown in Figure 1.

The three motifs analysed by site-directed mutagenesis are between unique *Kpn*I and *Bam*HI targets in the *hsdR* gene of plasmid pSB2. The oligonucleotide primers used in the PCR to amplify this 550 bp region were 5′CGATGGCGACCGGTACCG and 5′GGCGGATCCTGGTCG-ATC, in which the *Kpn*I (*Asp*718) and *Bam*HI targets respectively are underlined. The *hsdR* fragment was excised by *Bam*HI in combination with either *Kpn*I or *Asp*718.

The site-directed mutagenesis of motifs II and III used the recombinant PCR technique as described by Higuchi (1990). One of the primers used to change the coding sequence for each motif is given in Figure 2; the second primer was the complement. In each case, degeneracies were introduced at one position (see Figure 2). For motif I the mutations were sufficiently close to the *Kpn*I site that the oligonucleotide primer included both the target site and the sequence to be mutated. The sequence of this primer, and the degeneracies associated with the codon for the lysine (K) residue, are also shown in Figure 2. $Vent_R$ DNA polymerase (New England BioLabs) was used, and for each mutation the nucleotide sequence of the entire *Kpn*I–*Bam*HI fragment was determined to identify the mutation and confirm the absence of other mutations.

The mutations were transferred to a λ vector to facilitate their transfer to the *E.coli* chromosome. The 3.6 kb *Eco*RI–*Sma*I fragment containing all but the first few codons of *hsdR* (see Figure 1) was excised from mutant derivatives of pSB2 and used as a linker to join the left arm of the *Pam* phage λNM1265 to the right arm of the *Eam* phage λNM1347. The genome of the former phage was cut with *Sma*I, and that of the latter with *Eco*RI. Phage genomes were recovered by *in vitro* packaging (Epicentre Technologies). The required recombinants, which in contrast to both parental phages lack amber mutations, were selected on a *sup*° *hsd*Δ host NM679. The presence of the insert was confirmed by analysis of digests of phage DNA.

Transfer of mutations to the bacterial chromosome

The λ phages including *hsdR* encode a temperature-sensitive repressor (*c*I857) and are defective in their attachment site (*b*527). Lysogens selected as immune colonies at 32°C frequently result from homology-dependent recombination, but they occasionally lose their prophage by homologous recombination. These 'cured' clones may be selected at 42°C. The *b*527 mutation greatly reduces the frequency of site-specific integration but does not entirely prevent it. Lysogens resulting from site-specific recombination only rarely produce cured clones and liberate no or few progeny phage.

λ phages in which the *hsdR* mutation has inactivated restriction activity gave both r_k^+ and r_k^- lysogens of AB1157, depending on the location of the integrative cross-over with respect to the mutation. Roughly 50% of the cured colonies selected at 42°C have replaced the chromosomal *hsdR* allele with that of the phage. The presence of the required mutation on the chromosome was confirmed by determination of the nucleotide sequence using the PCR. One mutant (A619G) failed to give r_k^- lysogens in an $hsdR^+$ host but, as expected, some cured derivatives acquired the mutation.

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